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Title: Searching for the gut microbial contributing factors to social behavior in rodent models

Running title: Gut microbiota and social behavior

Brittany D. Needham[1], Weiyi Tang[1], Wei-Li Wu[1*]

[1]Division of Biology and Biological Engineering, California Institute of Technology, 1200 E. California Boulevard, Pasadena, CA, 91125, USA

[*]To whom correspondence should be addressed:

Wei-Li Wu, Ph.D.

Division of Biology and Biological Engineering

California Institute of Technology

1200 East California Blvd. MC 156-29

Pasadena, CA 91125

Phone: (626) 395-8979

wlwu@caltech.edu

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Abstract

Social impairment is one of the major symptoms in multiple psychiatric disorders, including autism spectrum disorder (ASD). Accumulated studies indicate a crucial role for the gut microbiota in social development, but these mechanisms remain unclear. This review focuses on two strategies adopted to elucidate the complicated relationship between gut bacteria and host social behavior. In a top-down approach, researchers have attempted to correlate behavioral abnormalities with altered gut microbial profiles in rodent models of ASD, including BTBR mice, maternal immune activation (MIA), maternal valproic acid (VPA) and maternal high-fat diet (MHFD) offspring. In a bottom-up approach, researchers use germ-free (GF) animals, antibiotics, probiotics or pathogens to manipulate the intestinal environment and ascertain effects on social behavior. The combination of both approaches will hopefully pinpoint specific bacterial communities that control host social behavior. Further discussion of how brain development and circuitry is impacted by depletion of gut microbiota is also included. The converging evidence strongly suggests that gut microbes affect host social behavior through the alteration of brain neural circuits. Investigation of intestinal microbiota and host social behavior will unveil any bidirectional communication between the gut and brain and provide alternative therapeutic targets for ASD.

Keywords: Autism spectrum disorder (ASD); Gut microbiota; Gut-brain axis; Social behavior; Germ-free (GF)

INTRODUCTION

Social behavior deficit is a primary symptom of autism spectrum disorder (ASD), combined with restricted interests and repetitive behaviors. So far, there is no effective therapy for the social behavior deficit in ASD, partly because etiology of the disorder is still not clear. ASD is an early onset, neurodevelopmental disorder that is predominately genetic and more common in males. Its prevalence has increased dramatically in the past 50 years, with recent estimates rising to 1 in 68 children, although controversy about whether this trend is simply increased diagnosis exists (Investigators, 2014). The pattern of increased ASD diagnosis is reminiscent of the recent increase of allergies and autoimmune diseases, which according to the hygiene hypothesis, can be attributed to alterations in the microbiota due to an industrialized lifestyle, such as the rise in antibiotics, and environmental hygiene conditions (Bach, 2002). This parallel timing raises the possibility that overly hygienic environments and the decrease of microbial diversity in humans might be associated with the increasing prevalence of ASD (Becker, 2007). In addition, gastrointestinal (GI) complications and an altered gut microbiome are common comorbidities in ASD (Adams et al., 2011; Buie et al., 2010; Coury et al., 2012; Parracho et al., 2005). A small analysis of infant microbiomes has suggested that gut microbial composition could predict cognitive performance, including communicative behavior (Carlson et al., 2017). These associations have spurred investigations over the past decade into the intertwined relationships between the gut microbiota, brain, and behavior. Numerous review articles have extensively discussed this gut-brain hypothesis, focusing on the beneficial and detrimental consequences of bi-directional communication between systems. However, the specific roles of the gut microbiota in the social deficiencies in ASD are still not well understood.

Throughout the classes of life, examples of the microbiota affecting social behavior abound. Even in cell culture, single-celled chenoflagellates begin to cluster in the presence of certain bacteria

(Alegado et al., 2012). From flies to hyenas, mating preference can be determined by the microbiota (Sharon et al., 2010; Theis et al., 2013). Infection by a certain virus can cause bees to be aggressive and sharing gut microbiota between nest mates can protect against it (Koch and Schmid-Hempel, 2011). In baboons and chimpanzees, the microbiota is defined by social structure and interaction, and it is unlikely that the effects are not reciprocal on primate behavior as well (Moeller et al., 2016; Tung et al., 2015). In fact, in the hologenome theory of evolution, the host and all of its associated microbes are considered a single unit of selection in evolutionary change (Brucker and Bordenstein, 2013). Defining the mechanisms involved in such phenomenon has remained elusive for the gut-brain field, in part due to the complexity of the microbial community. The mammalian GI tract harbors a unique ecosystem, with hundreds to thousands of species of bacteria packed denser than any other known ecosystem (Human Microbiome Project, 2012). Exchange of microbiota through social interaction is advantageous for the host in numerous ways, such as improved resistance to pathogens, diversification of metabolic functions, and increased development of the immune system (Browne et al., 2017). It is of interest to understand the function of each bacteria to host social behavior. However, there are at least over 500 species symbiotically live in mouse gut (Xiao et al., 2015). It is extremely difficult to screen the functionality for each individual bacteria, so thus far it has been a struggle to define the specific qualities of the microbiota that directly affect social behavior, in animals or humans.

This review will focus on the literature using rodent models to tackle questions of gut microbes' link to social behavior deficit in ASD. Social behavior is an interaction among conspecific individuals and can be classified into several different forms- social approach/investigation behavior, aggressive behavior, social defeat, social avoidance behavior, and sociosexual behavior etc (Sandi and Haller, 2015). Impairment of social investigation and social novelty behavior are

the most common and consistent phenotypes in animals without gut microbiota and are also the convergent phenotypes among different mouse models of ASD. These are typically tested using the 3-chamber social and reciprocal social interaction tests, which are performed by allowing a subject mouse to explore an area containing a novel mouse over an object or a familiar mouse, and by scoring the social interactions between a subject and novel mouse in a cage, respectively (Silverman et al., 2010). Although a social behavior deficit can be observed in other psychiatric disorders in addition to ASD, such as social phobia, schizophrenia, and depression, this review will focus on ASD-related research due to the availability of literature studying the gut microbiota in ASD.

These behavioral paradigms can be tested using either a top-down or bottom-up approach (Fig. 1A). In a top-down approach, researchers focus on the GI abnormalities in mouse model of ASD and whether these abnormalities correlate with the gut microbial compositions and behavior phenotypes. In bottom-up approach, researchers consider the gut as a vessel and are able to exploit extreme conditions that would be impossible in humans, such as gnotobiotic animals that allow complete control over which, if any, microbes are colonizing the gut. Additionally, antibiotics, probiotics and pathogens can be used as tools to eliminate or supplement the gut microbiota to validate their functional effects on the host. In such studies, various brain regions are affected by depletion of gut microbiota. Likewise, these brain regions have been implicated as control hubs for social behavior. Understanding the contributions of gut microbes to host behavior will advance the current therapeutic strategy for ASD and provide alternative and safe practices to ameliorate the disorder.

TOP-DOWN APPROACH: RODENT MODEL OF RISK FACTORS FOR ASD WITH GUT MICROBIAL DYSBIOSIS

Most cases of ASD are idiopathic, with strong evidence that environmental factors are involved (Modabbernia et al., 2017). Because there is no definitive pathology or a single genetic mutation for ASD, animal models mostly rely on the display of behavioral abnormalities that correspond to the core symptoms of ASD. In this section, we review four models of ASD that not only exhibit social impairment or communication deficit, but also show gut microbial dysbiosis. These models include one genetic model of ASD- BTBR; and three non-genetic models for ASD- maternal immune activation (MIA), valproic acid (VPA) and maternal high-fat diet (MHFD)(Fig. 1A; Table 1).

Various studies have used a top-down approach to ask whether the animal model of ASD possesses differential gut microbiota and whether these alterations in gut microbiota contribute to social deficits. Several reports indicate that children with ASD have been found to have different microbiota than neurotypical children (Adams et al., 2011; De Angelis et al., 2013; Finegold, 2011; Krajmalnik-Brown et al., 2015; Parracho et al., 2005; Williams et al., 2012). These changes in the microbiota inevitably lead to changes in gut and serum metabolites as well as other systems such as immune changes and GI symptoms. Interestingly, most of the ASD models we discussed in this review show a "leaky gut" phenotype, where the intestinal epithelial barrier is compromised (Coretti et al., 2017; Hsiao et al., 2013). Whether the leaky gut phenotype is a universal feature in mouse models of ASD that contributes to the etiology of social impairment is still unknown. A complementary analysis of whether a leaky gut phenotype is sufficient to result in ASD-like social behaviors is warranted. Although the leaky gut hypothesis is promising, more work will need to be done in order to elucidate the cause–effect relationship.

BTBR

BTBR is an inbred mouse strain with the full spectrum of ASD-like behavioral phenotypes, the causes of which remain unclear. Although striking brain pathologies are obvious in this model, with reduced hippocampal commissure and absent corpus callosum, why and how the BTBR strain develops ASD-like behaviors and neuropathologies are open questions (Wahlsten et al., 2003). BTBR was first found to be associated with autism when researchers ran a series of ASD behavioral tests in 10 different inbred strains (Moy et al., 2007). BTBR mice showed decreased anxiety in a standard test, the elevated-plus maze, but also decreased sociability in 3-chamber social test. Interestingly, the ability to differentiate novel and old strangers is normal in BTBR mouse, indicating there is no deficit in social recognition in the BTBR mouse (Moy et al., 2007). Later, a more comprehensive social behavior tests, including juvenile play test and reciprocal social interaction, were performed to examine the social behavior in BTBR strain (McFarlane et al., 2008). BTBR mice displayed social impairment in most social tests. Moreover, BTBR mice emit an unusual pattern of ultrasonic vocalizations (USVs) during infancy (Scattoni et al., 2008) and fewer USVs in adulthood when presence with social cues (Wohr et al., 2011). This evidence strongly suggests that BTBR serves as a suitable model to study the social development in ASD.

