Altered brain-gut axis in autism: Comorbidity or causative mechanisms?

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The concept that alterated communications between the gut microbiome and the brain may play an important role in human brain disorders has recently received considerable attention. This is the result of provocative preclinical and some clinical evidence supporting early hypotheses about such communication in health and disease. Gastrointestinal symptoms are a common comorbidity in patients with autism spectrum disorders (ASD), even though the underlying mechanisms are largely unknown. In addition, alteration in the composition and metabolic products of the gut microbiome has long been implicated as a possible causative mechanism contributing to ASD pathophysiology, and this hypothesis has been supported by several recently published evidence from rodent models of autism induced by prenatal insults to the mother. Recent evidence in one such model involving maternal infection, that is characterized by alterations in behavior, gut physiology, microbial composition, and related metabolite profile, suggests a possible benefit of probiotic treatment on several of the observed abnormal behaviors.

Keywords:
brain gut interactions; gut microbiome; intestinal permeability; neurodevelopment disorder

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Abbreviations:
ASD, autism spectrum disorder; **4EPS,** 4-ethylphenylsulfate; **GF/CF,** gluten-free/casein-free; **GI,** gastrointestinal; **MIA,** maternal immune activation; **OUT,** operational taxonomic units; **VPA,** valproic acid.

Introduction

The designation "autism spectrum disorder" (ASD) refers to a group of heterogeneous neurodevelopmental disorders with multiple causes and courses, a significant range in severity of symptoms, and several associated co-morbid disorders, including anxiety and gastrointestinal (GI) symptoms. The presence of GI symptoms has led to many speculations about a possible etiological role of alterations in gut-brain interactions, starting with the infamous and later retracted Lancet report by Wakefield et al. [1], and resulting in several (failed) treatment strategies. More recently, the interest in brain gut interactions in ASD has led to an appreciation that altered interactions between the gut microbiome and the brain (Fig. 1) might not only play a role in ASD pathophysiology, but are likely to significantly contribute to the disease burden *in general* in affected children. This renewed interest in a role of brain gut microbiome interactions in ASD and several other brain disorders has been driven by converging evidence from gut microbiology [2], microbial endocrinology [3], behavioral studies [4–6], and human brain imaging studies [7] all supporting important bidirectional interactions between the brain and the gut microbiome.

ASD is defined by several core symptoms, including persistent deficits in social communication and interaction, and restricted repetitive patterns of behaviors and interests. Symptoms must be present in the early developmental period and must cause significant impairment in social and occupational functioning [8]. There has been a dramatic increase in the reported prevalence of ASD, from 4.5 in 10,000 children in 1966 [9, 10] to 1 in 110 in 2006 (http://www.cdc.gov/mmwr/preview/mmwrhtml/ss5810a1.htm) to 1 in 68 children aged 8 years in 2010 (http://www.cdc.gov/ncbddd/autism/data.html). Even though a greater awareness and changes in the definition of the disorder are likely responsible for part of this dramatic increase, various environmental factors have also been implicated, including toxic exposures, perinatal insults, and prenatal infections [11]. The increase in prevalence is surprising, because ASD is among the most heritable disorders evidenced by family and twin studies, displaying a concordance rate of 70–90% for monozygotic twins compared to 0–10% for dizygotic twins [12]. The fact that

