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PRINCIPAL INVESTIGATOR:

Paul H. Patterson

CONTRACTING ORGANIZATION:

California Institute of Technology, Pasadena, CA 91125

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14. ABSTRACT Although autism spectrum disorder (ASD) is defined by core behavioral impairments, gastrointestinal (GI) symptoms are commonly reported. Subsets of ASD individuals display dysbiosis of the gut microbiome, and some exhibit increased intestinal permeability. We demonstrate GI barrier defects in a mouse model of an important ASD risk factor, maternal immune activation (MIA). Remarkably, oral treatment of MIA offspring with the human commensal <i>Bacteroides fragilis</i> corrects gut permeability and ameliorates defects in communicative, stereotypic, anxiety-like and sensorimotor behaviors. MIA offspring also display an altered serum metabolomic profile, and <i>B. fragilis</i> normalizes levels of several of the serum metabolites. These findings suggest a gut-microbiome-brain connection in autism, and identify a potential probiotic therapy for ASD.					
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Introduction

Autism is a neurodevelopmental disorder characterized by stereotypic behavior and deficits in language and social interaction. The incidence of autism has rapidly increased to 1 in 88 births in the United States (1), representing a significant medical and social burden in the coming decades. However, therapies for treating the core symptoms of autism are limited, and reproducible molecular diagnostics have not been developed. Much research into autism spectrum disorder (ASD) has focused on genetic, behavioral and neurological aspects of the illness, but primary roles for environmental risk factors (2), immune dysregulation (3) and additional peripheral disruptions (4) in the pathogenesis of ASD have recently gained significant attention. Of several potential contributions to ASD, gastrointestinal (GI) distress is of particular interest, given its prevalence and correlation with the severity of core autism behavioral abnormalities (5, 6). A significant subset of ASD children display GI abnormalities, including abdominal cramps, chronic diarrhea or constipation and increased intestinal permeability (7, 8). Moreover, antibiotic treatment and restricted diet are reported to provide behavioral improvements for some autistic children (5). The causes of these GI problems are unclear, but may be linked to gut bacteria, as the intestinal microbiome is altered in ASD individuals (9, 10).

Humans are colonized with a diverse gut microbiota, which plays a critical role in obesity and inflammatory bowel disease (IBD) (11). In animal models, the microbiota not only modulates the development and function of the enteric immune system, but also impacts neuroinflammation (12, 13). In particular, the human commensal *Bacteroides fragilis* exhibits therapeutic properties in mouse models of both colitis and multiple sclerosis (MS) (13, 14). Recent studies have revealed that commensal bacteria also affect a variety of behaviors, including emotional, nociceptive and anxiety-like behaviors, and may contribute to brain development in mice (15-17). Based on an emerging appreciation of a gut-brain connection (18, 19), we are exploring whether commensal bacteria, and *B. fragilis* in particular, affects behaviors relevant to ASD.

Maternal immune activation (MIA) is an important environmental risk factor for ASD. Several large epidemiological studies link maternal viral and bacterial infection with increased autism risk in the offspring (20, 21). We have shown that modeling this risk factor in mice by injecting pregnant dams with the viral mimic poly(I:C) yields offspring that exhibit the core behavioral symptoms of autism, as well as a common autism neuropathology (22, 23). MIA offspring also display altered peripheral immune responses (24), which aligns well with recent studies highlighting a role for immune dysregulation in ASD (3). Although several environmental and genetic risk factors have been investigated in mice, GI abnormalities have not been reported in preclinical models of ASD.

