Chapter 14
Deciphering the Gut Microbial Contribution to the Etiology of Autism Development

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Abstract Autistic spectrum disorder (ASD) is a spectrum of early-onset lifelong neurodevelopmental disorders that severely impact social and behavioral functioning. It is a debilitating disorder that affects 1 % of the global children population with increasing prevalence and presented huge economic burden to the family and the nation. Current diagnosis for ASD is very subjective mainly because of the multifactorial nature of the disorders. The etiology of ASD is highly complex and multifaceted involving the gene, environment, and diet and is associated with various abnormalities that include immunologic, metabolic, and, more recently, the host–gut microbiome stability (Fig. 14.1). The gut microbiota is a consortium of bacteria that coexisted and coevolved with the host from the time of birth. As such the gut microbiome–mammalian "superorganism" represents a level of biological evolutionary development where true symbiosis is characterized by extensive "transgenomic" modulation of metabolism and functions between the two entities. The gut microbiota is involved in various mammalian biological processes including defense against pathogens, immunity, intestinal microvilli development, and recovery of metabolic energy through fermentation of otherwise nondigestible dietary fiber. In addition, the gut microbiota has been shown to communicate with the brain via the gut–brain axis to modulate brain development, function, and behavior. Recent evidence indicated that the gut microbiota influenced central nervous system development and responses to stress. Current understanding on the potential and extend of gut microbe involvement in brain development and host metabolic signaling is still in its infancy. Coupled with ever-increasing awareness on the importance of the gut microbiome in health and disease particularly autism, understanding the fundamental mechanistic interaction between host brain development and gut microbiota is crucial for unraveling the mystery behind the etiopathology of autism.

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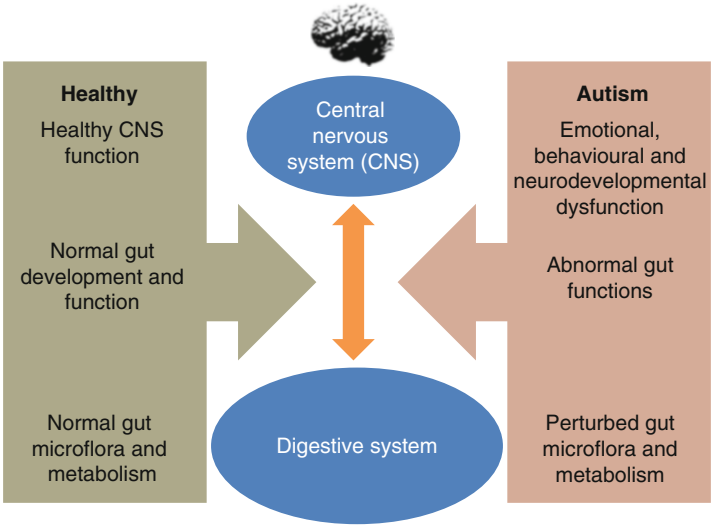

Fig. 14.1 Scheme summarizing the main factors related to gut–brain axis associated with brain development and autism

Keywords Autism • Disease • Gut–brain axis • Gut microbiota • Health • Mass spectrometry • Metabonomics • Metagenomics • Nuclear magnetic resonance spectroscopy • Nutrition • Systems biology

14.1 Gut–Brain Axis in Health and Disease

The term "gut–brain" or "brain–gut" axis is increasingly employed to define a bidirectional neurohumoral communication system. It comprised of neural pathways and humoral pathways, which include cytokines, hormones, and neuropeptides as signaling molecules [1]. The brain–gut–enteric microbiota axis includes the central nervous system, the neuroendocrine and neuroimmune systems, the sympathetic and parasympathetic arms of the autonomic nervous system, the enteric nervous system, and the intestinal microbiota [2]. Through this bidirectional communication network, signals from the brain can influence the motor, sensory, and secretory modalities of the GIT, and reciprocally, visceral messages from the GIT can influence brain function [2]. Recent evidences showed that gut microbiota communicates with the brain via the gut–brain axis to modulate brain development and behavioral phenotypes. In particular, this system provides the intestinal microbiota and its metabolites with a potential route towards the brain. Consequently, the gut microbiota could associate with brain functions as well as neurological diseases via the gut–brain axis [3], and further insights would require a better characterization of the composition and the metabolic activities of the gut microbiota. However,

identification of microbes constituting gut microbiota has been the main technological challenge currently due to massive amount of intestinal microbes and the difficulties in culture of gut microbes [3]. In parallel, many challenges remain to better assess, understand, and modulate gut microbial metabolic activities and their influence at panorganismal scale [4].

