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Intestinal microbiota, metabolome and gender dimorphism in autism spectrum disorders

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ABSTRACT

There is a male predominance in autism, with a male/female ratio of 4:1 and an even higher ratio (11:1) in individuals with high functioning autism. The reasons for gender differences in ASD are unknown. Genetic and environmental factors have been implicated, but no definitive evidence exists to explain male predominance. In this review, evidence is presented to support a hypothesis that the intestinal microbiota and metabolome play a role in gender dimorphism in children with autism. Metabolic products may affect not only gastrointestinal (GI) tract and the central nervous system, but also behavior, supporting communication between GI tract and central nervous system. Furthermore, mood and anxiety may affect intestinal function, indicating bidirectional flow in the gut-brain axis. Several hormone-based hypotheses are discussed to explain the prevalence of autism in males. Observations in animal models and studies in humans on the intestinal microbiome and metabolome are reviewed to support the proposed gender dimorphism hypothesis. We hypothesize that the intestinal microbiome is a contributing factor to the prevalence of ASD in boys either directly, through microbial metabolites and/or epigenetic factors capable of regulating host gene expression through DNA methylation and/or histone modification.

1. Introduction

Autism is a neurodevelopmental disorder, but GI problems including constipation, diarrhea and abdominal pain are common. Recent data from the CDC demonstrated that the frequency of autism spectrum disorders (ASD) in American boys is 1:42, but only 1:189 in girls (Home, 2014), with a male/female ratio of 4:1 (Fombonne, 2003). In high-functioning autism, the male/female ratio is even higher, at 11:1 (Gillberg, Cederlund, Lamberg, & Zeijlon, 2006). In a subgroup of individuals diagnosed with ASD without any physical or brain abnormalities as measured by MRI, the ratio was as high as 23:1 (Miles & Hillman, 2000).

The reason for gender differences in ASD is unknown. This observation is well recognized, but the reasons are poorly understood (Schaafsma & Pfaff, 2014). Investigators have sought a genetic explanation for the male prevalence in ASD, since up to 2.5% of genes in the brains of men and women are differentially expressed or spliced (Trabzuni et al., 2013). Although many genes are implicated in ASD, there are also many individuals who have alterations in those same genes and are not symptomatic (Weiss et al., 2008). Furthermore, the majority of genes implicated in autism are not located on the sex chromosomes. However, it remains possible that genes on the Y chromosome interact with ASD susceptibility genes to contribute to autism in males.

Recently, environmental factors or their interaction with human genetics were considered to play important roles in ASD etiology,

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especially in subjects with vulnerable phenotypes. Factors such as early exposure to androgenic hormones and early maternal immune activation could affect gender-specific susceptibility to ASD (Schaafsma & Pfaff, 2014; Pfaff, Rapin, & Goldman, 2011; Schaafsma et al., 2017).

In this review, a hypothesis involving role of the intestinal microbiota on gender dimorphism in individuals with autism is considered. The impact of three factors: (i) the intestinal microbiome; (ii) the intestinal metabolome; and (iii) gut-brain interactions in model animals and in humans contribute to the gender dimorphism hypothesis.

2. Environment and intestinal microbiota in ASD

Specific prenatal and perinatal environmental factors are implicated to increase ASD risk (Gardener, Spiegelman, & Buka, 2011), to the extent that some investigators suggest that in ASD environmental factors may be more important than genetic factors (Hallmayer, Cleveland, & Torres, 2011). Environmental factors include not only external factors such as xenobiotics, hormones, inflammatory agents and early stress, but also host environmental factors such as intestinal microbiota. There are more than 1000 microbial species living in the human intestine and dysbiosis of the intestinal microbiota may be associated with various diseases (metabolic diseases, intestinal disorders, cancer, etc.) including autism. Malabsorption in patients with autism might be related to a disruption of the indigenous microbiota promoting the overgrowth of potentially pathogenic microorganisms, such as *Clostridia.* Some of these bacteria are known to produce toxins, including neurotoxins, lethal toxins, oxygen-liable hemolysins, binary toxins and ADP-ribosyltransferases (Hatheway, 1990). Using traditional cultivation methods that provide information on a limited number of bacterial species inhabiting the gastrointestinal tract, Finegold et al. (2002) demonstrated nine species of *Clostridium* in the stool of children with autism that were not found in stool from healthy subjects. Implementation of modern molecular methods including next generation sequencing techniques allowed more extensive evaluation of the microbiome. Subsequent work by Finegold's group using real-time PCR quantified some *Clostridium* clusters and the species *Clostridium bolteae* in the stool of children with ASD (Song, Liu, & Finegold, 2004). The higher incidence of *Clostridium*, specifically *C. histolyticum*, in children with autism as compared with a healthy control group was subsequently confirmed by Parracho et al. (Parracho, Bingham, Gibson, & McCartney, 2005) using fluorescence *in situ* hybridization with 16S rRNA-based oligonucleotide probes. They suggested a possible link between clostridial levels and GI function in ASD patients.