Experimental Results Relevant for Tasks 1 and 2

As previously reported (22), maternal poly(I:C) injection yields offspring with increased anxiety in the open field, increased stereotyped marble burying, decreased number and duration of ultrasonic vocalizations, deficient pre-pulse inhibition (PPI) of acoustic startle, and low sociability and social preference (Fig. 1; compare saline to poly(I:C)). Remarkably, oral treatment with *B. fragilis* ameliorates many of these ASD-associated behavioral abnormalities in MIA offspring. Probiotic-treated poly(I:C) offspring are protected from anxiety-like behavior in the open field (Fig. 1A) and also exhibit significantly decreased levels of stereotyped marble burying and restored number of ultrasonic vocalizations, with the duration per call exceeding that observed in control offspring (Fig. 1, B and D; compare poly(I:C) to poly(I:C)+BF). *B. fragilis* treatment also improves sensorimotor gating as indicated by the restoration of PPI to levels comparable to controls (Fig. 1C). Interestingly, behavioral improvement in response to *B. fragilis* treatment is not associated with changes in systemic immunity in MIA offspring (data not shown) and is not dependent on polysaccharide A (PSA), the capsular component identified to confer immunomodulatory effects of *B. fragilis* in experimental autoimmune encephalomyelitis (EAE) and colitic mice (data not shown) (13, 14). Furthermore, amelioration of behavior is not specific to *B. fragilis*, as similar treatment with *Bacteroides thetaiotaomicron* also significantly improves anxiety-like, repetitive and communicative behavior in MIA offspring (data not shown). While we cannot exclude the possibility of a tissue-specific effect of *B. fragilis* on immunity, these data suggest a novel

beneficial effect of *B. fragilis* in the autism model as compared to models of MS or IBD. Notably, *B. fragilis* does not alter baseline behavior in saline offspring (Fig. 1; compare saline to saline+BF) in all tests but PPI, suggesting that the diseased state elicits a physiological response to *B. fragilis* that differs from that found in treated control mice.

Although *B. fragilis*-treated poly(I:C) offspring exhibit improved communicative, repetitive, anxiety-like and sensorimotor behavior, they retain deficits in sociability, as indicated by decreased preference for a novel mouse versus a novel toy, and in social preference for an unfamiliar versus familiar mouse (Fig. 1E). Interestingly, this parallels the inability to improve social behavior by administration of risperidone to CNTNAP2 knockout mice, a genetic mouse model for ASD (27), and by irradiation and bone marrow transplant of MIA offspring (24). These data suggest that there are fundamental differences in the circuitry or circuit plasticity governing social behavior as compared to the other behaviors, and that *B. fragilis* modulates specific pathways during amelioration of ASD-related behavioral defects in MIA offspring.

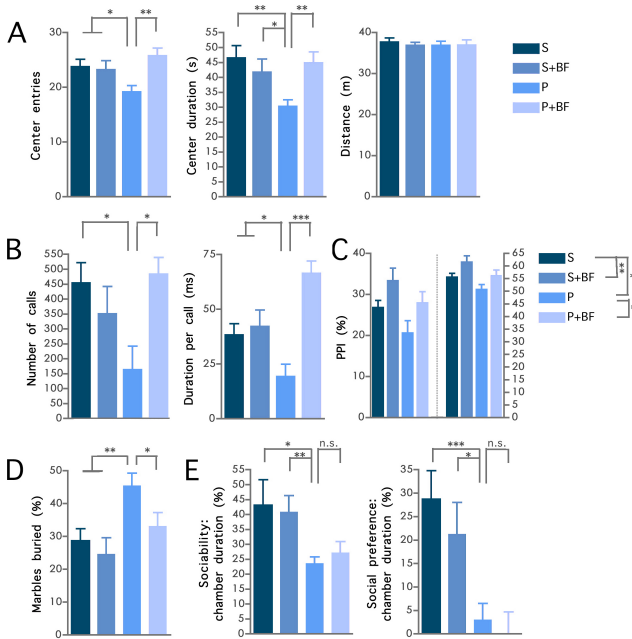


Fig. 1. *B. fragilis* treatment ameliorates autism-related behaviors in MIA offspring. (A) Poly(I:C) offspring exhibit anxiety-like behavior, as measured by decreased entries into and duration spent in the center of an open field, which is restored to control levels by *B. fragilis* treatment. There is no difference between experimental groups in total distance traveled. $n = 35-75$. (B) *B. fragilis* treatment corrects deficits in communicative behavior, as indicated by the total number and duration of ultrasonic vocalizations produced by adult male poly(I:C) offspring in response to a female. $n = 7-10$ animals (C) *B. fragilis*-treated poly(I:C) and saline offspring display increased pre-pulse inhibition (PPI) of an acoustic startle, indicating improved sensorimotor gating (PPI5, 5 db pre-pulse; PPI15, 15 db pre-pulse). $n = 35-75$ (D) Poly(I:C) offspring engage in elevated stereotypic marble burying, and this phenotype is corrected by *B. fragilis* treatment. $n = 16-35$ (E) *B. fragilis* is not effective in treating social abnormalities in MIA offspring, having no impact on the decreased preference for a novel mouse versus novel toy (sociability) or the decreased preference for an unfamiliar versus familiar mouse (social preference) observed in MIA offspring. $n = 10$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. = not significant