The gut microbiota of BTBR mice was recently profiled in young and aged animals (Golubeva et al., 2017; Klein et al., 2016; Newell et al., 2016). Two reports focused on the caecal and fecal microbiota during the young adult stage (7 weeks of age). They found that, compared to its wild-type genetic background strain, C57BL/6 mice, microbiota profiles were dissimilar in both fecal and caecal samples, and the total bacterial abundance was decreased in caecal but not fecal samples in BTBR male mice (Klein et al., 2016). The common features of microbiota in BTBR mice are an increase in *Akkermansia muciniphila*, and a decrease in *Bifidobacterium spp.*, *Clostridium cluster XI*, *Enterobacteriaceae*, and *Methanobrevibacter spp.* Additionally, caecal microbiota in BTBR mice have lower levels of *Bacteroides/Prevotella spp.* and *Clostridium*

cluster I (Klein et al., 2016; Newell et al., 2016). Furthermore, one recent report analyzed the caecal microbiota in BTBR mice at adult stage (14 weeks of age)(Golubeva et al., 2017). Interestingly, they found that *Akkermansia* is increased, but *Bifidobacterium* and *Lachnospiraceae (Clostridiales)* are decreased in BTBR mice. In addition, they also identify that the reduction of *Rikenella, Parabacteroides, Odoribacter, Desulfovibrio, Blautia* and *Bifidobacterium* species and the increase of *S24–7 family*, *Bilophila* and *Bacteroides* in the cecum of BTBR mice are associated with the sociability, anxiety-like behaviors and repetitive behaviors (Golubeva et al., 2017).

Another group found that in aged mice (12 months old), the C57BL/6 and BTBR fecal microbiota differ as well. Bacteroidetes and Firmicutes were the major contributing factors driving the difference of gut microbiota profiling (Coretti et al., 2017). The relative abundance of identified operational taxonomic units (OTUs) classified at bacterial phylum level was not different between male BTBR and male C57BL/6 mice. However, several microbial changes were identified at the level of genus: in male mice, BTBR samples were increased in *Bacteroides*, *Parabacteroides*, *Lacobacillus*, *Coprobacillus*, and *Helicobacteraceae*, but decreased in *Dehalobacterium*, *Ruminococcus*, and *Desulfovibrio* (Coretti et al., 2017). Interestingly, the BTBR mice have deficits in intestinal integrity. The intestinal tight junction proteins- *Ocln* and *Tjp1* are reduced in male BTBR mice while comparing to C57BL/6 mice (Coretti et al., 2017). In addition, the difference of gut microbiota in BTBR mice could be further associated with the host metabolites, gut permeability, inflammatory cytokines and behavioral deficits. The effect of sex and diet to gut microbiota are also in a notion in BTBR mice (Coretti et al., 2017; Klein et al., 2016; Newell et al., 2016). These results indicate that the gut microbiota in BTBR mice is indeed different from C57BL/6 mice. The changes remain pronounced in aged mice between the two

strains. Causative links between the altered microbiota, brain, and behavior in BTBR mice have not been established.

Maternal Immune Activation (MIA)

Epidemiological studies of maternal databases have highlighted a correlation between infection during pregnancy and ASD (Atladottir et al., 2010; Gorrindo et al., 2012; Zerbo et al., 2015). In the MIA mouse model of ASD, the maternal immune response is activated during gestation with an immunogen such as polyinosinic:polycytidylic acid (polyI:C), a viral mimic (Boksa, 2010; Chow et al., 2016; Meyer and Feldon, 2010; Meyer et al., 2005; Patterson, 2009). As a result of the downstream inflammatory cytokine response, the exposed offspring grow up with comprehensive behavioral abnormalities that are similar to ASD and schizophrenia (Estes and McAllister, 2016; Patterson, 2011). Overall, MIA offspring show lower social behavior observed in the 3-chamber social test, and they display altered USV (Careaga et al., 2017; Choi et al., 2016b; Hsiao et al., 2012; Hsiao et al., 2013; Malkova et al., 2012; Schwartzer et al., 2013; Wu et al., 2017).

Similar to the common GI comorbidities of people with ASD, MIA offspring display decreased intestinal barrier integrity and an altered gut microbiota (Hsiao et al., 2013). Variations in OTUs within the classes Clostridia and Bacteroidia account for most of the dysbiosis in the fecal samples of these offspring. OTUs from various bacterial families were enriched in MIA samples, including *Lachnospiraceae, Porphyromonadaceae*, *Prevotellaceae*, unclassified Bacteriodales, while others were higher in the control samples, such as *Ruminococcaceae, Erysipelotrichaceae*, and *Alcaligenaceae.* Interestingly, when MIA offspring are treated with a live bacterial strain, *Bacteroides (B.) fragilis,* fecal levels of OTUs from the *Lachnospiraceae* family are restored and several ASD behavioral abnormalities are normalized. However, social behavior is unaffected by

this treatment and corresponding change in the microbiota (Hsiao et al., 2013). Further work by Kim, et al. has shown that the MIA model is dependent on the presence of IL-17 inducing microbial species. Colonization of female mice with segmented filamented bacteria (SFB) or a mix of human commensal bacteria known to promote an IL-17 response leads to exacerbated ASD-like phenotypes in MIA offspring, while the absence of these microbes inducing IL-17 limits the effects of MIA (Kim et al., 2017).

Multiple groups have worked to defined the mechanisms of the MIA model, and its links to the microbiota. For instance, MIA dysregulates genes associated with ASD, such as neurogenesis pathways and early brain development (Lombardo et al., 2017). Offspring shows cortical abnormalities, altered synaptic proteome, altered synaptic organization, and altered neurotransmitter levels (Gyorffy et al., 2016; Kim et al., 2017; Kirsten et al., 2012; Pendyala et al., 2017). Furthermore, cytokine levels in the gut and brain (Garay et al., 2013; Hsiao et al., 2013; Pendyala et al., 2017; Wu et al., 2015; Wu et al., 2017) and the microglia transcriptome (Mattei et al., 2017) are involved.

Understanding the connections between these mechanistic insights, specific social behavioral abnormalities, and the gut microbiota will be crucial to improving therapeutics. Multiple groups have explored the possibility of treating social deficits with diet or oral drugs that could be functioning through or acting on the microbial community. Oral vitamin D and poly unsaturated fatty acids in the maternal diet improved social outcome for MIA offspring (Vuillermot et al., 2017; Weiser et al., 2016). Treatment in offspring directly with poly unsaturated fatty acids, a ketogenic diet, minocycline, and antipurinic therapy also improved social interactions (Fortunato et al., 2017; Li et al., 2015; Mattei et al., 2017; Naviaux et al., 2014; Ruskin et al., 2017).

Whether these molecules have direct effects on the nervous system or are mediated by the microbiota will be an interesting question to answer with further work.

Valproic acid (VPA)

Prenatal exposure to valproic acid, an antiepileptic and mood stabilizing drug, is a significant risk factor for the development of cognitive defects and ASD in humans, including but not limited to social developmental delay, deficits in social functioning, and impaired communication (Bromley et al., 2013; Christensen et al., 2013; Nadebaum et al., 2011). VPA administration in rodents results in ASD-like social tendencies in the offspring (Dufour-Rainfray et al., 2010). A decrease in social play and reciprocal social behavior was first observed in rats (Schneider and Przewlocki, 2005). Since then more groups have observed that in both rats and mice, VPA exposure consistently decreases sociability (Kim et al., 2014; Kim et al., 2011; Moldrich et al., 2013; Roullet et al., 2010). Additionally, VPA exposure diminishes olfactory discrimination, which can be related to social recognition and development (Bienenstock et al., 2017; Melo et al., 2006; Roullet et al., 2010; Terry and Johanson, 1996).

In spite of the fact that VPA is a short-chain fatty acid (SCFA), a class of molecules under scrutiny in connection to the gut microbiota and host health, the gut microbiota of children exposed to VPA *in utero* has yet to be characterized. In fact, thus far only one study in mice has compared the gut microbiota between VPA-exposed and control offspring (de Theije et al., 2014b). de Theije *et. al* observed at the phyla level a decrease in *Bacteroidetes* and an increase in *Firmicutes* in VPA offspring. Such a shift is seen in other disease states such as obesity (Ley et al., 2006). At the order level, the VPA microbiota had higher levels of Clostridiales and Desulfovibraionales, echoing observations in some human ASD studies (Kang et al., 2013; Louis, 2012).

At the OTU level, those assigned to the genera *Alistipes, Eterorhabdus, Mollicutes*, and *Erysipelotrichales* were especially associated with male VPA offspring. Social behavior and the gut-produced neurotransmitter serotonin were also lower in male offspring, and *Alistipes* and *Erysipelotrichales* have been speculated to affect serotonin levels (de Theije et al., 2014a). Although no particular taxa were correlated with social behavior in this work, gut microbiota have been shown to be a critical regulator of serotonin production and serotonin increases sociability in a separate mouse model of ASD (Nakai et al., 2017; Yano et al., 2015). Further work in this area could reveal direct connections between specific bacteria and neurotransmitters, along with their effects on social behavior.