In other studies investigators demonstrated significant microbial dysbiosis in the stool of children with autism as compared with that of unaffected children. Finegold et al. (Finegold, Dowd, & Gontcharova, 2010), using bacterial amplicon pyrosequencing technology, reported decreased *Firmicutes* and *Actinobacteria* and increased *Bacteriodetes* and *Proteobacteria* in the stool of children with autism when compared to controls. In addition, *Desulfovibrio* species and *Bacteroides vulgatus* were present in significantly higher numbers in stools of children with severe autism when compared with controls. The stool of children with autism also contained lower numbers of *Bifidobacterium* species and the mucolytic bacterium *Akkermansia muciniphila* (Wang et al., 2011). Kang et al. (2013) demonstrated a less diverse microbiome in the stool of individuals with autism than in unaffected controls, with lower levels *of Prevotella, Coprococcus*, and unclassified Veillonellaceae. They noted that the simplified microbiome in the study population was associated with the presence of symptoms of autism rather than the severity of GI symptoms. A less diverse gut bacterial population in children with ASD compared with neurotypical controls was confirmed in a recent study by the same group (Kang et al., 2017). Changes in microbiome diversity were found also by De Angelis, Piccolo, and Vannini (2013), who analyzed the intestinal microbiome and metabolome in children with autism and pervasive developmental disorder not otherwise specified (PDD-NOS) in comparison with neurotypical controls. They found more changes in the intestinal microbiota and metabolome in PDD-NOS individuals, and especially children with autism, than in healthy controls, and hypothesize that the degree of microbial alteration correlates with the severity of the disease. Other investigators reported that the stools of children with autism contain lower levels of beneficial bacteria such as *Bifidobacterium*, slightly lower levels of *Enterococcus*, and much higher levels of *Lactobacillus* in comparison with unaffected children (Adams, Johansen, Powell, Quig, & Rubin, 2011). More detailed information on changes in the stool microbiota of children with autism in comparison with controls can be found in recent reviews (De Angelis, Francavilla, Piccolo, De Giacomo, & Gobbetti, 2015; Krajmalnik-Brown, Lozupone, Kang, & Adams, 2015; Ding, Taur, & Walkup, 2017; Li, Han, Dy, & Hagerman, 2017).

Compositional dysbiosis was found also in the ileum of children with autism. Metagenomic analysis demonstrated a decrease in *Bacteroidetes*, increased ratio of *Firmicutes* to *Bacteroidetes* and an increase in Betaproteobacteria (Williams et al., 2011). In another study, the same authors reported that *Sutterella* represented a major component of the ileal mucosal microbiota in over half of the children with autism along with gastrointestinal dysfunction, but was absent in neurotypical children with GI disorders (Williams, Hornig, Parekh, & Lipkin, 2012). Bacterial dysbiosis or an overgrowth of a specific population of bacteria was found not only in the colon and ileum, where microbiota represented mostly by Gram-negative species, but also in the duodenum, which is populated predominantly by Gram-positive microorganisms from the oropharynx (Williams et al., 2012; Riordan et al., 2001; Simon & Gorbach, 1986). A significant number of non-spore-forming anaerobes and microaerophilic bacteria were identified in the duodenal fluid of children with autism in contrast to the total absence of such bacteria in control children (Finegold et al., 2002). The mucosal adherent bacterial in the duodenal mucosa of children with autism demonstrated different populations of bacteria. Bacteria belonging to the genus *Burkholderia* were more abundant in subjects with autism, while members of the genus *Neisseria* were higher in unaffected controls. At the species level, a relative decrease in abundance of two *Bacteroides* species and *Escherichia coli* was found in individuals with autism. Interestingly, in individuals with autism, disaccharidase activity correlated with the abundance of *Clostridium* species (Kushak et al., 2016).

Thus, data from multiple studies show that an abnormal gut microbiota is related to ASD. Microbial dysbiosis in children with autism was found not only in the stool and colonic mucosa, but also in mucosa of the small intestine. Observations about the

composition of the intestinal microbiota underscore the complexity of microbial ecosystem in individuals with ASD; however, the clinical significance of these alterations to the neurologic changes in autism remains undetermined. Variations in sampling, microbiological assays, bioinformatic analysis as well as dietary factors and characterization of GI disorders all contribute to the challenges of interpreting the relevance of the data to autism.

