INSIGHTS INTO THE MOLECULAR MECHANISMS OF BACTERIAL METABOLITES IN THE PATHOGENESIS OF AUTISM SPECTRUM DISORDER

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Insights into the molecular mechanisms of bacterial metabolites in the pathogenesis of autism spectrum disorder

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Chapter 1

General introduction

and

thesis outline

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairments in social interaction and communication, deficits in learning and memory activities and the presence of stereotyped behaviors [1]. ASD is generally diagnosed in early childhood and might persist across the whole lifespan [2]. Nowadays, ASD clinical diagnosis mainly relies on behavioral assessment due to the absence of reliable biomarkers [3, 4], leading to different prevalence of ASD across countries [5]. The ASD prevalence increased over past years, an epidemiological survey has recently estimated that the worldwide prevalence of ASD is 1% [6]. The 1% worldwide prevalence of ASD has been further demonstrated by Zeidan et al. Very recently using a systematic literature analysis published in peer-reviewed journals from 2012 onwards [7]. In addition, ASD prevalence shows a sex-difference and occurs more in male than in female with an approximate ratio of 4:1 [6, 7].

ASD etiology is thought to be associated with both genetic and environmental factors [8]. Genetic factors include many susceptibility genes, like Neuroligin-1 [9, 10] and Neural Glial-Related Cell Adhesion Molecules (NrCAM) [11], as well as Phosphatase and tensin homolog (PTEN) [12], which account for up to 20% of clinical ASD cases [13]. Environmental factors include prenatal exposure to medicine, such as the anti-epileptic drug Valproic acid (VPA), which has been widely used as a murine model to mimic ASD core symptoms [14]. In recent years, the abnormal changes at the level of the gut-brain axis has been considered to play an important role in the pathogenesis of ASD, which includes microbial dysbiosis, impaired intestinal and blood brain barriers, dysregulated synaptic function and derailed neuroimmune responses [15, 16].

In addition to behavioral deficits, ASD children have gastrointestinal (GI)comorbidities, which include abdominal pain, and constipation or diarrhea. These GI symptoms are related to impaired intestinal barrier and intestinal inflammation that are associated with changes in gut microbiota composition and activity [17-19]. E-cadherin is a junction protein and is one of the most important molecules involved in the maintenance of intestinal epithelial integrity. Decreased E-cadherin leads to the compromised intestinal barrier [20, 21]. In addition, Cyclooxygenase-2 (COX-2) as an inflammatory enzyme in the intestinal tract, and enhanced COX-2 expression causes intestinal inflammation [22, 23]. Changes in bacterial composition in ASD have been described repeatedly including changes in the relative abundances of specific intestinal bacteria, such as the families Clostridiaceae, Lachnospiraceae and Ruminococcaceae, which trigger the subsequent changes in levels of bacterial metabolites that include *para*-cresol (*p*-cresol) and 4-ethylphenol [24-27]. These two metabolites are produced by intestinal bacterial fermentation of the amino acids tyrosine and phenylalanine. The aforementioned bacterial metabolites are mainly transformed in the host into *Para*-cresyl sulfate (*p*CS) and 4-ethylphenyl sulfate (4EPS) by O-sulfonation primarily occurring in the host liver [24, 26, 28].

In recent years, *p*CS and 4EPS have been suggested to be involved in ASD. Increased levels of both metabolites in feces, urine and serum are reported in children diagnosed with ASD [29, 30]. Moreover, *p*CS has been proposed to be used as a biomarker for ASD diagnosis [31]. *P*-cresol administration in drinking water induces social behavior deficits and repetitive behavior in mice [32]. 4EPS administration into mice by intraperitoneal injection causes anxiety-like behavior that is a common co-morbidity and might contribute to core ASD symptoms [33]. In addition, in a clinical trial the GI-restricted adsorbent AB-2004 has shown to decrease urine *p*CS and 4EPS levels and to ameliorate ASD-like behaviors [34]. However, the molecular mechanisms of *p*CS and 4EPS in the pathogenesis of ASD remain to be investigated.

Both *p*CS and 4EPS can reach the brain since they have been detected in murine brain tissues and therefore might impact brain functions, such as synapse development and neuroimmune function [33, 35, 36]. Several researches have demonstrated that *p*CS can attenuate LPS-induced inflammation in macrophages and inhibits Th1-type immune responses in mice [37-

39], indicating that pCS might play a role in the derailed neuroimmune regulation associated with ASD. A Disintegrin And Metalloprotease 10 and 17 (ADAM10 and ADAM17) play important roles in brain function through controlling synapse development and neuroimmune function [16]. ADAM10 and ADAM17 are two single-pass type I transmembrane proteins that cleaves membrane-bound proteins [16, 40]. ADAM10 controls synaptogenesis through cleaving many synaptic molecules into soluble fragments that includes neuroligin-1 and NrCAM [41, 42]. As ASD susceptibility genes, both Neuligin-1-deficient mice and NrCAM-deficient mice show impaired social interaction and compromised cognitive function [9, 43]. Additionally, ADAM10 also regulates intestinal permeability through cleaving E-cadherin into soluble fragments [21, 44]. ADAM17 is also called TNF-α Converting Enzyme (TACE) and it regulates neuroimmune response via cleaving membrane-bound Tumor Necrosis Factor α (TNF-α) into soluble fragment [45]. In addition to cleaving TNF-α, ADAM17 regulates the production of Transforming Growth Factor beta 1 (TGF-β1) [46, 47]. TGF-β1 levels are decreased in the serum of children diagnosed with ASD and plays important roles in neuroimmune response, synaptic plasticity and brain development [48-51]. Furthermore, ADAM17 can be cleaved by other ADAMs to generate soluble ADAM17, and this soluble form has shown to be increased in in the brain tissues of ASD children [52, 53]. The inhibition of ADAM10 or ADAM17 showed to increase microglial phagocytosis that prunes synapse during postnatal development to maintain neuronal circuits and synaptic function [54, 55]. The elevated phagocytosis capacity of microglia early in life is associated with ASD-like behaviors in mice [56]. Additionally, ADAM10 and ADAM17 cleaves other ASD-associated neuroinflammatory cytokines including IL-6 receptor (IL-6R) and Tumor necrosis factor receptor 1 (TNFR-1) and other ASD-associated synaptic molecules including amyloid precursor protein (APP) and Neurexins [16, 41, 57, 58]. Therefore, investigating whether the interaction between the ASD-associated bacterial metabolites and ADAM10 and ADAM17 plays a role in ASD will contribute to unravelling the mechanisms of ASD pathogenesis and potentially identify novel targets for treatment of ASD-associated problems.