The same study also found that the altered VPA microbiota correlated with increased butyrate, especially in male animals, and butyrate was inversely correlated with social behavior. However, precise levels or ratios of particular SCFAs are likely important, as levels of lactic acid were positively associated with social behavior (de Theije et al., 2014b). Furthermore, social deficits in offspring exposed to VPA, which itself is a potent HDAC inhibitor, can persist in the subsequent generation, indicating that epigenetic mechanisms are possibly at play (Choi et al., 2016a).

Maternal high-fat diet (MHFD)

Maternal obesity during pregnancy is associated with neurodevelopmental disorders and social deficits. In epidemiological studies, maternal metabolic conditions such as obesity increase the risk of ASD in children (Krakowiak et al., 2012; Lyall et al., 2013), and in animal models, MHFD-induced obesity causes ASD-like behavioral phenotypes in offspring (Buffington et al., 2016; Kang et al., 2014). In fact, causal relationships between MHFD, gut microbiota, brain circuitry and social behavior in offspring have been found. MHFD causes microbial dysbiosis in

the gut and a reduction in social interaction, sociability and social novelty in the mice offspring by testing with reciprocal social interaction and 3-chamber social test.

MHFD affects neuronal functions through multiple mechanisms. For example, dietary components regulate maternal metabolism, which interacts with the neuroendocrine pathway to perturb the programming of fetal brain circuitry (Sullivan et al., 2015). Moreover, several studies indicate that MHFD and maternal obesity could alter the gut microbiota in offspring of both humans (Chu et al., 2016; Galley et al., 2014) and animal models (Ma et al., 2014). Since the gut microbiota interacts with the brain, MHFD-induced changes in the gut microbiota could be an important mediator of atypical neurodevelopment in this model. Interestingly, transfer of microbiota from regular maternal diet to MHFD mice by co-housing could rescue the social deficit observed in MHFD offspring. Mechanistically, the study found that MHFD causes defects in ventral tegmental area (VTA) dopaminergic neuron plasticity and a reduction of oxytocin^{+} cells in the hypothalamus.

Analyzing the gut microbiota by unweighted analyses of Unifrac distances showed pronounced differences in the structure of the bacterial communities between MHFD and maternal regular diet treated offspring. The diversity of microbiota in MHFD offspring was decreased while comparing to maternal regular diet treated offspring. Metagenomic shotgun sequencing revealed that the abundance of several bacterial species was reduced in MHFD fecal sample (>2-fold), including *Lactobacillus (L.) reuteri* (>9-fold reduction), *Parabacteroides distasonis*, *Helicobacter hepaticus*, *B. uniformis*. Interestingly, *L. reuteri* treatment is able to rescue sociability and social novelty in MHFD offspring, as well as the associated deficit in synaptic plasticity and molecular phenotypes in the brain. The effect is specific to MHFD offspring, because only MHFD offspring shows defective social behavior compared to the maternal regular

diet offspring; *L. reuteri* treatment does not change sociability or social novelty in maternal regular diet treated offspring (Buffington et al., 2016).

The effect of MHFD on social behavior could be complicated and intertwine with other environmental factors. One study suggests that MHFD modulates early-life stress in maternal separation (MS) model (Rincel et al., 2016). MS has been shown to alter colonic microbiota, induce anxiety-like behavior in a microbiota-dependent manner (De Palma et al., 2015) and decrease social interaction (Rincel et al., 2016). However, MHFD could normalize the social deficit caused by MS, suggesting that maternal nutrition could affect offspring behavior (Rincel et al., 2016). The mechanism is highly likely to be through the gut-brain axis, because both MS and MHFD affected the gut microbiota (Buffington et al., 2016; De Palma et al., 2015). Together, these evidences highlight the microbiota as a mechanism by which MHFD modulates social communication development.

In summary, ASD is a multifaceted neurodevelopmental disorder where MIA combined with environmental risk factors and/or genetic mutations can lead to a variety of neurological conditions (Schaafsma et al., 2017). In addition to allowing the study of the altered microbiota in ASD, these models provide interesting possibilities to combine genetic and environmental models. For example, inducing MIA in the BTBR mouse will expand into the complexity that potentially occurs in human ASD (Schwartzer et al., 2013). In fact, MIA is worse in combination with risk alleles (Abazyan et al., 2010). MIA is likely one part of a multifaceted neurodevelopmental onslaught that if combined with environmental risk factors and/or genetic mutations can lead to a variety of neurological conditions (Knuesel et al., 2014).

BOTTOM UP APPROACH: WHAT DO WE LEARN FROM BACTERIAL INTERVENTION STUDIES IN SOCIAL DEVELOPMENT?

Gnotobiotic animals and antibiotic treatments have become powerful tools to examine the physiological, immunological and behavioral features that are impacted by gut microbiota (Fig. 1B). Total deprivation of any microbiota (termed germ-free) during the entire lifespan of an animal allows the study of bacterial colonization effects in a controlled, albeit extreme, environment. Mono-colonization of a single bacterial species illuminates the effect of specific bacteria to the host. Manipulation of complex gut microbiota by antibiotics gives researchers the flexibility to control the microbes' presence or absence in a temporal fashion. Probiotic supplements provide a way to understand how a single bacterial species changes the microbial community and the physiological functions of the host. Microbial manipulation is an attractive therapeutic target due to its accessibility and its status as a potentially malleable "organ". In fact, once crucial bacterial taxa are better identified and understood, bacterial treatment and transplants, or prebiotics (dietary components like fiber that increase growth of certain beneficial bacteria) in the diet could be used to select for desired bacterial communities (Buffington et al., 2016; Burokas et al., 2017; Hsiao et al., 2013; Kang et al., 2017).

Germ-Free (GF)

GF animals are carefully controlled to have a total lack of any microbes in or on their bodies, and they exhibit abnormal social behavior. This illustrates the point that absence of bacterial colonization during development exerts a detrimental effect to mouse social behavior (Desbonnet et al., 2014). GF Swiss-Webster mice spend more time investigating a novel object than a stranger mouse in 3-chamber social test. Further, GF mice showed no preference to a novel stranger over a familiar mouse, indicating that GF mice not only displayed impairment of sociability, but also social recognition deficit. Colonizing GF mice with normal fecal microbiota

during the juvenile stage can reverse the sociability defect. These results suggest that gut microbiota play a pivotal role in social development (Desbonnet et al., 2014). However, a report with a similar approach found an opposite finding. By testing GF mouse in 3-chamber social test, they found that GF mice spent more time investigating the stranger mouse than specific-pathogen free (SPF) control group (Arentsen et al., 2015). The conflicting results between the two studies could be due to the differences in experimental design- different strain for stimulus mice and different age of testing (Arentsen et al., 2015).

Later observations of GF social behavior in C57BL/6 mice supported the conclusion that the lack of gut microbiota during development perturbs social development. Social impairment was observed in both 3-chamber social and reciprocal social interaction tests, and colonization with a normal gut microbiota at 4 but not 8 weeks of age restored social behavior (Buffington et al., 2016). A social deficit phenotype has also been observed in GF rats in the reciprocal social interaction test (Crumeyrolle-Arias et al., 2014).

These studies clearly point out that mice devoid of gut microbiota during their entire lifespan are impaired in conspecific social interaction and social preference. The developmental stage for gut microbial colonization is also critical for developing social behavior. Later in this review we give a comprehensive discussion of potential mechanisms involved for the GF phenotypes described above.

Antibiotics

Broad-spectrum antibiotic cocktails (ABX) provide another way to eliminate gut bacteria, mimicking the results seen in GF animals. However, the particular antibiotics used, as well as the dosage, schedule, delivery route, and age of mice during ABX administration are all critical

factors influencing the outcome of behavioral phenotypes. Therefore, the effect of ABX on mouse social behavior is still under debate (Table 2).

Regardless of the inherent variations in study design, ABX treatment during certain perinatal stages does appear to affect social behavior. Administration of succinylSulfaThiazole, a sulfonamide antibiotic, in the diet starting one month before pregnancy until embryonic day (ED) 15 decreased rat social behavior in the reciprocal social interaction test (Degroote et al., 2016). Treatment of penicillin V in drinking water from ED 12-14 to postnatal 21 days decreased both sociability and social novelty in BALB/c mice by 3-chamber social test (Leclercq et al., 2017). Another report shows that administration of neomycin trisulfate salt hydrate, bacitracin, pimaricin in the drinking water from ED 9-16 did not change the sociability and social novelty behavior in the male offspring by 3-chamber social test (Tochitani et al., 2016). In the MIA model, vancomycin treatment in mothers prevented USV and social deficit phenotypes (Kim et al., 2017). These studies suggest that the perinatal stage is critical for social development, and determining the connections between the depletion of certain bacteria sensitive to specific antibiotics, versus depletion of the entire community, and the corresponding mechanisms remain to be systematically studied in the context of social behavior.