Antibiotic treatment for GI problems led clinicians to evaluate how antibiotics might affect symptoms in children with autism. Sandler et al. reported that treatment with vancomycin, which targets Gram-positive bacteria including *Clostridia* resulted in improvement not only of gastrointestinal symptoms in children with regressive autism, but also in cognitive skills (Sandler, Finegold, & Bolte, 2000). However, the effects of vancomycin did not persist and efficacy was lost shortly after termination (Sandler et al., 2000). Significant improvement in the behavior of children with autism was also observed during a 10-day course of amoxicillin (Rodakis, 2015). However, antibiotics disrupt the microbiome and could theoretically result in ongoing dysbiosis (Power, O'Toole, Stanton, Ross, & Fitzgerald, 2014).

Probiotics are used to treat inflammatory bowel disease and irritable bowel syndrome with variable efficacy (Dong, Teng, Wei, Gao, & Wang, 2016; Currò, Ianiro, Pecere, Bibbò, & Cammarota, 2017); studies in individuals with ASD demonstrated increases in the colony counts of Bifidobacteria and Lactobacilli, changes in the stool metabolome as well as improvements in gastrointestinal symptoms and behavior (Adams et al., 2011; Navarro, Liu, & Rhoads, 2016). However, randomized controlled trials are needed to determine the efficacy of probiotics in ASD (Shaaban et al., 2017).

The effects of probiotics were seen more clearly in mouse models of ASD. Hsiao et al. (2013) demonstrated that *Bacteroides fragilis* treatment altered gut microbiota, the metabolomic profile in the blood, gut permeability and improved ASD-associated behavior. Even more promising was a recent study with a fecal microbial transplant in a small number of children with ASD (Kang et al., 2017). Fecal transplant increased diversity of the gut microbiome making it indistinguishable from that of controls and significantly improving behavior. All these observations suggest that antibiotics, probiotics and fecal transplant might alter microbial abnormalities and behavior in children with autism. However, the methodological limitations of these studies, lack of a clear mechanism and long-term outcomes limit microbial therapies as treatments for ASD at this time.

3. Metabolic role of microbiota

Human metabolome contains metabolites produced by the host as well as by the microbiome. Metabolomic analysis was helpful in finding specific biomarkers for type 2 diabetes, cancer, stroke, and other diseases (Zhang, Sun, Wang, Han, & Wang, 2012; Zhang, Sun, Wu, & Wang, 2012). Metabolic profiling has been applied to characterize IBD (Marchesi et al., 2007), celiac disease (Bertini, Calabrò, & De Carli, 2009), and vegetarian diets (Pettersson et al., 2008). Variations in the metabolome could help understand the heterogeneity of clinical manifestations and lead to novel approaches to treatment. Studies of metabolites from urine, plasma (Cai, Ding, Zhang, Xue, & Wang, 2016; Shimmura, Suda, & Tsuchiya, 2011; Arnold, Hyman, Mooney, & Kirby, 2003; El-Ansary, Bacha, & Al-Ayahdi, 2011) and stool (Adams et al., 2011; Wang, Christophersen, & Sorich, 2012) from children with autism demonstrated many changes in metabolic pathways in comparison with neurotypical children. They include changes in amino acids, fatty acids, organic acids, porphyrins and other compounds metabolism (Table 1).

Metabolic disorders appear to be more severe in children on restricted diets. In children on gluten-free, casein-free diets, Arnold et al. (Arnold et al., 2003) found an increased prevalence of amino acid deficiencies and lower plasma levels of essential amino acids including the neurotransmitter precursors tyrosine and tryptophan. Metabolic phenotyping in individuals with autism demonstrated perturbations in the tryptophan-nicotinic acid metabolic pathway as well as in urinary levels of the free amino acids glutamate and taurine. These observations differentiated children with autism from their unaffected siblings and controls (Yap et al., 2010; Ming, Stein, Barnes, Rhodes, & Guo, 2012). Increased glutamate was also found in plasma of affected children. Glutamic acid may play an important role in the pathogenesis of autism, since excitatory neurotransmitter signaling modulates cognitive functions such as memory and learning (Cai et al., 2016).