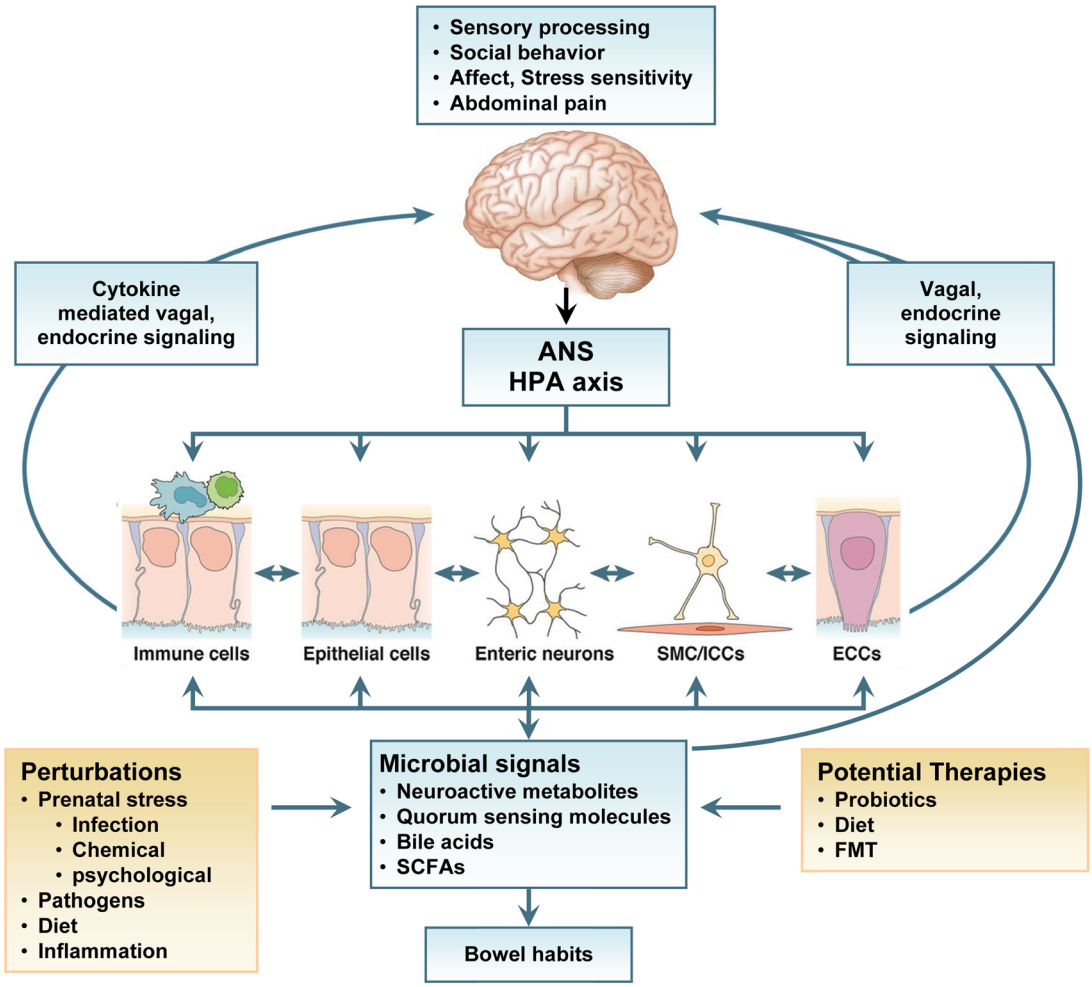

Figure 1. Brain gut microbiome interactions with possible relevance to ASD. The brain modulates a network formed by intestinal target cells and the gut microbiome via the autonomic nervous system (ANS) and the hypothalamic pituitary adrenal (HPA) axis. Signaling molecules generated within this network by enteroendocrine cells and mucosal immune cells, as well as by the gut microbiome feedback to the brain via endocrine and neurocrine signaling pathways. Perturbations of the gut microbiome by prenatal, postnatal, and adult influences can change the pattern of these microbial signals, while potential ASD therapies can normalize such alterations. Altered microbiome related signaling to the brain may play a role in brain development or result in neuroplastic and functional alterations in the adult. SCFAs, short chain fatty acids; FMT, fecal microbial transplant. Reproduced with modifications from Mayer et al. [38] with permission.

dizygotic twins showed no concordance for narrowly defined autism suggests that environmental factors (including those that can affect the gut microbiome, including mother to infant transmission and transmission amongst household members) and epigenetic factors largely determine the *degree* of expression of autism related traits, though not of the core symptoms per se.

GI disorders in ASD – Evidence for altered brain gut interactions

There has been a growing awareness of GI symptoms as an important comorbidity in ASD patients [13]. Even though the underlying mechanism(s) are not known, there is a realization that when untreated, these GI symptoms can give rise to behavioral difficulties, ranging from inattentive or irritable behaviors to self-injury. GI morbidity often remains undiagnosed, and hence untreated, because of the communicative impairments of affected patients.

