The Microbiome and Host Behavior

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Abstract

The microbiota is increasingly recognized for its ability to influence the development and function of the nervous system and several complex host behaviors. In this review, we discuss emerging roles for the gut microbiota in modulating host social and communicative behavior, stressor-induced behavior, and performance in learning and memory tasks. We summarize effects of the microbiota on host neurophysiology, including brain microstructure, gene expression, and neurochemical metabolism across regions of the amygdala, hippocampus, frontal cortex, and hypothalamus. We further assess evidence linking dysbiosis of the gut microbiota to neurobehavioral diseases, such as autism spectrum disorder and major depression, drawing upon findings from animal models and human trials. Finally, based on increasing associations between the microbiota, neurophysiology, and behavior, we consider whether investigating mechanisms underlying the microbiota-gut-brain axis could lead to novel approaches for treating particular neurological conditions.

Contents

INTRODUCTION

The brain integrates complex sensory information and responds to the needs and experiences of each body system. The microbiota, comprising communities of bacteria, viruses, fungi, and other microorganisms that live mutualistically in and on animals, is increasingly recognized as an essential component of normal physiology, with important roles in health and disease. As the first life forms on the planet, microorganisms are integrated fundamentally across biological scales. They regulate the biogeochemical cycling of elements essential for life; form the likely endosymbiotic origins of genomic elements, eukaryotic organelles, and multicellular organisms; and maintain homeostatic interactions within and across plant, animal, and atmospheric ecosystems. Recent advances in sequencing, mass spectrometric, bioinformatic, and gnotobiotic technologies have enabled investigations into roles for host-associated microbiota in modulating physiological systems, including gastrointestinal function, immunity, and metabolism, as well as select host behaviors. Perturbations in the microbiota have been associated with changes in social, communicative, stress-related, and cognitive behavior in lab animals and animals in the wild. Additional evidence suggests that the microbiota influences core neurological processes, including neurogenesis, synaptic plasticity, neurotransmitter signaling, neurodevelopment, and neuroinflammation. Interactions between the microbiome, neurophysiology, and behavior in animal models displaying endophenotypes of neurological disease have been corroborated by limited human studies linking microbial dysbiosis to such conditions as autism spectrum disorder (ASD), major depressive disorder, and Parkinson's disease. These findings supporting a microbiota-gut-brain axis are conceptually intriguing, raising the question of whether microbial effects on host brain and behavior represent evolutionarily conserved processes that impact the fitness of both host and microbiota. Emerging evidence for microbial influences on chemical communication, social interactions, stress-related behavior, and performance in learning and memory tasks could contribute

to the notion of a unified holobiont in which animals and their microbiomes have coevolved together as a primary unit of natural selection. Notably, however, further investigation is required to test the reproducibility and enhance the rigor of studies in this nascent field, and additional studies are needed to identify clear molecular and cellular mechanisms underlying interactions between the microbiota and nervous system.

SOCIAL AND COMMUNICATIVE BEHAVIOR

Chemical Communication

Chemical communication is the most widespread communication modality across kingdoms, from bacteria and fungi to plants and animals (Steiger et al. 2011). Scents carry information regarding the individual's age, sex, group membership, reproductive status, and other socially relevant variables (Steiger et al. 2011). As such, olfactory signals facilitate several social communicative behaviors, including territorial marking, mating, and foraging. A substantial body of literature supports the ability of bacteria and other microorganisms to produce a variety of volatile chemicals (Kai et al. 2009). In addition, field and laboratory studies have examined microbial communities in scent glands, secretions, and excretions and their potential to modify olfactory signals (Ezenwa & Williams 2014). Findings from these efforts raise the question of whether an animal's microbiome may influence communication.

Evidence suggests that microbiome-related changes in odorant profiles regulate social isolation versus attraction in insects. Raising levels of bacterial colonization on red harvester ants increased the likelihood that an ant would be attacked and ejected from the colony (Dosmann et al. 2016). In contrast, antibiotic-swabbed ants did not induce this aggressive response, suggesting that an ant's normal external microbiota is not necessary for nestmate recognition. Similar effects were seen in the lower termite, *Reticulitermes speratus*, suggesting that colonization with foreign microbes promotes unfamiliar scents that identify intruders to the colony (Matsuura 2001). Additional studies reveal that the gut microbiota can stimulate aggregation responses that attract organisms to conspecifics. German cockroaches that lacked bacteria in the alimentary tract exhibited depleted levels of volatile carboxylic acids in their feces, which were subsequently less attractive to conspecifics than were feces from conventionally colonized controls (Wada-Katsumata et al. 2015). Inoculating sterile cockroaches with control microbiota corrected these deficits, wherein levels of attractiveness covaried with bacterial diversity. Similar effects on aggregation of locusts into vast swarms were attributed to the production of the pheromone guaiacol by indigenous gut microbes (Dillon et al. 2000). In *Drosophila melanogaster*, adult flies and larvae preferred food that had previously been used by other larvae compared to unused, fresh food (Venu et al. 2014). This preference was abolished when the food was used by axenic larvae and was further restored by mixing sterile used food with particular *Lactobacillus* spp. from the normal fly gut microbiota, suggesting that deposition of normal flora on food can influence feeding behavior. Another *D. melanogaster* study revealed a microbial role in mating preference (Sharon et al. 2010). Flies fed a molasses-based versus a starch-based medium exhibited different microbiomes and mated preferentially with flies reared on the same food. Antibiotic treatment eliminated this preference and decreased levels of cuticular hydrocarbons (CHCs), whereas colonization with *Lactobacillus plantarum* restored mating preference and particular CHCs. Although the majority of these studies imply that microbiome-based changes in chemical signals are mediated by direct synthesis of particular odorants or pheromones by bacteria, additional studies are required to determine fully whether host-microbe interactions may be involved.

Several mammals exhibit variations in microbiota composition that correlate with changes in odorant profiles. Scent gland secretions from badgers contained microbiomes that discriminated between cubs and adults (Sin et al. 2012). Similar observations revealed that the meerkat microbiome was predictive of age, sex, and group differences (Leclaire et al. 2014). In a field study of social spotted hyenas versus solitary striped hyenas, scent pouch secretions contained chemical and microbial profiles that sufficiently distinguished males, pregnant females, and lactating females (Theis et al. 2013). Alterations in specific microbial taxa consistently covaried with particular volatile fatty acids in the social versus solitary hyenas, revealing correlations between microbiome composition, odorant profiles, and social behavior in mammals. Consistent with this, in laboratory mice, strain, background, and variations in the major histocompatibility complex gene correlated with differences in both volatile odor profiles and the microbiome (Zomer et al. 2009). Despite olfactory communication being less prevalent among primates compared to other mammals, there is some evidence that changes in the human skin microbiota are associated with differences in odorant profiles (Verhulst et al. 2011). However, whether the microbiome influences the production of pheromones with consequences for human behavior remains poorly understood. Overall, additional research involving transplant of ecological microbiome samples into laboratory model organisms would be useful for testing causal effects of microbiome differences on communicative behavior.

