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## Gut-immune-brain dysfunction in Autism: Importance of sex

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### Abstract

Autism Spectrum Disorder (ASD) is characterized by social behavior deficits, stereotypies, cognitive rigidity, and in some cases severe intellectual impairment and developmental delay. Although ASD is most widely identified by its neurological deficits, gastrointestinal issues are common in ASD. An intimate and complex relationship exists between the gut, the immune system, and the brain, leading to the hypothesis that ASD may be a systems-level disease affecting the gut and immune systems, in addition to the brain. Despite significant advances in understanding the contribution of the gut and immune systems to the etiology of ASD, there is an intriguing commonality among patients that is not well understood: they are predominantly male. Virtually no attention has been given to the potential role of sex-specific regulation of gut, peripheral, and central immune function in ASD, despite the 4:1 male-to-female bias in this disorder. In this review, we discuss recent revelations regarding the impact of gut-immune-brain relationships on social behavior in rodent models and in ASD patients, placing them in the context of known or putative sex specific mechanisms.

### Keywords

Autism Spectrum Disorder; Gut-immune-brain; Microbiome; Sex differences

## 1. Introduction

An intimate and complex relationship exists between the gut, the immune system, and the brain. From the emerging evidence implicating the microbiome in psychiatric and neurodegenerative disease to the immune system “programming” of brain development, it is clear that the brain not only heavily influences, but is also *heavily influenced by*, the rest of the body. This notion has opened a new line of inquiry to determine how different bodily systems fit together as pieces of the same puzzle, in health and disease.

Autism Spectrum Disorder (ASD) is characterized by social behavior deficits, stereotypies, cognitive rigidity, and in some cases severe intellectual impairment and developmental

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delay. Although ASD is most widely identified by its social behavior deficits, gastrointestinal issues are common in ASD, and alterations of the gut microbiome, known as dysbiosis, has been widely reported in ASD patients (Kohane et al., 2012; Vuong and Hsiao, 2017). Recent reports suggest the intestinal epithelial barrier may also be dysfunctional in ASD patients (Fiorentino et al., 2016), resulting in increased intestinal permeability (de Magistris et al., 2010). Consistent with this notion, higher serum zonulin levels, a protein demonstrated to critically impact intestinal permeability (El Asmar et al., 2002), were reported in ASD patients relative to controls, which was positively correlated with ASD symptom severity (Esnafoglu et al., 2017). Collectively, these data have led to the hypothesis that ASD is a systems-level disease affecting the gut and immune systems, in addition to the brain. Several excellent reviews discussing the role of gut microbial products and their potential role in ASD etiology, symptomology, and treatment exist (Li et al., 2017; Vuong and Hsiao, 2017). However, virtually no attention has been given to the potential role of sex-specific regulation of the gut and its interactions with peripheral and central immune function in ASD, despite the 4:1 male-to-female bias in this disorder, an understanding of which is needed to inform future inquiry into the pathogenesis of ASD. In this review, we briefly discuss recent revelations regarding the impact of gut-immune-brain relationships on social behavior in rodent models, and in ASD patients, placing them in the context of known or putative sex specific mechanisms.

## 2. Diverse etiology for social behavior deficits: Evidence for a dysfunctional gut-immune-brain relationship

Several rodent models exist for the etiological characterization of social behavior abnormalities. Interestingly, germ free mice, which lack a gut microbiome, have impaired sociability (Buffington et al., 2016; Desbonnet et al., 2014). Normal social behavior in germ free mice can be restored via fecal microbiota transfer from sex-matched mice (with normal bacterial colonization) if the transfer is completed within a specific time frame: microbiota transfer at 4 weeks of age, but not 8 weeks, restored social behavior to normal levels (Buffington et al., 2016). These data suggest that the microbiome's influence on social behavior, or at least peer-induced sociability and social novelty seeking, has a critical period of development and plasticity between 4 and 8 weeks in mice. This notion will be important to explore in humans, as they suggest that microbiome transfer as a therapy may be less effective for individuals with social behavior deficits, like ASD patients, after a certain age. Importantly, *how* deficits in social behaviors are produced may dictate the efficacy of microbiome manipulations. In a model of maternal high-fat diet, assessment of male offspring revealed impaired social behavior and synaptic plasticity in the ventral tegmental area of the dopaminergic reward circuitry, and decreased oxytocin-expressing neurons in the hypothalamus; disrupted neural signaling and social behavior deficits (but not other behavioral abnormalities) were restored by probiotic treatment with *L. reuteri* (Buffington et al., 2016).