Increased prevalence of GI symptoms in ASD patients

Based on clinical and parents' observations, and a series of small, generally poorly controlled studies, it had long been known that ASD is associated with a range of chronic GI symptoms. These include alterations in bowel habits, chronic abdominal pain and discomfort and food intolerances [13]. The reported prevalence of GI symptoms in ASD varies widely depending on the type and size of patient population studied, and depending on the control group [14–17]. In one of the most comprehensive and best controlled studies performed in 589

subjects with familial ASD and their unaffected sibling controls, GI problems (based on parents' reports) were identified in 42% of children and 12% of controls, constipation (20%) and chronic diarrhea (19%) being the most common symptoms. Prevalence was highest in patients with "Full" Autism as compared to "Spectrum" [18]. Increased autism symptom severity was associated with increased odds of having GI problems. In contrast, in a study in patients with high functioning autism using a different symptom assessment strategy, no correlation between autism symptom severity and GI symptoms was observed, despite a similar high prevalence of such symptoms (61%). However, patients with GI symptoms (primarily abdominal pain [41%] and early satiety [40%]) had significantly higher levels of affective problems [19].

Are GI symptoms the consequence of altered brain to gut signaling in ASD?

The study of GI comorbidity in ASD has been complicated by the fact that symptom reports of altered bowel habits (notoriously unreliable even when directly obtained from patients without impairment in communication) are generally obtained indirectly through the parents, and may not reflect underlying biological mechanisms. Based on the observation of increased prevalence of anxiety in more than 40% of ASD children [20], a model has been proposed that links anxiety with the highly prevalent trait of sensory over-responsitivity [21, 22]. This model is analogous to models proposed for visceral hypersensitivity in functional GI disorders [23] (see also Fig. 1). In both ASD and functional GI disorders, the behavioral abnormality is accompanied by brain imaging abnormalities in sensory and emotion regulation regions [24, 25]. Even though various alternative mechanisms may play a role in the pathophysiology of GI symptoms in ASD or in subsets of ASD, one may speculate that some of these symptoms are the result of centrally driven sensory augmentation, including visceral hypersensitivity. Up-regulation of brain networks concerned with sensory processing, emotion regulation, and stress responsiveness may play an important role in this central sensory amplification. For example common ASD symptoms such as early satiety, bloating, and feeling constipated may all reflect this perceptual abnormality, rather than a primary dysfunction of the GI tract. According to such a brain-centric model of brain gut alterations, altered autonomic nervous system activity associated with anxiety and enhanced stress responsiveness may also play a role in the observation of increased epithelial permeability in ASD patients (as evidenced by lactulose/mannitol permeability assays) as well as in animal models of ASD [26].

Are GI symptoms the consequence of altered gut regulation by the enteric nervous system?

However, it has to be emphasized that a range of alternative mechanisms could underlie GI symptoms in ASD or in special subsets of ASD, including genetic alterations in neural or endocrine elements of the enteric nervous system [27]. Such alterations have been reported both in ASD patients and several rodent models in the form of mutations in the gene for the serotonin transporter (SERT) [28], or of genes related to postsynaptic adhesion molecules affecting excitatory and inhibitory neurotransmission such as Neuroligin-3 and Shank-3 [29]. In summary, based on currently available evidence, it is likely that the GI symptoms in ASD patients are caused by multiple factors. These include central sensory augmentation, altered modulation of GI function (motility, secretion, and epithelial permeability) by the autonomic nervous system, altered regulation of motility and secretion by the enteric nervous system, or a combination of these factors.

Does the gut microbiome play a role in ASD pathophysiology and GI symptoms?