Social Interactions

In addition to research on the effects of the microbiome on chemical communication, an increasing number of studies of laboratory rodents highlight possible roles for the gut microbiome in modulating intrinsic motivation for social interactions (**Table 1**). To date, five independent studies have examined effects of microbiome depletion on social behavior in animal models, with some conflicting results. In addition to these, two additional studies on effects of probiotic treatment on social behavior in animal models have been compelling in testing causality and identifying potential cellular mechanisms for microbial effects on behavior. All studies were modestly powered and used standard methods for rodent social testing with automated tracking and analysis software. Most included examination of both male and female animals, but some examined only males. In a social approach assay, germ-free (GF) rats, raised in the absence of microbial colonization, exhibited reduced social investigation of an unfamiliar age-, sex-, weight-, and microbiome-matched rat than did conventionally colonized [specific pathogen–free (SPF)] controls (Crumeyrolle-Arias et al. 2014). This deficit was seen only during the first two minutes of the social task, suggesting increased initial social stress that diminished with habituation. In the three-chamber social interaction task, mice were given the choice to interact with a novel mouse contained in an unfamiliar enclosure (novel object) or the novel object alone. Whereas SPF mice exhibited a typical preference to interact with the mouse over the object, GF mice displayed a substantial preference for the object over the mouse, denoting deficient sociability and increased social avoidance (Desbonnet et al. 2014). Similarly, when given the choice to interact with an unfamiliar versus familiar mouse, GF mice exhibited an abnormal decrease in preference for social novelty compared to SPF controls. Interestingly, conventionalization of GF mice with an SPF microbiome at weaning sufficiently corrected deficits in sociability but not in social novelty, suggesting that social avoidance behavior in particular can be modulated postnatally with microbiome-based interventions. In contrast to these studies linking GF status with decreased social behavior, one study of behavioral performance reported the opposite finding: increased sociability in GF mice compared to SPF controls (Arentsen et al. 2015). Causes for the discrepancy between the two experiments, which compared adult male GF versus SPF mice of the same strain (Swiss Webster) in the same social paradigm

Table 1 Interactions between the microbiota and social behavior

Subject	Perturbation	Age	Sample size and sex	Test	Result	Reference
Microbiome depletion						
Rat: F344	GF versus SPF	81–85 days	$n = 12$, male	Reciprocal social interaction	Decreased social sniffing by GF mice during first two minutes of test	Crumeyrolle-Arias et al. 2014
Mouse: Swiss Webster	GF versus SPF	55–60 days	$n = 5$–13, male and female	Three-chamber social assay: sociability and preference for social novelty	Decreased sociability and social preference in GF mice	Desbonnet et al. 2014
Mouse: Swiss Webster	GF versus SPF	>60 days	$n = 10$, male	Three-chamber social assay: sociability	Increased sociability in GF mice	Arentsen et al. 2015
Rat: Wistar	Maternal oral treatment with 1% succinylsulfathiazole (antibiotic)	25 days	$n = 8$, male and female	Reciprocal social interaction	Decreased social investigation by offspring from antibiotic-treated mothers	Degroote et al. 2016
Mouse: C57BL/6 and NOD	Antibiotic treatment, intestinal microbiota transfer	7 weeks old	$n = 11$, male and female	Social approach	No effect of adult antibiotic treatment on social behavior	Gacias et al. 2016
Probiotic or bioactive treatment						
Mouse: C57Bl/6, maternal high-fat diet	*Lactobacillus rhamnosus*	Adult	$n = 5$–14, male and female	Reciprocal social interaction and three-chamber social assay: sociability and preference for social novelty	Decreased social interaction in offspring from high-fat diet–fed mothers corrected with probiotic treatment	Buffington et al. 2016
Mouse: C57Bl/6, maternal immune activation	*Bacteroides fragilis*	Adult	$n = 16$–45, male and female	Three-chamber social assay: sociability and preference for social novelty	No effect of probiotic treatment on social interaction	Hsiao et al. 2013

Abbreviations: GF, germ-free; NOD, nonobese diabetic; SPF, specific pathogen–free.

(the three-chamber social assay), are unclear. However, differences in the specific ages of the adult mice tested, the strains of the stimulus mice used, the baseline microbiota of SPF mice, and the housing conditions used for SPF mice (i.e., in gnotobiotic isolators or in microisolator cages) could be contributing factors. Additional studies are needed to demonstrate the reproducibility of microbiota-dependent social behavioral alterations across testing conditions and experimental designs. More extensive quantitation of social behavioral parameters across additional paradigms (e.g., ultrasonic vocalizations, aggression, mating, juvenile play) would provide greater insight into the nature of social behaviors affected by the microbiota.

Links between the microbiota and social behavior are supported by additional studies that examine disruptions in the composition of the gut microbiota, rather than the complete absence of microbes, as in GF mice. Adolescent offspring of antibiotic-treated rats exhibited altered microbiomes and decreased social investigation (Degroote et al. 2016). Similar correlations between deficient social behavior and altered gut microbiota profiles were observed in offspring of pregnant mice exposed to maternal immune activation (Hsiao et al. 2013) or valproic acid (de Theije et al. 2014), but whether microbiota changes are involved in the etiopathogenesis of impaired social behavior in these mouse models remains unclear. One recent study provided strong evidence for a causal effect of microbial dysbiosis on the manifestation of deficient social behavior by using gnotobiotic transplant and treatment approaches combined with rigorous behavioral, neuroimaging, and electrophysiological testing (Buffington et al. 2016). Pregnant mice fed a high-fat diet yielded offspring with substantial alterations in the microbiota that correlated with abnormal behavior in tests for reciprocal social interaction, sociability, and preference for social novelty. Transplantation of the microbiota from offspring of high-fat diet–fed mothers into GF mice recapitulated social deficits, whereas transplantation of standard microbiota into GF mice corrected social deficits. Furthermore, restoration of a conventional microbiota in offspring of high-fat diet–fed mothers corrected impairments in social behavior. This effect of the conventional microbiota on regulating social behavior could be mediated by the indigenous gut bacterium *Lactobacillus reuteri*, which was reduced in the gut microbiota of high-fat diet offspring compared to controls. Treatment of high-fat diet offspring with live cultures of *L. reuteri* was sufficient to correct deficiencies in social behavior and to induce long-term potentiation in dopaminergic neurons of the ventral tegmental area. Notably, however, treatment of offspring of immune-activated mothers with another commensal bacterium, *Bacteroides fragilis*, had no effect on social interaction in the three-chamber sociability paradigm, despite ameliorating deficits in ultrasonic vocalizations produced in response to social novelty (Hsiao et al. 2013). These studies point to the importance of distinguishing between motivated social investigation versus responsive vocal communication. Altogether, these findings suggest that specific bacterial species from the gut microbiota can influence social communicative behavior in a postnatally inducible and reversible manner and highlight the need to uncover biological bases for bacterial species–specific responses. Additional studies are needed to elucidate clear molecular and cellular signaling pathways between gut bacteria and the central nervous system and to determine whether microbial modulation of social behavior is host or context dependent.

Reciprocal Interactions Between Social Behavior and the Microbiota

In addition to studies that support an effect of the microbiota on modulating social behavior, there is also evidence that social behavior itself shapes the microbiota. In wild baboons, social group membership predicted gut microbiome composition. Within social groups, individuals that interacted physically through social grooming harbored more similar communities of gut bacteria to each other (Tung et al. 2015). The degree of social interaction explained variation in the gut

microbiota, even after controlling for diet, host genetics, and shared environment. Similarly, social interactions in chimpanzees were associated with homogeneity in microbial community profile, and these microbiota appeared to be transmitted socially to infants through successive generations (Moeller et al. 2016). Consistent with this, cohoused humans and their pets were identifiable by similarities in their microbiomes (Lax et al. 2014). Social transmission of the microbiota may be beneficial for propagating the microbes themselves, and some evidence suggests it could confer beneficial effects for the host communities as well. In bumble bees and honey bees, for example, social transmission of the microbiota through exposure to feces from nest mates was associated with host protection against parasitic infection (Koch & Schmid-Hempel 2011). Although additional research is needed to test the causality and directionality for interactions between the microbiota and social behavior, these initial studies have raised the question of whether microbiota-mediated changes in social behavior affect social transmission of the microbiota and whether there are consequences on both host and microbial fitness. Social interactions could also propagate disease-causing microorganisms, highlighting a need to examine whether pathogenic and mutualistic microbes have differential effects on the manifestation of social behavior.

The Microbiota and Autism Spectrum Disorder

Emerging research linking the microbiota to social behavior, in addition to nutrition, immunity, and gastrointestinal function, has motivated examinations of the microbiota in ASD, a neurodevelopmental disease characterized by impaired social communication and stereotyped behavior and associated with various medical comorbidities, including gastrointestinal issues and immune dysfunction. There is evidence that the microbiota may contribute to abnormal behavior in select animal models that exhibit stereotypies and impairments in social communication (Buffington et al. 2016, de Theije et al. 2014, Hsiao et al. 2013). In addition, several studies reveal microbiome abnormalities in ASD individuals relative to neurotypical controls (Krajmalnik-Brown et al. 2015, Vuong & Hsiao 2016). Importantly, however, there is little consensus and sometimes disagreement across these studies on a precise microbiota signature for ASD. Many factors could be confounding, including heterogeneity in the study cohort with regards to symptom severity, diet, medical comorbidities, age, sex, and pharmacological exposures. Nonetheless, a few studies reported beneficial effects of antibiotic treatment for improving behavioral abnormalities in ASD (Krajmalnik-Brown et al. 2015). Whether these effects are mediated by off-target signaling of antibiotics, rather than through primary depletion of the microbiome, is unclear. These clinical studies support an association between microbial dysbiosis and ASD, but caution should be taken against inferring reverse causality. Controlled trials that test the effects of microbiome transplant and probiotic treatment in ASD will be important for determining whether abnormalities in the ASD microbiota could contribute to core symptoms of the disorder and whether microbiome-based treatments could ameliorate symptom severity.