If recent studies have highlighted the depth of microbiota influence on the development and function of the host brain, one of the first observations resulted from the beneficial impact of orally administered antibiotics in reversing encephalopathy in patients with decompensated liver disease [5]. In addition, accumulating evidence further describes a relationship between psychiatric and gastrointestinal tract (GIT) disorders, such as irritable bowel syndrome (IBS) or inflammatory bowel disease (IBD), that are also associated with disturbances of the intestinal microbiota [6]. As highlighted in Chap. 14 of this book, there is compelling evidence that the brain may influence gastrointestinal functions (such as motility, secretion, and mucin production) as well as immune functions, and therefore being a direct vehicle for mediating the effects of emotional factors such as stress or depression influence [7–10].

These GIT disorders are also intimately related with gut dysbiosis [11], illustrating the potential brain influences on the microbial composition and activity along the GIT and reciprocally for the microbiota to modulate host metabolism [1]. Moreover, the gut microbiota, the intestinal mucosa, and the intestinal immune system issue multiple signals from the gut to the brain carried by sensory neurons, immune mediators, gut hormones, and microbiota-derived signaling molecules [12]. For instance, the influence of the gut microbiota on the development of the central nervous systems and stress responses was recently documented [13]. In this, two specific interactive systems are being highlighted, namely, the hypothalamus–pituitary–adrenal axis and the vagus nerve, as important means of communicating signals from gut microbes to the central nervous systems. Furthermore, recent efforts focused on the members of the neuropeptide Y (NPY) family of biologically active peptides, NPY, peptide YY (PYY), and pancreatic polypeptide (PP) [12]. PYY and PP are exclusively expressed by endocrine cells of the digestive system, whereas NPY is found at all levels of the gut–brain and brain–gut axis. Recent studies have extensively described how PYY is influenced by the intestinal microbiota, with particular interest in appetite regulation in the context of obesity pandemic. Due to its multilevel homeostatic mechanism, pharmacological manipulation of NPY–Y receptor system may have considerable therapeutic efficacy in many common metabolic and GIT disease in addition to psychiatric disorders.

Several lessons learned so far are mainly based and limited to preclinical studies [7], especially using gnotobiotic and germfree animal models. Such systems models have enabled rediscovering the multiple and complex facet of the interaction with the gut microbiota at multi-compartmental levels [4, 14–16]. The influence of the gut microbiota on the nervous system, brain development, and behavior, in particular during microbial colonization of the host, has recently been receiving profound interest [17]. In particular, the metabolic modulation of metabolites influencing functions of the nervous system, such as tryptophan and kynurenine levels, further illustrates the functional microbiota–neurohumoral relationship during gut colonization.

Moreover, novel evidence describes how gut microbiota type and presence can impact the cerebral biochemical profiles [18], including cerebral glycolytic metabolism.

