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Review

Pathways underlying the gut-to-brain connection in autism spectrum disorders as future targets for disease management

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ABSTRACT

Autism spectrum disorders (ASDs) are pervasive neurodevelopmental disorders, characterized by impairments in social interaction and communication and the presence of limited, repetitive and stereotyped interests and behavior. Bowel symptoms are frequently reported in children with ASD and a potential role for gastrointestinal disturbances in ASD has been suggested. This review focuses on the importance of (allergic) gastrointestinal problems in ASD. We provide an overview of the possible gut-to-brain pathways and discuss opportunities for pharmaceutical and/or nutritional approaches for therapy.

Contents

1. Introduction

Autism spectrum disorders (ASDs) comprise autism, pervasive developmental disorder not otherwise specified (PDD-NOS) and Asperger's disorder. These pervasive neurodevelopmental disorders are characterized by impairments in social interaction and communication and the presence of limited, repetitive and stereotyped interests

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and behaviors (Johnson and Myers, 2007; Vandereycken, 2003). So far, no biomarkers for ASD have been identified. Therefore, diagnosis of ASD is entirely dependent on behavioral observations, according to the DSM-IV criteria (American-Psychiatric-Association, 2000). Literature suggests that the prevalence of ASD has increased 20 times, from a rate around 1:2500 in the mid 1980s to a rate of 9:1000 at present (Genuis, 2009; MMWR, 2009). Nowadays, early diagnostic tools are available (Luyster et al., 2009) and diagnostic stability has been established (Zwaigenbaum et al., 2009). Although many believe that the ASD escalation is a consequence of better and earlier diagnosis, improved awareness and expanding criteria to fulfill the diagnosis, some believe that these changes do not adequately account for the rapid rise (Hertz-Picciotto and Delwiche, 2009).

Current interest in research on ASD is boosted, but the underlying pathophysiology of the disorder remains unknown. Despite the importance of genetic factors, as indicated by the high concordance rates among twins (Bailey et al., 1995), ASD is most likely a multifactorial disease, in which a combination of genetic disturbances and environmental factors play a role in the expression of the autistic phenotype. Currently, many environmental factors, both pre- and postnatal, are found to be associated with ASD, including gastrointestinal disturbances. Although data are conflicting and more studies are required to establish the prevalence of gastrointestinal disorders in the autistic population, bowel symptoms in autistic patients are repeatedly reported. This review focuses on the importance of (allergic) gastrointestinal disturbances in ASD. We provide an overview of the possible gut-to-brain pathways and discuss opportunities for pharmaceutical and/or nutritional approaches for therapy.

2. Gastrointestinal disturbances in ASD

Due to social and communicative impairments, identifying gastrointestinal problems in patients with ASD is extremely challenging. Many autistic patients have verbal impairments, which makes it almost impossible for them to express their discomfort. Even autistic individuals who are verbally skilled may be less able to express their feelings, because of their social disabilities. Therefore, it is difficult to determine the true prevalence of gastrointestinal disturbances in the autistic population. The reported prevalence ranged from 9% to 91.4% (Black et al., 2002; Galli-Carminati et al., 2006; Ibrahim et al., 2009; Mouridsen et al., 2009; Parracho et al., 2005; Smith et al., 2009; Valicenti-McDermott et al., 2006), an immense dispersion that is partially due to different interpretations of 'gastrointestinal problems'. Frequently observed symptoms among autistic patients include chronic constipation or diarrhea, abdominal pain and pathological observations such as food allergy, gastroesophageal reflux disease (GERD), enteric colitis, lymphoid hyperplasia and oesophagitis (Horvath et al., 1999; Wakefield et al., 2005). Pang and Croaker (2011) determined the incidence of ASD among patients presented to their Paediatric Surgical Constipation clinic. ASD appeared to be almost 10 times more common in the constipation clinic (8.5%) than in the general population (0.9%). Even more recently, Peeters et al. (2011) performed a similar study, determining the prevalence of ASD in children presented at their clinic with functional constipation or functional non-retentive fecal incontinence. Remarkably, 18% of the children had scores indicative for ASD. The study of Pang and Croaker (2011) also showed that the onset of constipation was earlier in patients suffering from autism and moreover, earlier than the average onset of autism in a different study (Ibrahim et al., 2009). From this, they suggested that constipation is an intrinsic rather than secondary factor in the development of ASD. Ibrahim et al. (2009) were unable to find significant differences between the overall prevalence of gastrointestinal problems in ASD compared to controls, but they did identify a higher prevalence of constipation (ASD: 33.9% vs. controls: 17.6%; $P = 0.003$). In a different study, diarrhea was linked to ASD as well (Sandhu et al., 2009). At 30 and 42 months of age, children with ASD were more likely to have two or more stools a day and the incidence of diarrhea was significantly enhanced in the autistic group compared to controls (ASD: 58% vs. controls: 44%; $P = 0.039$).

3. Gastrointestinal pathology in ASD

Three studies investigated enteric lymphocyte infiltration in biopsies of children with ASD and found remarkable results (Ashwood et al., 2003; Furlano et al., 2001; Torrente et al., 2002). Compared to histologically non-inflamed controls, there was a higher number of infiltrated helper and cytotoxic T cells and $CD19^+$ B cells in biopsies of the duodenum, terminal ileum and colon of autistic patients with gastrointestinal disturbances (Ashwood et al., 2003; Furlano et al., 2001; Torrente et al., 2002). Furthermore, even compared to histologically inflamed controls, there was more infiltration of helper T cells and $CD19^+$ B cells in all three intestinal compartments of these autistic children (Ashwood et al., 2003). Even more surprisingly, helper T cell infiltration was also more enhanced in the terminal ileum and colon of these children with autism, compared to children suffering from inflammatory bowel disease (Ashwood et al., 2003). In a different study, enhanced density of dendritic T cells was observed in the colon of ASD children with gastrointestinal disturbances compared to histologically non-inflamed controls, and even compared to controls suffering from lymphoid nodular hyperplasia, Crohn's disease and ulcerative colitis. Basement membrane thickness was enhanced as well, compared to all other groups. However, histopathology demonstrated that lymphocytic colitis was less severe in autistic children than in classical inflammatory bowel disease (Furlano et al., 2001). Furthermore, on the basolateral enterocyte membrane of autistic children with gastrointestinal disturbances, deposition of IgG1 and IgG4 was shown to be accumulated compared to normal controls and celiac patients (Torrente et al., 2002).