Apart from ADAM10 and ADAM17, Phosphatase and tensin homolog (PTEN) as a ASD susceptibility gene plays an important role in ASD etiology and might be a target for treatment of ASD-associated detrimental symptoms [12]. Up to 15% ASD children presents with macrocephaly that has been used for clinically diagnosing ASD [59, 60]. Germline mutation in PTEN occurs in up to 20% of adolescents diagnosed with macrocephalic ASD [61]. PTEN is a classical a classical tumor suppression gene that attenuates the expressions of COX-2 and E-cadherin to inhibit tumor growth through antagonizing phosphatidylinositol 3-phosphate kinase (PI3K)/AKT signaling [62, 63]. COX-2 and E-cadherin are associated with intestinal inflammation and permeability, respectively [20, 22]. It is still not clear whether PTEN is involved in enhanced COX-2 and reduced E-cadherin expression that possibly regulate the GI symptoms in ASD. In addition to these, PTEN is necessary to maintain brain development, neuronal circuit, synaptic function and microglial function [64, 65]. PETN deficient mice showed changes in the expression of synaptic proteins and exhibit impairments in social interaction together with repetitive behavior [66]. Furthermore, PTEN expression is shown to be decreased in the *in utero* VPA-induced murine model of ASD [67]. In addition, PTEN inactivation elevates both neuroinflammation and microglial phagocytosis activity in primary microglia and mice, which may be responsible for of ASD-like behaviors development in these in mice [65, 68]. Therefore, investigating whether bacterial-derived metabolites *p*CS and 4EPS affect PTEN expression will shed light on the mechanism of how the bacterial metabolites induce ASD.

Taken together, bacterial-derived metabolites *p*CS and 4EPS cause ASD-like behaviors, but the underlying mechanisms are not known. ADAM10, ADAM17 and PTEN affect synaptic function and neuroimmune response of microglia, which are vitally associated with ASD. For these

reasons, the dysregulation of these targets triggered by bacterial metabolites *p*CS and 4EPS might lead to ASD pathogenesis, which can direct us to better understand the mechanisms of ASD pathogenesis. In addition, ADAM10 regulates intestinal permeability through cleaving E-cadherin, leading ADAM10 as a 'linking pin' at the level of gut-brain axis.

Aims and outlines of this thesis:

This thesis aims to find out whether ADAM10, ADAM17 and PTEN, can be regulated by bacterial-derived metabolites *p*CS or 4EPS *in vivo* and *in vitro* to gain insight into the molecular mechanisms behind ASD. Furthermore, this thesis aims to identify ADAM10 as a potential therapeutical target for the treatment of detrimental symptoms in ASD. To this end, an *in vivo in utero* VPA-induced mouse model of ASD and an *in vitro* model of LPS-stimulated neuroinflammation in microglial cells were employed. All together the major objectives of this thesis are:

1. To investigate the involvement of ADAM10 and ADAM17 in ASD pathogenesis of *in utero* VPA-exposed male mice and in the *p*CS-induced neuroimmune response of microglial cells.
2. To investigate the effects of ADAM10 inhibition on ASD like symptoms including impaired intestinal barrier, compromised synaptic function and disturbed behavior.
3. To investigate the involvement of PTEN in ASD pathogenesis using a VPA induced mouse model for ASD as well as an *in vitro* model to study *p*CS- or 4EPS-induced neuroimmune response of microglial cells.

Chapter 2 gives a detailed background of the role of bacterial-derived metabolites *p*CS or 4EPS in ASD, including their generation from intestinal bacteria and their pathobiological functions in relation to ASD. **Chapter 3** elaborately introduces how ADAM10 and ADAM17 possibly can contribute to ASD pathogenesis by being the linking targets of the gut-brain axis and points out that targeting ADAMs might be a promising treatment for detrimental symptoms in ASD. **Chapter 4** demonstrates that *p*CS decreases both ADAM10 and ADAM17 in microglial cells through which *p*CS induces a derailed immune response. *In vivo* results further demonstrates that ADAM10 maturation is increased and ADAM17 maturation is decreased in the brain of *in utero* VPA-induced murine model for ASD which is associated the elevated *p*CS in the circulation. **Chapter 5** demonstrates that inhibiting ADAM10 might improve the compromised cognitive function while not affecting the disturbed social interaction in *in utero* VPA-exposed male mice. On molecular level, the inhibition of ADAM10 reduced the VPA-induced increases in Neuroligin-1 cleavage in the prefrontal cortex and E-cadherin cleavage in the colon to ameliorate synaptic dysfunction and impaired intestinal barrier, respectively. **Chapter 6** demonstrates that PTEN expression is decreased in the hippocampus but is not changed in the distal ileum of the *in utero* VPA-induced murine model for ASD. The *in vitro* results demonstrate that *p*CS and 4EPS reduces PTEN expression of microglial cells. However, PTEN is not involved in the *p*CS and 4EPS-induced dysregulated microglial immune responses. **In chapter 7**, we summarized main findings, discussed them, and provide suggestions for future studies.

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Chapter 2

The role of bacterial-derived aromatic amino acids metabolites relevant in autism spectrum disorders: a comprehensive review

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Abstract

In recent years, the idea of the gut microbiota being involved in the pathogenesis of autism spectrum disorders (ASD) has attracted attention through numerous studies. Many of these studies report microbial dysregulation in the gut and feces of autistic patients and in ASD animal models. The host microbiota plays a large role in metabolism of ingested foods, and through the production of a range of metabolites it may be involved in neurodevelopmental disorders such as ASD. Two specific microbiota-derived host metabolites, *p*-cresol sulfate and 4-ethylphenyl sulfate, have been associated with ASD in both patients and animal models. These metabolites originate from bacterially produced *p*-cresol and 4-ethylphenol, respectively. *P*-cresol and 4-ethylphenol are produced through aromatic amino acid fermentation by a range of commensal bacteria, most notably bacteria from the *Clostridioides* genus, which are among the dysregulated bacteria frequently detected in ASD patients. Once produced, these metabolites are suggested to enter the bloodstream, pass the blood-brain-barrier and affect microglial cells in the central nervous system, possibly affecting processes like neuroinflammation and microglial phagocytosis. This review describes the current knowledge of microbial dysbiosis in ASD and elaborates on the relevance and synthesis pathways of two specific ASD-associated metabolites that may form a link between the microbiota and the brain in autism. While the two discussed metabolites are promising candidates for biomarkers and (nutritional) intervention targets, more research into the role of these metabolites in ASD is required to causally connect these metabolites to ASD pathophysiology.

Keywords: autism, gut–brain axis, bacterial metabolites, *p*-cresol sulfate, 4-ethylphenyl sulfate

INTRODUCTION

Autism spectrum disorders (ASD) consist of a set of heterogeneous neurodevelopmental conditions, characterized by early-onset deficits in social communication and interaction, and unusually restrictive or repetitive behavior and interests [1]. According to data from the Autism and Developmental Disabilities Monitoring network from the U.S. Centers for Disease Control and Prevention (CDC), approximately 1 in 54 children has been identified with ASD [2]. A recent, large-scale European study estimated an average prevalence of 1 in 89 children in Europe having ASD (Autism Spectrum Disorders in the European Union, ASDEU consortium, 2018). Fombonne et al. have recently estimated the current worldwide ASD prevalence is 1% based on a thorough epidemiological review [3], this makes ASD one of the most frequently occurring neurodevelopmental disorders in childhood. The past decades have seen a vast increase in ASD cases, most likely due to changes in the definitions, diagnostic criteria and increased awareness of ASD, however, a true increase in ASD incidence currently cannot be ruled out [4].

Autism diagnosis currently relies on behavioral evaluations, therefore there is a need for valid and clinically useful biomarkers [1]. Biomarkers can aid diagnosis and can be used to validate effectiveness of interventions [5]. Currently, there are no pharmacological treatments targeting the core symptoms of ASD, but behavioral therapies are common practice [6]. Various psychological and educational interventions are used to address the behavioral and functional deficits that are associated with ASD [1]. While no pharmacotherapies addressing the disorder itself exist, two antipsychotic drugs (risperidone and aripiprazole) are approved for the treatment of ASD-associated irritability and aggression [7].