14.2 Brain–Gut–Microbe Communication in Health and Disease

Autistic spectrum disorder (ASD) is a spectrum of neurological disorders characterized by a complex lifelong neurodevelopmental and sociological disorder with poorly defined etiology. ASDs are associated with an array of disabilities such as social withdrawal, speech impairment, and repetitive behavior [19] (Fig. 14.1). According to a recent estimate from the Centers for Disease Control and Prevention (CDC)'s Autism and Developmental Disabilities Monitoring (ADDM) Network, about 1 in 88 children, from the 14 communities of the network within the United States, have been identified with an ASD [20]. A recent global estimate by Elsabbagh *and coworkers* puts the global prevalence estimate to be about 62 in 10,000 [21] or 1 % of the global children population had an ASD [20]. More alarmingly, there has been a significant increase in incidence of ASD worldwide, and within the United States, between 2002 and 2008, there has been a 57 % increase in incidence of ASD [20]. ASD places a large economic burden on society with the cost of the disorder on the UK economy estimated to be £2.7 billion [22]. The lifetime cost for someone with ASD and intellectual disability is estimated at approximately £1.23 million and for someone with ASD without intellectual disability is approximately £0.80 million [22]. It has been reported that the ASDs affect all racial, ethnic, and socioeconomic group and that boys are five times more likely to have ASDs as compared to girls [20]. Studies have shown that among identical twins, if one child has an ASD, then the other will be affected about 36–95 % of the time. In nonidentical twins, if one child has an ASD, then the other is affected about 0–31 % of the time [23–25]. Parents who have a child with an ASD have a 2–18 % chance of having a second child who is also affected [26, 27]. ASDs tend to occur more often in people who have certain genetic or chromosomal conditions. About 10 % of children with autism are also identified as having Down syndrome, fragile X syndrome, tuberous sclerosis, and other genetic and chromosomal disorders [28–31]. A number of known disorders such as phenylketonuria and Smith–Lemli–Opitz syndrome have been shown to be associated with the ASD behavioral traits in children. These metabolic disorders are mainly autosomal recessive genetic disorders that present within the first 3 years of life. Current diagnosis for ASD is through a set of criteria defined in the Diagnostic and Statistical Manual of Mental Disorders 4th Edition (DSM-IV-TR) and behavioral observations made by clinician thus making diagnosis very subjective. The ongoing controversy about the precise definition of ASD stems from the current lack of understanding of the underlying causes of ASD and its multifactorial nature.

14.3 Gut Microbiota and ASD

Gastrointestinal dysfunction has been reported in ASD children [32], and studies have suggested that the condition may be associated with abnormal gut microbiota. Given the importance of the microbiome in mammalian metabolism, e.g., bile acid metabolism, there is a possibility of previously unrecognized etiologic connections between microbiome disorders and childhood developmental problems. Individuals with ASD are commonly exposed to repeated courses of multiple antibiotic therapies, and this may contribute to the complex relationships between gastrointestinal symbiosis and ASD by altering the composition or stability of their gut microbiota.

One of the very first suggestions of the potential involvement of bacteria in ASD was the publication by Bolte et al. where it was hypothesized that ASD may be linked to low-grade intestinal infection with *Clostridium tetani* [33]. It is well known that majority of children with ASD undergo extensive antibiotic therapy. Oral antibiotics can disrupt the stability and integrity of the "normal" gut microbiota thus resulting in an environment for opportunistic pathogens to colonize. One of this opportunistic pathogen is *C. tetani. Clostridium* belongs to the phylum of *Firmicutes*. They are rod-shaped obligate anaerobes that produce endospores [34]. Some of the most important biological pathogens belong to this genus of bacteria, namely, *C. botulinum*, *C. difficile*, and *C. tetani*, associated with botulism, antibiotic-associated diarrhea, and tetanus, respectively. *Clostridium* species such as *C. botulinum* and *C. tetani* are known to produce neurotoxins, which trigger the very clinical pathological manifestation that they are associated with. It has been shown that toxin produced by *C. tetani* in the intestine of experimental animals can be transported to the central nervous system via the vagus nerve resulting in the disruption of neurotransmitters release [35, 36]. It was suggested that such inhibition may lead to the myriads of behavioral deficits observed in children with ASD.