A factor that might contribute to the gastrointestinal disturbances among autistic individuals is an abnormal composition of gut microbiota. Several groups have studied the intestinal microbiota of the autistic population and found a different composition of several microbial species compared to healthy controls. These ASD-related microbial species mainly comprised various *Clostridium* strains, *Ruminococcus*, *Bacteroidetes*, *Bacteroides*, *Firmicutes* and *Desulfovibrio* species (Finegold et al., 2002, 2010; Parracho et al., 2005; Song et al., 2004). A recent paper by Adams et al. (2011) demonstrated lower levels of *Bifidobacterium* and higher levels of *Lactobacillus* (all strains) in ASD, both considered to be beneficial bacteria (Adams et al., 2011). Colonization of *Clostridum* species to the expense of *Bifidobacterium* have been associated with higher risks of food allergy in children and with the development of (pediatric) inflammatory bowel diseases as well (Adlerberth et al., 2007; Schwiertz et al., 2010; Vanderploeg et al., 2010; Willing et al., 2010). Interestingly, antibiotic treatment of ASD children did not only lead to gastrointestinal improvements, but also improvements in cognitive skills (Sandler et al., 2000).

The data on gastrointestinal disturbances, such as changes in gut microbiota and T cell infiltration, strongly indicate an altered immune status in the intestine of autistic individuals. It is unknown whether the association between autistic behavior and gastrointestinal disturbances is a cause-and-effect relationship and what factor could be the intrinsic one. Given the fact that gastrointestinal disturbances are strongly correlated with the severity of autistic behavior (Adams et al., 2011), we hypothesize that the presence of gastrointestinal inflammation makes a child with a genetic predisposition for ASD more prone to express the autistic phenotype or that it increases the severity of autistic behavior. In Fig. 1, the possible pathways are depicted in which immune factors of gastrointestinal origin can influence neuronal functioning and thereby behavior.

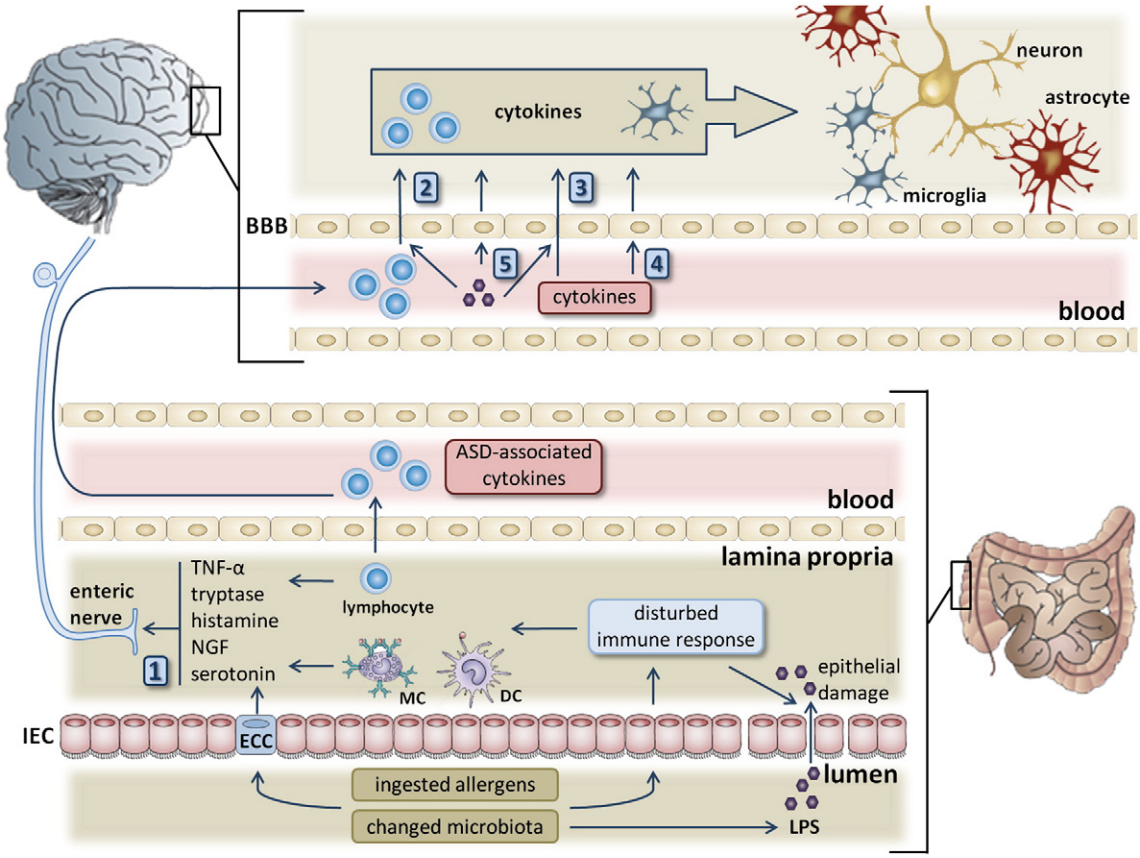

Fig. 1. *Possible pathways involved in neuroimmune interactions in ASD.* Upon immune disturbance in the gastrointestinal tract, intestinal epithelial cells (IECs) become more permeable and enterochromaffin cells (ECCs), lymphocytes, mast cells (MCs) and dendritic cells (DCs) secrete all kinds of neuroimmune factors that can stimulate enteric nerves (1). In addition, ASD-associated cytokines (IL-1β, IL-4, IL-5, IL-6, IL-12, IL-13, IFN-γ, TNF-α) and lymphocytes are present in the circulation. Subsequently, lymphocytes can pass the blood-brain barrier (BBB) (2), serum cytokines (IL-1β, IL-6, IFN-γ, TNF-α) can pass the blood-brain barrier (3) and cytokines (IL-1β, TNF-α) can bind to brain endothelial cells inducing an immune response at the brain side (4). LPS can increase the permeability of the blood-brain barrier, enhancing cytokine and lymphocyte infiltration, or bind to brain endothelial cells inducing an immune response at the brain side (5). The immune response in the brain can consist of an increased number of lymphocytes and cytokines (IL-1β, IL-6, CXCL-8, IL-10, IFN-γ, TNF-α, CCL-2 and GM-CSF), also produced by neuroglia, resulting in changed neuronal homeostasis.