STRESS-RELATED RESPONSES

Stressor-Induced Behavior and Anxiety

Animals have evolved flexible mechanisms to adapt their behavior in response to integrated environmental and physiological cues. Situated at the interface of gene-environment interactions, the composition and function of the microbiota is dependent on host genetics and shaped critically by environmental factors, including diet, infection, pharmacological treatments, and stress. Across various experimental paradigms, physical and psychosocial stressors sufficiently induced abnormal behavior in laboratory animal models concomitant with altered gut microbiota profiles

(Aguilera et al. 2013, Bailey et al. 2011, Bendtsen et al. 2012). Although these findings suggest that exposure to stressors can alter the composition of the gut microbiota, many studies indicate that the microbiota can in turn influence stress-related behavior, such as freezing, reduced exploration and thigmotaxis, as manifestations of the fight-or-flight response (**Table 2**). To date, exploratory drive and risk avoidance have been the most frequently studied behaviors in microbiome depletion and probiotic treatment paradigms. These studies appear to be appropriately powered and have implemented standard behavioral methodology, using automated tracking software where applicable. Findings have been widely reproduced across experimental paradigms, behavioral assays, and model organisms, with a few exceptions.

Alterations in stress-related behavior have been replicated across several studies of microbiota-depleted animal models. In the open field test and elevated plus maze, GF mice exhibited increased exploration of the center of the open field and open arms of the plus maze as compared to SPF controls (Diaz Heijtz et al. 2011, Neufeld et al. 2011a, Sudo et al. 2004, Clark et al. 2013, Campos et al. 2016, Zheng et al. 2016). This increase in exploratory behavior at baseline was also seen in a GF zebrafish model (Davis et al. 2016). In response to physical or psychosocial stressors, however, GF mice and rats displayed elevations in plasma corticosterone and adrenocorticotropic hormone and reduced exploratory behavior compared to stressed SPF controls across various tasks typically used to screen for anxiolytics (Crumeyrolle-Arias et al. 2014, Diaz Heijtz et al. 2011, Sudo et al. 2004). Likewise, treatment of conventionally colonized mice with antibiotics increased baseline exploration of the light chamber in the light-dark behavioral assay but resulted in negative thigmotactic behavior following restraint stress (Desbonnet et al. 2015). Interestingly, baseline differences in exploration during the step-down task between two strains of mice, NIH Swiss and BALB/c, were transferable by cross-transplantation of the gut microbiota (Bercik et al. 2011). These studies similarly reveal that GF animals exhibit high exploratory behavior at baseline but hyperresponsive stress-induced inhibition of exploratory behavior. Taken together, these findings reveal potential bidirectional interactions between the microbiota and stress behavior that may affect host responses to situational stressors.

Conventionalization of young, but not adult, GF mice with standard SPF microbiota sufficiently reversed abnormalities in exploratory behavior (Diaz Heijtz et al. 2011, Neufeld et al. 2011a). Similarly, colonization of GF mice with SPF microbiota at 6 weeks was more efficient at reversing hypothalamic-pituitary-adrenal responses to stress compared to reconstitution at 14 weeks (Sudo et al. 2004). These studies suggest that the microbiota influences behavioral networks for stress during a critical time window (Diaz Heijtz et al. 2011).

The importance of the early-life microbiota on programming later-life behavior is supported further by studies on the effects of maternal insults on the development of offspring microbiota and behavior. Several recent reports have examined the roles of the maternal and early postnatal microbiota in mediating detrimental effects of maternal diet, pharmaceuticals, infection, or stress on offspring exploratory, social, and sensorimotor behaviors (Buffington et al. 2016, Degroote et al. 2016, Hsiao et al. 2013, Zijlmans et al. 2015). Maternal-to-offspring transmission of microbiota that impacts stress-related behavior and physiology was supported by evidence that maternal stress altered the maternal vaginal microbiota and that the inheritance of such microbiota abnormalities was sufficient to induce negative thigmotactic behavior in the offspring (Jasarevic et al. 2015). These findings highlight the importance of early-life microbiota in regulating normal exploratory behavior and stress responses in animals.

Emerging studies reveal positive effects of probiotics on modulating stress-related behaviors. In a rat model of chronic stress, treatment with *Lactobacillus helveticus* NS8 improved exploratory behavior in the open field and elevated plus maze and reduced corticosterone and adrenocorticotropic hormone levels (Liang et al. 2015). Moreover, in mouse models of inflammatory bowel

Table 2 Interactions between the microbiota, stress-related behavior, and anxiety

Microbiome depletion						
Subject	**Perturbation**	**Age**	**Sample size and sex**	**Test**	**Result**	**Reference**
Zebrafish	GF versus SPF	6 days postfertilization	$n = 36–87$	Locomotion and thigmotaxis, cortisol	Increased locomotor activity, reduced thigmotactic behavior, and reduced cortisol levels in stress in GF fish	Davis et al. 2016
Mouse: BALB/c	GF versus SPF versus gnotobiotic	9 weeks	$n = 6–11$, male	Acute restraint stress, ACTH and corticosterone levels	Decreased ACTH and corticosterone levels in GF mice	Sudo et al. 2004
Mouse: Swiss Webster	GF versus SPF	8 weeks	$n = 12$, female	Elevated plus maze, locomotor activity, plasma corticosterone	Increased exploratory behavior, increased plasma corticosterone in GF mice	Neufeld et al. 2011a,b
Mouse: NMRI	GF versus SPF	8–10 weeks	$n = 6–14$, male	Open field, elevated plus maze, light-dark box	Increased exploratory behavior in GF mice	Diaz Heijtz et al. 2011
Mouse: Swiss Webster	GF versus SPF	6–9 weeks	$n \geq 9$, male and female	Light-dark box	Increased exploratory behavior in GF mice	Clarke et al. 2013
Mouse: BALB/c	GF versus EX-GF	7–16 weeks	$n = 10$	Open field, marble burying	Increased locomotor and marble burying behavior in GF mice	Nishino et al. 2013
Rat: F344	GF versus SPF	81–85 days	$n = 28$, male	Open field	Decreased exploratory behavior in GF rats after acute stress	Crumeyrolle-Arias et al. 2014
Mouse: BALB/c	Antibiotics, microbiota transplant	8–10 weeks	$n = 15–47$, male	Step-down, light-dark box	Increased exploratory behavior in antibiotic-treated mice; altered stress behavior after transplant of NIH Swiss versus BALB/c mice	Bercik et al. 2011
Mouse: Swiss Webster	Antibiotics	>3 weeks	$n = 15$, male	Object recognition, light-dark box, social transmission of food preference, restraint stress	Increased exploratory behavior and elevated serum corticosterone in stressed antibiotic-treated mice	Desbonnet et al. 2015

(*Continued*)

Table 2 (*Continued*)