Germ free mice and the assessment of offspring from mothers on high fat diet yield important insights into how the gut affects social behavior, but they, like all models, have their drawbacks. The former model is not biologically plausible and the latter model is not

widely considered a model for ASD-like behaviors, though maternal obesity is correlated with increased ASD incidence (Modabbernia et al., 2017; Sanchez et al., 2017). One of the most prominent rodent models for studying ASD-like phenotypes is induced by delivering an immune challenge to a pregnant dam, known as maternal immune activation (MIA; Bilbo et al., 2017). Recently, the maternal immune response that results in ASD-like behaviors in offspring has been dissected. Prenatal poly(I:C) injection, which mimics a viral infection, results in maternal immune cells, specifically T helper 17 (T<sub>H</sub>17) cells, to increase production of IL-17a in the placenta via an IL-6-dependent mechanism, resulting in abnormal cortical development and social behavior deficits in mixed sex offspring (Choi et al., 2016; Shin Yim et al., 2017). Moreover, injection of IL-17a itself into the fetal forebrain, with no MIA procedure, resulted in similar cortical and behavioral abnormalities (Choi et al., 2016). Interestingly, the microbiome has a critical role in T cell maturation and response (Lathrop et al., 2011; Mazmanian et al., 2005), and pregnant mice colonized with gut bacteria that induce T<sub>H</sub>17 cells are more likely to produce offspring with ASD-like behaviors (Kim et al., 2017). In a separate report, prenatal poly(I:C) treatment in mothers induced increased intestinal permeability, abnormal intestinal tight junction protein and cytokine levels, and an altered serum metabolite profile, paralleled by gut dysbiosis, in the off-spring (Hsiao et al., 2013). Incredibly, all gut and immune symptoms in offspring from this model could be ameliorated corrected by postnatal treatment (at weaning) with the human gut bacteria *B. fragilis*. However, while probiotic manipulation in afflicted off-spring improved intestinal integrity, microbiome dysbiosis, repetitive behaviors, and ultrasonic vocalization abnormalities, it did not improve social behavior (Hsiao et al., 2013). These data suggest the role of the gut and microbiome in neurodevelopment and behavior is a combination of maternal and offspring influences. In humans, fecal microbiota transfers have been piloted in small groups of mixed-sex ASD patients. After antibiotic treatment, bowel cleanse, and 7–8 week treatment with a standardized human gut bacteria extracted from stool, improvements in GI and ASD symptoms as well as increases in microbial diversity persisted 8 weeks after treatment (Kang et al., 2017). Taken together, these data are a promising step forward for the potential of microbiome transfers as a therapeutic course.

Despite significant advances in understanding the contribution of the gut and immune systems to the etiology of ASD, there is an intriguing commonality among patients that is not well understood: they are predominantly male. ASD presents with a 4:1 male bias, and there is also considerable evidence for a female “protective” factor (Jacquemont et al., 2014; Robinson et al., 2013). For example, deletion of *shank1* in males results in an ASD phenotype, while in female family members with the same deletion, there is no ASD symptomology (Sato et al., 2012), raising the possibility that biological sex alters the body’s *interpretation of and response to* identical stimuli.

In the following section, we draw parallel themes from the ASD literature with exploration of sex-specific regulation of gut-immune-brain relationships. Though the literature remains sparse, this discussion can serve as a platform on which to evaluate how gut-immune-brain systems work, or fail to work, together in ASD.