Autism has been shown to have a strong association with various metabolic abnormalities, but the significance of these observations is unknown. Some metabolic abnormalities might be related to the composition of the gut microbiota (Finegold et al., 2002). Specifically, increased levels of urinary dimethylamine and lower levels of hippurate and 4-cresol sulfate in children with autism might be associated with colonization of certain bacteria in the gastrointestinal tract such as *Bacteroides* and/or *Clostridia* (Yap et al., 2010). Elevated levels of p-cresol may come from *Clostridia* species and *Pseudomonas stutzeri* (Altieri et al., 2011). Show et al. (Shaw, 2010) found higher levels of urinary 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in children with ASD compared with controls, and suggested that the source of this compound might be multiple species of anaerobic bacteria of the *Clostridium* genus. The composition of microbiota also determines the levels of short chain fatty acids (SCFA), and changes in microbial composition can alter the levels of these metabolites. Stool analysis demonstrated that children with autism had much lower levels of total SCFA, including acetic, propionic, and valeric acids than unaffected children (Adams et al., 2011). However, Wang et al. (Wang et al., 2012) reported that levels of these SCFA and ammonia in stool were significantly increased in children with autism when compared with controls. The difference between these two studies might be explained by the use of probiotics by some participants in the first study.

Some products of abnormal bacterial metabolism may affect not only GI health but also behavior, indicating communication between the GI tract and central nervous system (gut-brain axis) (Bravo et al., 2011). For example, fructose and lactose malabsorption may be associated with depression (Ledochowski et al., 2000). Of particular interest are recent studies linking the microbiota to both the hormonal regulation and the neurologic function. Yano et al. (Yano et al., 2015) uncovered a role for the microbiota in controlling the production of a major neurotransmitter, serotonin, and showed that its changes correlate with the presence of spore-forming bacteria, primarily from the *Clostridium* genus. Interestingly, certain microbes themselves can produce serotonin (O'Mahonya,

Table 1
Metabolomic changes in individuals with ASD in comparison with unaffected controls.

Source	Metabolite	Changes	References
Urine	Oxalate	Increased	Konstantynowicz et al. (2012)
Urine	Porhyrins	Increased	Heyer, Echeverria, and Woods, (2012)
Urine	Homocysteine	Increased	Kałużna-Czaplińska, Michalska, and Rynkowski (2011)
Urine	p-Cresol	Increased	Altieri et al. (2011)
Urine	Organic acids	Increased (16 acids) Decreased (5 acids)	Kałużna-Czaplińska, Żurawicz, Struck, and Markuszewski (2014)
Urine	Tryptophan	Decreased	Kałuzna-Czaplinska, Michalska, and Rynkowski (2010)
Urine	N-methyl-2-pyridone-5-carboxamide; N-methylnicotinic acid; N-methtlnicotinamide; taurine	Increased	Yap et al. (2010)
	Glutamate	Decreased	
Urine	Glycine, Serine, Threonine, Alanine, Histidine, Glutamic Acid, Taurine	Decreased	Ming et al. (2012)
Urine	Succinate, Glycolate	Increased	Emond et al. (2013)
	Hippurate, 3-hydroxyphenylacetate; Vanillylhydracrylate; 3-hydroxyhippurate; 4-hydroxyphenyl-2-hydroxyacetate; 1H-indole-3-acetate; phosphate; palmitate; stearate; 3-methyladipate	Decreased	
Urine	3-(3-Hydroxyphenyl)-3-hydroxypropionic acid,	Increased	Shaw (2010)
Urine	3-(3-Hydroxyphenyl)-3-hydroxypropanoic acid; 3,4-Dihydroxybutyric acid; Glycolic acid; Glycine; cis-Aconitic acid; Phenylalanine; Tyrosine; p-Hydroxyphenylacetic acid; Homovanillic acid	Increased	Noto, Fanos, and Barberini (2014)
Plasma	Glutamate	Increased	Cai et al. (2016)
Plasma	Glutamate	Increased	Shimmura et al. (2011)
	Glutamine	Decreased	
Plasma	Amino acids (essential)	Decreased	Arnold et al. (2003)
Plasma	Fatty acids (FA)	Increased (saturated FA, except propionic acid) Decreased (unsaturated FA)	El-Ansary et al. (2011)
Stool	Free amino acids, 3,7-dimethyl-2,6-octadien-1-ol, phenol, 4-(1,1,3,3-tetramethylbutyl)-phenol, p-cresol	Increased	De Angelis et al. (2013)
	Ethanol, 2-propyl-1-pentanol and 1-pentanol, Ketones	Decreased	
Stool	Acetic acid, Propionic acid, Valeric acid	Decreased	Adams et al. (2011)
Stool	Acetic acid, Propionic acid, Valeric acid, ammonia	Increased	Wang et al. (2012)

Clarkea, Borrea, & Dinana, 2015). The potential association of biologically active metabolites with brain function is presented in greater detail below.