There is growing evidence that subsets of autistic children display GI abnormalities, including increased intestinal permeability or “leaky gut” (5, 28). Remarkably, we find that MIA offspring display a significant deficit in intestinal barrier permeability, as reflected by increased translocation of orally administered FITC-dextran across the intestinal epithelial layer and into the circulation (Fig. 2A, left panel). A similar deficit is present in 3-week-old MIA offspring (Fig. 2A, right panel), demonstrating that this abnormality is established early during development and prior to *B. fragilis* treatment. Notably, *B. fragilis* treatment restores this MIA-associated increase in intestinal barrier permeability to levels comparable to those observed in saline controls (Fig. 2A, left panel). These data demonstrate that, in addition to improving autism-related behaviors, *B. fragilis* ameliorates leaky gut in MIA offspring. The presence of GI defects prior to probiotic administration suggests that *B. fragilis* may treat, rather than prevent, this ASD-related pathology in MIA offspring.

To assess the molecular basis for increased intestinal permeability in MIA offspring, we examined the colons of MIA offspring for levels of the tight junction components ZO-1, ZO-2, ZO-3, occludin and claudins 1, 2, 3, 4, 7, 8, 12, 13 and 15 (29). Consistent with the leaky gut phenotype, colons from adult poly(I:C) offspring exhibit decreased expression of ZO-1, ZO-2, occludin and claudin 8, and increased expression of claudin 15 mRNA (Fig. 2B). Furthermore, *B. fragilis* treatment ameliorates MIA-associated changes in expression of claudin 8 and 15, but has no restorative effect on expression of ZO-1, ZO-2 or occludin mRNA. Similar changes in colon claudin 8 and 15, with restoration by *B. fragilis* treatment, are also observed at the protein level (Fig. 2C). Interestingly, both decreased claudin 8 and increased claudin 15 are associated with alterations in sodium paracellular permeability in the mouse intestine (30, 31), and sodium electrochemical gradients across the intestinal epithelia are known to regulate sodium-dependent ion and nutrient absorption as well as GI motility (32). No such effects of *B. fragilis* on tight junction expression are observed in small intestines from MIA offspring (data not shown), consistent with reports showing that *B. fragilis* colonizes the colonic mucosa and that *Bacteroides* species are predominantly found in the colon (33). Collectively, discovery of GI defects in

MIA offspring recapitulates intestinal co-morbidities found in subsets of ASD individuals, and correction of leaky gut and behavioral abnormalities by *B. fragilis* supports emerging evidence for a gut-brain link in autism.