Subject	Perturbation	Age	Sample size and sex	Test	Result	Reference
Microbiome depletion						
Mouse: Swiss Webster	GF versus SPF: LPS injection	10 weeks	$n = 5$–10, male	Open field	Increased exploratory behavior in GF mice	Campos et al. 2016
Mouse: Kunming	GF versus SPF	NA	$n = 15$–21	Open field	Increased exploratory behavior in GF mice	Zheng et al. 2016
Rat: Wistar	1% SST maternal diet	3–7 weeks	$n = 20$, male and female	Open field, social interaction, marble burying, elevated plus maze, prepulse inhibition	Decreased exploratory behavior in SST-treated mice	Degroote et al. 2016
Mouse: C57BL/6 and NOD	Antibiotics, microbiota transplant	7 weeks	$n = 11$, male and female	Elevated plus maze	Decreased exploratory behavior in NOD mice, with no difference after antibiotic treatment	Gacias et al. 2016
Mouse: C57BL/6	GF versus SPF: maternal separation (P4–weaning)	11–13 weeks	$n = 11$–16, male and female	Light-dark box, step-down, open field	Increased anxiety-like behavior in stressed SPF mice, but not GF	De Palma et al. 2015
Probiotic or bioactive treatment						
Zebrafish	*Lactobacillus plantarum*	6 days postfertilization	$n = 36$–87	Thigmotaxis	Decreased thigmotactic behavior after probiotic treatment	Davis et al. 2016
Mouse: BALB/c	*Mycobacterium vaccae*	38 days	$n = 8$–10, male	Hebb-Williams complex maze, zero maze	Increased exploratory behavior after probiotic treatment	Matthews & Jenks 2013
Mouse: CD1	Nondigestible galactooligosaccharides	6–8 weeks	$n = 6$, male	LPS injection, locomotor, marble burying, light-dark box	Increased exploratory behavior after probiotic treatment	Savignac et al. 2015
Rat: Sprague-Dawley	*Lactobacillus helveticus* NS8	Adult	$n = 8$, male	Elevated plus maze, open-field, object recognition	Increased exploratory and cognitive behavior after probiotic treatment	Liang et al. 2015

(*Continued*)

Table 2 (*Continued*)

Microbiome depletion						
Subject	**Perturbation**	**Age**	**Sample size and sex**	**Test**	**Result**	**Reference**
Mouse: BALB/c	*Blautia coccoides* or *Bifidobacterium infantis*	7–16 weeks	$n = 10$	Open field, marble burying	Increased exploratory behavior after *B. coccoides* treatment	Nishino et al. 2013
Rat: Wistar	*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175	NA	$n = 36$, male	Conditioned defensive burying	Increased exploratory behavior after probiotic treatment	Messaoudi et al. 2011
Mouse: BALB/c	*Lactobacillus rhamnosus*	Adult	$n = 36$, male	Open field, elevated plus maze, fear conditioning, stress-induced hypothermia	Increased exploratory behavior and decreased stress-induced corticosterone after probiotic treatment	Bravo et al. 2011
Rat: Wistar	*Lactobacillus farciminis*	Adult	NA	Partial restraint stress	Decreased stress-induced HPA activation after probiotic treatment	Ait-Belgnaoui et al. 2012
Mouse: C57Bl/6	*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175	6–8 weeks	$n = 8$, male	Water avoidance stress	Increased exploratory behavior after probiotic treatment	Ait-Belgnaoui et al. 2014
Mouse: BALB/c	*Bifidobacterium longum* 1714 and *Bifidobacterium breve* 1205	7 weeks	$n = 19$–22, male	Stress-induced hypothermia, defensive marble burying, elevated plus maze, open field	Increased exploratory behavior after probiotic treatment	Savignac et al. 2014
Mouse: C57Bl/6 + 3% DSS	*Lactobacillus helveticus* R0052 and *Lactobacillus rhamnosus* R0011	6–8 weeks	$n = 80$, male and female	Novel object recognition, light-dark box	Increased exploratory behavior after probiotic treatment	Emge et al. 2016

(*Continued*)

Table 2 *(Continued)*

Subject	Perturbation	Age	Sample size and sex	Test	Result	Reference
Microbiome depletion						
Mouse: C57Bl/6 Rag1$^{-/-}$	*Lactobacillus helveticus* R0052 and *Lactobacillus rhamnosus* R0011	6–8 weeks	n = 6–8, male and female	Water avoidance stress, novel object recognition, light-dark box	Increased exploratory behavior after probiotic treatment	Smith et al. 2014
Rat: Sprague-Dawley + ampicillin	*Lactobacillus fermentum* NS9	>4 weeks	n = 30, male	Elevated plus maze, Morris water maze	Increased exploratory behavior and serum corticosterone after probiotic treatment	Wang et al. 2015
Mouse: C57Bl/6, maternal immune activation	*Bacteroides fragilis*	Adult	n = 35–75, male and female	Open field, marble burying	Increased exploratory behavior after probiotic treatment	Hsiao et al. 2013
Human trials and associations						
Healthy adults	*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175	Average 42 years	n = 26–29, male and female	HADS	Decreased HADS score after probiotic treatment	Messaoudi et al. 2011
Anxiety disorder	None	>18 years	n = 63–133, male and female	Medical Expenditure Panel Survey	Association between infection and onset of anxiety disorder	Bruch 2016
Healthy infants	Maternal prenatal stress	Birth through 110 days	n = 56, male and female	State-Trait Anxiety Inventory, Pregnancy-Related Anxiety Questionnaire, 16S rRNA	Altered microbiota in infants of maternally stressed mothers	Zijlmans et al. 2015

Abbreviations: ACTH, adrenocorticotropic hormone; DSS, dextran sodium sulfate; EX-GF, ex-germ-free (conventionalized with SPF microbiota); GF, germ-free; HADS, Hospital Anxiety and Depression Scale; HPA, hypothalamic-pituitary-adrenal; LPS, lipopolysaccharide; NA, not applicable; NOD, nonobese diabetic; SPF, specific pathogen–free; SST, succinylsulfathiazole.

disease and immunodeficiency ($Rag1^{-/-}$ mice), treatment with *L. helveticus* R0052 and *Lactobacillus rhamnosus* R0011 corrected light-aversion behavior in the light-dark box (Emge et al. 2016, Smith et al. 2014), suggesting that behavioral improvement conferred by probiotics can occur in diverse physiological contexts. Reduced thigmotaxis was also observed in zebrafish treated with the commensal bacterium *L. plantarum* (Davis et al. 2016), pointing to the ability of select *Lactobacillus* species to promote exploratory behavior across model organisms. Corresponding human trials are lacking, but in a randomized double-blind placebo-controlled study, treatment of healthy humans with *L. helveticus* R0052 and *Bifidobacterium longum* R0175 decreased anxiety-related scores on the Hospital Anxiety and Depression Scale (Messaoudi et al. 2011). More research is needed to evaluate the effects of particular bacterial taxa, their mechanistic interactions with behavioral neurocircuits, any additional physiological side effects that may be elicited, and, ultimately, the efficacy of microbe-based treatments for behavioral disorders.

Stressor-Induced Behavior and Depression

In addition to stress-related behaviors that measure exploratory drive and risk avoidance, exposure to stress often induces abnormal performance in tasks used to measure learned helplessness and anhedonia. Some recent studies link changes in these behaviors to alterations in the composition of the gut microbiota in animal models (Dash et al. 2015, Dinan & Cryan 2013) (**Table 3**). These investigations have been fewer in number than those examining stress-induced exploratory and thigmotactic behavior, with four independent studies on microbiome depletion models and four additional studies on probiotic treatment. However, the studies appear to be rigorous in methodology, following standard behavioral protocols and rendering similar overall findings. Compared to SPF controls, GF mice displayed reduced immobility time in the forced swim and tail suspension tests, common assessments for screening antidepressants (Campos et al. 2016, Zheng et al. 2016). Nonobese diabetic (NOD) mice subjected to daily gavage stress exhibited microbial dysbiosis and increased immobility in the forced swim test, which was reversed by antibiotic treatment (Gacias et al. 2016). Similarly, maternal separation–induced stress increased immobility in the tail suspension test in SPF mice but not in GF mice (De Palma et al. 2015). Furthermore, treatment of SPF mice with the bacterium *L. rhamnosus* decreased immobility time in the forced swim test, revealing a beneficial effect of probiotic treatment on stress-induced learned helplessness (Bravo et al. 2011). Together, these studies suggest that the microbiome plays an important role in modulating host behavioral responses to stress.