4. Gut-brain interactions

There are multiple ways in which microbiota could impact the central nervous system. Enteric neurons innervate the gut and transmit signals from it to the brain. Conversely, the brain is connected to the gut through the enteric nervous system, which contains between 200 and 600 million neurons (Furness, 2006) and innervates the complete length of the GI tract. The microbiota produce metabolites that when absorbed may cross the blood–brain barrier and affect behavior. Neurotransmitters secreted by microorganisms are the same substances that central nervous system neurons use to communicate and regulate mood. These include dopamine, serotonin and GABA (gamma-aminobutyric acid), which are associated with psychiatric and functional intestinal disorders. About 50% of dopamine and most serotonin originate in the intestine and regulate appetite, satiety and digestion. Recently, Lyte (2013, 2014) reviewed the role of microbes in the synthesis of these neurotransmitters and the interactions between the neuroendocrine system and the microbiota.

Studies on animals have indicated that the intestinal microbiota is involved in the host's response to noxious stimuli that interfere with the behavioral response. For example, rat pups stressed by separating them from their mothers for 3 h daily showed markedly altered fecal microbiota (O'Mahony, Marchesi, & Scully, 2009). Similar findings have been reported in infant rhesus monkeys separated from their mothers and in adult rats exposed to chronic psychological stress (Bailey & Coe, 1999; Cui, Gai, She, Wang, & Xi, 2016). Studies on animal models demonstrated that communications in the gut-brain axis are bi-directional (Bercik et al., 2011). Not only does the microbiota affect behavior, but also the brain may influence microbiota, rapidly changing its composition through the release of neurochemicals into the gut lumen. A shift in the composition of the microbiota was observed within 24 h following 6-hydroxydopamine administration, during which time the Gram-negative bacteria increased over 5 logs in total population (Lyte & Bailey, 1997).

Analysis of gut-brain interaction demonstrated that germ-free (GF) animals had an increased stress response compared with their conventionally colonized (CC) counterparts, and that their aberrant endocrine profile could be partially reversed by reconstitution of the microbiota (Clarke et al., 2013). The exaggerated stress-anxiety behavior in GF mice is associated with increased plasma corticosterone and adrenocorticotropic hormone levels in comparison with specific-pathogen-free (SPF) mice. GF mice colonized with the stool of SPF mice partially normalized their behavior, while animals treated with probiotics (*Bifidobacterium infantis*) totally

reverted to a normal behavior. A decrease in brain-derived neurotrophic factor, which is often associated with a change in brain plasticity, was found in the cortex and hippocampus of GF animals but not in SPF controls (Sudo et al., 2004). In another study on GF and SPF mice that underwent vagotomy or chemical sypathectomy, the link between intestinal microbiota and behavior was dependent on these connections (Bercik et al., 2011). The GF mice also showed greater exploratory activity when compared with SPF mice, suggesting decreased anxiety-like behavior and increased motor activity. This conclusion was supported by elevated noradrenalin and dopamine levels and serotonin turnover rates in the striatum, all of which have been previously associated with anxiety-like behavior (Diaz Heijtz, Wang, & Anuar, 2011).

Thus, the bacterial colonization of the intestine has a major role in the postnatal development and maturation of the endocrine and immune systems (Grenham, Clarke, Cryan, & Dinan, 2011), both of which are associated with central nervous system function (Clarke et al., 2013; Cryan & O'Mahony, 2011) and are of particular relevance to the developing serotonergic system (Clarke et al., 2013; Leonard, 2006). These results suggest that changes in microbiota could alter motor activity and anxiety-like behavior and indicate that bacterial dysbiosis might contribute to psychiatric (neurologic) disturbances seen in patients with intestinal diseases.

Lee, Manezes, Umesaki and Mazmanian (2011) confirmed the existence of gut-brain connections by describing how changes in gut microbial composition affect the brain. They characterized the microbiota during the induction of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis. Mice maintained under GF conditions develop significantly attenuated EAE and lower levels of proinflammatory cytokines compared with CC mice. However, when GF animals were colonized with segmented filamentous bacteria (common commensal inhabitants of the mouse gut) they developed the disease following immunization with central nervous system antigens.