Sandler and colleagues conducted a small cohort study of oral vancomycin in autistic children which subsequently proved the hypotheses [37]. It was reported in the study that children receiving vancomycin treatment showed improvement in gastrointestinal problems such as abdominal pain, constipation, and/or diarrhea. In addition, behavioral improvements were observed with significant reduction in aggressive behavior, increased eye contact, and significant improvement in language and speech. However, such changes were dependent on vancomycin treatment and all children relapsed after discontinuation of the antibiotic. The work by Sandler et al. presented one of the first clear scientific evidences on the link between gut–brain axis and ASD. Several studies had subsequently indicated that children with ASD have perturbed gut microbiota as compared to typically developing children. To investigate further the potential involvement of *Clostridium* species (spp.) in ASD, Finegold et al. studied the feces of children with ASD and compared with healthy controls. It was found that children with ASD have higher levels of *Clostridium* spp. as well as greater species variation [38]. A subsequent study by the same group showed that children with ASD have elevated C. *bolteae* and *Clostridium*

clusters I and XI [39]. Around the same time, a study conducted by Parracho et al. comparing feces of children with ASD versus healthy siblings and healthy unrelated controls showed that children with ASD has higher levels of certain *Clostridium* spp. and, more interestingly, the healthy siblings had an intermediate level between their siblings with ASD and those of the healthy unrelated controls [40]. Further, a recent pyrosequencing study on fecal microbiota composition between typically developing controls versus ASD children and their normal functioning siblings showed that children with ASD had higher level of *Bacteroidetes* and lower level of *Firmicutes* as compared to the controls [41]. In addition, children with ASD also had lower level of several *Bifidobacterium* species while *Desulfovibrio* was higher [32, 41]. A study by Wang et al. also showed lower level of *Bifidobacterium* and *Akkermansia muciniphila* in ASD children [42].

In fact, the study by Finegold et al. showed that *Desulfovibrio* was present in half of the autistic subjects and in some siblings. More interestingly, none of the control subjects had *Desulfovibrio* [32]. Children with ASD are known to be sulfur deficient. Aldred et al. showed that individuals with autism have lower levels of plasma sulfate but considerably elevated levels of urinary sulfate as compared to normal individuals [43, 44]. These data suggest that autistic individuals may have impaired detoxification potential involving sulfation as evidenced by their inability to sulfate acetaminophen [44]. The presence of sulfate-reducing bacteria such as *Desulfovibrio* in ASD children could be one of the reasons behind the abnormality observed in sulfur metabolism. Moreover, the severity of ASD behavior is positively correlated with increased *Desulfovibrio* species [32].

14.4 Metabonomics in ASD

Finding the cause of ASD has proved challenging due to the multifactorial nature of the disorder, which also means that finding biochemical markers for ASD has remained elusive thus far. However, successful discovery of a set of specific and accurate biomarkers for ASD would not only help in understanding the pathophysiology of the condition but would, together with behavioral assessment, immensely help in the diagnosis of ASD thus allowing the possibility of early detection and thereby allowing early targeted intervention, which could possibly reduce severity of ASD [19]. Metabonomics or metabolic profiling approach is becoming increasingly important in identifying biomarkers of disease progression and drug intervention and can provide additional information to support or aid the interpretation of genomic and proteomic data. Since metabolic phenotypes are the results from the interaction between host genome [45] and the environment including diet and host microbiome [46, 47], perturbation in such complex interactions will lead to altered metabolic profiles, which can be studied using metabonomic approaches [48–52].

One of the earlier works on urinary phenotyping of autistic children was carried out by Lis et al. [53], where urine samples from autistic ($n = 19$) and normal (n = unknown) children were analyzed using anion exchange chromatography.