The effect of broad-spectrum ABX treatment post-weaning on social behavior is less studied. Applying ampicillin, vancomycin, neomycin, and metronidazol in the drinking water from weaning till adulthood produces minimal effect to social memory to Swiss-Webster mice as demonstrated by social transmission of food preference test. In this test, a demonstrator mouse is allowed to choose between two foods, then later spends time with the testing subject mouse. When the testing subject mouse is then allowed to choose between the two foods, it usually picks the one the demonstrator mouse liked (cued food). On the contrary, a subject mouse with social

deficit would fail to differentiate the two foods, so that the preference to cued food would not be significant. Interestingly, ABX treatment decreased the preference to cued food, which was consumed by demonstrator mouse, 24 hours after social interaction. But there was no difference between control and ABX mice in the preference to cued food immediately after social interaction (Desbonnet et al., 2015). Another study showed that there was no change to C57BL/6 mouse social interaction when orally gavaged with broad-spectrum ABX, including vancomycin, neomycin, metronidazol, with ampicillin supplemented in the water. However, the gavage with vehicle decreased the social interaction in non-obese diabetic (NOD) mouse and ABX was able to normalize the social avoidance behavior that was caused by gavage stress (Gacias et al., 2016). Interestingly, selective depletion of the gut microbiota by oral enrofloxacin decreased aggressive behavior in hamster, but unchanged the investigation behavior. Female hamsters were more susceptible to enrofloxacin treatment. The female aggressive behavior decreased at the first 7 day ABX treatment and the effect lasted even after withdraw the enrofloxacin treatment. However, male hamsters only showed lower aggressive behavior at the second time ABX treatment. The effect did not persist after withdrawal of the ABX treatment. On the contrary, the investigative behavior was not changed by enrofloxacin treatment in hamsters (Sylvia et al., 2017).

ABX are a powerful tool to address the role of microbiota in the brain and behavior. Nonetheless, ABX could have confounding effects to the host. However, it appears that the effect of oral administration of ABX, such as ampicillin and vancomycin, is limited to circulation and does not result in increasing the levels of ABX in the brain (Frohlich et al., 2016). Rodents are sensitive animals and very vulnerable to stressful handling. The intrinsic behavior could be masked when animals faces stressful situation. Therefore, it is crucial to optimize the methods to deliver ABX to the animal with a stable and harmless way.

Probiotics

Bacteria that confer benefits on the host are termed probiotics, and those with beneficial effects on mental health have been termed psychobiotics (Dinan et al., 2013). In this emerging field, only a few have been identified to date, but the complex mammalian gut microbial community is replete with functional redundancy (Lozupone et al., 2012). It is possible that entire classes of bacteria will be identified that can serve to activate the same mechanisms as known psychobiotics, whatever those mechanisms are. However, specificity exists, since in some cases close relatives of psychobiotics have been shown to lack activity (Buffington et al., 2016; Perez-Burgos et al., 2013). There also seems to be specificity for bacteria affecting certain behavioral pathways but not others, as seen in the MIA model, where the bacterial strain *B. fragilis* ameliorates several behavioral deficits, such as anxiety, repetitive behavior, and USV during social encounter, but does not improve social behavior in the 3-chamber social test (Hsiao et al., 2013).

Most of the published evidence for psychobiotics inducing social behavior is with *Lactobacillus (L.) spp. reuteri* and *rhamnosus*. In microbiota of MHFD offspring, *L. reuteri* was the most drastically reduced taxa (9-fold). Administering *L. reuteri* in the drinking water of MHFD weanlings for 4 weeks improved sociability and preference for social novelty in the 3-chamber social test, but a related strain *L. johnsonii* could not. The *L. reuteri* probiotic treatment restored the low levels of oxytocin-producing neurons in the model, and it also restored social-interaction-induced long-term potentiation (Buffington et al., 2016).

L. rhamnosus also seems to affect social behavior. *L. rhamnosus* JB-1 has been the most extensively studied strain. It partially prevented the preference for social novelty in antibiotic treated mice when co-administered, especially in females (Leclercq et al., 2017). In the chronic social defeat model, mice are repeatedly submitted to dominance by an aggressor. As a result, the

defeated mouse has diminished social preference in the 3-chamber social test. JB-1 treatment by gavage for 4 weeks improved the behavior to show no preference for social or non-social. However, JB-1 did not affect aggressor avoidance following social defeat, and in this model, did not prevent the altered microbiota caused by social defeat (Bharwani et al., 2017). Furthermore, JB-1 increases the neurons firing in the mesenteric nerve bundle, while *L. salivarius* did not (Perez-Burgos et al., 2013).

Some small pilot clinical studies have moved forward to test the effects of *L. rhamnosus* in humans, with modest results (Kelly et al., 2017; Partty et al., 2015; Scalabrin et al., 2017; Slykerman et al., 2017). However, these studies included healthy participants, so future studies with at-risk populations might be more meaningful. Whether these psychobiotic effects are direct, the result of restoration of other bacterial taxa, or affecting metabolism of other bacteria in the community has not been worked out. In the antibiotic treated mice, JB-1 was sufficient to maintain wild type levels of several families including *S24-7, Lachnospiraceae, Erysipelotrichaceae*, and *Enterobacteriaceae* (Leclercq et al., 2017). The molecular mechanisms involved are yet to be elucidated, although related evidence indicates that the vagal nerve is likely involved (Bravo et al., 2011; Perez-Burgos et al., 2013).

Pathogens

Evolution in the presence of microbes has likely shaped social behavior. Pathogens, especially those that require contact for transmission, benefit from the increased transmission rate of group living (Antonovics et al., 2011; Moller, 1993). They could even have driven it through mechanisms like increased oxytocin production or RNA regulation necessary for brain development (Insel, 2010; Lukas and Clutton-Brock, 2013; Meyer-Lindenberg and Tost, 2012; Skuse et al., 2014). Fortunately for the host, the benefits gained by social living outweigh the

costs of increased exposure to pathogens, not the least of which is the exchange of microbiota that provide numerous benefits (Koch and Schmid-Hempel, 2011; Stilling et al., 2014). As such there are clear costs to isolation, and social deprivation or maternal neglect disturb the microbiota (Bailey and Coe, 1999; Bailey et al., 2011; O'Mahony et al., 2009). Additionally, social living comes with its own costs, it requires energy/evolution to produce skills like recognition of conspecifics, empathy, which important for hunting in animals, and is disrupted in ASD (Baron-Cohen, 2009). So there is a balance to social living in groups versus the avoidance of pathogens transmitted in groups, and it is possible that both the social drive to form groups as well as the structural size limits of these same groups are both heavily impacted evolutionarily by bacteria, both commensal and pathogenic.

Although pathogens likely affected evolution of social behavior, it has been difficult to pinpoint specific pathogens that induce it outright in mice. Pathogens cause sickness behavior, lowering locomotion and health, which can confound the study of sociability mechanisms (Dantzer R, 2000). In an indirect way, infection does affect social behavior through sickness avoidance, where mice spend less time with a conspecific in an acute inflammatory state, and the oxytocin gene is involved (Boillat et al., 2015).

One type of behavior, anxiety, which is a common comorbidity of ASD and has connections with social behavior, has been observed in connection to infection by a handful of pathogens. Infection by the nematode parasite *Trichuris muris* affects brain neurotrophic factor BDNF in the hippocampus as well as circulating cytokines and metabolites. Treatment with *B. longum* normalized behavior (Bercik et al., 2010). Mice infected with *Campylobacter jejuni*, the most common cause of diarrheal disease in the US (Allos, 2001), or *Citrobacter rodentium*, a mouse pathogen that is comparable to infection in humans by food-borne illness *Escherichia coli*,

showed anxiety in as little as 4-8 hours after infection. These infected mice showed anxiety in the open field arena and hole-board test, both of which show exploratory behavior of mice, with differential neuronal activation in anxiety-circuit brain regions even before immune mediators were induced (Goehler et al., 2005; Goehler et al., 2008; Lyte et al., 2006). *L. rueteri* is able to reduce the stress response caused by *C. rodentium* (Mackos et al., 2013).

Besides single pathogens altering social tendencies, it is possible that a dysbiotic microbiota as a pathogenic community could affect behavior. Much work is currently devoted to identifying particular bacterial taxa involved in susceptibility and resistance to various disease states, such as in chronic social defeat models (Szyszkowicz et al., 2017; Yang et al., 2017). The answer may lie in the altered metabolites, SCFA, and regulatory features of the community as a whole. For instance, the levels of some microbe-associated molecular patterns (MAMPs) and their detection by host pathogen recognition receptors (PRRs) can relate to social behavior. The peptidoglycan-sensing molecule, *Pglyrp2* detects peptidoglycan from the commensal gut microbiota. Its knockout affects expression of an autism risk gene, c-Met, and also causes changes in social behavior. Expression of *Pglyrp2* is affected by antibiotic treatment or germ-free status in offspring (Arentsen et al., 2017). Clearly, microbial dysbiosis is associated with altered social behavior, but the extent to which a disrupted bacterial community is pathogenic is yet to be determined.

BRAIN COMPARTMENTS INVOLVED IN SOCIAL CIRCUITS ARE AFFECTED BY THE LOSS OF GUT MICROBIOTA

The brain is the main organ controlling the output of social behavior. Therefore, understanding the brain regions that contribute to social behavior will be crucial to understanding the

interactions between the gut microbiota and the brain. The loss of microbiota in the gut results in profound perturbation of brain development (Fig. 2).