Interestingly, chronic treatment with *Lactobacillus rhamnosus* has been shown to reduce anxiety and also reduce stress-induced corticosteroid responses when given to adult CC animals. The effect of the probiotic was not present in vagotomized mice, thus implicating the vagus nerve as one of the important pathways for bidirectional communication between the intestinal microbiota and the brain (Bravo et al., 2012). The effect of probiotics was also demonstrated in a mouse model of autism. The study by Hsiao et al. (2013) describes improvement in the animals' intestinal permeability, communication, as well as stereotypic and anxiety-like behaviors when they were treated with the commensal organism *Bacteroides fragilis.* Experimental mice exhibiting abnormal communication and repetitive behaviors improved when they were given one of the two strains of *Bacteroides fragilis.* In another study, introduction of *Lactobacillus reuteri* into drinking water of mice from mothers fed high fat diets increased oxytocin level in the offspring and improved their sociability and preference for social novelty (Poutahidis et al., 2013).

Research suggests that the microbiota may be implicated in neurological conditions and that some products of bacterial metabolism may affect not only animal but also human behavior (Collins & Bercik, 2013). Analysis of brain responses in a healthy woman using fMRI brain imaging demonstrated that ingestion of fermented milk with probiotics affects activity regions of the brain that control emotion and sensation (Tillisch et al., 2013). In addition, alterations in microbial composition or in their metabolic products may play a role in the pathophysiology of psychiatric disease or in chronic abdominal pain syndromes such as irritable bowel syndrome (Rao et al., 2009; Jeffery et al., 2012). Thus, the effect of substances produced in the intestinal microbiome on brain function is reported not only in animals but also in humans.

Collins, Surette, and Bercik (2012) discussed the mechanisms by which bacteria access the brain and influence behavior. They include bacterial products that gain access to the brain via: (i) the bloodstream and the area postrema; (ii) cytokine release from mucosal immune cells; (iii) the release of gut hormones such as serotonin from the enteroendocrine cells; or (iv) via afferent neural pathways including the vagus nerve. The critical role of microbiota in regulating host serotonin level was confirmed by Yano et al. (2015), who demonstrated that indigenous spore-forming bacteria from the mouse and human microbiota promote serotonin biosynthesis from colonic enterochromaffin cells. They also proposed a model in which microbial-derived metabolites, such as short-chain fatty acids (butyrate, acetate) or secondary bile acids act directly upon enterochromaffin cells, inducing transcription of the rate-limiting serotonin biosynthetic enzyme.

Taken together, these studies lend strong support to the concept that the presence of intestinal bacteria influences the development of neuronal circuitry that is relevant to a broad spectrum of activities, including anxiety-like behavior, motor control, memory and learning. They support the hypothesis that intestinal microbiota influence the gut–brain axis and may be implicated in neurological conditions such as autism. However, it is still not clear whether gut dysbiosis affects brain development and function, or whether changes in the intestinal microbiota are regulated by neural regulation of gut function.

5. Gender dimorphism hypotheses

Many genes have been associated with ASD (Abrahams & Geschwind, 2008). As gender difference is one of the most apparent features of ASD, attempts have been made to identify genes that could explain the male-biased prevalence; (Schaafsma & Pfaff, 2014; Pfaff et al., 2011; Lai, Baron-Cohen, & Buxbaum, 2015; Werling & Geschwind, 2013) however, the mechanism(s) underlying the gender-dependent effect of a particular gene(s) is not known and perturbations in gene expression alone are not able to explain the gender-specific prevalence of ASD (Schaafsma & Pfaff, 2014).

The prevalence of autism in boys stimulated studies of androgens in children with ASD. Some of these studies demonstrated a positive correlation between fetal testosterone in amniotic fluid with behaviors associated with autism in boys ages 18–24 months or 6 to 10 years (Baron-Cohen et al., 2011; Auyeung, Taylor, Hackett, & Baron-Cohen, 2010). These observations supported the "extreme male brain" hypothesis by Baron-Cohen et al. (Baron-Cohen, 2002) that fetal testosterone may drive cognitive hyper-masculinization in ASD and that this hormone level correlates positively with traits associated with autism and inversely with social development and empathy. Subsequent studies have found elevated androstenedione (intermediate in testosterone biosynthesis)

levels in serum from adults with ASD compared with controls irrespective of gender (Ruta, Ingudomnukul, Taylor, Chakrabarti, & Baron-Cohen,2011) and increased free androgen index in females with Asperger's syndrome as compared with controls (Schwarz et al., 2011).