Findings in animals are corroborated by a few human studies revealing correlations between the microbiome and depression. Across various paradigms, pre- or probiotic treatment positively affected emotion-related scores and reduced feelings of aggression and rumination (Pärtty et al. 2013, Schmidt et al. 2015, Steenbergen et al. 2015). Fecal microbiota from major depressive disorder patients were substantially altered relative to those from healthy controls, with notable increases in Actinobacteria and Bacteroidetes (Zheng et al. 2016). Notably, transplant of microbiota from depression patients into GF mice was sufficient to induce elevated immobility times in the forced swim and tail suspension tasks as compared to transplantation of healthy control microbiota (Zheng et al. 2016), suggesting a possible role for gut microbial dysbiosis in the manifestation or persistence of stress-related behavior in human depression. Metabolomic profiling of mice colonized with the depression microbiota revealed alterations in hippocampal carbohydrate and amino acid metabolism (MacQueen & Frodl 2011). However, it remains unclear how the microbiota induces metabolic changes in specific brain regions and whether these effects are relevant to behavioral modulation. Overall, this provides evidence that microbiome changes observed in humans with depression can cause endophenotypes of the disorder in mice.

Table 3 Interactions between the microbiota, stress-related behavior, and depression

Subject	Perturbation	Age	Sample size and sex	Test	Result	Reference
Microbiome depletion						
Mouse: Swiss Webster	GF versus SPF: LPS injection	10 weeks old	$n = 5–10$, male	Open field, forced swim, tail suspension, sucrose preference	Decreased stress-related behavior in GF mice	Campos et al. 2016
Mouse: Kunming	GF versus SPF	NA	$n = 15–21$	Forced swim, tail suspension, Y-maze	Decreased stress-related behavior in GF mice	Zheng et al. 2016
Mouse: C57BL/6	GF versus SPF: maternal separation (P4–weaning)	11–13 weeks	$n = 11–16$, male and female	Tail suspension	Maternal separation increased immobility time in SPF mice but not GF mice	De Palma et al. 2015
Mouse: C57BL/6 and NOD	Antibiotic treatment, intestinal microbiota transfer	7 weeks old	$n = 11$, male and female	Social interaction and forced swim	High immobility time in NOD mice abrogated by antibiotic treatment and transferable by microbiome transplant	Gacias et al. 2016
Probiotic or bioactive treatment						
Mouse: BALB/c	*Bifidobacterium longum* 1714 and *Bifidobacterium breve* 1205	7 weeks old	$n = 19–22$, male	Tail suspension and forced swim	Decreased immobility times after *B. longum* treatment	Savignac et al. 2014
Rat: Sprague-Dawley	*Lactobacillus helveticus* NS8	Adult	$n = 8$, male	Sucrose preference test and object recognition test	Decreased stress-related behavior and cognitive dysfunction after probiotic treatment	Liang et al. 2015
Rat: Sprague-Dawley, maternal separation	*Bifidobacterium infantis* 35624	>6 weeks	$n = 7–11$, male	Forced swim test	Decreased immobility time after probiotic treatment	Desbonnet et al. 2010

(*Continued*)

Table 3 (*Continued*)

Microbiome depletion						
Subject	**Perturbation**	**Age**	**Sample size and sex**	**Test**	**Result**	**Reference**
Mouse: BALB/c	*Lactobacillus rhamnosus*	Adult	$n = 36$, male	Forced swim test, fear conditioning, and stress-induced hypothermia	Decreased stress-related behavior after probiotic treatment	Bravo et al. 2011
Human trials and associations						
Healthy human	Prebiotic: fructooligosaccharides or galactooligosaccharides	18–45 years	$n = 22$–23, male and female	Salivary cortisol, emotional processing tasks, attentional dot-probe, facial expression recognition, emotional categorization, and memory	Decreased salivary cortisol, increased positive versus negative vigilance after prebiotic treatment	Schmidt et al. 2015
Mood disorder	4-week probiotic [*Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, and *Lactococcus lactis* (W19 and W58)]	Average 20 years	$n = 20$, male and female	Cognitive reactivity to sad mood in revised Leiden index of depression sensitivity scale	Reduced cognitive reactivity to sad mood (reduced rumination and aggressive thoughts) after probiotic treatment	Steenbergen et al. 2015
Healthy adult	Treatment with *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175	Average 42 years	$n = 26$–29, male and female	HADS	Decreased HADS score after probiotic treatment	Messaoudi et al. 2011

(*Continued*)

Table 3 (*Continued*)

Microbiome depletion						
Subject	**Perturbation**	**Age**	**Sample size and sex**	**Test**	**Result**	**Reference**
Healthy infants	Perinatal *Lactobacillus rhamnosus*	Infants	$n = 89$, male and female	Infant crying and fussing	Decreased fussing and crying after prebiotic treatment	Pärtty et al. 2013
Chronic fatigue syndrome	*Lactobacillus casei* strain Shirota	18–65 years	$n = 35$, male and female	Beck Depression and Beck Anxiety Inventories	Increased *Lactobacillus* and *Bifidobacterium* in chronic fatigue patients	Rao et al. 2009
Major depressive disorder	None	Average 40 years	$n = 58$–63 male and female	16S rRNA sequencing	Increased Firmicutes, Actinobacteria, and Bacteroidetes in depression patients	Zheng et al. 2016
Healthy children	None	18–27 months	$n = 77$, male and female	Early Childhood Behavior Questionnaire and bTEFAP	Association between phylogenetic diversity and increased surgency/extraversion and temperament	Christian et al. 2015
Healthy adult	None	27, 29, and 32 years	$n = 3$, male and female	16S rRNA sequencing and profile of mood states questionnaire	Correlation of *Faecalibacterium* with depression and anger, correlation of *Parasutterella* with tension	Li et al. 2016
Depression	None	Not reported	$n = 18$–37	16S rRNA	Increased Bacteroidales and decreased Lachnospiraceae in depression patients	Naseribafrouei et al. 2014
Schizophrenia	None	Average 34.5 years	$n = 16$, male and female	Shotgun metagenomics of oropharyngeal microbial composition	Increased abundance of Ascomycota, *Lactobacillus*, and *Bifidobacterium* in schizophrenia patients	Castro-Nallar et al. 2015

Abbreviations: bTEFAP, bacterial tag-encoded FLX amplicon pyrosequencing; GF, germ-free; HADS, Hospital Anxiety and Depression Scale; LPS, lipopolysaccharide; NA, not applicable; NOD, nonobese diabetic; SPF, specific pathogen–free.

Sensory Nociception

Nociception or the sensation of pain is an evolutionary trait that is essential for adaptation to harmful stimuli, such as physical stressors. However, dysregulated nociception (e.g., hypernociception) is a key symptom in numerous chronic disorders. Developing evidence suggests that nociception is linked to dysbiosis of the intestinal microbiota and could influence pain behavioral responses (Moloney et al. 2016, Theodorou et al. 2014). Early indications that indigenous microbes can modulate pain came from microbiota manipulation studies in mice subjected to colorectal distension. Mice pretreated with antibiotics displayed enhanced visceral hypersensitivity, whereas supplementation of antibiotic-treated mice with a *Lactobacillus* strain normalized this response (Verdu et al. 2006). These findings were corroborated by a separate study demonstrating altered pain responses due to early-life perturbation of the intestinal microbiota by vancomycin treatment (O'Mahony et al. 2014). Collectively, these data provided the first demonstrations that the microbiota can modulate enteric pain responses.

In addition to visceral pain, peripheral pain responses also appear to be controlled by intestinal bacteria. In one study, hypernociception induced by injection of inflammatory stimuli in the paw was attenuated in GF compared to SPF mice and was restored following microbiota conventionalization (Amaral et al. 2008). Inflammatory hypernociception triggered by carrageenan was associated with elevated local expression of the anti-inflammatory cytokine *Il10* in GF mice compared to SPF mice, and neutralization of IL-10 was sufficient to restore pain sensitivity. In support of a role for proinflammatory responses in promoting pain sensitivity, researchers demonstrated that $CD11b^+$ myeloid cells but not neutrophils or inflammatory monocytes control mechanical hypersensitivity in a model of tissue injury–induced inflammatory pain (Ghasemlou et al. 2015). These studies suggest a critical function of the intestinal microbiota in modulating peripheral pain responses through interactions with the immune system.