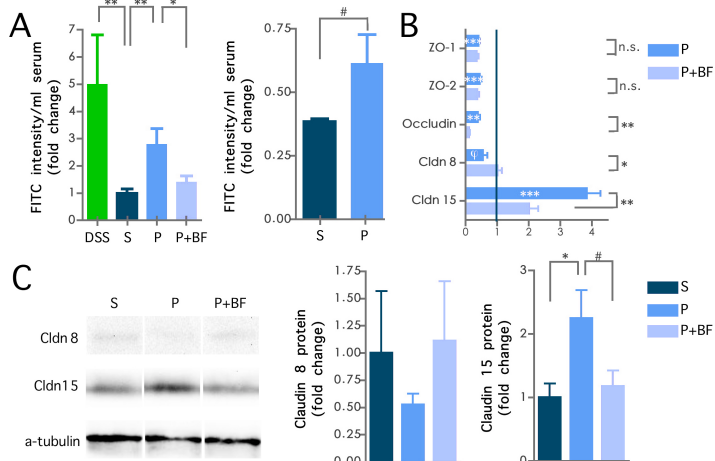


Fig. 2. MIA offspring exhibit a deficit in GI barrier function and expression of tight junction components, which are corrected by *B. fragilis* treatment. (A) *Left panel:* Adult poly(I:C) offspring display increased serum FITC fluorescence intensity after oral FITC-dextran gavage, indicating elevated intestinal permeability. This is restored to levels comparable to saline controls by *B. fragilis* treatment. DSS-treated colitic mice are used as a positive control for leaky gut. n = 12-13 for poly(I:C) and saline, n = 6 for DSS and poly(I:C)+BF. *Right panel:* 3 week old MIA offspring also exhibit increased intestinal permeability, demonstrating that this GI barrier abnormality is present prior to probiotic treatment. n = 4 (B) The abnormal intestinal barrier integrity in MIA offspring corresponds to altered colon tight junction expression of ZO-1, ZO-2, occludin, claudin 8 and 15 mRNA.

The changes in claudin 8 and 15 expression are ameliorated by *B. fragilis* treatment. n = 8 (C) Similar alterations in colon claudin 8 and 15 are seen at the protein level, with trending decreases in claudin 8 and significant increases in claudin 15, which are both corrected by *B. fragilis* treatment. n = 3. # $p < 0.07$, $\phi p = 0.05$, * $p < 0.05$, ** $p < 0.01$, n.s. = not significant

Cytokines are signaling molecules used widely by both the immune and nervous systems. Levels of a variety of cytokines, including interleukin-6 (IL-6), are altered in the serum and cerebrospinal fluid of autistic individuals (3, 34), but whether they also exhibit altered gut expression patterns is unknown. Remarkably, colonic tissues of adult MIA offspring contain increased levels of IL-6 mRNA and protein, and *B. fragilis* treatment restores elevated IL-6 to levels found in control mice (data not shown). Levels of other cytokines are also altered in both colons and small intestines of MIA offspring (data not shown), but these are not affected by *B. fragilis* treatment, revealing specificity for IL-6. This is interesting in light of a previous study showing that induction of IL-6, but not other cytokines, in response to MIA is required for the development of behavioral deficits in the offspring (35). Nonetheless, altered intestinal cytokines may be the basis for the increased intestinal permeability observed in MIA offspring, as several cytokines including IL-6 regulate tight junction expression and intestinal barrier integrity (32, 36). Consistent with the notion that IL-6 affects intestinal permeability in MIA offspring, we find that recombinant IL-6 treatment can modulate colon levels of both claudin 8 and claudin 15 in *in vivo* and in *in vitro* colon organ cultures (data not shown). This suggests that *B. fragilis*-mediated restoration of colonic IL-6 levels regulates changes in colon tight junction protein expression. Overall, these findings demonstrate that MIA offspring exhibit defective GI barrier integrity, with corresponding changes in colon tight junction and cytokine profiles, which are reversed by *B. fragilis* treatment.

Metabolomic studies have shown that gut microbial products are found in many extra-intestinal tissues, and molecules derived from the microbiota may influence metabolic, immunologic and behavioral endophenotypes in mice and humans (12, 25, 26, 37-39). Given that MIA offspring display altered intestinal permeability and tight junction expression, we utilized liquid chromatography/gas chromatography with mass spectrometry (LC/GC-MS)-based metabolomic profiling to identify MIA-associated changes in serum metabolites. 2400 metabolites were assayed and of these, 322 metabolites, spanning amino acid (94), peptide (15), carbohydrate (22), energy (10), lipid (128), nucleotide (23), xenobiotic (19) and cofactor and vitamin (11) super pathways were detected in sera from adult mice. Interestingly, MIA leads to statistically significant alterations in 8% of all serum metabolites detected (data not shown). Furthermore, postnatal *B. fragilis* treatment has a significant effect on the serum metabolome, altering 34% of all metabolites detected (fig. S6 and additional data table S1). The majority (72%) of *B. fragilis*-mediated changes are found in MIA offspring and not in control mice, demonstrating that the particular effects of postnatal *B. fragilis* treatment on the serum metabolome depend on the disease status of the host. This is consistent with our finding that *B. fragilis*-mediated improvement of many ASD-related behaviors is specific to MIA offspring and is not observed in controls (Fig. 1). In particular, *B. fragilis* treatment lowers serum levels of several fatty acids and increases levels of molecules associated with purine catabolism (data not shown). In light of our finding that *B. fragilis* itself does not permanently colonize the GI tracts of treated MIA and control offspring (data not shown), these widespread changes suggest that *B. fragilis* disrupts the composition and/or functional activity of the resident intestinal microbiota. Moreover, that vehicle-treated MIA offspring do not display many of these changes in fatty acid and purine metabolites suggests that these disruptions do not play a significant role in the onset of