GF mice exhibit ASD-like social deficits (Desbonnet et al., 2014). Since social behavior is controlled collectively by multiple brain regions that regulate emotion, social cognition, learning and stress, the question then becomes: does the alteration in gut microbiota affect the brain? If so, what are the consequences to the brain at the morphological, transcriptional and functional levels when the gut microbiota is depleted?

Brain morphology

Evidence shows that the amygdala and hippocampus are targets for altered brain morphology in GF animals. Compared to SPF mice, GF mice show enlarged amygdala and hippocampal volume, with the total brain volume unchanged. Specifically, GF mice display larger lateral amygdala (LA), basolateral amygdala (BLA), central amygdala (CeA), hippocampal CA2 and CA3 (Luczynski et al., 2016). CA2 activation is known to enhance social memory and its malfunction is implicated in ASD (Hitti and Siegelbaum, 2014; Leroy et al., 2017; Smith et al., 2016). Another study showed that in GF mice, there is increased dorsal hippocampal neurogenesis in the adults compared to SPF mice, which can potentially explain the altered hippocampal volume (Ogbonnaya et al., 2015).

At the level of single neurons, GF mice exhibit altered neuronal dendrite length and branching. In the BLA aspiny and pyramidal interneurons, dendrites are longer with more branching points. Ventral hippocampus pyramidal neurons display shorter dendrites, smaller spine density, and fewer stubby and mushroom spines. Under GF condition, there are more synaptic connections in the BLA region and less synaptic connection in the hippocampus (Luczynski et al., 2016).

Besides the amygdala and hippocampus, GF mice exhibit enlarged periaqueductal gray (PAG), smaller anterior cingulate cortex (ACC), and increased basilar dendritic length in ACC pyramidal neurons (Luczynski et al., 2017). The changes in volume and dendritic morphology in the brain subregions of GF mice could contribute to the altered stress response and social impairment.

Transcriptional analysis

GF mice display a unique transcriptome signature in the brain that might underlie behavioral changes. Several studies have attempted to understand the transcriptional profiles between GF and SPF mice. The first study profiling the transcriptional level in GF mice identified several different subsets of genes that are differed from SPF mice in different regions, including hippocampus, cortex, striatum, cerebellum and hypothalamus. They concluded that four canonical pathways in the brain could be mediated by gut microbiota, including citrate cycle, synaptic long-term potentiation, C21-steroid hormone metabolism and cAMP-mediated signaling (Diaz Heijtz et al., 2011). Therefore, differential gene expression in the regions mentioned above implicates alterations in their activity and can potentially explain the changes in social behavior in GF mice.

Later, the transcriptional profile in the amygdala of GF compared to SPF mice showed up-regulation of genes related to neurogenesis, synaptic transmission, cognition and nervous system development (Hoban et al., 2017; Stilling et al., 2015). Immediate early genes (*Fos*, *Fosb*, *Arc, Egr2 and Nr4a1*), synapse and neuronal transmission-related genes (*Drd2*, *Syt2*, *Chat*, *Adora2a*, *Sigmar1* and *Pde10a*), chemokines (*Ccl27a*) and the MAP kinase signaling pathway were up-regulated, and neuropeptides (*Bdnf*) were down-regulated in GF animals (Hoban et al., 2017; Stilling et al., 2015). Clearly, the lack of gut microbiota could cause pre-activation of neuronal-related genes and alterations in neuropeptides in the amygdala.

In another study, a GF state was associated with myelination-related gene upregulation in the prefrontal cortex. A lack of microbiota could result in the increased myelin plasticity and neuronal activity in the prefrontal cortex. Several genes that are related to myelination (*Mag*, *Mbp*, *Mobp*, *Mog*, *Plp1*) are upregulated only in prefrontal cortex of GF mice, but not in other regions. In addition, oligodendrocyte-specific gene (*Olig1*) and genes involved in myelin regulation (*Egr2*, *Sox10*) were also found to be upregulated in the absence of microbiota. Indeed, the myelin sheath thickness is dramatically increased in the prefrontal cortex of GF mice. Interestingly, recolonizing the GF mice with conventional gut microbiota at weaning could reverse the effect on the transcription profile, suggesting a causal relationship between microbial colonization and gene expression in the brain. The recolonization experiment also implied a time window in which the developing brain is especially sensitive to modulation by gut microbiota (Hoban et al., 2016).

The hippocampus participates in social cognition and shapes social behaviors. In GF mice, microarray analysis revealed that the cAMP responding element-binding protein (CREB) signaling was one of the most highly dysregulated pathways in GF mice. They further confirmed that phosphorylated CREB (pCREB) expression is upregulated and protein kinase C beta (*Prkcb*), AMPA receptor subunits (*Gria1-4*), and serine/threonine-protein kinase (*Akt1*) are downregulated in the hippocampus of GF mice. The changes of CREB and pCREB level can be restored by colonization with SPF microbiota (Zeng et al., 2016).

Since the amygdala, hippocampus and prefrontal cortex are target regions for social communication, the evidence that gut microbiota can affect transcriptional profile in these regions has a profound implication for the mechanism by which the gut communicates with the

brain to control social behavior. However, the exact time window in which the microbiota modulates brain development and the corresponding behavioral effects on adult social communication remains to be elucidated. Together, the above evidence suggests the role of gut microbiota in modulating neuronal related gene expression and behavior.

Neuronal molecules

Neurotransmitters, neurotrophic factors and other signaling molecules could modulate brain function in response to behavior and external stress. These molecules in the brain can be altered in the absence of gut microbiota (Table 3) and can potentially be the mechanisms by which gut microbiota modulate brain neuronal activity and social behavior. Several neurotransmitters and their receptors are differentially expressed in GF mice, which suggests an alteration in both the level and the activity of these neurotransmitters.

The brain serotonergic system can potentially be mediated through the gut microbiota. Serotonin, serotonin metabolite, and the serotonin precursor tryptophan are increased in GF mice in the hippocampus and striatum (Clarke et al., 2013; Diaz Heijtz et al., 2011). However, no significant change in the level of serotonin transporters or receptors was detected in the hippocampus (Clarke et al., 2013). Indeed, another study specifically focusing on the dentate granule of the hippocampus found a decreased level of serotonin 1A receptor (Neufeld et al., 2011), and in GF rats, a lower serotonin level in the hippocampus was observed (Crumeyrolle-Arias et al., 2014).

Alterations in the dopaminergic pathway were also reported in GF animals. In GF mice, increased dopamine turnover rate and elevated dopamine D1 receptor levels were found in the striatum and hippocampal dentate gyrus (DG), respectively (Diaz Heijtz et al., 2011). On the contrary, dopamine turnover rate is decreased in GF rat, with a lower dopamine metabolite

detected in the frontal cortex, hippocampus and striatum (Crumeyrolle-Arias et al., 2014). Another canonical monoamine neurotransmitter, norepinephrine, has not been extensively studied in GF mice, although one report indicated that the turnover rate of norepinephrine was increased in the striatum of GF mice (Diaz Heijtz et al., 2011). Besides monoamine neurotransmitter, glutamate receptors are also regulated by microbial state (Neufeld et al., 2011; Sudo et al., 2004).

Brain derived neurotrophic factor (BDNF), a major neurotrophic factor in the brain, has been extensively investigated in GF mice. In general, the level of BDNF is lowered in GF mice compared to SPF mice across brain regions including the cortex (Sudo et al., 2004), amygdala (Arentsen et al., 2015), BLA (Diaz Heijtz et al., 2011), hippocampus (Clarke et al., 2013; Sudo et al., 2004) and hippocampal CA1 (Diaz Heijtz et al., 2011; Gareau et al., 2011). Although most studies indicate that BDNF is reduced in the brain of GF mice, one study showed that BDNF expression is increased in hippocampal DG in female GF mice (Neufeld et al., 2011), suggesting that the effect of gut microbiota on brain activity can be sex-specific.

GF mice also display higher circulated corticosterone level in baseline (Neufeld et al., 2011) and under stress (Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Sudo et al., 2004). Interestingly, the receptor for corticosterone, glucocorticoid receptors (GR), was lower in the hippocampus GF rat. Meanwhile, the activator of corticosterone, corticotropin-releasing factor (CRF), was increased in the hypothalamus of GF rat (Crumeyrolle-Arias et al., 2014). These results indicate an increased stress response and explain the corresponding neurochemical changes in the brain. This effect can be partially rescued when GF mice are colonized with SPF microbiota during early (9 week) but not late (17 week) ages (Sudo et al., 2004). These results show that the gut microbiota interacts with the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the stress response and participates in programing of brain circuits in specific developmental time

points. The hypothalamus as a component of the HPA axis is also implicated in controlling social behavior, suggesting a correlation between stress response, anxiety and social interaction (Liu et al., 1997).

The abundance of postsynaptic density protein (PSD-95) and synaptophsin is increased in the striatum of GF mice, implicating changes in synaptic plasticity in GF mice. Altered levels of both these markers of synaptogenesis suggest a long-term programming of synaptic connections that could lead to change in behaviors in adults (Diaz Heijtz et al., 2011). Synaptic molecule-NGF1-A, an immediate early gene, was reduced at the basal level in the amygdala, anterior olfactory region and orbital frontal cortex (Arentsen et al., 2015; Diaz Heijtz et al., 2011), while another immediate early gene, c-Fos, was decreased in the hippocampus (Gareau et al., 2011). The transcriptional factor- ΔFosB, a mediator of long-term neuronal plasticity, is upregulated in dorsal raphe nucleus (DRN) in GF mice (Campos et al., 2016).