The pathophysiology of ASD remains elusive, but it is thought to be caused by an interplay between genetic and environmental factors [8]. The disorder is highly heritable and a wide range of susceptibility genes have been identified, accounting for 10-20% of ASD cases [9]. Possible environmental factors include both pre- and postnatal factors such as prenatal maternal exposure to certain medications, toxins or infections, epigenetic influences and immune abnormalities [8]. In addition to systemic immune dysregulation, abnormalities in neuroimmune function have frequently been associated with ASD [10]. The state of chronic neuroinflammation often observed in ASD, is characterized by increased levels of pro-inflammatory cytokines and chemokines in the cerebrospinal fluid (CSF) and activation of microglial cells in the brain tissues of autistic patients [10, 11].

Gastrointestinal problems, including abdominal pain, diarrhea, chronic constipation and gastro-esophageal reflux occur frequently in ASD patients [12, 13]. Possibly related to such intestinal problems, are the changes in composition and activity of intestinal bacteria in ASD, which has now been reported by multiple studies [14-17]. This is in line with the recognition of the intestinal microbiota and its metabolites as a player in neurodevelopmental disorders [18]. Moreover, increased intestinal permeability is linked to intestinal dysbiosis in ASD, and may present a potential route for intestinal metabolites into systemic circulation [19]. In addition, several studies have suggested that the severity of GI symptoms is associated with ASD symptoms severity in ASD children [20-22]. These findings link the gut microbiota, brain and behavior together in the form of a microbiota -gut-brain axis in ASD.

As the intestinal microbiota and its metabolites are emerging as important environmental factors in ASD, this report is intended to provide an overview of the current understanding of ASD gut microbial composition and its contribution to the production of two specific ASD-associated bacterial metabolites: 4-methylphenyl sulfate (*p*-cresyl sulfate or *p*CS) and 4-ethylphenyl sulfate (4EPS). Furthermore, the aim of this review is to answer the question of how these ASD-associated metabolites are produced and how they may be involved in ASD pathophysiology.

COMPOSITION OF THE GUT MICROBIOTA IN AUTISM SPECTRUM DISORDER

Human Autism Spectrum Disorder Studies

As with the disorder itself, findings with regards to microbial dysregulation in ASD patients are highly heterogeneous. There is currently no consensus on the composition of an ASD-specific microbiota, but some bacterial taxa are frequently reported to have either increased or decreased abundances in ASD patients compared to neurotypical controls.

Clostridioides Bacteria

One of the most frequent and interesting findings is the significantly elevated levels of *Clostridioides* species in fecal samples from ASD patients [15, 17, 23, 24]. Bacteria from this genus are suggested to be associated with autism in various ways. Already in 1998, Bolte suggested a possible role for *Clostridia* in ASD through tetanus neurotoxin (TeNT) release by *C. tetani,* and subsequent transport of TeNT to the central nervous system (CNS) [25]. In addition to TeNT, *Clostridia* are known to produce a range of toxins and potentially toxic metabolites, such as phenols, 4-methylphenol (*para*-cresol or *p*-cresol) and indole derivatives [15].

Another noteworthy association with regards to this genus is the one between ASD and a history of extensive antibiotic use during infancy [26]. Oral antibiotics can disrupt the protective intestinal microflora and thereby create an environment that is favorable for colonization by opportunistic, toxin-producing bacteria, such as *Clostridioides* species [25, 27]. Infection with *C. difficile* and subsequent diarrhea and colitis are associated with broad-spectrum antibiotic therapy, as this bacterium is able to proliferate enterically during use of certain antimicrobials [28]. Treatment of *C. difficile* infection involves the specific antibiotics metronidazole or vancomycin [28]. The effects of the latter have been investigated in a group of patients with regressive ASD, in which prior use of broad spectrum antibiotics was followed by the development of chronic diarrhea and the manifestation of autistic features [27]. Treatment with vancomycin improved autistic symptoms in these patients, however, after treatment discontinuation, the benefits and symptom improvements disappeared. A possible explanation for this relapse is the presence of clostridial spores, which are resistant to antimicrobial agents and allow for clostridial recolonization of the gut [29]. While vancomycin is not feasible as a treatment strategy for ASD, this study further underlines the existence of a connection between the gut, intestinal bacteria such as *Clostridia*, and symptoms of ASD.

Other Abnormalities

In addition to *Clostridia*, a range of other taxa are reported to be increased in the feces of autistic individuals compared to controls. This includes elevated levels of *Lactobacillus* [14] *Ruminococcus* [15] and *Bacteroides* [23]. Members of the *Lachnospiraceae* family (e.g. *Roseburia* and *Dorea*) have also been associated with ASD [23]. Finegold et al. (2010) found that *Desulfovibrio* species and *Bacteroides vulgatus* were present in high numbers in autistic patients and suggested that these bacteria could be important contributors to ASD [30]. In line with this, a study in a cohort of autistic children in Slovakia found an association between *Desulfovibrio* species and autism severity [31]. Reports of decreased abundances of certain bacteria are also common and include *Prevotella, Coprococcus and Veillonellaceae*, all of which are important for carbohydrate fermentationb[32]. Several studies have also found lower relative abundances of *Bifidobacterium* species [14, 23, 30, 33].

There are reports of changes in the *Bacteroidetes/Firmicutes* ratio in the stool of autistic children, but these are inconclusive. Finegold et al. (2010) found increased levels of the

phylum *Bacteroidetes* in severely autistic patients, while levels of phylum *Firmicutes* were higher for controls [30]. Conversely, another study reported a decrease in the *Bacteroidetes/Firmicutes* ratio in the feces of autistic patients compared to control, which was normalized by probiotic diet supplementation [31]. This inconsistency might be related to the severity of ASD and probiotic intervention, the latter never occurred in first study as the ASD subjects have been excluded if they had been on antibiotics or probiotics during the preceding month. Yet another study found an increase in *Firmicutes* and an accompanying decrease in *Bacteroidetes* in autistic patients with gastrointestinal symptoms [34]. This discrepancy might be attributed to the ASD children with or without gastrointestinal symptoms. Similar discrepancies are found for other bacteria, for example for *Akkermansia* species; one study found a low relative abundance of the mucolytic bacterium *Akkermansia municipalia* in autistic children [33], while others reported the genus *Akkermansia* to be present at high levels in autistic subjects [23, 32]. Obviously, the ratio of female to male in these studies varies very largely. Additionally, one study shows the relative abundance in species level, but the others show the relative abundance of *Akkermansia* in genus level. Such inconsistent findings seem to reflect the current state of this field of research, possibly due to differences among studies or due to the intrinsic heterogeneity of the disorder. More studies with adequate sample sizes and standardized sequencing techniques are required to pinpoint specific bacterial communities or species that are involved in ASD symptoms and possibly pathology.

Autism Spectrum Disorder Animal Models

Animal models of autism can provide additional insights and information about the intestinal microbiota and its contribution to pathophysiology of ASD. Various animal models exist and show not only behavioral abnormalities, but often also intestinal and microbial changes consistent with ASD in humans [35].