4. Neuroimmune interactions at the side of intestinal inflammation

4.1. Pathway of intestinal inflammation

The gastrointestinal tract continuously encounters dietary antigens and bacteria and their products. Therefore, it is a crucial site of innate and adaptive immune regulation. Ingested antigens enter the gut mucosa through the microfold (M) cells in the Peyer's patch or through damaged epithelium, from where they are transferred to or directly taken up by antigen presenting cells (APCs). APCs, most likely dendritic cells (DCs), move to T cell areas, such as the Peyer's patch or mesenteric lymph node (MLN), where they interact with naïve lymphocytes to initiate an adaptive immune response. Upon repeated encounter of the antigen, memory T and B cells are activated, resulting in a proliferative response and cytokine release, leading to gastrointestinal inflammation (Mowat, 2003). Chronic inflammation in the gut can damage the epithelial cell layer and thereby increase intestinal permeability, resulting in a higher antigenic load. Intestinal permeability was found to be enhanced in autistic patients (D'Eufemia et al., 1996). Recently, de Magistris et al. (2010) confirmed these findings by demonstrating significantly increased intestinal permeability in children with ASD and their first-degree relatives (de Magistris et al., 2010). Abnormal high intestinal permeability was observed in 36.7% of the patients with ASD, compared with none of the age-matched controls. Among the first-degree relatives, 21.2% showed abnormal high intestinal permeability compared with 4.8% of the adult controls. The enhanced intestinal permeability observed in the autistic population could be both the cause and the result of inflammation in the gastrointestinal tract of these children. Nevertheless, high intestinal permeability enhances gastrointestinal inflammation and thereby worsens gastrointestinal discomfort.

4.2. Serotonin: neurotransmitter and mediator of inflammation

The serotonergic system has been implicated in the pathogenesis of ASD since increased levels of blood serotonin (5-hydroxytryptamine; 5-HT) were first described in children with autism (Schain and Freedman, 1961). Subsequent studies demonstrated that about one-third of the patients with ASD has blood hyperserotonemia (Anderson et al., 1987; Hanley et al., 1977). On the other hand, the capacity of 5-HT synthesis in the global brain was decreased in children with autism (Chugani et al., 1999), indicating a lower brain 5-HT availability. The cause of ASD-related hyperserotonemia is thought to arise from genetic (Coutinho et al., 2007), gastrointestinal (Minderaa et al., 1987; Mulder et al., 2010) or immune (Burgess et al., 2006; Warren et al., 1986) changes. Based on intestinal low-grade inflammation, blood hyperserotonemia and low 5-HT synthesis in the brain, we propose the following hypothesis. During an inflammatory response in the gut, 5-HT is produced and released by enterochromaffin cells and intestinal inflammatory cells such as mast cells and platelets, resulting in a faster moving gut and an increase in secretion, vasodilatation and vascular permeability. This, in turn, leads to problems in functional

dysmotility, stool consistency (diarrhea or constipation) and infiltration of leukocytes in the intestinal wall. Because of the increased utilization of dietary tryptophan by the gut, there will be less tryptophan available for passage through the blood-brain barrier. As a result, brain 5-HT levels are reduced and this may lead to mood and cognitive dysfunctions found in ASD. Indeed, the availability of tryptophan was demonstrated to be important, since depletion of tryptophan from the diet increased autistic behavior in affected adults (McDougle et al., 1996). More research is required to establish whether 5-HT metabolism can be a therapeutic target in ASD, either by providing dietary tryptophan or by pharmaceutical treatments such as selective serotonin reuptake inhibitors (SSRIs). Recently, it was reported that there is no evidence for a beneficial effect of treatment with SSRIs in autistic children and only limited evidence exists for the effectiveness of SSRIs in adults suffering from ASD (Williams et al., 2010). Perhaps, targeting the ASD-associated low-grade intestinal inflammation might be more successful in restoring the availability of tryptophan for 5-HT synthesis in the brain.

4.3. Food Allergy in ASD

A disturbed intestinal immune reaction can be directed against food particles, initiating an allergic response. Food allergy has often been suggested to be present among autistic individuals. Parental reports indicate that food allergy is more common in the autistic population compared with healthy controls (Gurney et al., 2006; Jyonouchi et al., 2008). It is important to take into account that ASD children are likely underdiagnosed for food allergies, because of their impaired ability to express their discomfort. Lucarelli et al. (1995) observed that an oral challenge with cow's milk protein led to worsening of some of the behavioral symptoms specific for ASD. They also found significantly higher serum levels of IgA, IgG and IgM for casein and IgA for lactalbumin and β-lactoglobulin in children with ASD compared with healthy controls (Lucarelli et al., 1995). Furthermore, the intake of milk protein was a significant predictor of constipation in the autistic population (Afzal et al., 2003). Therefore, patients with ASD often exclude gluten and milk protein from their diet, better known as a gluten-free, casein-free diet. Some publications on gastrointestinal disturbances in ASD compared ASD patients on a gluten and milk free diet with ASD patients on an unrestricted diet. For instance, eosinophil infiltration in intestinal biopsies of children with regressive autism and gastrointestinal disturbances was significantly less abundant in those on a gluten and milk free diet compared with those on an unrestricted diet (Ashwood et al., 2003). Moreover, the ASD patients that excluded gluten and milk proteins, showed a significant reduction in the enhanced intestinal permeability compared with ASD patients on a unrestricted diet (de Magistris et al., 2010). In addition to the beneficial effects on gastrointestinal disturbances, a gluten and milk free diet was claimed to improve autistic behavior as well. Indeed, parents reported improvements in social behavior and linguistic skills (Elder et al., 2006). Few studies have been performed on the efficacy of a gluten and casein elimination diet in autistic individuals, showing improvements in rituals, verbal communication, interpersonal relations and learning (Hsu et al., 2009; Knivsberg et al., 2002; Millward et al., 2008; Whiteley et al., 2010). Unfortunately, these studies comprised either small cohort studies or case reports and could therefore not confirm the beneficial outcome of a gluten-free, casein-free diet. More research is necessary to strengthen these findings.