autism-related symptoms in the MIA model. Whether *B. fragilis*-mediated changes in fatty acid uptake and purine salvage serve a protective role in ameliorating MIA-induced autism-related behaviors remains to be investigated.

In line with the notion that increased gut permeability leads to leakage of metabolites into the bloodstream, we hypothesized that *B. fragilis*-mediated improvement of intestinal barrier integrity prevents alterations in serum metabolite levels. We therefore focused on serum metabolites that are significantly altered by MIA treatment and completely restored to control levels by *B. fragilis* treatment (Fig. 3). MIA offspring display a striking, 45-fold increase in serum levels of a recently identified metabolite, 4-ethylphenylsulfate (4EPS), which is dramatically reduced by *B. fragilis* treatment (Fig. 3A). This metabolite is of particular interest because of the reported production of 4EPS by GI microbes and proposed role of 4EPS in communication by mice (40). Moreover, we find that, compared to conventionally colonized mice, germ-free mice display nearly undetectable levels of serum 4EPS, indicating that serum 4EPS is derived from or critically modulated by the commensal microbiota (Fig. 4E). Interestingly, 4EPS is suggested to be a uremic toxin, as is *p*-cresol, a metabolite identified as a possible urinary biomarker for human autism (41). MIA offspring also exhibit elevated levels of serum *p*-cresol (4-methylphenol), though the increase does not reach statistical significance (data not shown).

In addition to 4EPS, MIA offspring display significantly increased levels of serum indolepyruvate, a key molecule of the tryptophan metabolism pathway, which is restored to control levels by *B. fragilis* treatment (Fig. 3B). Indolepyruvate is generated by tryptophan catabolism and, like 4EPS, indolepyruvate is known to be produced by gut microbes (42). Moreover, the elevation in serum indolepyruvate observed in MIA offspring is analogous to the increase in another major tryptophan metabolite observed in human autism, indolyl-3-acryloylglycine (IAG), which was suggested to be a urinary biomarker for ASD (43). Interestingly, IAG is involved in GI homeostasis and is produced by bacterial tryptophan metabolism (44). Although IAG was not detected in our metabolomic screen, it is notable that MIA offspring exhibit increased levels of serum serotonin ($0.05 < p < 0.10$; additional data table S1), which reflects an alteration in another pathway of tryptophan metabolism and is reminiscent of the hyperserotonemia endophenotype of autism (45). Importantly, the commensal microbiota is known to impact serum levels of indole-containing tryptophan metabolites and serotonin (46). MIA also leads to altered serum glycolate, imidazole propionate and N-acetylserine levels (Fig. 3, C-E), which are corrected by *B. fragilis* treatment. How changes in these metabolites may be relevant to ASD or GI dysfunction, however, are unclear. Overall, we demonstrate that MIA elevates, and *B. fragilis* treatment normalizes, serum levels of 4EPS and indolepyruvate, two molecules modulated by the intestinal microbiota with potential relevance to autism.