Valproic Acid Model

In mice, *in utero* exposure to the anticonvulsant valproic acid (VPA), leads to developmental and behavioral deficits in offspring that are similar to ASD [36]. De Theije et al. (2014) used this model to assess intestinal microbial composition and found altered microbial colonization as well as an intestinal inflammatory phenotype in VPA-exposed mice compared to control [37]. On the phylum level, a decrease in *Bacteroidetes* and an increase in *Firmicutes*, mainly consisting of *Clostridiales*, was found in the VPA-exposed offspring. But Liu et al. have shown an increase in *Bacteroidetes* in male rats prenatally exposed to VPA compared to control. This difference might be attributed to different host species [38]. This is interesting in light of the findings with regards to *Clostridioides* species in individuals with autism. Additionally, significant effects were found for *Desulfovibrionales*, which, as stated above, has also been associated with ASD in children [30, 37]. Furthermore, this study has also shown a significant increase in caecal levels of butyric acid, one of short chain fatty acids, in male offspring with correlation to the affected microbial abundance by prenatally exposure to VPA [37]. In addition, in a valproic acid model in rats it is shown that the changed fecal microbiota and altered metabolic potential is similar to that observed in ASD [38]. However, more studies are need to measured real changes in microbiota-associated metabolites in these rodent models for ASD.

BTBR Mice

The intestinal microbiota composition of BTBR mice, an inbred strain with multiple ASD-like behavioral phenotypes, also shows abnormalities compared to control. Golubeva et al. (2017) reported a reduction in the relative abundance of *Bifidobacterium* and *Blautia* species. This was associated with deficient bile acid and tryptophan metabolism in the intestine, gastrointestinal dysfunction, and impaired social interactions in male BTBR mice [39]. *Akkermansia* bacteria were increased, as well as the *Bacteroidetes/Firmicutes* ratio. Both can be matched to findings from autistic children [30, 32]. Furthermore, there was a reduction in *Bifidobacterium* and *Desulfovibrio* in the BTBR mice, with the reduction in *Bifidobacterium* in accordance with the reduction seen in ASD children [14, 39]. Two other studies with BTBR mice found a decrease in *Bifidobacterium* and an increase in *Akkermansia* as well [40, 41]. Exposure of BTBR mice to a high glycemic index diet during pregnancy and after birth induced higher levels both in the brain and blood of phenolsulfate, a tyrosine metabolite of bacterial origin. Other bacterial phenolic amino acid metabolites were also enhanced in high glycemic index diet fed BTBR mice. These metabolic effects were accompanied by reduced social behavior and cognition and enhanced repetitive behavior when compared to low glycemic index diet fed BTBR mice [42]. Of interest is the recent study demonstrating that i.v. injection of the gut bacteria produced aromatic metabolite *p*-cresol significantly increased ASD-like behavior in BTBR mice [43]. This study demonstrated a possible causal relation between bacteria derived aromatic metabolites and ASD-like behavior. Overall, the gut microbiota composition and activity of these mice are altered compared to control mice, with similar findings among studies. While some differences compared to human microbiota studies exist, BTBR mice appear to present with microbial dysregulation that is fairly representative for observations from human studies so far.

Maternal Immune Activation

A different approach to modeling autism in mice is through maternal immune activation (MIA). Epidemiological studies have associated maternal infections during late pregnancy with a higher risk of ASD in the child [44]. MIA offspring present with behavioral abnormalities, decreased intestinal barrier integrity, microbiota alterations and altered serum metabolomic profile, including elevated level of 4EPS, serotonin, indolepyruvate, glycolate and imidazole proprionate in serum [19]. MIA fecal samples differ in bacterial composition from control animals, with the main driver being changes in the diversity, and not overall abundance, of *Clostridia* and *Bacteroidia*. Increased abundance of bacteria from the families *Prevotellaceae, Porphyromonadaceae,* and *Lachnospiraceae* were found for MIA offspring, whereas abundances of *Ruminococcaceae, Erysipelotrichaceae* and *Alcaligenaceae* were higher in controls. Hsiao et al. suggest a pathogenic role for *Lachnospiraceae* and other *Bacteroidal* species in MIA, whereas other taxa may instead provide protective effects [39]. In this study, treatment with *Bacteroides fragilis* improved barrier function and restored levels of *Lachnospiraceae, Bacteriodales* and several metabolites, such as 4EPS, indolepyruvate, glycolate and imidazole proprionate, as well as ASD-associated behavioral abnormalities in MIA offspring [19]. Pre-conception microbiota transplantation from MIA mice can transfer susceptibility to neurodevelopmental abnormalities to control mice [45]. These MIA studies imply that the maternal microbiota may indeed be a risk factor for the development of neurodevelopmental disorders in offspring from mothers subject to immune activation. To what degree these findings translate to humans remains to be studied.

Fecal Microbiota Transplant

A recent study found that transplanting human gut microbiota from ASD patients to germ free (GF) mice promotes behavioral ASD-like symptoms in these mice [46]. Fecal microbiota transplant (FMT) from ASD donors in mice led to impaired social communication and interaction, and increased repetitive behavior in offspring, which was not found for FMT from neurotypical donors. Not only behavior, but also intestinal microbial composition was different for offspring of the mice that had undergone different FMTs. A decrease in *Bacteroidetes*, *Bacteroides* and *Parabacteroides* was reported for ASD-FMT offspring, with an increase in *Lachnospiraceae*, *Sutterella* and *Akkermansia* [46]. The increase in *Akkermansia* matches with reports from autistic subjects [32] and ASD animal models (VPA and BTBR) [37, 39]. Interestingly, metabolomic analysis of serum and colon contents of FMT offspring mice indicated different metabolomic profiles for ASD-FMT offspring and control [46]. The taurine and 5-aminovaleric acid were found lower in colon contents from ASD-FMT mice. When these two GABA receptor agonists were administered to BTBR mice, ASD-like behaviors improved [46]. Recently, Needham et al. have shown different metabolic profiles in plasma and feces of bacterial phenolic metabolite levels, between ASD children and typical developing controls. Furthermore, FMT from ASD donors with higher 4EPS level in serum and TD donors into male germ-free mice did not result in differences of this specific metabolite in serum [47]. 4EPS is only produced by bacteria. Hsiao et al. have previously shown the 4EPS production has a gender difference in both GF mice and SPF mice, it is much higher in female SPF mice than male SPF mice. It cannot be detected in male GF mice while it still presents in female GF mice [19, 47]. Very recently, Xiao et al. have shown the fecal microbiota transplantation of ASD children alters microbial community as well as tryptophan and serotonin metabolism in GF mice, but the causality between induced ASD-like behaviors and altered microbial metabolites remains to be further confirmed [48].

Perinatal Antibiotic Treatment

Epidemiological studies have revealed that early-life antibiotic exposure can increase the risk of neurodevelopmental later in life [26, 49, 50]. Indeed, perinatal treatment with low dose penicillin induced impaired social behavior in mice, which was associated with changes in gut microbiota composition, increases cytokine expression in frontal cortex, modifies blood-brain barrier integrity [51]. More recent studies, revealed that prenatal low dose penicillin exposure led to abnormal social behavior only in the male offspring. The penicillin-induced changes in microbiota composition clustered differently in both sexes [52]. Antibiotic exposed male mice had significant increased bacteria diversity compared to control and female mice, characterized by increases levels of amongst others *Staphylococcus, Parabacteroides, Enterococcus* , *Streptococcus*, *Dehalobacterium*, and *Blautia*. The control male offspring had significantly more *Prevotella*, *Bacteroides,* and *Mucispirillum*. Low dose penicillin treatment during lactation induced similar social deficits in male mice, which was associated with increased abundance of *Proteobacteria* and *Firmicutes* at the expense of *Bacteroidetes*. Concurrent treatment with the probiotic strain *L. Rhamnosus* prevented the behavioral deficits and normalized microbiota composition [53].