The majority of allergies is characterized by a T helper (Th) 2-type immune reaction. Th2 effector cells produce Th2 cytokines (interleukin (IL)-4, IL-5 and IL-13) and can activate memory B cells to secrete immunoglobulins (Valenta, 2002). Supporting the suspected role of allergy in ASD, there seems to be an imbalance in Th1 and Th2 cytokines in these patients. Indeed, peripheral blood mononuclear cells (PBMCs) of children with ASD produced significantly higher levels of IL-4, IL-5 and IL-13 than their matched controls (Molloy et al., 2006). In blood of ASD children, interferon (IFN)-γ and IL-2 positive helper and cytotoxic T cells were less abundant than in blood of healthy controls. In contrast, IL-4 positive helper and cytotoxic T cell numbers were enhanced (Gupta et al., 1998). In addition to these data on a disturbed Th1/Th2 balance, a lower IFN-γ/IL-10 ratio was observed in male rats prenatally exposed to valproic acid, a well-characterized animal model for autism (Schneider et al., 2008). In response to cow's milk protein, PBMCs from ASD children with and without gastrointestinal disturbances produced more tumor necrosis factor (TNF)-α and IL-12 than those from control subjects (Jyonouchi et al., 2005). Furthermore, there were less IL-10 positive T cells present in both the periphery and the gut mucosa of ASD children with gastrointestinal symptoms, compared with non-inflamed controls and children with Crohn's disease (Ashwood and Wakefield, 2006). T cells that produce the anti-inflammatory cytokine IL-10 are mainly inducible T regulatory cells. Allergen-specific T regulatory cells are predominantly present in healthy individuals to suppress an allergic response. Less IL-10 positive T cells are therefore associated with enhanced Th2 responses. Plasma levels of another T regulatory cytokine transforming growth factor (TGF)-β, were decreased as well, as observed by two groups (Ashwood et al., 2008; Okada et al., 2007). Low TGF-β levels were inversely correlated with behavioral scores (Ashwood et al., 2008). This indicates that regulatory T cell responses are decreased in individuals with ASD and that the lack of suppressive capabilities of the immune system could be involved in the expression of autistic behavior.

During an allergic reaction, immunoglobulins activate mast cells and basophils, causing the release of various mediators, including histamine and cytokines. Mast cell activation has been suggested to play a role in autistic disorders as well. This hypothesis is supported by a preliminary report, indicating that ASD is more prevalent in patients with mastocytosis than in the general population (Theoharides, 2009). Not only immunoglobulins, but also several neuropeptides can trigger mast cell activation, including substance P, nerve growth factor (NGF), vasoactive intestinal peptide (VIP) and neurotensin (Theoharides et al., 2004). Neurotensin was significantly increased in serum of children with ASD (Angelidou et al., 2010). Upon activation, mast cells can express various substances that can trigger enteric neurons, such as tryptase, histamine, 5-HT, NGF and TNF-α (Rijnierse et al., 2007) (Fig. 1: pathway 1). Mast cell–neuron interactions occur in the gastrointestinal tract, for instance in inflammatory bowel disease and irritable bowel syndrome (Rijnierse et al., 2007). Therefore, an allergic reaction in the gut might influence behavior via mast cells or other immune cells, which are able to trigger enteric neurons to convey information through afferent pathways in vagal and spinal nerves to the central nervous system (CNS).

4.4. Association between ASD and maternal allergic diseases

Cumulating to the importance of allergy in the pathophysiology of ASD is the finding that mothers, diagnosed for asthma or allergies (such as atopic eczema and rhinitis) during the second trimester of their pregnancy, had a greater than two fold elevated risk for ASD in their offspring (Croen et al., 2005). In addition, there was an enhanced association observed between allergic conditions and autism in families with more than one ASD-affected child. This observation suggests that genes underlying atopy may be related to the etiology of ASD. (Croen et al., 2005). Recently, King (2011) hypothesized that epigenetic disruption of brain development is caused by gestational exposure to allergy-associated inflammatory mediators (for example IL-6 and histamine) (King, 2011). These mediators promote retinoic acid and estradiol gene transcription, resulting in overexposure of the fetus to retonic acid and estradiol. Retinoic acid (a vitamin A metabolite) is required for growth and development. An excess in vitamin A or retinoic acid is associated with brain abnormalities reminiscent of those present in ASD, such as cerebellar malformations, cranial nerve abnormalities and abnormalities of the dopaminergic system (London, 2000). Estradiol is known to defeminize the fetal brain, playing an important

role in sexual differentiation. Overexposure to estrogen affects a wide range of cognitive functions which are characteristic for autistic individuals such as anxiety, motor deficits, stereotype and repetitive movements, hyperactivity and attention deficits (King, 2011; McEwen et al., 1999).

4.5. Other immune processes in ASD

Although many studies support the hypothesis that ASD is associated with a Th2-skewed immune response, there are also studies that indicate the involvement of other immune pathways. For instance, plasma levels of IL-12 and IFN-γ were shown to be increased in autistic individuals, suggesting rather an enhanced Th1 response instead of Th2 (Singh, 1996). Reduced cytotoxic activity of natural killer (NK) cells was also suggested (Vojdani et al., 2008). Recently, Ashwood et al. (2011a,b) reported increased plasma levels of a heterogeneous group of cytokines, including IL-1β, IL-6, CXCL8 and IL-12p40, making it even more difficult to identify a specific type of immune response. Furthermore, macrophage migration inhibitory factor (MIF), which is also constitutively expressed in brain tissues (Bacher et al., 1998) was enhanced in peripheral blood of autistic individuals compared to typically developing controls. The high plasma MIF levels were positively correlated to autistic behavior (Grigorenko et al., 2008). Chemokines CCL2, CCL5 and CCL11 were also enhanced in plasma of children with ASD, compared with healthy controls. The increased chemokine levels were associated with higher aberrant behavior scores (Ashwood et al., 2011b). The heterogeneity of autistic disorders may be the reason behind these conflicting data.

5. Neuroimmune interactions at the side of the blood-brain barrier

Immune cells produce all kinds of substances upon gastrointestinal inflammation, such as cytokines and chemokines. These immune cells and their substances are not restricted to the gut, but enter the circulation and will therefore pass all organs in the body, including the brain. The brain is a highly vascularized organ, but brain cells are protected from harmful compounds in the blood by means of the blood-brain barrier. This barrier is a layer of endothelial cells, cemented together with tight junctions. The cells lack intracellular fenestrations and have very little ability to undergo pinocytosis (Reese and Karnovsky, 1967). The uniquely modified endothelial cells prevent free transport of most soluble substances between blood and brain. However, cytokines are still able to cross the barrier by active transport and even immune cells can pass through tight junctions by diapedesis (Banks and Erickson, 2010). Therefore, gastrointestinal inflammation in autistic patients may influence the brain and thus behavior through many different pathways, as indicated in Fig. 1.