It is increasingly assumed that changes in the human gut microbiome related to the "Western" diet, medication use and overall hygiene may be partly responsible for the hitherto unexplained rise in the developed world of various chronic diseases over the last 50 years, including autoimmune and brain diseases. Hence, it has been speculated that such microbiome-related factors may also be responsible for the increase in ASD prevalence [5, 30]. Many patients with ASD experience benefit on various dietary regimen, even though controlled evidence for the effectiveness of such popular diets as the gluten-free/casein-free diet (GF/CF) is not available. It has been suggested that such diets may exert their beneficial effects by modulating the gut microbiome in addition to having other effects on the brain [31]. Several studies have provided evidence for alterations in gut microbiome composition [32, 33], including a greater representation of members of the family of Clostridiales, and with greater abundance of the phyla of Bacterioidetes over Firmicutes in more severe ASD [34] (Table 1). Another study revealed an increased Firmicutes to Bacterioides ratio when comparing an ASD group with GI symptoms and a non-ASD control group with similar GI symptoms [35]. Some studies have also provided evidence for abnormal levels of mammalian-microbial cometabolites, including dimethylamine, hippurate, and phenylacetyl glutamine [36, 37]. Besides the small sample size and limitations of sequencing of bacterial 16sRNA, it remains to be determined if the observed increase in Bacteroidetes-Firmicutes ratio in more severe ASD patients is a consequence of stress- and anxiety-induced altered autonomic control of the microbial environment, analogous to observations in non-ASD patients with irritable bowel syndrome [38]. In addition, none of the published studies include a control for the unique individual dietary habits of affected patients, which are likely to have a major effect on microbiome parameters. Several pieces of evidence emphasize the potential importance of dietary factors on autism symptoms and associated GI functional alterations. Two small randomized controlled trials of a gluten-free and/or casein-free (GF/CF) diet showed clinical improvement in some patients [39, 40], and subjects on the GF/CF diet had lower intestinal permeability. However, neither probiotics nor

Table 1. Reported gut microbiome alterations in ASD.

Author	Study groups	Microbiome analysis method	Key findings	Limitations
Parracho et al. [32]	ASD ($n=58$) vs. ASD siblings ($n=12$) vs. healthy control ($n=10$)	FISH analysis of stool bacteria	Increased *Clostridia* species in ASD group	Patient heterogeneity in terms of age, sex, bowel habits, dietary habits, group differences in terms of diet, probiotic/prebiotic intake, antibiotic exposure
Song et al. [33]	ASD ($n=15$) vs. control ($n=18$)	16S rRNA PCR strategy	Increased *Clostridia* species in ASD group	Lack of clinical data
Finegold et al. [34]	ASD ($n=33$) vs. ASD siblings ($n=7$) vs. control ($n=8$)	Bacterial tag encoded FLX amplicon pyrosequencing	Increased Bacteroides in ASD group. Decreased Firmicutes	Mixed male/female sample, group differences in terms of male:female ratio, special diets, antifungal agents; no classification according to presence of GI symptoms
Wang et al. [48]	ASD ($n=23$) vs. ASD siblings ($n=22$) vs. control ($n=9$)	Quantitative real-time PCR of bacterial stool	Increased *Sutterella* species in ASD group	Lack of clinical data
De Angelis et al. [49]	ASD ($n=10$) vs. control ($n=10$)	Bacterial tag encoded FLX amplicon pyrosequencing	Lower Firmicutes in ASD group. Increased Bacteroides in ASD group	Small sample size, exclusion of ASD subjects with GI symptoms, mixed male/female sample
Kang et al. [50]	ASD ($n=20$) vs. control ($n=20$)	Pyrosequencing of 16SrDNA	Less diverse gut microbiome in ASD group	Heterogeneous sample in terms of age, sex, GI symptoms, diet, and supplements
Gondalia et al. [51]	ASD with GI sx ($n=28$) vs. ASD w/o GI sx ($n=23$) vs. sibling control ($n=53$)	Bacterial tag encoded FLX amplicon pyrosequencing	No clinically meaningful differences between groups	Heterogeneous sample in terms of age, sex; group difference in probiotic use; no formal dietary assessment
Williams et al. [35]	ASD with GI sx ($n=15$) vs. control with GI sx ($n=7$)	16SrRNA pyrosequencing of cecal and ileal biopsies	Lower levels of Bacteroides in ASD group. Increased ratio of Firmicutes to Bacteroides and Clostridia to Bacteroides	Small sample size

special diets have had a consistent effect on ASD core symptoms or associated GI comorbidity.