However, a study of unaffected Japanese adults (Takagishi et al., 2010) challenged this hypothesis. The purpose of this study was to examine the relationship between salivary testosterone levels and traits associated with autism in adults using Japanese version of Autism-spectrum Quotient. In a study of 92 individuals, no correlation was found between these indices in males and females. There are also observations that children with high levels of prenatal testosterone did not develop ASD, indicating that prenatal testosterone alone may not be sufficient to be the trigger for ASD (Whitehouse et al., 2012). Plasma levels of testosterone and the adrenal androgen dehydroepiandrosterone (DHEA) sulfate were similar in pre-pubertal and post-pubertal subjects with autism and in unaffected controls (Tordjman, Anderson, & McBride, 1995). Also, no relationship was found between early postnatal testosterone concentrations in saliva and traits associated with autism in 18 to 30-month-old children (Kung, Constantinescu, Browne, Noorderhaven, & Hines, 2016). Jamnadass et al. (2015) compared traits associated with autism in young adults with concentrations of testosterone, androstenedione, DHEA, and estrogens in umbilical cord blood, but did not find any association with androgens or the androgen/estrogen ratio. In summary, these data indicate that the relevance of androgens during the prenatal or early postnatal period of life is undetermined, and may not contribute to the subsequent development of autism.

Another hypothesis to explain gender dimorphism in ASD, is the combine elevated levels of prenatal testosterone with prenatal stress. Triggers might include maternal stress that activates the maternal immune system, exposure to high level of gonadal hormones, inflammatory agents, endocrine disrupting chemicals or other environmental factors that may induce epigenetic changes (Schaafsma & Pfaff, 2014; Stilling, Dinan, & Cryan, 2014).

Pfaff et al. (2011) suggested the so-called "three hit" hypothesis of autism where the first hit is genetic, the second hit is prenatal stress and the third hit is the testosterone surge. According to this hypothesis, high levels of androgens in the male will increase the vulnerability to early stressors in genetically vulnerable individuals. This neurobiological theory is based on animal studies to explain male predominance in ASD. "In young male animals, testosterone binds to androgen receptors in brainstem neurons responsible for enhancing brain arousal. As a consequence, arousal-related neurotransmitters bombard the amygdala hypersensitized by testosterone acting though androgen receptors. Arousal-related inputs prime amigdaloid mechanisms for fear and anxiety, with resultant social avoidance." The authors suggest that similar mechanisms contribute to the observed male predominance in people with autism. Thus, this ASD dimorphism theory is based on two interacting factors: the molecular effects of testosterone in genetically vulnerable boys and environmental stresses they might experience *in utero,* in the perinatal period, or during the first years of life.

Many brainstem neurons are androgen-sensitive and secrete specific arousal-related neurotransmitters such as norepinephrine, dopamine and serotonin. Boys are exposed to higher levels of testosterone from uterine life causing arousal-related neurotransmitters to increase activation of the amygdala, making them more sensitive to the detrimental effects of early stressors (Hamson, Jones, & Watson, 2004). Pfaff et al. (2011) hypothesize that unlike boys, girls do not face the problem of their amygdala being stimulated by testosterone-driven arousal-causing inputs, and, additionally, are protected by estrogenic hormones, oxytocin and oxytocin receptors. Interestingly, children with autism express low plasma oxytocin levels (Modahl et al., 1998), and oxytocin administration improves sociability and decreases repetitive movements in children with high functioning autism (Andari et al., 2010; Green & Hollander, 2010).

There are also other gender-specific differences between boys and girls with autism. Recent proteomic analysis of males and females with Asperger's syndrome found sex-specific protein changes in their serum. Adult females had alterations in proteins involved mostly in lipid transport and metabolism pathways; whereas, adult males showed changes predominantly in inflammatory signaling (Steeb, Ramsey, & Guest, 2014). Earlier studies of intestinal disaccharidases in children with autism demonstrated that boys below 5 years of age had statistically lower lactase activity than girls of the same age (Kushak, Lauwers, Winter, & Buie, 2011).

The preponderance of ASD-like behaviors in males including defective social interaction similar to that of patients with autism was demonstrated in experiments on rats in the valproic acid model of autism (Kim, Kim, & Go, 2013). Another study using the valproic acid autism mouse model found disturbed social interaction, increased expression of neuroinflammatory markers, deficits in the serotonergic system in brain and intestinal tissue, and increased levels of cecal butyrate in males as compared with females (de Theije, Koelink, & Korte-Bouws, 2014).

The intestinal microbiota have been associated with many inflammatory disorders (Simon & Gorbach, 1986; Power et al., 2014). Some believe that gut microbiome may interact with sex hormones to modulate innate and adaptive immunity, disease progression and autoimmune disorders (Gomez, Luckey, & Taneja, 2015; Rubtsova, Marrack, & Rubtsov, 2015). We hypothesize that microbiota are associated with ASD prevalence in boys either directly or through microbial metabolites and/or epigenetic factors capable of regulating host gene expression through DNA methylation and/or histone modification (Stilling et al., 2014). Intestinal microbiota and/or its metabolites might also interact with human androgens. If this were the case, the intestinal microbiota hypothesis would support the "three hit hypothesis". Perturbations in the microbiome could represent an environmental stress whose significance has thus far not been appreciated.