Overall, reports of microbial dysbiosis in ASD, both in human patients and animal models, are abundant and point towards a role for the gut microbiota in the disease. A well-defined ASD-microbiota profile has, however, not been established yet. Bacterial taxa that appear to be of interest in the context of ASD include *Clostridioides*, *Bifidobacterium*, *Bacteroides*, *Lachnospiraceae* and *Desulfovibrio.* Whatever the exact changes in composition may be, they

will inevitably affect gut and serum metabolites with a bacterial origin, which may have further downstream effects involved in ASD pathophysiology.

AUTISM SPECTRUM DISORDER-ASSOCIATED BACTERIAL METABOLITES *p*-CRESYL SULFATE AND 4-ETHYLPHENYL SULFATE

Along with the intestinal composition, the metabolites derived from intestinal bacteria are gaining more attention in ASD research, as various metabolites have been associated with symptoms, severity or pathophysiology of the disease [19, 46, 48]. Such microbiota-derived metabolites are *p*-cresol, *p*CS, 4-ethylphenol and 4EPS. **Figure 1** represents an overview of the intestinal microbial and host metabolism involved in the production of *p*-cresol, *p*CS, 4-ethylphenol and 4EPS [19, 43]. While there are some studies supporting a role for these metabolites in ASD pathophysiology, a clear connection has not been established so far.

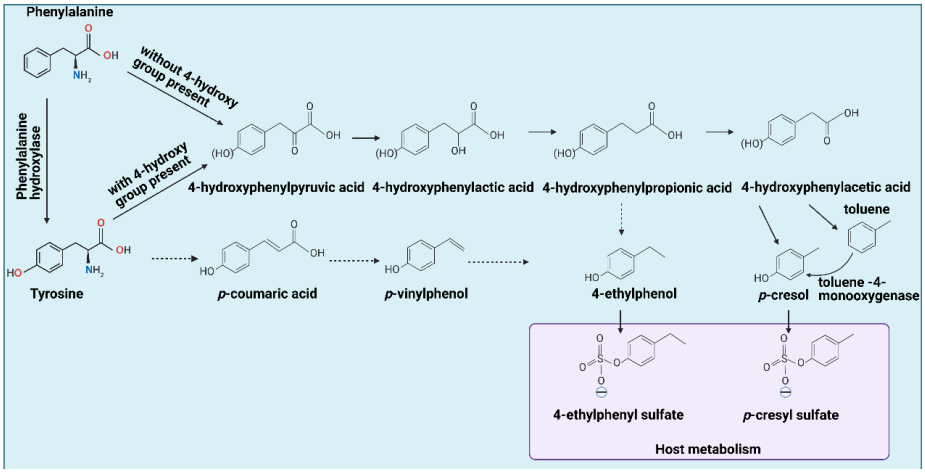

Figure 1. Tyrosine by gut bacteria leading to formation of 4-ethylphenol and *p*-cresol, which can be metabolized by the host into 4-ethylphenyl sulfate and *p*-cresol sulfate, respectively, indicated by the blue box. Solid lines represent established reactions, dotted lines represent presumed reactions taking place. Phenylalanine metabolism occurs similarly, but without the 4-hydroxy group present. In addition, phenylalanine can be converted to tyrosine first, then goes through same procedures with tyrosine metabolism. This figure is adapted from Hsiao et al., 2013; Smith & Macfarlane, 1996, Schuck et al., 2015. [19, 54, 55].

p-Cresol and *p*-Cresyl sulfate

Levels of the bacterial metabolite *p*-cresol and its derivative *p*CS are elevated in urine [56, 57] and feces of autistic children [23, 58, 59]. Urinary *p*-cresol has been suggested as a biomarker for autism liability in small children because of its significant elevation [56]. Moreover, *p*-cresol has been hypothesized to play a role in ASD pathogenesis [60]. The compound is generated by intestinal bacteria, as serum levels of *p*-cresol and its sulfate conjugate *p*CS are significantly decreased in germ free mice [61]. Of interest is the recent finding that microbiota transfer therapy lowered enhanced fecal *p*CS levels in ASD similar to those of the typically developing controls [59]. Furthermore, mice chronically exposed to *p*-cresol in the drinking water

demonstrated an ASD-like behavioral phenotype which was shown to be dependent on *p*-cesol-induced changes in the intestinal microbiota composition [62]. This highlights *p*-cresol and pCS as intriguing ASD-associated molecules with possible implications in the microbiota - gut-brain axis in ASD.

Environmental exposure to *p*-cresol through inhalation, skin contact or food ingestion is also relatively common, with natural sources of exposure including plants, rainwater, petroleum and tar. Artificial sources of exposure include disinfectants, preservatives, paints, solvents and perfumes and cosmetics, among others [60]. The most important and significant source of *p*-cresol exposure however, is formed by certain intestinal bacteria that ferment the aromatic amino acids tyrosine and phenylalanine [60].

Once produced by bacteria, 95% of *p*-cresol is metabolized by the host into *p*CS via O-sulfonation, a process that occurs primarily in the liver, and to a smaller extent in colonic epithelial cells [60]. Approximately 3-4% is metabolized to *p*-cresyl glucuronide and the remaining 1% of *p*-cresol exists in free form in serum and urine [57], as for the content of *p*-cresol itself in feces or intestine, it remains to be investigated. All three forms are filtered from the blood by glomerular filtration and can be detected at low concentrations in the urine of all individuals [63]. It is not *p*-cresol, but rather its conjugate *p*CS that is more abundant in serum and urine.

Studies reporting on urinary and serum *p*-cresol levels mostly rely on detection methods that first require acidification of the biological sample, which hydrolyses the conjugates *p*CS and *p*-cresyl glucuronide to *p*-cresol. This means that measured levels of *p*-cresol from biological samples may actually represent levels of *p*CS, and to a lesser extent, *p*-cresyl glucuronide as well as *p*-cresol itself. This indirectly determined *p*-cresol concentration is largely representative of the *p*CS concentration, while it is not responsible for the physiological effects and toxicity that have been attributed to *p*-cresol [57, 63]. In the study that first found elevated levels of *p*-cresol in the urine of autistic children, urine samples were first heated to hydrolyze sulfate and glucuronide conjugates [56], pointing towards an actual increase in *p*CS rather than *p*-cresol. An alternative sample preparation method is methanol deproteinization, which preserves *p*-cresol conjugates, allowing for detection of *p*CS without hydrolysis to *p*-cresol [64, 65]. This latter method may present a better method of sample preparation for accurate measurements of *p*CS and *p*-cresol in serum and urine. Currently, there is no standard method of measuring *p*-cresol and *p*CS and therefore concentrations may differ largely among studies.

Urinary *p*-cresol concentration does not correlate with fecal *p*-cresol concentrations, which limits the use of urinary concentrations in making statements about actual intestinal production of *p*-cresol [66]. However, urinary concentrations can reflect plasma concentrations of free *p*-cresol and its conjugates [56]. Since *p*-cresol is not present at significant concentrations in human serum, physiological effects of *p*-cresol are less relevant than those of *p*CS. However, being produced by bacteria in the colon, the effects of *p*-cresol on intestinal epithelial cells and gut bacteria may still be relevant, as one study has found *p*-cresol to interfere with metabolic activities in colonic epithelial cells, in addition to being genotoxic to these cells [67].