### 3. Sex differences in the relationship between the gut, immune system, and brain

The health and development of the immune system is tightly coupled with that of the gut and its commensal bacteria, the microbiome. Immune cells have receptors that respond to “danger” signals, such as bacterial cell wall motifs. For the immune system and natural gut bacterial ecosystem to co-exist without extraneous inflammatory processes, they must form a homeostatic relationship. Dysregulation of the immune response can cause many problems, including autoimmune disorders, which present with a strong female bias (Jacobson et al., 1997). Indeed, the female immune system in general mounts a more robust inflammatory response, and this is at least in part due to sex hormones (Klein and Flanagan, 2016). The autoimmune disorder type 1 diabetes (T1D) occurs when pancreatic  $\beta$  cells that secrete insulin are destroyed by autoantibody-mediated immune attack. Surprisingly, Markle and colleagues demonstrated that the microbiome regulates testosterone production and thus confers male protection to T1D in the non-obese diabetes (NOD) mouse model Markle et al., 2013. Germ-free NOD mice do not have a sex-bias in T1D incidence, and colonization of young female NOD mice with adult male NOD cecal contents increased testosterone levels, decreased autoantibody levels, and protected from T1D (Markle et al., 2013). Moreover, sex hormone manipulation (neonatal masculinization or ovariectomy later in life) in female rats altered the gut microbiome even more than nutritional interventions, resulting in overall lower bacterial diversity (Moreno-Indias et al., 2016). Interestingly, in a study of oesophageal squamous cell carcinoma, Dong and colleagues discovered that androgen receptors directly bind to the IL-6 promoter and enhance its transcription, and IL-6 can increase androgen receptor expression (Dong et al., 2017), suggesting that there may be feedback loops between male sex hormones and IL-6, which could drive the IL-17a response reviewed above. Collectively, these data suggest that bidirectional sex hormone-microbiome communication is critical in sexually dimorphic outcomes and sex-specific vulnerability.

Paradoxically, early in life, the male immune response is more robust than in females, and male infants are more likely to succumb to infection than females (Klein and Flanagan, 2016). In fact, it has been postulated that this age- and sex-specific immune sensitivity may account for the male prevalence in early-onset neurodevelopmental disorders and the female prevalence in later-life neuropsychiatric disorders (Schwarz and Bilbo, 2012). In support of this notion, there is a large body of literature indicating early life infection in males can lastingly “prime” microglia, the resident immune cells of the brain, such that a later-life immune challenge results in an exacerbated pro-inflammatory response from microglia and impaired learning and memory (Bilbo et al., 2005; Williamson et al., 2011), an effect that is absent in females (Bilbo et al., 2012). Interestingly, gene expression analysis of neurotypical cortical tissue indicates that ASD risk genes are not sex-specifically expressed, but rather are transcribed at higher levels in males than in females; of note, these genes were highly enriched in glial cells, including microglia (Werling et al., 2016).

Microglia not only exhibit sex differences in their development and activity (reviewed in Lenz and McCarthy, 2015), but also are critical for the masculinization of the brain. Around the time of birth, there is a surge of male sex hormones (androgens), which serve to

masculinize the developing organism, including the brain (McCarthy, 2010). Surprisingly, this is accomplished not directly through androgen receptor signaling, but rather indirectly through the classic immune pathway, cyclooxygenase 1/2 → prostaglandin E2 (COX1/2 → PGE2; Amateau and McCarthy, 2004). As such, male microglia respond by amplifying PGE2 activity, ultimately resulting in sex-specific neural architecture and behavior (Lenz et al., 2013). While the microbiome is established at birth and unstable early in life (Jasarevic et al., 2016), how microbiome-mediated androgen production affects this process in development has not yet been explored.

In the mouse hippocampus, a brain region that does not have sex-specific neural circuitry, a recent report indicates that male and female microglia follow a similar developmental trajectory very early in life, until around weaning, when female microglia continued to mature (assessed in young adulthood) and male microglia plateau (Hanamsagar et al., 2017). Peripheral immune activation was sufficient to increase the developmental profile of male microglia, while the female microglia remained unchanged (Hanamsagar et al., 2017), suggesting that sex-specific developmental trajectories may serve to make male and female microglia differentially responsive to immune challenge, which in turn alters their developmental path. Interestingly, microglial maturity and development of their immune response is regulated by the microbiome. Adult mixed-sex groups of germ free mice have perpetually “immature” microglia characterized by increased proliferation and decreased immune reactivity (Erny et al., 2015). Microbiome regulation of microglia appears to be a constitutive process, as antibiotic depletion of the microbiome in normally raised mice results in the same immature profile, and can be reversed by re-colonization with a diverse (but not limited) bacterial population or by treatment with short-chain fatty acids, metabolites derived from commensal bacteria (Erny et al., 2015).

Taken together, interactions between the microbiome, immune system, and sex hormones (coined the “microgenderome;” Flak et al., 2013) may collectively regulate sexually-dimorphic peripheral and central immune responses and thus sex-specific vulnerability or resilience. Importantly, sex-biases in disease, be it ASD or other disorders, likely means that sex-specific treatments and therapies will be required. Thus, we argue that examining the mechanisms underlying ASD requires a cross-disciplinary approach with mucosal biologists, microbiologists, geneticists, neuroscientists, and immunologists working in parallel to develop and test novel system-wide hypotheses, in males and females.

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