New evidence from rodent models of ASD

In a recent publication, Hsiao et al. used a rodent model of ASD to test the hypothesis that alterations in the gut microbiome and associated changes in serum metabolites, play an important role in behavioral manifestation of autism-like behaviors and GI function, and that these changes were rapidly reversible by ingestion of a probiotic [41]. Several rodent models with potential relevance for ASD pathophysiology have been reported [27]. These models fall into three general categories: (i) Naturally occurring rodent strains that demonstrate ASD-relevant behavioral traits (including Balb/c mice); (ii) models expressing a human genetic mutation associated with ASD (including NLG3 and Shank3 mutations); and (iii) models with acquired behaviors resulting from various environmental insults either affecting the developing animal directly [31, 42] or affecting the mother of ASD offspring. In one such prenatally induced model, pregnant mice were treated with valproic acid (VPA) resulting in ASD-like behavior in the offspring, which was associated with inflammatory and endocrine changes in both intestinal tract and in the nervous system. Interestingly, the changes were primarily seen in male offspring [43]. The maternal immune activation (MIA) model used by Hsiao et al. [41] also falls into the latter category, and has previously been used as a rodent model for other neurodevelopmental disorders. These include schizophrenia in the rat and the rhesus monkey [44]. Hsiao et al. used a mouse model of MIA to evaluate the effect of the gut microbiome on autism-like behaviors. Pregnant mice were injected with a viral mimetic, polyinosinic:polycytidylic acid (poly(I:C)) to produce offspring with stereotypic ASD-like behaviors. Analysis of the MIA model offspring revealed

changes in gene expression of intestinal barrier integrity genes and functional defects in intestinal permeability, as well as robust differences in the membership of gut bacteria between MIA offspring and controls. Changes in the diversity of the classes Clostridia and Bacterioidia operational taxonomic units (OTUs) were the primary drivers of these microbiota differences. The authors were able to show that a limited set of 67 bacterial OTUs was able to discriminate experimental samples from controls, and some of the identified organisms mirrored those reported in the feces of subjects with ASD [45]. By introducing the probiotic *Bacteroides fragilis*, the MIA model offspring displayed significant restoration in the relative abundance of six out of the 67 OTUs found to discriminate MIA from control offspring. Additionally, introduction of *B. fragilis* was shown to dramatically attenuate the stereotypic behaviors seen in the offspring. Behavioral mouse experiments evaluating anxiety, communication, and repetitive behaviors all showed improvement or normalization in MIA offspring treated with the commensal organism. The authors had previously characterized the anti inflammatory effects of *B. fragilis* on experimental models of colitis which is mediated by the purified capsular polysaccharide A (PSA) on intestinal Foxp3 + CD4 regulatory T cells (Tregs) and IL-10 production. In the current study, PSA apparently was not involved in the mediation of the probiotic effect on ASD-related behavioral or biological alterations.

Will probiotics be capable of treating ASD symptoms and pathophysiology?

Given that the experimental model displayed increased gut permeability, tight junction defects, and alterations in the gut microbiome composition, Hsiao et al. hypothesized that the gut bacteria may affect the serum metabolites in mice. Indeed, metabolomic studies have shown that gut microbial products may influence metabolic, immunologic, and behavioral phenotypes in mice and humans. It is important to emphasize that prebiotic fibers contained in the diet can have pronounced effects on both microbiome composition as well as on the gut immune system. Using mass spectrometry profiling, the group was able to identify MIA-associated changes in serum metabolites. MIA offspring had statistically significant alterations in 8% of all serum metabolites detected. *B. fragilis* treatment in the experimental mice had a significant effect on the serum metabolome, altering a significant portion of all metabolites detected. The group focused on serum metabolites that were significantly altered by MIA treatment and restored to control levels by *B. fragilis* treatment. One metabolite of particular interest was 4-ethylphenylsulfate (4EPS). 4EPS is thought to be a uremic toxin, as is *p*-cresol (4-methylphenol), a chemically related metabolite reported to be a possible urinary biomarker for autism [46]. This study showed a 46-fold increase in 4EPS in the MIA model and normalization upon *B. fragilis* treatment. By using germ-free mice as controls, it was suggested that the gut bacteria may exclusively generate 4EPS since germ-free mice have undetectable serum concentrations of 4EPS. Mice were treated with 4EPS potassium salt or vehicle, daily from three weeks of age to six weeks of age. Surprisingly, systemic administration of the single metabolite, 4EPS, to naive wild-type mice was sufficient to induce anxiety- (but not autism-) like behavior similar to the MIA offspring.