Most of what is known about *p*-cresol and *p*CS originates from their well-established role as toxic retention solutes, as they reach high concentrations in uremic patients where they can exert various toxic effects, for example on the immune system, cardiovascular system or the brain [68-70]. In the context of ASD, especially the neurological symptoms occurring in uremic patients are of interest, as uremic toxins such as *p*CS may be involved [43]. Patients with chronic kidney disease are at a larger risk of developing cognitive disorders, dementia and stroke [71]. A recent study found high levels of *p*CS and indoxyl sulfate, a related metabolite

derived from tryptophan, in the cerebral spinal fluid of patients with Parkinson's disease and associated these uremic toxins with Parkinson's pathogenesis and progression [72]. This indicates that uremic toxins may indeed contribute to neurological disorders, but the mechanisms behind a causal connection remain to be established.

Physiological effects of *p*-cresol and *p*CS seem to be almost opposite [63], so keeping in mind that *p*CS is presumably the largest contributor is important when considering toxicity. Physiological effects of *p*-cresol include decreased endothelial proliferation [73], impaired endothelial barrier function [74] and inhibition of leukocyte effector functions *in vitro* through reduced production of reactive oxygen species [75, 76]. In contrast, *p*CS can induce free radical producing leukocytes, thereby boosting oxidative stress instead of suppressing it [77]. Similar inflammation-inducing effects of *p*CS were found in human monocyte-derived macrophages, in which low concentrations of *p*CS (10, 25 µg/mL; mean uremic concentrations) increased phagocytosis and production of reactive oxygen species [78]. On the other hand, the highest concentration tested in this study (50 µg/mL), corresponding with the maximum uremic concentration, actually decreased the ability to activate the immune cells and initiate a proper immune response to toxins such as lipopolysaccharide (LPS) [78]. Similar suppression of LPS-induced anti-microbial immune responses was observed in *p*CS-exposed murine macrophages [79]. Again, especially the highest concentrations of *p*CS (250, 1000 µM) suppressed immune responses by increasing IL-10 and decreasing IL-12 p40. *p*CS can also suppress Th1-type cellular immune responses both *in vitro* and *in vivo*, a mechanism that may be involved in immune dysfunction in patients with chronic kidney disease, as they typically have high concentrations of *p*CS in their blood [80].

It is interesting to expand this hypothesis of microbiota-derived *p*CS being a risk factor for immune dysfunction to include ASD patients, considering the elevated levels of *p*-cresol, and thus *p*CS, that have been found for these patients [59]. Especially because immune abnormalities are implied in ASD pathophysiology [10]. The urinary levels of *p*-cresol/*p*CS, found in autistic children are within the range that is found in uremic patients [57], suggesting that similar effects as those observed in uremic patients could occur in autistic patients as well.

4-Ethylphenol and 4-Ethylphenyl Sulfate

Another ASD-associated metabolite is 4EPS, which is structurally related to *p*-cresyl sulfate, but is derived from 4-ethylphenol. Like *p*-cresol and *p*CS, 4EPS is derived from, or at least modulated by intestinal bacteria, demonstrated by the finding that germ-free mice have very low serum levels of 4EPS compared to conventionally colonized animals [19]. Elevated concentrations of 4-EPS in serum of children with ASD have been reported [47]. In a MIA mouse model of autism, serum levels of 4EPS were significantly elevated in autistic-like mice compared to control, and when the animals were treated with the probiotic *B. fragilis*, these elevated levels of 4EPS were fully reduced [19]. This same study showed that systemic 4EPS administration induced anxiety-like behavior in wild-type mice that was comparable to behavior observed in MIA autistic-like mice. While other ASD-characteristic behaviors were not affected, it opens up the possibility of microbiota-derived metabolites playing a role in autism through direct modulation of behavior. Other biological effects of 4EPS have not been studied, and a lot remains unknown about this metabolite. Like *p*CS, it is also a uremic toxin [81].

As 4EPS and *p*-cresol have a similar chemical structure, they are thought to have similar effects on the body. However, apart from it being a uremic toxin like *p*CS, little is known about the biological effects of 4EPS. While some studies support the potential relevance of *p*-cresol and

*p*CS in ASD, data for 4-ethylphenol and 4EPS is scarce and more studies are required [47], for example on toxicity and its effects on the immune system. Further research on these specific ASD-associated bacterial metabolites may elucidate new pathways involved in the disease and possibly present new targets for intervention.

Aromatic Amino Acid Metabolism

Both *p*CS and 4EPS are products of microbial degradation of aromatic amino acids (AAA) in the gut. With intestinal microbial dysregulation being frequent in ASD patients and models, it is possible that there is a connection between specific intestinal dysregulation and increased levels of metabolites such as *p*CS and 4EPS [19, 59]. In order to connect these two, it is necessary to first look at which bacterial taxa are involved in the production of *p*-cresol and 4-ethylphenol, the precursors of *p*CS and 4EPS, and then whether these bacteria can be linked to ASD. As *p*CS and 4EPS are sulfonated by the body from *p*-cresol and 4-ethylphenol, respectively, this section will describe the bacterial production of these precursor molecules, both of which originate mainly from bacterial fermentation of the amino acid tyrosine, and to a smaller extent phenylalanine.

Tyrosine and phenylalanine can undergo reductive as well as oxidative metabolism by intestinal bacteria [82]. The first step for both processes is an aminotransferase reaction, yielding 4-hydroxyphenylpyruvic acid and phenylpyruvic acid, respectively. Reductive metabolism yields propionic acids: 4-hydroxyphenyl propionic acid (4-HPPA) for tyrosine and phenylpropionic acid for phenylalanine. 4-ethylphenol can be produced via 4-hydroxyphenyl propionic acid or via *p*-vinylphenol. Oxidative metabolism yields 4-hydroxyphenylacetic acid (4-HPA) and phenylacetic acid [82], the former being the direct precursor of *p*-cresol. As tyrosine is the main source of *p*-cresol and other hydroxy-phenolic products, the focus will be on tyrosine metabolism **(Figure 1)**.

The production of cresols from tyrosine (and phenylalanine) has been attributed to various intestinal anaerobes, including species of *Clostridioides, Bacteroides*, *Bifidobacterium* and various others [54, 83]. Through cross-feeding pathways, end products of some species can act as substrates for other species, meaning that even species that do not produce *p*-cresol itself can still contribute to its overall production through synthesis of precursor molecules [84].

Bacterial *p*-Cresol Production

At least two metabolic pathways for *p*-cresol are known, the first one being oxidation of tyrosine to 4-hydroxyphenylacetic acid, which is then decarboxylated to form *p*-cresol [60]. Only bacteria that express 4-HPA decarboxylase, such as various species of *Clostridioides* and one genre of *Lactobacillus*, are able to use this pathway [85]. The second known synthetic pathway involves oxidation of toluene to *p*-cresol through toluene monooxygenase activity [86]. Of these two pathways, the one involving tyrosine fermentation by 4-HPA decarboxylase is thought to be more important, as there is much more tyrosine than toluene available as a substrate in the gut lumen and there is a broader distribution of strains that may have the required enzyme for this reaction [60]. However, the ability to metabolize tyrosine (and phenylalanine) into *p*-cresol or precursor molecules is not limited to *Clostridioides* and *Lactobacillus* bacteria. This section elaborates on bacteria that may be involved in the production of *p*-cresol from aromatic amino acids, and highlights some of them as points of

interest for future research into the microbiota -gut-brain axis in ASD. An overview of the bacterial taxa producing *p*-cresol and/or 4-HPA and 4-HPPA is presented in **Table 1**.