Although ASDs are considered neurodevelopmental disorders, the neuropathology remains poorly understood. Brain growth abnormalities are the most prominent findings in the neuropathology of ASD. The brain undergoes a period of rapid growth, followed by slow growth later in development (Courchesne et al., 2003). In addition to the abnormal growth patterns of the brain, one of the most consistent findings of neuroimaging studies in autistic individuals is the presence of abnormalities in the cerebellum, such as loss of Purkinje cells, increased cerebral white matter and thickening of cerebral cortex (Bauman and Kemper, 2003; Ecker et al., 2010; Schumann et al., 2010).

5.1. Lymphocytes enter the brain and influence neurons via the production of immune factors

The endothelial cell layer of the blood-brain barrier is surrounded by a basal lamina, which is in direct contact with pericytes and astrocytes, with microglia in close attendance. Physiological changes in neuroglial cells can influence the blood-brain barrier integrity and make it more permeable for lymphocytes (Banks and Erickson, 2010). Immune factors can also alter blood-brain barrier permeability. TNF-α, for instance, can disrupt the barrier by increasing P-glycoprotein expression (Bauer et al., 2007) and by altering brain endothelial cell cytoskeletal architecture (Deli et al., 1995). Lymphocyte migration over the blood-brain barrier occurs under healthy circumstances and lymphocytes are consistently present in the brain, but infiltration is highly increased upon immune activation (Fig. 1: pathway 2). After infiltration into the brain, lymphocytes secrete cytokines and chemokines that can activate microglia and thereby alter neuronal functioning, as described in section 5.2. One group studied the presence of lymphocytes in postmortem brains of autistic children, but could not identify lymphocyte infiltration or immunoglobulin deposition (Vargas et al., 2005). Therefore, it could be rather cytokines than lymphocytes crossing the blood-brain barrier and initiating an immune response.

5.2. Cytokines enter the brain and influence neurons via neuroglia

Numerous cytokines are able to cross the blood-brain barrier, for example IL-1β, IL-6, and TNF-α (Banks et al., 1994; Gutierrez et al., 1993; McLay et al., 2000; Pan et al., 1997). As mentioned, IL-1β and IL-6 plasma levels were shown to be enhanced in autistic patients and PBMC stimulation with cow's milk resulted in an enhanced TNF-α response (Ashwood et al., 2011a; Jyonouchi et al., 2005). Because these cytokines are able to cross the blood-brain barrier, they are important in neuroimmune interactions (Fig. 1: pathway 3). In the brain, these cytokines can interact with neuroglial cells to induce neuroinflammation. In the healthy CNS, astrocytes and microglia play important roles in neuronal function and homeostasis, as they are both fundamentally involved in cortical organization, neuroaxonal guidance and synaptic transmission (Fields and Stevens-Graham, 2002). Furthermore, astrocytes and microglia are also crucial for the regulation of immune responses in the CNS. Microglia are the macrophages of the brain and therefore, involved in immune surveillance (Aloisi, 2001). Astrocytes and microglia are able to produce neurotrophic factors, cytokines and chemokines (Bauer et al., 2001; Watkins and Maier, 2003) and are important in regulating the integrity of the blood-brain barrier (Prat et al., 2001). In response to an immune challenge, activated astrocytes and microglia can induce neuronal and synaptic changes, which modify CNS homeostasis and contribute to neuronal dysfunction during disease processes.

In postmortem brains of autistic patients, enhanced activation of astrocytes and microglia was observed (Vargas et al., 2005). Astrocyte activation was identified in the subcortical white matter of the midfrontal gyrus and the anterior cingulate gyrus and in the granular cell layer, the Purkinje cell layer and the white matter of the cerebellum. In addition, enhanced astrocyte activation was observed in the striatum, hippocampus and cerebral cortex of mice with fragile X syndrome (highly related to autism) (Yuskaitis et al., 2010). Microglia activation was predominantly observed in granular cell layer and white matter of the cerebellum of ASD brains (Vargas et al., 2005). It is unclear when or how neuroglia become activated in the brain of autistic patients. To investigate this, Vargas et al. (2005) additionally characterized cytokine and chemokines profiles in the midfrontal gyrus, the anterior cingulate gyrus and the cerebellum of ASD brains and cerebrospinal fluid. Enhanced levels of IL-6, IFN-γ, CCL2, CCL4, CXCL8 and CXCL10 were found in the cerebrospinal fluid of autistic children. In postmortem brains of autistic individuals, TGF-β was increased in all three brain regions (midfrontal gyrus, anterior cingulate gyrus and cerebellum) and pro-inflammatory chemokines CCL2 and thymus and activation-regulated chemokine (TARC) were increased in the anterior cingulate gyrus and the cerebellum. Furthermore, the anterior cingulate gyrus showed increased levels of a wide range of pro-inflammatory cytokines and chemokines, including IL-6, IL-10, CCL7, CCL22, CCL23, CXCL9, and CXCL13 (Vargas

et al., 2005). Another group measured cytokine profiles in the frontal cerebral cortex of ASD brains and observed enhanced levels of pro-inflammatory cytokines IL-6, TNFα and granulocyte macrophage colony stimulating factor (GM-CSF), Th1 cytokine IFN-γ and chemokine CXCL8 (Li et al., 2009). Because no enhanced lymphocyte infiltration was observed in the brains of autistic individuals, it may be more likely that neuroglia become activated upon stimulation by infiltrated cytokines. The activated neuroglia can produce immune factors, as described above, consequently adapt neuronal homeostasis and functioning, leading to alterations in behavior.

5.3. Immune factors influence neurons by binding to brain endothelial cells

Brain endothelial cells function as a barrier between blood and brain and regulate the infiltration of immune factors. In addition to this barrier function, brain endothelial cells are known to be activated by cytokines and to produce cytokines themselves (Fig. 1: pathway 4). IL-1β (Cao et al., 1996) and TNF-α (Bugno et al., 1999), two cytokines that are also relevant in ASD, can bind to brain endothelial cells and induce an immune response (Stanimirovic and Satoh, 2000). In turn, brain endothelial cells are important sources of pro-inflammatory mediators, such as prostaglandins, leukotrienes, cytokines and chemokines (Stanimirovic and Satoh, 2000; Vadeboncoeur et al., 2003; Verma et al., 2006). The factors that they produce, including cytokines such as IL-6, GM-CSF and TNF-α and chemokines like CCL2 and CXCL8 (Verma et al., 2006; Zhang et al., 1999), can be released both at the side of the brain and the blood vessel. When brain endothelial cells secrete immune factors at the brain side, astrocytes and microglia become activated and consequently influence neuronal functioning and thereby behavior.