There are some experimental data that support this hypothesis. Studies on animal models indicate that microbial exposure affects sex hormone levels and that both exert an effect on autoimmune diseases. Markle, Frank and Mortin-Toth, (2013) demonstrated that early-life microbial exposure determines sex hormone levels and modifies progression to autoimmunity in a non-obese diabetic mouse model of type 1 diabetes. Colonization by commensal microbes elevated serum testosterone and protected male mice from diabetes. Transfer of gut microbiota from adult males to immature females altered the recipients' microbiota, resulting in elevated testosterone. Thus, the commensal microbial community is capable of altering sex hormone levels and regulating the development of autoimmune disease.

Another study reported that bacterial colonization of the intestine has a major role in the development and maturation of the immune and endocrine systems and eventually central nervous system signaling. Studying the effect of microbiome on the serotonergic system, Clarke et al. (2013) found that only male GF animals have a significant elevation in the hippocampal concentration of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid, its main metabolite, compared with conventionally colonized control animals. Gender specificity was shown also for tryptophan, the precursor of serotonin, whose concentration was increased in the plasma of male GF animals. These observations support a humoral route through which the microbiota can influence CNS serotonergic neurotransmission. In the same study the reduced anxiety in GF animals was normalized following restoration of the intestinal microbiota. In a study using the valproic acid autism model in mice, the microbiota differences in males deviated from those observed in females. These differences were positively associated with increased levels of cecal butyrate as well as ileal neutrophil infiltration and inversely associated with intestinal levels of serotonin and social behavior scores (de Theije, Wopereis, & Ramadan, 2014). The most direct demonstration of the effect of microbiota or its products on gender dimorphism came from a recent study by Schaafsma et al. (Schaafsma et al., 2017) on a mouse model of ASD. In this study pregnant mice were treated with lipopolysaccharide (LPS), components of the cell wall of Gram-negative bacteria, that induced maternal immune activation. This treatment affected social responses in the offspring of these mice, but only in the males. The authors conclude that interaction between genetic model for ASD (contactin-associated protein-like 2 mouse) and environmental factors (LPS) are associated with the male predominance in ASD.

Information on the effect of gender on intestinal microbiota in humans is very limited. In a cross-sectional study on 230 healthy subjects at four European locations in France, Germany, Italy, and Sweden, gender effects were observed for the *Bacteroides-Prevotella* group, with higher levels in males than in females (Mueller, Saunier, & Hanisch, 2006). In another study on 200 patients with enteric infections, the genus *Bacteroides* was higher in females while *Escherichia* predominated in males (Singh & Manning, 2016). Thus, gender seems to affect the intestinal microbiota in both animal models and humans. The observed effects of the role of the microbiome on behavior, may offer a novel explanation for the striking difference in the incidence of autism spectrum disorders in males and females and suggest an area of research in need of new knowledge.

6. Conclusion

A strong male prevalence in ASD is well known, but no mechanism for this phenomenon has been established. Multiple studies suggest that hormones, particularly testosterone, might be involved in male bias; however, there are observations indicating that androgens alone *per se* might not explain the behavioral abnormalities in boys with autism. Some authors hypothesize, based on animal research, that a combination of prenatal testosterone and prenatal stress in genetically susceptible individuals may explain male prevalence in ASD (“three hit hypothesis”). Multiple studies highlight the importance of the effect of environmental factors, including intestinal microbiota and its metabolites, in the etiology of ASD. Some investigators suggest that genetic and environmental factors are responsible for ASD. In both cases, the involvement of intestinal microbiota appears to be relevant, since it plays a significant role in gut-brain relationships. The gender dimorphism hypothesis of autism presented in this review is based on differences in intestinal microbiota observed in male and female laboratory animals and in humans. The confirmation of this hypothesis can be achieved through a careful analysis of intestinal microbiota and the metabolome in boys and girls with autism in comparison with neurotypical individuals. Understanding the pathogenesis for the preponderance of males with autism may open new vistas for ASD prevention and treatment and provide greater insight into the neurobiology of the disorders.

Conflicts of interest

RIK does not have conflict of interests. HSW has potential sources of conflict of interests in the past three years:
Entity/Company Type of conflict
Pediatric IBD Foundation Scientific advisor, grant support
Janssen Pharmaceutical Consultant, grant support
Prometheus Consultant
Salix Consultant
AstraZeneca Consultant, grant support
Shire Consultant, grant support
UCB Consultant, grant support
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