Table 1: Overview of bacterial taxa producing *p*-cresol and/or 4-hydroxyphenylacetic acid (4-HPA) and 4-hydroxyphenyl propionic acid (4-HPPA).

Family	Genus	Species	*p*-cresol	4-HPA or 4-HPPA	Reference
Clostridiaceae	*Clostridioides*	*C. bartletti*	X	X	Russell et al., 2013
		C. bifermentans		X	Smith & Macfarlane, 1996
		C. butyricum	X		Bone, Tamm & Hill, 1976
		C. clostridioforme	X	X	Van der Meulen et al., 2008
			X		Saito et al., 2018
		C. difficile	X	X	Elsden et al., 1976; Dawson et al., 2011; Harrison et al., 2020
			X		Saito et al., 2018
		C. paraputrificum	X	X	Bone, Tamm & Hill, 1976; Smith & Macfarlane, 1996
		C. perfingens	X		Smith & Macfarlane, 1996; Van der Meulen et al., 2008;
			X		Saito et al., 2018
		C. saccharolyticum	X	X	Russell et al., 2013
			X		Saito et al., 2018
		C. scatologenes	X	X	Elsden et al., 1975
		C. septicum	X	X	Bone, Tamm & Hill, 1976; Elsden et al., 1975
		C. sporogenes	X	X	Bone, Tamm & Hill, 1976; Elsden et al., 1975
	Peptostreptococcus	*P. anaerobius*		X	Lambert & Moss, 1980
Bacteroidaceae	*Bacteroides*	*B. eggerthii*	X	X	Russell et al., 2013
		B. fragilis	X	X	Bone, Tamm & Hill, 1976; Russell et al., 2013; Smith & Macfarlane, 1996; Van der Meulen et al., 2008
		B. ovatus		X	Russell et al., 2013; Smith & Macfarlane, 1996
			X		Saito et al., 2018
		B. thetaiotaomicron	X	X	Russell et al., 2013; Smith & Macfarlane, 1996; Van der Meulen et al., 2008
		B. uniformis	X	X	Russell et al., 2013
			X		Saito et al., 2018
Bifidobacteriaceae	*Bifidobacterium*	*B. adolescentis*	X	X	Russell et al., 2013; Smith & Macfarlane, 1996
		B. animalis		X	Van der Meulen et al., 2008
		B. bifidum	X	X	Smith & Macfarlane, 1996
		B. infantis	X	X	Russell et al., 2013; Smith & Macfarlane, 1996
			X		Saito et al., 2018
		B. pseudolongum	X	X	Smith & Macfarlane, 1996
Lachnospiraceae	*Anaerostipes*	*A. caccae*	X	X	Russell et al., 2013
		A. hadrus	X	X	Russell et al., 2013
	Butyrivibrio	*B. fibrisolvens*	X	X	Russell et al., 2013
	Roseburia	*R. intestinalis*	X	X	Russell et al., 2013
		R. inulinivorans	X	X	Russell et al., 2013
Ruminococcaceae	*Faecalibacterium*	*F. prausnitzii*	X	X	Russell et al., 2013
	Ruminococcus	*R. obeum*	X	X	Russell et al., 2013
		R. torques	X	X	Russell et al., 2013
			X		Saito et al., 2018
Eubacteriaceae	*Eubacterium*	*E. cylindroides*		X	Russell et al., 2013
		E. halii		X	Russell et al., 2013
		E. rectale	X	X	Russell et al., 2013
			X		Saito et al., 2018

Clostridiaceae

Within the family of *Clostridiaceae*, especially species from the genus *Clostridioides* are involved in the metabolism of AAAs. Various species of this genus are involved in *p*-cresol production, either by performing the final decarboxylation step of 4-HPA or by producing precursor molecules of *p*-cresol that may be further metabolized by cross-feeding bacteria. The species that has attracted most attention with regards to *p*-cresol production is *C. difficile*, which expresses 4-HPA decarboxylase in order to catalyze the formation of *p*-cresol [85, 87, 88]. Interestingly, *C. difficile* has a high tolerance for *p*-cresol, whereas *p*-cresol is toxic to many other microbes via its ability to interfere with metabolism and inhibit growth [89, 90]. This ability to produce and tolerate *p*-cresol is thought to provide the bacterium with a competitive advantage over other intestinal bacteria [91]. High *p*-cresol tolerance has also been reported for two other *Clostridioides* species, *C. perfingens* and *C. sordellii*, even though these species produce little to no *p*-cresol themselves. This indicates overlap in tolerance pathways amongst various species of this genus [89, 92]. As discussed earlier, infant antibiotic use has been associated with ASD, which has led to speculations about antibiotics disrupting the protective commensal microflora, thereby creating an environment that allows for colonization by opportunistic bacteria like *Clostridioides* species [25]. This could be accompanied by increased levels of *Clostridioides*-derived *p*-cresol, which in turn could maintain suppression of (parts of) the intestinal microbiota due to high *p*-cresol concentrations.

Production of *p*-cresol is not unique to *C. difficile* as it has also been demonstrated for *C. scatologenes* [87] and other *Clostridioides* species including *C. paraputrificum, C. perfringens*, *C. septicum* and more (**Table 1**) [54, 93]. Other *Clostridioides* species can metabolize tyrosine and/or phenylalanine to phenolic compounds that can be used by other species to form *p*-cresol. Various molecules that can act as precursors for *p*-cresol can be produced (**Figure 1**), but especially the production of 4-HPA and 4-hydroxyphenyl propionic acid are likely to be contributors to *p*-cresol production further down the line. Species known to produce 4-HPA include *C. difficile* and *C. scatologenes* among others (**Table 1**) [83, 87]. *C. sporogenes, C. paraputrificum, C. bifermentans* and *C. septicum* have been found to produce 4-HPPA, which may act as a cross-feed precursor of both *p*-cresol and 4-ethylphenol, which is the precursor of 4EPS [54, 87].

These multiple *Clostridioides* species involved in *p*-cresol production, as well as the associations between *Clostridioides* abundances and ASD, make this genus and its capacity to produce ASD-associated metabolites interesting for future research in ASD. At least it warrants further research into a possible (direct) link between Clostridia and autism, for example by assessing fecal and urinary *p*-cresol and serum *p*CS levels as well as intestinal microbiota composition of autistic patients. Additional behavioral testing can provide further evidence on such an association.

Another genus of *Clostridiaceae* involved in AAA metabolism is *Peptostreptococcus*. *P. anaerobius* can metabolize tyrosine to 4-HPPA through deamination of tyrosine into *p*-coumaric acid and subsequent reduction [94]. Both products can be used as precursors for *p*-cresol and 4-ethylphenol production (**Figure 1**). However, *P. anaerobius* does not produce *p*-cresol itself, and thus is presumably only a cross-feeder [95].