Although accumulating literature suggests that pain is triggered by inflammation, bacteria themselves regulate the activity of nociceptive sensory neurons. In a subcutaneous *Staphylococcus aureus*–infection mouse model, infection-induced pain hypersensitivity was independent of innate immune activation but correlated with bacterial load (Chiu et al. 2013). Treatment of dorsal root ganglia neurons with multiple strains of bacteria, including *Staphylococcus*, *Streptococcus*, *Helicobacter*, and *Pseudomonas*, induced action potentials in nociceptor-expressing neurons, suggesting direct neuronal activation by bacteria. Indeed, these neurons could be activated by bacterial-derived *N*-formylated peptides and pore-forming toxins such as α-hemolysin (Chiu et al. 2013). The inhibitory neurotransmitter γ-aminobutyric acid (GABA) is a key negative regulator of nociceptive sensory neuron activation. One study of a rat fecal retention model of intestinal pain showed that GABA-producing *Bifidobacterium* had analgesic effects that were dependent on GABA biosynthesis (Pokusaeva et al. 2016). Although pain is thought to be secondary to immune activation, these data highlight at least two immune-independent pathways by which bacteria can modulate nociceptor activity and suggest alternative mechanisms by which the intestinal microbiota regulates peripheral and visceral pain.

COGNITIVE BEHAVIOR

Learning and Memory

Learning and memory are active processes of acquiring, interpreting, and retaining sensory information. There is growing evidence that changes in the gut microbiome alter rodent performance in visual-spatial learning and memory tasks (**Table 4**). These studies include five on microbiome depletion and seven on probiotic treatment. Standard behavioral assays for spatial memory and

Table 4 Interactions between the microbiota and learning and memory behavior

Microbiome depletion						
Subject	**Perturbation**	**Age**	**Sample size and sex**	**Test**	**Result**	**Reference**
Mouse: Swiss Webster	GF versus SPF	5–6 weeks	$n = 3$–6, female	Novel object recognition	Decreased working memory in GF mice	Gareau et al. 2011
Mouse: C57Bl/6N	Antibiotic- versus vehicle-treated SPF	8–11 weeks	$n = 6$–8, male	Novel object recognition, Barnes maze	Decreased working memory in antibiotic-treated SPF mice; no effect on spatial memory	Frohlich et al. 2016
Mouse: C57Bl/6	Antibiotic- versus vehicle-treated SPF	6–8 weeks	$n = 12$, female	Novel object recognition	Decreased working memory in antibiotic-treated SPF mice	Möhle et al. 2016
Rat: Lister-Hooded	Antibiotic- versus vehicle-treated PCP-injected rats	Adult	$n = 11$–12, male	Novel object recognition	Corrected (increased) working memory in antibiotic-treated PCP rats	Pyndt Jørgensen et al. 2015
Rat: Sprague-Dawley	Antibiotic versus vehicle-treated SPF	Weanling	$n = 10$, male	Morris water maze	Decreased spatial memory in antibiotic-treated SPF rats	Wang et al. 2015

(*Continued*)

Table 4 (*Continued*)

Microbiome depletion						
Subject	**Perturbation**	**Age**	**Sample size and sex**	**Test**	**Result**	**Reference**
Probiotic or bioactive treatment						
Mouse: BALB/c	*Bifidobacterium longum* 1714 oral gavage for 11 weeks	7–8 weeks	n = 9–12, male	Novel object recognition, Barnes maze	Increased working and spatial memory in probiotic-treated mice	Savignac et al. 2015
Mouse: C57Bl/6	*Desulfovibrio vulgaris* fecal gavage on the day of experimentation	5 weeks	n = 10, female	Morris water maze; 8-arm radial maze	Decreased spatial memory in probiotic-treated mice	Ritz et al. 2016
Mouse: C57Bl/6 Rag1 knockout versus wild type	*Lactobacillus rhamnosus* and *Lactobacillus helveticus* in drinking water for 4 weeks	6–8 weeks	n = 4–6, male and female	Novel object recognition	Increased working memory in probiotic-treated Rag1 KO mice	Smith et al. 2014
Mouse: C57Bl/6, DSS-treated versus wild type	*Lactobacillus rhamnosus* and *Lactobacillus helveticus* oral gavage for 15 days	6–8 weeks	n = 9–12, male and female	Novel object recognition	Increased working memory in probiotic-treated DSS mice	Emge et al. 2016
Mouse: 129/SvEv, IL-10 knockout versus wild type, on Western diet	*Lactobacillus helveticus* oral gavage for 21 days	3 weeks	n = 5–6	Barnes maze	Increased spatial memory in probiotic-treated IL-10 knockout mice	Ohland et al. 2013
Rat: Sprague-Dawley	*Lactobacillus fermentum* NS9 in drinking water for 41 days	Weanling	n = 10, male	Morris water maze	Increased spatial memory in probiotic-treated mice	Wang et al. 2015
Mouse: Swiss Webster infected with *Citrobacter rodentium*	*Lactobacillus rhamnosus* and *Lactobacillus helveticus* in drinking water for 17 days	5–6 weeks	n = 8–10, female	Novel object recognition	Increased working memory in probiotic-treated *C. rodentium*–infected mice	Gareau et al. 2011

Abbreviations: DSS, dextran sodium sulfate; GF, germ-free; SPF, specific pathogen–free.

working memory were used, but many studies examined only male or female animals, and some appear to be underpowered. Nonetheless, results have generally been consistent across studies. Compared to SPF controls, GF mice exhibited decreased working memory behavior in the novel object recognition task (Gareau et al. 2011). SPF mice treated with a cocktail of antibiotics also displayed substantial deficiencies in object recognition memory (Frohlich et al. 2016, Möhle et al. 2016) but no difference in spatial memory behavior in the Barnes maze task (Frohlich et al. 2016). By contrast, rats treated with ampicillin exhibited impaired spatial memory behavior in the Morris water maze, suggesting differential effects based on rodent background, behavioral task, type of antibiotic and/or duration of antibiotic treatment (Wang et al. 2015). In rats treated with phencyclidine (PCP) to model endophenotypes of schizophrenia, microbial dysbiosis correlated with impaired performance in an object recognition memory test (Pyndt Jørgensen et al. 2015). Treatment with ampicillin restored memory-dependent behavior in the task, suggesting that PCP-induced changes in the microbiota contributed to abnormalities in cognitive behavior. Additional research is needed to evaluate the effects of GF status and specific antibiotic treatments across different mouse and rat strains and disease models that impact learning and memory.

Several studies suggest that select probiotic treatments can modulate learning and memory behavior in animals. Treatment of ampicillin-exposed rats with *Lactobacillus fermentum* NS9 sufficiently restored impairments in spatial memory behavior in the Morris water maze (Wang et al. 2015). BALB/c mice treated with the gut bacterium *B. longum* 1714 exhibited increased object recognition, fewer probe trial errors in the Barnes maze, and elevated context and cue-dependent freezing in response to fear conditioning, suggesting improved episodic, spatial, and long-term learning and memory (Savignac et al. 2015). Beneficial effects on object recognition memory, but not in spatial memory, were also seen after probiotic treatment with *Bifidobacterium breve* 1205, pointing to specificity of cognitive behavioral modulation to particular bacterial species. In addition, probiotic treatment with *L. helveticus* improved deficits in spatial memory behavior seen in mice fed a high-fat Western diet (Ohland et al. 2013). In contrast, treatment of mice with live, but not heat-killed, *Desulfovibrio vulgaris* impaired learning and memory-related behavior in the Morris water maze and 8-arm radial maze (Ritz et al. 2016), highlighting differential outcomes based on specific bacterial species, treatment methods, and behavioral task. Moreover, in a human clinical study, obese subjects exhibited abnormal microbiome profiles relative to matched controls, and select microbiota alterations covaried with performance in tasks measuring speed, attention, and cognitive flexibility (Fernandez-Real et al. 2015). Further studies are needed to examine the extent to which microbiome changes and particular bacterial species modulate quantitative parameters of cognitive behavior and to test whether such interactions contribute to or modify behavior in animal models displaying deficient learning and memory (e.g., in genetic mouse models for Alzheimer's disease). In addition, integration of microbiome profiling into clinical studies of cognitive disorders is needed to determine whether causal findings in preclinical models apply to human conditions.