5.4. LPS influences neurons via the blood-brain barrier

Lipopolysaccharide (LPS) plasma levels are enhanced in patients with severe autism. Moreover, LPS levels correlate with the severity of behavior in this subset of patients (Emanuele et al., 2010). LPS, the known TLR-4 ligand, is a major component of Gram-negative bacteria and high plasma levels of LPS are likely due to enhanced intestinal permeability. LPS is an important player in neuroinflammation, because of its influence on brain endothelial cells, which express TLR-4 (Nagyoszi et al., 2010). LPS can increase blood-brain barrier permeability through many different pathways (Jaeger et al., 2009; Wispelwey et al., 1988; Xaio et al., 2001) (Fig. 1: pathway 5). It can enhance endocytosis by brain endothelial cells (Banks et al., 1999) and facilitate immune cell trafficking (de Vries et al., 1994; Persidsky et al., 1997). Furthermore, LPS can stimulate brain endothelial cells to secrete cytokines (Reyes et al., 1999; Verma et al., 2006). Enhanced LPS levels in severe autistic patients may stimulate brain endothelial cells to secrete cytokines and can make the blood-brain barrier more permeable. This would enhance neuroinflammation and might therefore exacerbate behavioral deficits. This would explain the correlation between LPS levels and the severity of behavior in autistic individuals.

6. mTOR as a possible link between ASD-associated disturbances in immune system and CNS

The mammalian target of rapamycin (mTOR) is a highly conserved, intracellular serine/threonine kinase that regulates cell growth and metabolism in response to a wide variety of signals, including growth factors, nutrients, energy and inflammatory factors (Hay and Sonenberg, 2004; Sengupta et al., 2010; Wullschleger et al., 2006). mTOR belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and serves as the catalytic subunit of two structurally and functionally distinct multi-protein complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Fig. 2 depicts a schematic illustration of the major upstream and downstream mTOR signaling pathways. Rapamycin, which is a macrolide produced by soil bacterium *Streptomyces hygroscopicus* (Vezina et al., 1975), disrupts mTORC1 complex formation (Kim et al., 2002). mTORC2 shares several proteins with mTORC1 and was originally described as a rapamycin-insensitive complex, as acute rapamycin treatment is unable to inhibit mTORC2 (Sarbassov et al., 2005). However, subsequent studies have shown that, in some cell types, prolonged rapamycin treatment inhibits the assembly of mTORC2 (Sarbassov et al., 2006).

mTORC1 responds to growth factors such as insulin, through PI3K-AKT pathway, to regulate various cellular processes that are involved in cell growth and metabolism. The binding of insulin to its receptor on the cell membrane leads to the recruitment and phosphorylation of the insulin receptor substrate (IRS), which in turn, via a complex signal transduction, results in activation of Ras-like small GTP-ase, Rheb. Rheb was shown to directly bind to mTOR in mTORC1 and stimulate the catalytic activity of mTOR, inducing phosphorylation of specific targets that regulate protein synthesis and many other growth-related processes (Fingar and Blenis, 2004). Other upstream signaling cues of mTORC1 are nutrients, energy and inflammatory stress, such as cytokines and cross-linking of immunoglobulin receptors (Wullschleger et al., 2006). In contrast to mTORC1, relatively little is known about the signaling upstream of mTORC2. However, mTORC2 can indirectly activate mTORC1.

In patients with ASD, several mutations in genes are found that are strongly linked to the mTOR signaling pathway. Tuberous sclerosis is a genetic disorder caused by heterozygous mutations in the mTOR pathway related *Tuberous sclerosis complex* (*Tsc)1* or *Tsc2* genes and is commonly associated with the autistic phenotype. Mice with a heterozygous mutation in the *Tsc2* gene ($Tsc2^{+/-}$ mice) demonstrate enhanced mTOR signaling in hippocampus, which contributes to learning and memory impairments in $Tsc2^{+/-}$ mice. Treatment of adult $Tsc2^{+/-}$ mice with rapamycin reversed the learning and memory impairments (Ehninger et al., 2008). In addition, $Tsc1^{+/-}$ mice also displayed reduced levels of social behavior and cognitive function (Goorden et al., 2007). PTEN (phosphatase and tensin homolog deleted on chromosome ten) acts as a phosphatase that dephosphorylates one of the upstream TSC/mTOR-associated signal transduction molecules, resulting in enhanced activity of mTOR. Mutations in PTEN are associated with a wide variety of human neurological disorders, including ASD (Rosner et al., 2008). PTEN gene mutation analysis has been suggested for patients with macrocephaly, a condition that is observed in 20% of patients with ASD (Butler et al., 2005). *Pten* knock-out mice with deletion of *Pten* in neurons in the cortex and hippocampus develop autistic phenotypes such as macrocephaly and reduced social behavior. Moreover, changes in cell morphology have been observed, including neuronal hypertrophy and loss of neuronal polarity, which means that the establishment of axons and dendrites in these neurons is disrupted. Treatment with rapamycin in *Pten* knock-out mice reversed neuronal hypertrophy and macrocephaly and ameliorated ASD-related, abnormal behaviors (Zhou et al., 2009). Furthermore, mTOR is involved in protein synthesis-dependent synaptogenesis. Activation of mTOR pathway can increase the production of synaptic signaling proteins and the formation of new spine synapses in the prefrontal cortex of rats. mTOR inhibition with rapamycin blocked synaptic protein synthesis and antidepressant behavioral responses in rats (Li et al., 2010).