Together, the above evidence shows that the gut microbiome could alter the brain function at the morphological, transcriptional, and functional levels. Microbial regulation of neuronal molecules could modulate the neuronal activity in multiple brain regions, suggesting the mechanisms by which gut microbiota communicate with the central nervous system to control host social behavior.

What do the changes in the GF brain implicate in the control of social behavior?

Recently, the brain circuits controlling social behavior have been extensively investigated. Novel techniques, such as optogenetics and chemogenetics (also known as DREADD- Designer Receptors Exclusively Activated by Designer Drugs) have enabled significant advances toward understanding the machinery of social behavior. Optogenetics and chemogenetics are techniques

in which researchers deliver exogenous genes to the brain via viral infection. Later, these genes can be manipulated by either light (optogenetics) or synthetic chemicals (chemogenetics) that uninfected cells do not respond to (Boyden et al., 2005; Rogan and Roth, 2011). These advanced techniques enable researchers to pinpoint the brain regions that control social behavior. Since the impairment of social investigation and social novelty are drastic phenotypes in GF rodents, it is important to bridge the gap between social deficit and gut microbes at the level of neural circuits (Fig. 2).

As part of the emotion- and memory-controlling limbic system, the amygdala is crucial for the experience of multiple types of emotions, including social-anxiety. Two of its subregions, the BLA and LA, are responsible for regulating social behavior. The BLA and LA of GF mice has been found to show morphological changes and lower BDNF level (Diaz Heijtz et al., 2011; Luczynski et al., 2016). Whether these alterations result in the social deficit is still not clear. Activating the excitatory neurons of BLA through chemogenetic or optogenetic methods induces social anxiety-like behavior, with no social preference of a stranger mouse in the 3-chamber social test (Siuda et al., 2016). In addition, when excitatory neuron projections from the BLA to the ventral hippocampus brain region are inhibited, reduced social interaction is seen in the resident-juvenile-intruder and 3-chamber social tests. Likewise, activation of the same projections increased social interaction (Felix-Ortiz and Tye, 2014). The projection of LA neurons to the medial amygdala (MeA) has been found to be associated with transmission of social information. Inactivation of LA or MeA by DREADD diminished the social information that was delivered by another rat (Twining et al., 2017).

The hippocampus is another region in the mammalian limbic system, and it has been implicated in social memory storage. Several findings point out that the morphology, transcriptional

profiling, neurogenesis, synaptic neurotransmission, stress response and neuropeptide expression are altered in the hippocampus of GF mice (Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Diaz Heijtz et al., 2011; Gareau et al., 2011; Luczynski et al., 2016; Neufeld et al., 2011; Ogbonnaya et al., 2015; Sudo et al., 2004). Optogenetic inhibition of excitatory neurons in specific regions of hippocampus such as the ventral hippocampal CA1 disrupted the ability to discriminate a novel from a familiar mouse. Specific inhibition of the axonal terminals of these neurons in the nucleus accumbens (NAc) also disrupted the social memory storage (Okuyama et al., 2016).

The hypothalamus is responsible for many vital functions, such as the control of hormones and delicate emotional behaviors. Dysregulation of stress-response has been well documented in GF animals, as GF rodents display the increase of corticosterone when they are under stress conditions (Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Neufeld et al., 2011; Sudo et al., 2004). Furthermore, supplementation of the bacterial strain *L. reuteri* increases the oxytocin^{+} neurons in paraventricular nucleus of hypothalamus (PVN) in the MHFD model (Buffington et al., 2016), indicating that gut microbes might affect social behavior through the regulation of hypothalamic neurons. Optogenetically activating oxytocinergic neurons in the PVN facilitates anogenital exploration, a critical olfactory sampling of conspecific social investigation behavior. It also consolidates social memory and enhances social novelty seeking behavior (Oettl et al., 2016). Another study shows an increase of activity in oxytocin neurons in the PVN during social interaction, and optogenetic activation of oxytocin neurons promotes social behavior (Hung et al., 2017). In addition, the axon terminals of the PVN oxytocin neurons are located at the VTA, a region involved in social rewarding (Gunaydin et al., 2014). Manipulation the axon terminals from the PVN at the VTA also altered social behavior. Therefore, PVN oxytocinergic neurons can regulate social behavior through the enhancement of PVN-VTA social rewarding experiences

(Hung et al., 2017), which supports the mechanism by which *L. reuteri* promotes social behavior in MHFD mice through the increase of oxytocin in the PVN and restoration of synaptic plasticity in the VTA (Buffington et al., 2016).

The DRN has been well characterized as a source of neurotransmitters in the brain influencing our behaviors and physiology. The increase of particular transcription factors in the DRN in GF mice implicates an alteration of synaptic activity (Campos et al., 2016). One report suggests that dopaminergic neurons in the DRN are involved in controlling social behavior after social isolation. Optogenetic activation of dopaminergic neurons in the DRN increased social preference behavior in the 3-chamber social test. Moreover, inhibition of dopaminergic neurons in DRN can decrease social preference behavior only in a socially isolated mouse. These data suggest that dopaminergic neurons in DRN promote the social investigation behavior in order to counteract the aversive state of social isolation (Matthews et al., 2016).

Although the advance of technology in the neuroscience field has elucidated the intricate brain circuits controlling social behavior, the detailed wiring and circuits are still under investigation. Based on the understanding of brain circuits of social behavior, unveiling the mechanisms of how the loss or alteration of gut microbes affects social behavior will be an important step for the field to pursue.

CONCLUSIONS

The gut and our bacterial guests have received much attention recently. More and more researchers indicate that the living, co-existing microbes in our gut function far beyond digestion, but surprisingly regulate our immunity, metabolism, development, and even emotion. The microbiota-gut-brain axis theory has been postulated over a decade. However, the knowledge we

have in the intricate connections is still very limited. It is exceptionally challenging to understand whether there is a canonical mechanism underlying the communication among gut microbes, the brain, and social behavior. Social impairment is one of the major symptoms of ASD and has been associated with the dysbiosis of gut microbiota (Fig. 1). Gnotobiotic techniques and antibiotic treatments demonstrate the impact on the brain and host social behavior when gut microbes are completely or partially depleted (Buffington et al., 2016; Desbonnet et al., 2014; Desbonnet et al., 2015; Leclercq et al., 2017). Bacterial taxa that are identified from work with mouse models of ASD, such as *B. fragilis, L. reuteri, L. rhamnosus,* are great candidates to examine how the bacteria directly or indirectly regulate host social behavior (Bharwani et al., 2017; Buffington et al., 2016; Hsiao et al., 2013; Leclercq et al., 2017). The brain regions that are altered in GF rodents, such as prefrontal cortex, amygdala, hypothalamus, and hippocampus are likely directly involved in the circuits of social behavior (Fig. 2). Building on what is known, using the techniques being developed in the field, the mystery of what our microbiota do to our emotions will eventually become clear. Searching for a safe way to alleviate the social symptoms of ASD through manipulation of microbial communities will be the ultimate goal.

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Table 1. Summary of the primary gut microbial alterations across the different mouse models of ASD.