MICROBIAL EFFECTS ON NEUROPHYSIOLOGY

Although researchers are beginning to uncover molecular and cellular signaling mechanisms for how microbial factors alter gastrointestinal function and immunity, exactly how the microbiota modifies diverse behavioral responses remains unclear. Numerous pathways are implicated, including vagal nerve innervation, neuroendocrine signaling, and neuroimmune regulation, and several microbial effects on neurophysiology have been observed (**Figure 1**). As the notion of a microbiota-gut-brain axis is still in its infancy, reports of microbial effects on neurophysiology are

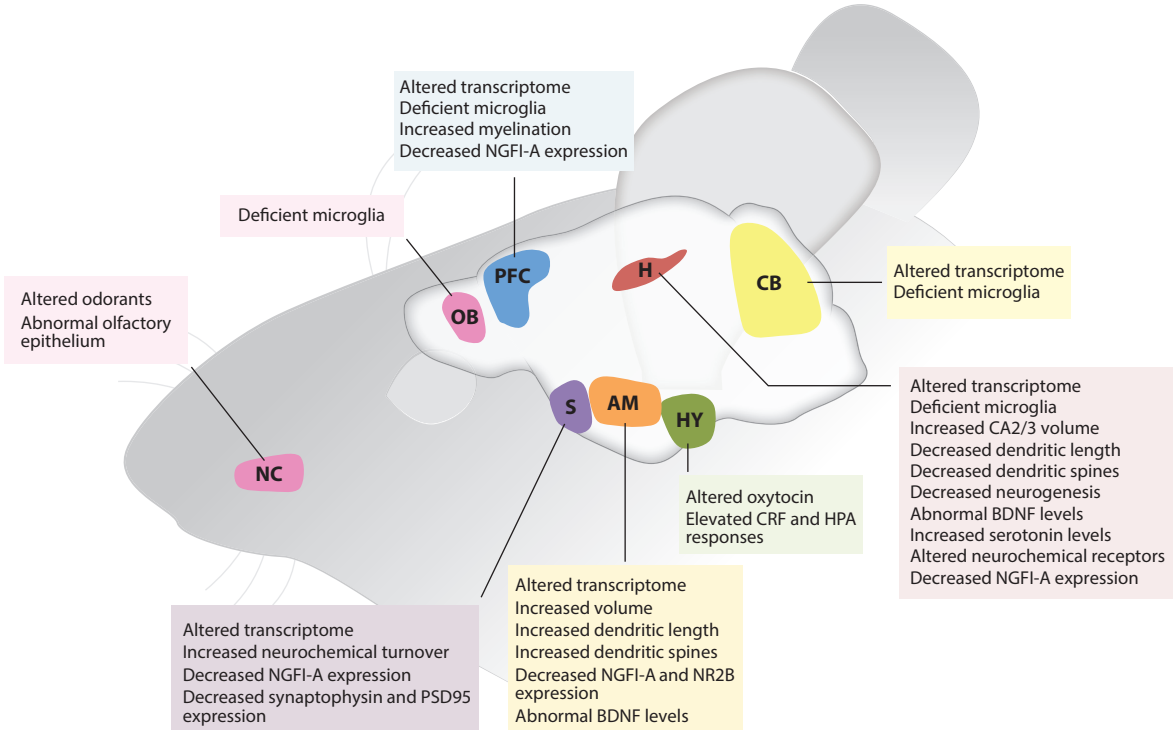

Figure 1

Neurophysiological abnormalities in microbiota-deficient animals. Abbreviations: AM, amygdala; BDNF, brain-derived neurotrophic factor; CB, cerebellum; CRF, corticotropin-releasing factor 1; H, hippocampus; HPA, hypothalamic-pituitary-adrenal; HY, hypothalamus; NC, nasal cavity; NGFI-A, nerve growth factor-inducible protein A; NR2B, *N*-methyl-D-aspartate receptor subtype 2B; OB, olfactory bulb; PFC, prefrontal cortex; PSD95, postsynaptic density protein 95; S, striatum.

recent, with the majority published after 2010. Although compelling, reproducibility across independent studies has not yet been firmly established, and further research in this area is warranted.

Global changes in the brain transcriptome were seen across the hippocampus, frontal cortex, and striatum of GF mice compared to SPF controls, with abnormal expression of genes relevant to synaptic long-term potentiation, steroid hormone metabolism, the citrate cycle, and cAMP-mediated signaling (Diaz Heijtz et al. 2011). Deficiencies in microglial maturation and function have also been reported across various gross brain regions, including cortex, corpus callosum, hippocampus, olfactory bulb, and cerebellum (Erny et al. 2015). These abnormalities contribute to a growing literature on microbiome-neuroimmune interactions that mediate behavioral and physiological abnormalities in mouse models for multiple sclerosis, depression, and stroke, among other disease conditions. Of relevance to the importance of the gut microbiome in modulating systemic metabolomic profiles, one study reported an effect of the microbiota on modulating integrity of the blood-brain barrier (BBB) (Braniste et al. 2014). Remarkably, GF-related defects in BBB permeability were corrected by postnatal colonization with a single *Clostridium* species or by supplementation with short-chain fatty acids—primary metabolic products of bacterial fermentation. Overall, the importance of the microbiome in modulating host behavior and neurophysiology raises the prospect of targeting endogenous host-microbiome interactions to develop novel microbe-based treatments for neurological disorders.

Amygdalar Structure and Gene Expression

Although the molecular mechanisms underlying how the microbiota modifies host social behavior are unclear, evidence suggests the microbiota alters the neurophysiology of brain areas considered key nodes for social and anxiety behavior networks, including the amygdala and hypothalamus (Goodson 2005). Stereological analysis revealed significantly increased volume across the lateral and basolateral amygdala and central nucleus of the amygdala in brains from GF mice compared to SPF controls (Luczynski et al. 2016). Basolateral amygdalar aspiny interneurons were hypertrophic, characterized by increased dendritic length and number of branch points. Increases in dendritic length were also observed in pyramidal neurons, with substantially elevated numbers of stubby and mushroom-type spines. RNA sequencing revealed global transcriptomic alterations in amygdala from GF versus SPF mice, with elevated expression of genes relevant for synaptic localization and immediate early transcriptional responses, and downregulation of genes relevant to neuronal projections and immune responses. In particular, decreases in nerve growth factor-inducible protein IA and *N*-methyl-D-aspartate (NMDA) receptor subtype 2B expression were observed in the GF amygdala, among several other brain regions (Arentsen et al. 2015, Diaz Heijtz et al. 2011, Neufeld et al. 2011a). Changes in expression of brain-derived neurotrophic factor (BDNF) have been reported in GF versus SPF mice across several studies. However, results have been conflicting: In one study, BDNF isoform IV was elevated in GF amygdala compared to SPF controls (Stilling et al. 2015); in two other studies, decreased levels of BDNF exon I, IV, VI, and IX transcript variants were seen in basolateral amygdala of GF mice compared to SPF controls (Arentsen et al. 2015, Diaz Heijtz et al. 2011). Similarly, BDNF protein was decreased in amygdala of antibiotic-treated SPF mice (Bercik et al. 2011). The bases for these discrepancies are unclear, but differences in amygdalar subregion or mouse strain, age, background, and experience could contribute. Interestingly, conventionalization of GF mice at weaning with a standard SPF microbiota restored only a subset of transcriptomic alterations, suggesting an important role for the microbiome during early development in programming adult baseline amygdalar gene expression. Modulation of these genes may be of particular relevance to specific behaviors that can be altered by microbiome interventions postweaning.

Hypothalamic Hormones and Neuropeptides

In addition to the amygdala, evidence suggests the microbiota alters the neuroanatomy and physiology of the hypothalamus, another important node in the behavioral network for stress and sociability. One particular study reports that the number of neuropeptide oxytocin-expressing cells in the paraventricular nucleus (PVN) of the hypothalamus are regulated by specific bacteria of the gut microbiota (Buffington et al. 2016). Adult offspring of mothers that were fed a high-fat diet exhibited deficient levels of oxytocin-immunoreactive PVN neurons, and treatment with the bacterium *L. reuteri* sufficiently increased oxytocin-positive cell counts in the PVN. This mechanism was thought to underlie the ability of *L. reuteri* to promote social behavior in the maternal high-fat diet mouse model. However, molecular mechanisms linking *L. reuteri* to changes in hypothalamic oxytocin expression remain unknown, and whether the effects of the microbiota on hypothalamic oxytocin levels and synaptic strength are specific to this bacterium in particular is unclear.