Currently, it is becoming more and more evident that mTOR also plays a central role in directing immune responses. A recent study suggests that Th1 and Th17 differentiation are specifically regulated by mTORC1 signaling. In contrast, Th2 differentiation is dependent on mTORC2 signaling, as T cells in which mTORC2 activity is eliminated failed to differentiate into Th2 cell both *in vitro* and *in vivo* but were able to differentiate into Th1 and Th17 cells (Li et al., 2010). Furthermore, it was shown that T cells differentiated into regulatory T cells in the presence of a conventional dose of rapamycin, which

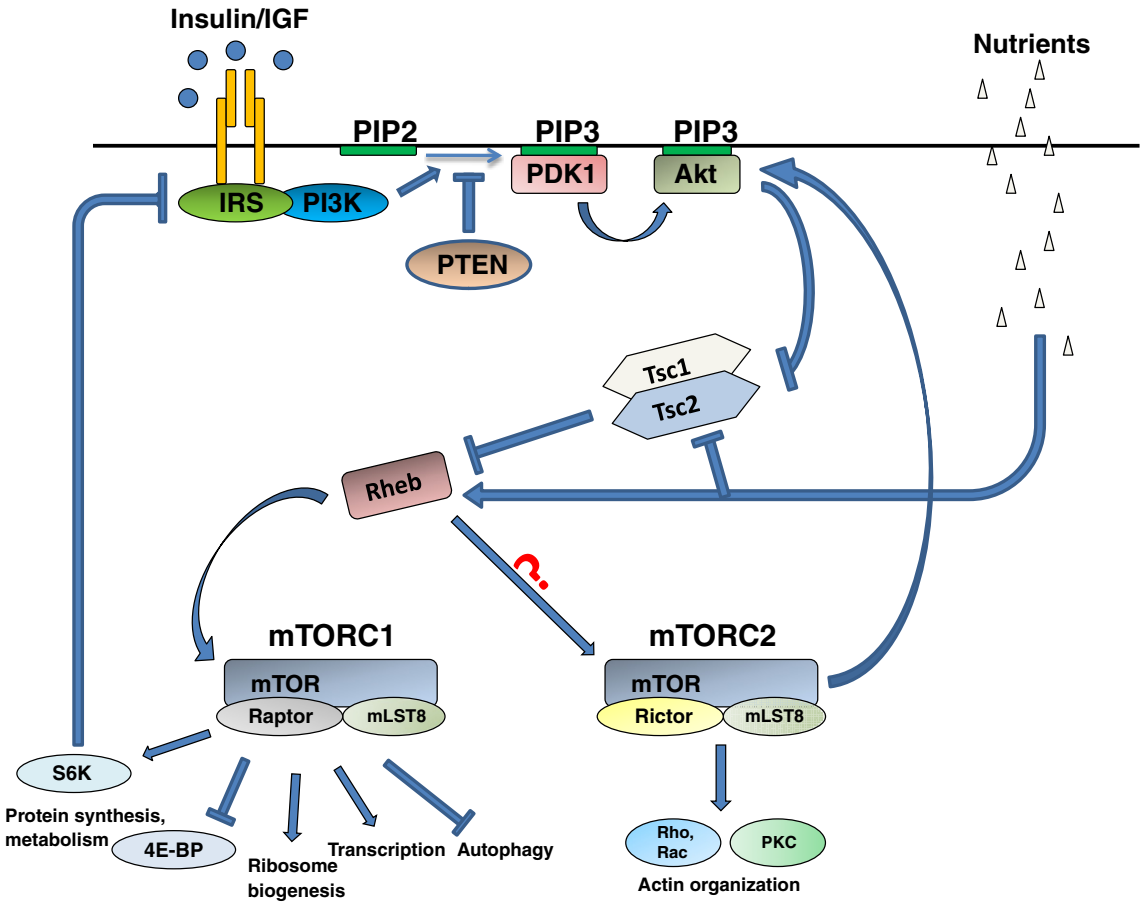

Fig. 2. *Schematic illustration of mTOR signaling pathway*. mTORC1 comprises four components apart from mTOR: regulatory associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8; also known as GβL), proline-rich AKT substrate 40 kDa (PRAS40), and DEP-domain-containing mTOR-interacting protein (Deptor). mTORC2 shares several proteins with mTORC1 and is composed of six different proteins: mTOR, rapamycin-insensitive companion of mTOR (Rictor), mammalian stressed-activated protein kinase interacting protein (mSIN1), protein observed with Rictor-1 (Protor-1), mLST8 and Deptor (Hay and Sonenberg, 2004; Sengupta et al., 2010). Two multi-protein complexes, mTORC1 and mTORC2, are centrally involved in the mTOR signaling network. mTORC1, which is rapamycin sensitive, is activated by growth factors through the PI3K/Akt signaling pathway and by nutrients, energy, stress, leading to the phosphorylation of S6K and 4EBP1 and thereby regulating protein synthesis and cell growth. In contrast to mTORC1, the upstream signaling of rapamycin insensitive mTORC2 is currently unknown. mTORC2 can directly phosphorylate Akt upstream of mTORC1 and thereby indirectly activate mTORC1. mTORC2 has also been involved in regulating cytoskeletal organization through the activation of PKC and RhoA and Rac1.

inhibits mTORC1 and mTORC2 (Li et al., 2010). Indeed, rapamycin-induced mTOR inhibition resulted in elevated Treg cells in tissue culture of nasal polyps obtained from patients suffering from chronic allergic rhinitis (Xu et al., 2009). Furthermore, mTORC1 activation in mast cells is associated with survival, differentiation, migration and cytokine production of the important 'allergic' cells (Kim et al., 2008). Finally, increased mTOR activity is shown to attenuate autophagy (Yu et al., 2010). This finding could explain the reduced clearance and maintenance of inflammatory cells at sites of allergic inflammation. In conclusion, because of its function in immune and neuronal pathways, mTOR may be a possible target for treatment in ASD.

7. Targeting the gastrointestinal tract in ASD

Many parents report that their autistic child suffers from gastrointestinal symptoms. This has led to research on the prevalence and characteristics of gastrointestinal disturbances in the autistic population. The contradictory results on the prevalence of gastrointestinal disturbances are likely due to different facts; interpretation of gastrointestinal symptoms, social and communicative impairments of patients and the heterogeneity of ASD. The severity of autistic behavior was shown to correlate with gastrointestinal disturbances, increased intestinal permeability, and enhanced serum LPS, cytokine and chemokine levels. Therefore, we hypothesize that children with a genetic predisposition are more susceptible for developing ASD when they suffer from immune disturbance or that the presence of gastrointestinal inflammation worsens behavior in children with ASD. This would mean that immunomodulatory dietary interventions (polyunsaturated fatty acids; PUFA and pre- or probiotics), allergen-free diets and pharmaceuticals (mast cell stabilizers and anti-inflammatory or immunosuppressive drugs) for the treatment of gastrointestinal inflammation could also be beneficial for the treatment of autistic behavior.