The study showed that autistic children have abnormal levels of urinary hippurate, 4-hydroxyhippurate, and *N*-methyl-2-pyridone-5-carboxamide (2PY) as compared to normal controls. It was postulated in the study that such observation in the urinary profiles could be due to several factors including involvement of gut microbiota and potential perturbation in endogenous metabolism. Hippurate is predominantly formed by hepatic glycine conjugation of dietary and gut microbial-derived benzoate, which is derived from plant phenolics [54]. Decreased urinary levels of hippurate could be an indication of reduced benzoic acid synthesis by the gut microbiota. The work by Lis et al. highlighted the potential of urine as a biochemical window into understanding ASD and a viable biomatrix for biomarker discovery and potentially diagnosis. The first metabonomic study on ASD was conducted by Yap et al. utilizing proton nuclear magnetic resonance (^{1}H NMR) spectroscopy-based metabonomic approach [55]. The study looked at the metabolic profiles of children diagnosed with ASD together with their non-autistic siblings and age-matched healthy volunteers. The main findings from the study showed alterations in nicotinic acid metabolism and gut microbe metabolism with increased urinary levels of 2PY, *N*-methyl nicotinic acid, and *N*-methyl nicotinamide and decreased levels of urinary gut microbe co-metabolites such as hippurate, phenylacetylglutamine, and 4-cresol sulfate. These observations were in agreement with the findings from Lis et al. [53] implicating the involvement of gut microbe in ASD and proved that metabonomic is an effective tool to aid understanding of the etiology of ASD and biomarker discovery. The study by Yap et al. also revealed differences in urinary amino acid levels such as glutamate, alanine, glycine, and taurine [55]. More intriguingly, the study also showed that the metabolic profiles of non-autistic siblings were quite different from their autistic siblings as well as the age-matched healthy volunteers. Such pattern was also observed by Parracho et al. when comparing the levels of *Clostridium* spp. between children with ASD versus healthy siblings and healthy unrelated controls, which showed that the healthy siblings had an intermediate level between their ASD siblings and the healthy unrelated controls. Such observation could indicate that the presence and levels of certain gut microbe could trigger the onset of ASD, which could lead to perturbation in the metabolic profiles and warrant further investigation.

Ming et al. utilized mass spectrometry-based metabonomic approach to further investigate metabolic perturbations in ASD children versus controls with the aim of identifying more specific biochemical disturbances linked to the pathogenesis of ASD [56]. The study showed that individuals with ASD showed differences in urinary amino acids such as glycine, alanine, and taurine as well as gut microbe co-metabolites such as propionic acid derivatives and bile acids. Results from this study validated the findings by Yap et al. [55], which showed perturbation in gut microbial co-metabolism. Furthermore, the study by Ming et al. also showed lower levels of urinary antioxidants carnosine and urate indicating potential increase in oxidative stress. More recently, Emond et al. evaluated the use of gas chromatography-coupled mass spectrometry metabonomic approach to study the urinary biochemical profiles of autistic versus healthy children [57]. The study

successfully differentiated autistic from healthy children, and several metabolites were identified to be significantly contributing the differences. Urinary metabolites succinate and glycolate were found to be higher in autistic children, whereas metabolites such as hippurate, 3-hydroxyphenylacetate, 3-hydroxyhippurate, and several other metabolites were found to be lower in autistic children. Interestingly, the urinary metabolites that were lower in autistic children were largely gut microbe co-metabolites.

Results from all four studies indicated a common factor that is the perturbation of gut microbe co-metabolites in ASD individuals [53, 55–57]. The later three studies [55–57] also demonstrated the potential of metabonomic as a noninvasive tool to study and understand the etiopathophysiology of ASD. In addition, the biochemical changes observed from these studies may provide novel biomarker information applicable for diagnostic and monitoring therapeutic interactions in the condition. The key findings on the role of the microbiome in brain development and the etiology and development of autism were summarized in Table 14.1.

Table 14.1 Role of the microbiome and microbial–host co-metabolites associated with brain development and autism: overview of key references