Caveats of extending the findings from rodents to ASD patients

The findings of this elegant and provocative study are significant for several reasons. They demonstrate: (i) The mother-to-offspring transmission of factors associated with a maternal infection that can induce a specific change in the gut microbiome and neuroactive metabolites in the offspring. These metabolite changes in turn appear to play a role in the development of autism-like behavior and gut epithelial changes. Even though observed in a different maternal insult model, these findings are consistent with the VPA model [43]. (ii) The prompt reversibility of many of these changes by the administration of a commensal organism, which previously had been shown to have systemic anti-inflammatory effects via stimulation of IL-10 from a subset of T lymphocytes, a mechanism not involved in the behavioral effects. (iii) The identification of a microbiome-related metabolite that was able to induce behavioral effects in control animals. Despite the fact that the study highlights an important mechanistic link in the brain-gut axis and opens new avenues of investigation in understanding the pathophysiology of psychiatric disorders, many questions remain, and caution is in order before extrapolating the findings to human ASD patients. Some ASD-relevant behavioral traits (deficits in sociability and social preference) were not reversed by *B. fragilis*, and the administration of the 4EPS did not reproduce the full spectrum of ASD-like behaviors, but promoted anxiety-like behavior. Finally, the study did not explore associated brain alterations but focused exclusively on behavioral changes. The MIA model is not specific for ASD, and rodent models of complex human traits have often been shown to have little or no predictive validity for the human disease. ASD is a heterogeneous group of disorders [17] and the identified mechanisms may only play a role in a subset of patients. For example not all patients show increased intestinal permeability, and documented maternal infections in ASD children are rare. Unless clinical trials use patient populations that are enriched based on microbiome characterization or clinical history, no significant clinical benefits can be demonstrated. Finally, mechanisms have yet to be determined: how does MIA result in alterations of the infant microbiome? How does the commensal *B. fragilis* promptly normalize many of the ASD-like abnormalities?

Conclusions

Ever since the publication of the first preclinical studies demonstrating a relationship between the gut microbiota, the brain and behavior [4], scientific, lay public, and patient interest in brain gut microbiome interactions in health and disease has dramatically increased. The recent demonstration

in healthy human subjects that modulation of the gut microbiome in healthy individuals can alter brain responsiveness to an emotion recognition task has further stimulated this interest [7]. In this context, the findings by Hsiao et al. not only provide an important piece of evidence supporting the long-held hypothesis that gut microbiome alterations may play a role in ASD pathophysiology, but they open the possibility for novel treatment approaches. The findings do not answer the question of the etiology of GI comorbidity, even though gut microbiome to brain signaling during brain development may play a role in the alterations in sensory and affect regulation networks. Gut microbial influences on the engagement of such networks has recently been demonstrated [7]. ASD is a neurodevelopmental disorder that likely starts to unfold in utero, and is characterized by neuroplastic remodeling of the brain. If gut microbiome-directed therapies are to prevent, reverse or attenuate such neuroplastic changes, they may have to be implemented in the perinatal period in high risk families, and may be ineffective once the full clinical phenotype has developed. Rigorous clinical trial design would be required to avoid the false hopes generated by previous gut-directed therapies, including those with secretin and broad-spectrum antibiotics. On the other hand, the findings by Hsiao et al. [41] provide hope that novel gut microbiome-directed therapies, including those based on fecal microbial transplantation, may one day translate into the long sought after clinical improvements in a clearly defined subsets of patients [47].

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