The use of Complementary and Alternative Medicine (CAM) practices for children with ASD is often reported (Golnik and Ireland, 2009; Hanson et al., 2007; Harrington et al., 2006; Levy et al., 2003; Levy and Hyman, 2003; Weber and Newmark, 2007; Wong and Smith, 2006). Examples of such treatments include the use of vitamin and mineral supplements, secretin, melatonin and gluten-free, casein-free diets (Levy et al., 2003). At this moment, approximately 50% of parents with an ASD child have tried CAM (Levy and Hyman, 2003), and half of these are using a gluten-free, casein-free diet (Levy et al., 2003). Results from a recent study indicate that gluten and milk free diets improve behavior in children with ASD (Whiteley et al., 2010). This result suggests the presence of food hypersensitivity or allergy in the autistic population. The gluten and milk elimination diet can be supported by the use of a free amino acid composition to avoid dietary insufficiencies. In addition to the elimination diet, dietary ingredients

such as omega-3 fatty acids and pre- and probiotics might be beneficial to the dietary management of autistic behavior and the associated gastrointestinal symptoms, because of their effects on CNS, immune system and/or on microbiota profile.

There is increasing evidence for prebiotics to have effects not only on enteric mucosa but also on systemic immunity. Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of *Bifidobacteria* and lactic acid bacteria in the colon, which are important markers of a healthy gut microbiota (Costalos et al., 2008; Frece et al., 2009; Langlands et al., 2004). The expertise on prebiotics originates primarily from the efforts to simulate the beneficial effects of breastmilk (Boehm et al., 2004a,b; van Hoffen et al., 2009). Human milk favors the growth of a "bifidus flora" which activates the immune system and defends from pathogens. A recent study shows that prebiotics have long bifidogenic effects in the intestines of infants (Salvini et al., 2011). Non-digestible oligosaccharides are examples of prebiotics and consist of naturally occurring sugar base units (e.g. glucose, fructose and galactose). These oligosaccharides are not hydrolyzed in the upper small intestine and reach the large intestine intact to serve as substrates for bacterial metabolism (Engfer et al., 2000). Non-digestible oligosaccharides were shown to be beneficial for disease progression and immune status in various studies, including murine models for allergy (Schouten et al., 2010) and clinical trials for treatment of allergy (van Hoffen et al., 2009). The immunomodulatory effects and potential working mechanisms of orally applied non-digestible carbohydrates are reviewed in Vos et al. (2007). Since the microbiota profile of ASD patients is enriched in pathogenic bacteria species (Finegold et al., 2002, 2010; Parracho et al., 2005; Song et al., 2004), which may exacerbate the disease (Sandler et al., 2000), these patients may benefit from dietary supplementation with a prebiotic mixture.

Probiotics may also be an option in the treatment of gastrointestinal problems observed in patients with ASD. Although no reliable conclusion can be drawn from the results on functional studies with probiotics, some evidence suggests that supplementation with probiotics is associated with a reduction in the risk of nonspecific gastrointestinal infections and lower frequency of colic or irritability (Braegger et al., 2011). Lower levels of the beneficial *Bifidobacterium* were observed in ASD patients. This bacterium has also been associated with food allergy and inflammatory bowel diseases, suggesting that the low levels might be an indication of gastrointestinal immune disturbances in ASD. Moreover, increased intestinal permeability was also observed in patients with ASD. Because probiotics were thought to reduce intestinal permeability and restore a 'healthy' gut, (Bodera and Chcialowski, 2009; Ramakrishna, 2009; Reid et al., 2011), there may be a beneficial effect of probiotics on gastrointestinal disturbances and behavioral deficits of autistic patients.

Another dietary intervention that may be beneficial for the ASD population is supplementation with PUFAs. Decreased levels of incorporated omega-3 PUFAs have been observed in peripheral blood cells of ASD patients repeatedly (Bell et al., 2004; Vancassel et al., 2001). After treatment with (omega-3 rich) fish oil, PUFA levels were enhanced and a decreased ratio of omega-6/omega-3 was observed (Meguid et al., 2008). Moreover, a significant improvement of behavior was observed after treatment of ASD patients with fish oil. The effect of PUFAs on autistic behavior may work via two different mechanisms. PUFAs are present in neuronal membranous phospholipids in the myelin sheath (Agostoni et al., 1995), where they modulate membrane fluidity and hence neuronal functioning, including receptor function and neurotransmitter release and uptake (Murphy, 1990). Indeed, deficiencies of omega-3 PUFAs lead to learning disabilities and memory loss (Lauritzen et al., 2001). Besides effects on the brain, omega-3 PUFAs have also been claimed to have a function in modulating the immune response. Omega-3 PUFAs can be incorporated in the membrane of immune cells, where they modulate intracellular pathways leading to an anti-inflammatory response (Calder, 2010). This anti-inflammatory response is mediated by a number of independent mechanisms. First, the effect of omega-3 is caused by replacing the pro-inflammatory omega-6 arachidonic acid. Second, omega-3 fatty acids give rise to the production of resolvins that can resolve inflammation (Serhan et al., 2008). Third, omega-3 fatty acids decrease the expression of adhesion molecules and prevent adherence of monocytes and macrophages (De Caterina and Libby, 1996; Miles et al., 2000). Finally, omega-3 fatty acids have been shown to decrease the production of inflammatory cytokines (Calder, 2008). This means that supplementation of omega-3 PUFAs could be beneficial for patients with ASD, because omega-3 PUFAs can act either directly on neuronal responses or indirectly via the immune system and gastrointestinal tract.

Nowadays, ASD treatment includes behavioral, educational and pharmacological therapy. No single drug has been proven to be effective for treating symptoms associated with autism. However, because many of the behavioral features are similar to serotonin-related disorders and because plasma hyperserotonemia is observed in about one third of the autistic population, SSRIs are often prescribed to ASD patients. It is hypothesized that gastrointestinal disturbances in ASD patients lead to high serotonin levels in the gut. This can be reflected by blood hyperserotonemia and consequently lead to reduced tryptophan availability for the brain, resulting in decreased serotonin synthesis in the brain. This hypothesis would suggest that it might be more effective to combine SSRIs with dietary interventions that reduce gastrointestinal disturbances. The strong gut-to-brain connection described in this review provides a compelling opportunity to target the brain via the gut and the immune system by using nutritional interventions.

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