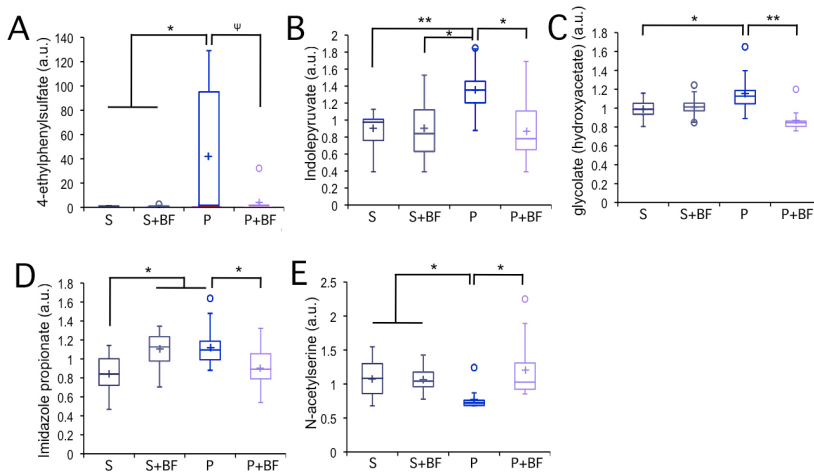


Fig. 3. *B. fragilis* treatment corrects MIA-induced alterations in serum metabolites. (A) Adult MIA offspring display a striking 45-fold increase in serum 4-ethylphenylsulfate levels, which is reduced to levels comparable to controls by *B. fragilis* treatment. MIA offspring also exhibit significantly increased serum indolepyruvate (B), glycolate (C) and imidazole propionate (D) levels, and decreased serum N-acetylserine levels (E), which are each corrected by *B. fragilis* treatment. $n = 8$. $\Psi = 0.08$, $*p < 0.05$, $**p < 0.01$

Key Research Accomplishments

- As in a very significant subset of ASD cases, the offspring of immune-activated pregnant mice display a leaky gut phenotype.
- Both the leaky gut and most of the ASD-like behaviors in these offspring can be prevented by administration of a probiotic bacterium.
- MIA offspring also display altered serum metabolites, some of which are corrected by probiotic treatment.

Reportable Outcomes

Hsiao EY, McBride SW, Hsien S, Codelli JA, Chow J, Reisman SE, Mazmanian SY, Patterson PH (2012) Gastrointestinal symptoms and probiotic treatment in a mouse model of an autism risk factor. Program No. 443.14. Neuroscience Meeting Planner. Washington DC: Society for Neuroscience, 2012. Online.

Hsiao EY, McBride SW, Hsien S, Chow J, Mazmanian SY, Patterson PH (2012) Gastrointestinal symptoms and probiotic treatment in a mouse model of an autism risk factor. 11th International Congress of Neuroimmunology, Boston, MA. Abstract 17.

Conclusions

While the impact of the microbiota on immunologic and metabolic disease is profound, little is known regarding a link to behavioral disorders. We propose that commensal bacteria of the microbiota can influence the gut-brain connection by modulating metabolites that alter behavior. We find that postnatal *B. fragilis* treatment corrects abnormal intestinal permeability and ameliorates communicative, stereotyped, sensorimotor and anxiety-like behavior in a mouse model of an ASD risk factor. While a number of microbes, including *Lactobacillus rhamnosus* (15) and *Bifidobacterium longum* (25), are known to alleviate anxiety and depressive behavior in animal models, our current findings represent the first evaluation of a microbial effect on core autism-related behaviors. We further demonstrate that, in addition to displaying cardinal behavioral and neuropathological symptoms of ASD (22, 23), offspring of immune-activated mothers exhibit altered serum metabolites and deficient GI integrity that is analogous to that seen in subsets of ASD individuals (5, 7, 8). Thus, the MIA model exhibits face and construct validity for particular co-morbid GI symptoms found in human autism. Consistent with the well-established role of GI microbes in regulating intestinal permeability and metabolic homeostasis (26, 32, 38, 46, 49, 50), we show that *B. fragilis* treatment corrects GI permeability and restores MIA-associated changes in blood metabolites. By this means, *B. fragilis* may prevent the leakage of deleterious molecules from the GI lumen and/or promote the synthesis of protective compounds in the periphery. It is intriguing that *B. fragilis* exerts beneficial behavioral and metabolomic effects in a disease-specific manner. This observation contributes to a growing number of studies emphasizing that microbial composition and function is critically influenced by differences in host genotype and environment (51-53). Taken together, we suggest a novel mechanism by which *B. fragilis* treatment can improve autism-related behavioral abnormalities and present compelling evidence for a probiotic-based therapy for ASD-associated symptoms. Validation of a specific metabolomic profile specific in human autism subjects with GI complications may serve as a novel molecular diagnostic and the basis for microbiome-mediated therapies.

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