Biological compartment	Main findings	Refs
Microbiome	Certain bacteria produce neurotoxin that can be transported to the central nervous system via the vagus nerve. Bacteria implicated: *Clostridium* species; *Clostridium tetani*	[35, 36]
	Vancomycin-treatment leads to short-term improvement in gastrointestinal problems and behavioral improvements	[37]
	Higher levels and greater variation of *Clostridium* species were found in children with ASD, and normal functioning siblings of autistic children have intermediate level of *Clostridium* species between ASD and healthy unrelated controls. Bacteria implicated: *Clostridium* species	[38–40]
	Children with ASD were reported to have higher level of *Bacteroidetes* and lower level of *Firmicutes*. Bacteria implicated: *Bacteroidetes*; *Firmicutes*	[41]
Metabolome	Abnormal levels of urinary hippurate, 4-hydroxyhippurate, and *N*-methyl-2-pyridone-5-carboxamide (2PY) found in ASD children	[53]
	First metabonomics study on ASD. Reported increased urinary levels of 2PY, *N*-methyl nicotinic acid, and *N*-methyl nicotinamide and decreased levels of urinary gut microbe co-metabolites such as hippurate, phenylacetylglutamine, and 4-cresol sulfate in ASD children	[55]
	Metabolic profiling showed differences in urinary amino acids, i.e., glycine, alanine, and taurine, as well as gut microbe co-metabolites, i.e., propionic acid derivatives and bile acids, between normal and ASD children	[56]
	Found levels of urinary succinate and glycolate higher in ASD children and levels of urinary hippurate, 3-hydroxyphenylacetate, 3-hydroxyhippurate lower in ASD children	[57]

The growing ASD incidence attracts interest in better defining the role of recent changes in food intake and exposure in the etiology of the disease. For instance, essential fatty acids taken in diets mediate brain functions and structures during development and are involved in many brain-related disorders like autism [58]. Among the various lipid species, cell membrane components, including mainly phospholipids, are very rich in PUFAs in brain tissue, with AA and DHA representing up to 20 % of the dry brain weight [58]. Abnormalities in the fatty acid compositions of phospholipids have been implicated in several neurodevelopmental disorders that manifest with psychiatric symptoms. In particular, alteration in fatty acids and phospholipids, including not only reduced levels of n-3 PUFAs but also increased levels of saturated fatty acids in the red blood cell membrane [59] or in plasma [21], was described in autistic subjects. In particular, blood plasma of autistic patients showed an increase in most of the saturated fatty acids except for propionic acid and a decrease in most of polyunsaturated fatty acids, which could relate to multifactorial processes ranging from oxidative stress to mitochondrial dysfunction and lead to induced metabolic alterations in Saudi autistic patients [60]. The concomitant alteration in phospholipase activity associated with decreased levels of AA, docosatetraenoic acid, and DHA in red blood cell membranes from autistic subjects further supports a fundamental role of the phospholipid metabolic regulation in autism and the potential role of nutritional intervention for future prevention strategies [61]. This was recently exemplified by El-Ansary et al. [58] in a study comparing the relative concentrations of essential fatty acids (linoleic and alpha-linolenic), their long chain polyunsaturated fatty acids, and phospholipids in plasma of autistic patients from Saudi Arabia with age-matching controls. They reported a significant modulation of the metabolism of fatty acids, as assessed via an alteration of the ratio between essential fatty acids/long chain polyunsaturated fatty acids and omega-3/omega-6 fatty acids, and a decrease in circulating levels of phospholipids. The authors provide particular emphasis on phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine lipid species which could be used as potential biomarkers for future treatment or prevention strategies.

14.5 Therapeutic Perspectives

The potentiality of using metabolic and gut microbial metabolic markers for future therapeutic perspectives is significant but so far at their infancy, due to the yet limited definition and understanding of the processes leading to the gut–brain axis dysfunction in ASD. One should separate the nutritional approaches aiming at prevention from management of specific conditions or disease stages. Once additional and more consolidated phenotype characterizations of the human host–microbiome are available, more studies should be dedicated to investigate metabolic features associated with the gradual development of the ASD dysfunction. In particular, family at-risk subpopulations should be defined and studied in order to generate further hypotheses on environmental and nutritional strategies for prevention and management.

14.6 Conclusions

Comprehensive and long-term phenotyping of populations at risk is envisioned to provide some key and still missing insights into understanding mechanisms involved into the pandemic development of ASD. In this, novel methodologies, enabling to rediscover the intimate relationships with the gut functional ecology and interactions along the gut–brain axis, are foreseen as a fundamental cornerstone of the molecular mechanisms at play. The molecular hypotheses about etiology of the metabolic phenotype are still highly debated, but they suggest that patients should be screened for their microbiota for therapeutic strategies or preventive programs, which could benefit from novel and minimally invasive systems biology approaches.

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