ASD model	Strain	Age	Sex	Vendor	Control	Sample source	Trend	Bacterial taxa	Fold	Reference
BTBR	BTBR T+tf/j	7 weeks	Male	JAX	C57BL/6	Caecal	Increase	Akkermansia muciniphila	919.00	Newell et al., 2016
								Methanobrevibacter spp.	3.85	
							Decrease	Bifidobacterium spp.	16.09	
								Enterobacteriaceae	3.34	
								Clostridium cluster I	1.77	
								Clostridium cluster XI	1.40	
						Fecal	Increase	Akkermansia muciniphila	6960.00	
								Clostridium cluster I	3.21	
								Bacteroides/Prevotella spp.	1.92	
							Decrease	Bifidobacterium spp.	10.95	
								Clostridium cluster XI	2.64	
								Enterobacteriaceae	2.18	
								Methanobrevibacter spp.	1.58	
		14 weeks	Male	JAX	C57BL/6J	Caecal	Increase	Verrucomicrobiaceae (Verrucomicrobiales)	13.77	Golubeva et al., 2017
								Akkermansia	13.77	
								Erysipelotrichaceae (Erysipelotrichales)	6.45	
								Bilophila	3.48	
								Bacteroidaceae (Bacteroidales)	2.91	
								Bacteroides	2.91	
								Coriobacteriaceae (Coriobacteriales)	2.06	
								S24-7 (Bacteroidales)	1.52	
								S24-7 Uncultured bacterium	1.52	
							Decrease	Porphyromonadaceae (Bacteroidales)	16.55	
								Odoribacter	16.19	
								Lachnospiraceae Incertae Sedis	5.26	
								Blautia	3.94	
								Coprococcus	3.63	
								Ruminococcus	2.52	
								Lachnospiraceae (Clostridiales)	2.16	
								Desulfovibrionaceae (Desulfovibrionales)	2.06	
								Family XIII (Clostridiales)	1.95	
								Ruminococcaceae Incertae Sedis	1.91	
								Bifidobacteriaceae (Bifidobacteriales)	Absence	
								Bifidobacterium	Absence	
								Parabacteroides	Absence	
								Rikenella	Absence	
								Acetitomaculum	Absence	
								Desulfovibrio	Absence	
		12 months	Male	JAX	C57BL/6J	Fecal	Increase	*Lactobacillus*	22.70	Coretti et al., 2017
								Coprobacillus	9.00	
								Bacteroides	8.70	
								U. Helicobacteraceae	4.20	
								Parabacteroides	3.70	
							Decrease	*Ruminococcus*	0.60	
								Dehalobacterium	0.40	
								Desulfovibrio	0.10	
			Female	JAX	C57BL/6J	Fecal	Increase	*Coprobacillus*	244.00	
								U. Enterobacteriaceae	109.00	
								U. Desulfovibrionaceae	17.30	
								Bacteroides	10.50	
								Parabacteroides	8.10	
								Sutterella	4.20	
								Akkermansia	3.10	
								Prevotella	2.80	
							Decrease	*AF12* (Rikenellaceae)	0.40	
								Oscillospira	0.40	
								Dehalobacterium	0.30	
								U. F16 (TM7)	0.20	
MIA	C57BL/6	3-6 weeks	Both	CR	C57BL/6	Fecal	Increase	Lachnospiraceae	4.04	Hsiao et al., 2013
								Porphyromonadaceae	N/A	
								Prevotellaceae	N/A	
								Bacteroidales	N/A	
							Decrease	Ruminococcaceae	N/A	
								Erysipelotricheaceae	N/A	
								Alcaligenaceae	N/A	
VPA	BALB/c	4 weeks	Both	CR	BALB/c	Caecal	Increase	*Erysipelotrichales*	Presence	de Thieje et al., 2014
							Decrease	*Desulfovibrionales*	2.00	
								Bacteroidales	1.84	
MHFD	C57BL/6J	7-8 weeks	Male	JAX	C57BL/6J	Fecal	Decrease	*Lactobacillus reuteri*	9.24	Buffington et al, 2016
								Parabacteroides distasonis	5.63	
								Helicobacter hepaticus	2.84	
								Bacteroides uniformis	2.65	
								Olsenella unclassified	1.90	
								Collinsella unclassified	1.75	
								Bifidobacterium pseudolongum	1.71	
								Lactobacillus johnsonii	1.43	

MIA: maternal immune activation; VPA: valproic acid; JAX: The Jackson Laboratory; CR: Charles River Laboratories

Table 2. The effect of antibiotics on rodent social behavior.

Animals				Antibiotics				Behavior		Reference
Species	**Strain**	**Age**	**Sex**	**Chemicals**	**Dosage**	**Route**	**Period**	**Test**	**Outcome**	
Rat	Wister	PD50	Both	SuccinylsulfaThiazole	1%	Diet (ad libitum)	Prenatal; One month before breeding to GD15	Reciprocal social interaction	Decrease	Degroote et al., 2016
Mouse	BALB/c	6 weeks	Both	Penicillin V	31 mg/kg per day	Water (ad libitum)	Perinatal; ED12-14 to PD21	3-chamber social test (Sociability; Social novelty)	Decrease	Leclercq et al., 2017
	Swiss	PD70	Male	Ampicillin;	1 mg/ml	Water (ad libitum)	Postnatal; PD21 to PD80	Social transmission of food preference test	Decreased of the preference to cued food 24 hr after social interaction	Desbonnnet al., 2015
				Vancomycin;	5 mg/ml					
				Neomycin;	10 mg/ml					
				Metronidazol;	10 mg/ml					
				Amphotericin-B	0.1 mg/ml					
	C57BL/6J	4 weeks	Male	Neomycin;	5 mg/ml	Water (ad libitum)	Perinatal; ED9-16	3-chamber social test (Sociability; Social novelty)	No change	Tochitani et al., 2016
				Bacitracin;	5 mg/ml					
				Pimaricin;	1.25 µg/ml					
				Acetic acid	0.075%					
	C57BL/6	~9 weeks	Male	Ampicillin;	1 g/L	Gavage (except ampicillin was given through drinking water)	Postnatal; PD49 to PD63	Social interaction test (test in open-field arena with the stranger mouse in wire cage)	No change	Gacias et al., 2016
				Vancomycin;	50 mg/kg					
				Neomycin;	100 mg/kg					
				Metronidazol;	100 mg/kg					
				Amphotericin-B	1 mg/kg					
	NOD	~9 weeks	Male	Ampicillin;	1 g/L	Gavage (except ampicillin was given through water)	Postnatal; PD49 to PD63	Social interaction test (test in open-field arena with the stranger mouse in wire cage)	No change compared to baseline social behavior, while the vehicle group decreased the social behavior	Gacias et al., 2016
				Vancomycin;	50 mg/kg					
				Neomycin;	100 mg/kg					
				Metronidazol;	100 mg/kg					
				Amphotericin-B	1 mg/kg					
	C57BL/6 Tac (Vehicle)	9 weeks	Male	Vancomycin	2.5 mg/kg	Gavage	Perinatal; 7 days before timed-mating to E18.5	3-chamber social test (Sociability)	No change	Kim et al., 2017
	C57BL/6 Tac (MIA)								Restore the social deficit	
Hamsters	-	> PD60	Both	Enrofloxacin	0.3 µl/g	Oral	Postnatal; >PD60; Treatment 1: Daily for 7 days (Day 1-7); Recovery 1: no treatment for 7 days (Day 8-14); Treatment 2: Daily for 7 days (Day 15-21); Recovery 2: no treatment for 7 days (Day 22-28)	Resident-intruder test	No change in investigation behavior. Male aggressive behavior is decreased in treatment 2. Female aggressive behavior is decreased in all treatment and recovery phase	Sylvia et al., 2016

PD: postnatal days; ED: embryonic days

Table 3. Neuronal molecules that are affected in GF rodents in different brain regions. All results are comparing to SPF control rodents.

Type of molecule	Species	Strain	Sex	Age	Brain subregion	Molecule	Level	Changes	Reference
Neurotransmission-related	Mouse	Swiss	Male	6-9 weeks	Hippocampus	5-HIAA	Metabolite	Increase	Clarke et al., 2013
		Swiss	Male	6-9 weeks	Hippocampus	5-HT	Neurotransmitter	Increase	Clarke et al., 2013
		NMRI	Male	8-10 weeks	Striatum	5-HIAA/5-HT	Ratio	Increase	Diaz Heijtz et al., 2011
		Swiss-Webster	Female	8 weeks	Hippocampus (dorsal DG)	5-HT1A	mRNA	Decrease	Neufeld et al., 2011
		Swiss	Male	6-9 weeks	Plasma	Tryptophan	Amino acid	Increase	Clarke et al., 2013
	Rat	F344	Male	11-13 weeks	Hippocampus	5-HT	Neurotransmitter	Decrease	Crumeyrolle-Arias et al., 2014
	Mouse	NMRI	Male	8-10 weeks	Striatum	DOPAC/DA	Ratio	Increase	Diaz Heijtz et al., 2011
		NMRI	Male	8-10 weeks	Hippocampus (dorsal DG)	Drd1a	mRNA	Increase	Diaz Heijtz et al., 2011
	Rat	F344	Male	11-13 weeks	Frontal cortex	HVA	Metabolite	Decrease	Crumeyrolle-Arias et al., 2014
						HVA/DA	Ratio		
					Hippocampus	HVA	Metabolite		
						HVA/DA	Ratio		
					Striatum	HVA	Metabolite		
						HVA/DA	Ratio		
	Mouse	NMRI	Male	8-10 weeks	Striatum	MHPG/NA	Ratio	Increase	Diaz Heijtz et al., 2011
	Mouse	BALB/C		9 weeks	Cortex	NR1	Protein	Decrease	Sudo et al., 2004
					Cortex	NR2A	Protein	Decrease	
					Hippocampus	NR2A	Protein	Decrease	
		Swiss-Webster	Female	8 weeks	Amygdala (CeA)	NR2B	mRNA	Decrease	Neufeld et al., 2011
Neurotrophic factor	Mouse	BALB/C	Male	9 weeks	Cortex	BDNF	Protein	Decrease	Sudo et al., 2004
		Swiss-Webster	Male	12 weeks	Amygdala	BDNF	mRNA	Decrease	Arentsen et al., 2015
						BDNF (exon I)			
						BDNF (exon IV)			
						BDNF (exon IX)			
						BDNF (exon VI)			
		NMRI	Male	8-10 weeks	Amygdala (BLA)	BDNF	mRNA	Decrease	Diaz Heijtz et al., 2011
		NMRI	Male	8-10 weeks	Hippocampus (dorsal CA1)	BDNF	mRNA	Decrease	Diaz Heijtz et al., 2011
		Swiss-Webster	Female	5-6 weeks	Hippocampus (dorsal CA1)	BDNF	Protein	Decrease	Gareau et al., 2011
		Swiss-Webster	Female	8 weeks	Hippocampus (dorsal DG)	BDNF	mRNA	Increase	Neufeld et al., 2011
		BALB/C	Male	9 weeks	Hippocampus	BDNF	Protein	Decrease	Sudo et al., 2004
		Swiss	Male	6-9 weeks	Hippocampus	BDNF	mRNA	Decrease	Clarke et al., 2013
Stress-related	Mouse	BALB/C	Male	9 weeks	Plasma	ACTH	Hormone	Restraint stress induced increase	Sudo et al., 2004
		BALB/C	Male	9 weeks		Corticosterone	Hormone	Restraint stress induced increase	Sudo et al., 2004
		Swiss	Both	6-9 weeks		Corticosterone	Hormone	Acute novel cage induced increase	Clarke et al., 2013
		Swiss-Webster	Female	8 weeks		Corticosterone	Hormone	Basal level increase	Neufeld et al., 2011
	Rat	F344	Male	11-13 weeks	Hippocampus (CA1)	GR	mRNA	Decrease	Crumeyrolle-Arias et al., 2014
					Hippocampus (DG)	GR	mRNA	Decrease	
					Hypothalamus (PVN)	CRF	mRNA	Increase	
					Serum	Corticosterone	Hormone	Open-field test induced increase	
Synaptic-related	Mouse	NMRI	Male	8-10 weeks	Anterior olfactory region	NGF1-A	mRNA	Decrease	Diaz Heijtz et al., 2011
					Orbital frontal cortex	NGF1-A	mRNA	Decrease	
					Striatum	PSD-95	Protein	Increase	
					Striatum	Synaptophysin	Protein	Increase	
		Swiss-Webster	Male	12 weeks	Amygdala	NGF1-A	mRNA	Decrease	Arentsen et al., 2015
		Swiss-Webster	Female	5-6 weeks	Hippocampus (dorsal)	c-Fos	Protein	Decrease	Gareau et al., 2011
		Swiss	Male	10 weeks	Dorsal raphe	ΔFosB	Protein	Increase	Campos et al., 2016
Tight junction protein	Mouse	C57BL/6J; NMRI	Both	E18.5 days	Fetal brain	Occludin	Protein	Decrease	Braniste et al., 2014
				8-10 weeks	Frontal cortex	Claudin-5			
						Occludin			
					Hippocampus (dorsal)	Claudin-5			
						Occludin			
					Striatum	Claudin-5			
						Occludin			