Other studies raise the notion that select metabolic products from the gut microbiota can influence social behavior. Intracerebroventricular injections of the short-chain fatty acid propionate induced deficient social interactions in mice and rats compared to vehicle-injected controls (Macfabe 2012). Although propionate is a primary product of bacterial fermentation and dependent on microbial metabolism, studies examining the effects of intestinal and systemic, rather than

intracerebroventricular, injection of the metabolite are warranted. Overall, there is some evidence that microbial metabolic products and downstream modulation of brain neuroactive peptides and transmitters could contribute to effects on social behavior. However, much remains to be discovered regarding the molecular and cellular signaling mechanisms by which the microbiota can modulate social interactions. Furthermore, how microbial effects on other processes, including the mesolimbic reward system, stress networks, and executive cognitive function, as described herein, could contribute to modifying social behavior remains poorly understood.

Hippocampal Structure, Neurogenesis, and Neurochemicals

Effects of the microbiota on the hippocampus could contribute to many behavioral phenotypes, including alterations in learning and memory. Hippocampal volume of the CA2/3 region was increased in GF mice compared to SPF controls (Luczynski et al. 2016). This correlated with dendritic atrophy of ventral hippocampal pyramidal neurons, characterized by decreased apical and basilar dendritic length, reduced branch points, and diminished numbers of stubby and mushroom spines. Dentate granule cells were also affected, exhibiting decreased numbers of branch points. Recent studies also suggest that the microbiota modulates hippocampal neurogenesis. Antibiotic-treated SPF mice displayed decreased numbers of proliferating BrdU- and NeuN-positive mature neurons and doublecortin-positive neuronal progenitor cells in the subgranular zone of the dentate gyrus (Möhle et al. 2016). Notably, these deficits in adult neurogenesis were corrected by postnatal treatment with the probiotic VSL3, which comprises eight bacterial strains: *Streptococcus thermophilus*, *Bifidobacterium breve*, *B. longum*, *B. infantis*, *Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei*, and *L. delbrueckii* subspecies *bulgaricus*. In contrast to this, however, a separate study revealed increased hippocampal neurogenesis in adult GF mice, which was not corrected by postnatal conventionalization (Ogbonnaya et al. 2015). The causes of these discrepancies are not clear, but differences between antibiotic treatment and GF status may contribute. Consistent with this, maternal exposure to peptidoglycans, components of the bacterial cell wall, were linked to altered neuroproliferation in the embryonic brain (Humann et al. 2016). In addition to these reports in rodents, one study of human brain microstructure by diffusion tensor imaging reported correlations between bacterial diversity of the gut microbiota and fractional anisotropy of the hippocampus as well as the hypothalamus and caudate nucleus (Fernandez-Real et al. 2015). Together, these studies suggest that the gut microbiota modulates hippocampal physiology and raises the question of whether such changes could underlie microbial effects on behavior.

Alterations in hippocampal BDNF expression have been reported widely in response to microbiome perturbations. Transcript levels of BDNF were decreased in the hippocampal CA1 region of GF brains compared to controls (Diaz Heijtz et al. 2011) and decreased similarly after antibiotic treatment of SPF mice (Frohlich et al. 2016). BDNF downregulation was specific to male GF mice, relative to female GF mice and SPF controls (Clarke et al. 2013). By contrast, a separate study reported elevated hippocampal BDNF protein in antibiotic-treated SPF mice (Bercik et al. 2011). Rats treated with prebiotic fructo- and galactooligosaccharides also exhibited elevated hippocampal BDNF levels (Savignac et al. 2013). The bases of these incongruities are unclear, but further studies that test effects of methodological variables are warranted.

Alterations in hippocampal neurochemical pathways have also been associated with changes in composition of the gut microbiota. GF mice exhibited substantial increases in hippocampal serotonin and 5-hydroxyindoleacetic acid compared to SPF controls (Clarke et al. 2013). Decreased expression of serotonin receptor subtype 1A was also seen in the dentate granule hippocampal subregion of GF mice (Neufeld et al. 2011b). Increases in expression of dopamine D1 receptor D1a were observed in the dentate gyrus of GF brains (Diaz Heijtz et al. 2011). Rats treated with

ampicillin exhibited decreased hippocampal levels of NMDA receptor, which was corrected by treatment with *L. fermentum* NS9 (Wang et al. 2015). Rats treated with prebiotic fructo- and galactooligosaccharides also exhibited elevated expression of NMDA receptor subunits NR1 and NR2A (Savignac et al. 2013). SPF mice treated with the bacterium *L. rhamnosus* exhibited sustained increases in hippocampal glutamate and *N*-acetyl aspartate beginning at one week posttreatment (Janik et al. 2016). Overall, disruptions in levels of brain neurotransmitters and their receptors have been observed in response to manipulations in the gut microbiota, but rigorous mapping of microbiome-dependent effects on neural circuitry is warranted to gain insight into the molecular basis of behavioral alterations.

Prefrontal Cortex Myelination and Gene Expression

Emotional states of fear, anxiety, depression, and stress are encoded by neural signaling of the limbic system. The mood-regulating limbic circuits consist of dynamic communication between several major brain structures including the nucleus accumbens, medial prefrontal cortex, amygdala, hippocampus, ventral tegmental area, and hypothalamus. RNA sequencing revealed overrepresentation of genes involved in myelination in prefrontal cortex from GF mice compared to SPF controls, with confirmed increases in expression of *Mag*, *Mbp*, *Mobp*, *Mog*, and *Plp1*, which were not seen in frontal cortex, hippocampus, cerebellum, amygdala, or striatum (Hoban et al. 2016). Electron micrographs corroborated these findings: GF mice exhibited increased myelin sheath thickness and increased number of laminae in the prefrontal cortex. These abnormalities were not corrected by conventionalization of GF mice with an SPF microbiota at weaning, suggesting an effect of the microbiota on myelination during developmental ages, which aligns with reports that myelin formation begins on postnatal day 10. Consistent with microbial effects on prefrontal cortical myelination, stress-exposed NOD mice exhibited altered prefrontal cortex myelin gene expression and amounts of myelinated fibers that were prevented by antibiotic treatment. Reduced myelination in medial prefrontal cortex of mice is associated with social avoidance behavior that occurs after prolonged social isolation (Liu et al. 2012, Makinodan et al. 2012). Notably, transplant of microbiota from NOD mice into wild-type mice was sufficient to induce social avoidance behavior and hypomyelination of the prefrontal cortex (Gacias et al. 2016). These findings suggest dynamic effects of the microbiome on cortical myelination that could contribute to key behavioral abnormalities.

CONCLUSIONS

Over the past decade, fundamental studies have revealed compelling effects of the microbiome on behavior and neurophysiology, inspiring further investigation of the microbiome-gut-brain axis. Although several neurological phenotypes have been characterized in response to microbial depletion, gnotobiotic interventions, and other microbiota-related stressors, principal questions regarding how microbiota changes modulate host behavior remain unanswered. Importantly, how do microbes communicate with the nervous system, and which microbial species confer particular host responses? How are the different routes of gut-brain signaling—neuroendocrine, neuroimmune, neuronal—affected, and which are most relevant? To what extent are microbial influences on host behavior dependent on concurrent alterations in nutrition, immunity, and metabolism, among other physiological processes? In addition, how are microbial effects on each mode of behavior impacted by the others; for example, are primary alterations in stress responses causal to abnormalities in social behavior or memory, and how might changes in sensory perception contribute? Finally, how will our understanding of microbiota-gut-brain communication shape

the development of novel therapeutics for treating behavioral and neurophysiological disorders? Future research is needed to integrate the various microbial interactions across body systems toward understanding how they collectively modify host behavior.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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