BLA: basolateral amygdala; DG: dentate gyrus; PVN: paraventricular nucleus of hypothalamus

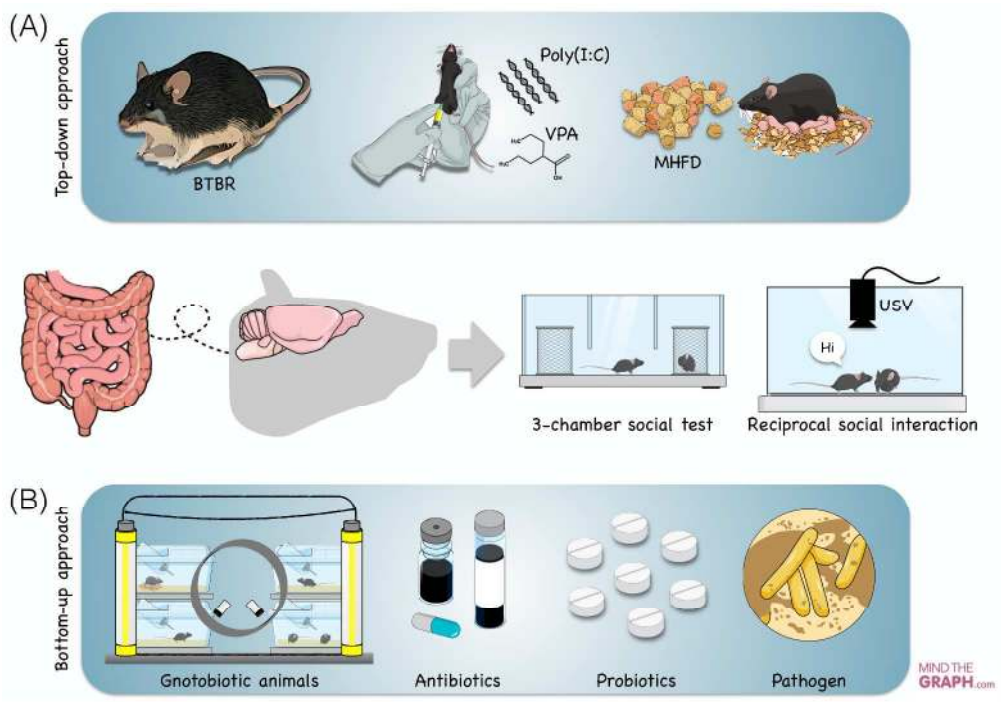

Figure 1. Bi-directional approaches toward understanding the complicated gut microbial contributing factors and gut–brain axis to social behavior in rodent models. (A) Top-down approach: Studying the gastrointestinal (GI) complications in the rodent models of ASD allows researchers to understand the association between the bacterial composition and the behavioral phenotypes. The models include BTBR mice, maternal poly(I:C) injection (termed maternal immune activation (MIA)), maternal valproic acid (VPA) and maternal high-fat diet (MHFD) offspring. (B) Bottom-up approach: Germ-free (GF) rodents, antibiotics, probiotics and pathogens treatment are tools to understand the effect of gut bacteria on social behavior by disrupting the bacterial colonization in the gut. USV: ultrasonic vocalization. A portion of this figure was created using the Mind the Graph platform: www.mindthe graph.com.

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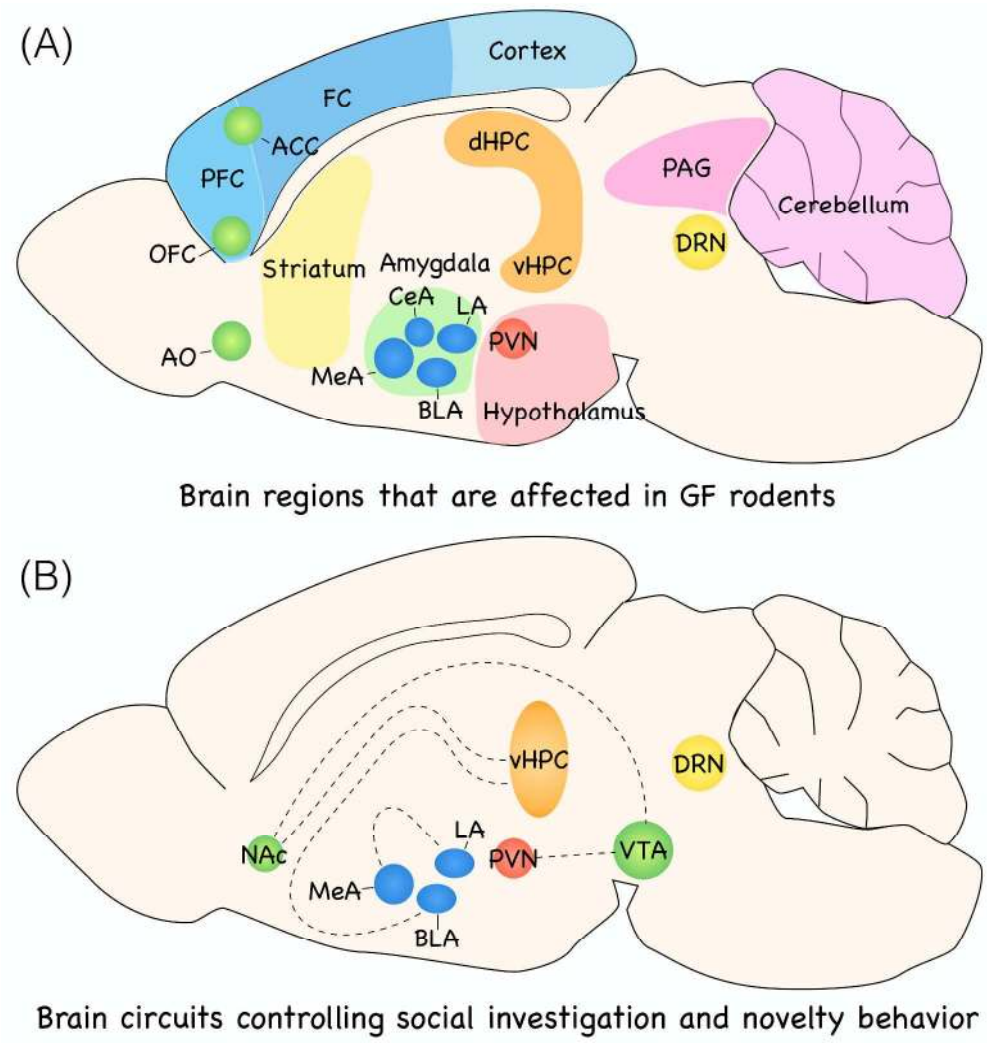

Figure 2. Brain circuits controlling social behavior and affected brain regions when loss of gut microbiota. (A) By comparing GF animals with SPF animals, morphology, transcriptional, and neuronal molecules are altered in several brain regions when loss of gut microbiota. (B) Optogenetics, chemogenetics and fiber photometry demonstrated that several brain subregions and circuits are responsible for social behavior. ACC: anterior cingulate cortex; AO: anterior olfactory region; BLA: basolateral amygdala; dHPC: dorsal hippocampus; DRN: Dorsal raphe nucleus; FC: frontal cortex; LA: lateral amygdala; MeA: medial amygdala; NAc: nucleus accumbens; OFC: orbital frontal cortex; PAG: periaqueductal gray; PVN: paraventricular nucleus of hypothalamus; PFC: prefrontal cortex; vHPC: ventral hippocampus; VTA: ventral tegmental area.

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