Bacteroidaceae

In addition to *Clostridioides*, some species of *Bacteroides* are able to ferment AAAs. *B. fragilis*, *B. thetaiotaomicron* and *B. uniformis* can produce *p*-cresol from tyrosine [54, 83]. Other

species such as *B. ovatus* may contribute to overall *p*-cresol through the production of 4-HPA or 4-HPPA (**Table 1**). According to Russell et al. (2013) there is a substantial population of AAA-metabolizing *Bacteroides* in the gut of most individuals and the AAA-metabolizing activity of these bacteria is high [83]. Overall, the major metabolite of *Bacteroides* fermentations is phenylacetic acid, but 4-HPA is also produced in significant amounts [83]. While direct *p*-cresol synthesis has only been proven for a few species of this genus, and only in low concentrations, its contribution to overall *p*-cresol through synthesis of precursor molecules may still be considerable.

Despite this possible contribution to the ASD-associated metabolite *p*-cresol, there is evidence that probiotic treatment with *B. fragilis* or the related *Bacteroides* species *B. thetaiotaomicron* and *B. vulgatus* has beneficial effects on ASD-like symptoms in MIA animal models of ASD [19, 96]. Probiotic administration of *B. fragilis* ameliorates ASD-like symptoms and normalizes serum levels of 4EPS and other metabolites characteristic for the ASD-like mice, possibly through improvement of intestinal barrier function [19]. So, while some species of this genus can contribute to the production of ASD-associated metabolites, they may also be protective through reducing serum levels of these metabolites. It may depend on the species involved or possibly a range of additional environmental factors.

Bifidobacteriaceae

Metabolites of tyrosine and phenylalanine have been detected for *Bifidobacterium* fermentations, with (hydroxy)phenyllactic acid being the major metabolite. *B. animalis* can produce hydroxyphenyllactic acid as well as low levels of 4-HPA [84]. While this study did not detect *p*-cresol in the fermentations with *Bifidobacteria*, Smith and Macfarlane (1996) earlier reported *p*-cresol production from tyrosine by various species of this genus, including *B. bifidum, B. adolescentis*, *B. infantis* and *B. pseudolongum*. Of the *Bifidobacterium* species, only *B. longum* fermented phenylalanine, thereby producing phenyllactate, and no *p*-cresol or direct precursors, indicating that *p*-cresol is predominantly formed by tyrosine-fermenting bacteria [54].

Bifidobacteria are considered to be beneficial commensal bacteria and have been reported to be decreased in autistic patients compared to control [14, 33]. A few trials have assessed the effects of probiotics including *Bifidobacterium* species on behavior and intestinal symptoms in autistic children. Some studies report amelioration of behavior and/or intestinal symptoms, but due to different probiotic combinations and different designs, studies are too heterogenous to draw conclusions about the real benefit of these bacteria [97].

Lachnospiraceae

One study found that bacteria within the *Lachnospiraceae* family are also able to metabolize tyrosine to *p*-cresol and 4-HPA although concentrations were low [83]. Within the genera *Anaerostipes*, *Butyrivibrio* and *Roseburia* there were five species able to produce both *p*-cresol and 4-HPA [83]. Increased abundances of *Lachnospiraceae* have been associated with ASD both in humans and an animal model of ASD [19, 23]. An *in vivo* study found that social avoidance behavior after microbiota transplantation was accompanied with elevated *Lachnospiraceae* and high levels of *p*-cresol, but the causalities remain to be further confirmed [98]. Apart from these findings, information about these bacteria is scarce, but as they are increased in autistic patients and models, and can contribute to production of ASD-associated

metabolites, a role in ASD pathophysiology or symptoms is possible and further research could be valuable.

Ruminococcaceae

Russell et al. (2013) detected low levels of *p*-cresol and 4-HPA from tyrosine fermentation for *Ruminococcus obeum* and *Ruminococcus torques*, as well as for *Faecalibacterium prausnitzii [83]*. In this study, these bacteria produced high amounts of benzoic acid and 4-hydroxybenzoic acid. Phenylpyruvic acid and phenyllactic acid, metabolites of phenylalanine, were also produced in significant amounts by these bacteria. While these molecules are not directly linked to *p*-cresol, contribution to cross-feeding pathways cannot be excluded. A potential association between increases in *Ruminococcaceae* and elevated *p*-cresol levels might be made with regards to social avoidance behavior in mice [98], if further direct evidences show this family can produce *p*-cresol in the mice gut.

Eubacteriaceae

In their elaborate study on AAA-metabolizing bacteria, Russel et al. (2013) found three *Eubacterium* species could produce phenylacetic acid and 4-HPA from phenylalanine and tyrosine, respectively (**Table** 1). Especially *E. hallii* was found to produce significant amounts of phenylacetic acid and 4-HPA. *P*-cresol was detected only for *E. rectale* fermentations, but concentrations were low [83]. With only one study assessing this family of bacteria, a firm connection cannot be made, but neither can involvement be excluded.

Bacterial 4-Ethylphenol Production

While multiple studies have investigated the production of *p*-cresol and 4-HPA as end products of bacterial AAA fermentation, less data on 4-ethylphenol, the presumed precursor of 4EPS, is available. Just as the other simple phenols (phenol and *p*-cresol), 4-ethylphenol is thought to originate from microbial metabolism of tyrosine [93, 99]. Tyrosine can be reductively deaminated to 4-HPPA, which in turn can be decarboxylated to form 4-ethylphenol (**Figure 1**) [99]. Through this synthetic pathway, essentially all bacterial taxa that have been found to produce 4-HPPA could be involved in the production of 4-ethylphenol, possibly through cross-feeding pathways as well. However, experimental data in support of this metabolic pathway is scarce. In fact, it has even been opposed by some studies that found that 4-HPPA could only be dehydroxylated to phenylpropionate and that no further metabolism occurred [100, 101].

An alternative synthetic pathway includes the formation of *p*-coumaric acid from tyrosine, which may be decarboxylated to form *p*-vinylphenol and subsequently reduced to 4-ethylphenol (**Figure 1**). There is limited literature elaborating on this synthetic pathway and whether commensal bacteria are involved. Instead, research on 4-ethylphenol is mainly centered around its production by yeasts in food fermentations [102].

A recent study reported that a whole range of intestinal bacteria can produce phenolic precursors of 4-ethylphenol, including *Coriobacteriaceae, Enterobacteriaceae, Fusobacteriaceae* and *Clostridioides* clusters I and XIVa [103]. Production of 4-ethylphenol by *Clostridial* species has also been suggested in earlier studies [93, 104]. Findings from the MIA

study from Hsiao et al. support this idea, as both elevated serum levels of 4EPS and increased levels of the *Lachnospiraceae* family of Clostridia were observed in MIA autistic mice [19]. While this is no direct evidence, it indicates an association between bacteria of the *Lachnospiraceae* family and serum 4EPS levels. In the same study, however, it has been shown that treatment of mice with 4EPS indeed resulted in anxiety-like phenotype demonstrating a causal relationship between exposure to 4-EPS and behavioral changes. Moreover, Hsiao et al. suggest that the structural similarity of 4EPS to the (mainly) *Clostridioides* -derived *p*-cresol and *p*CS could be an indicator of similar biosynthetic pathways, again pointing towards clostridial involvement in the production of 4-ethylphenol and 4EPS.

Recently, Santamaría and coworkers found that *Lactobacillus plantarum* is able to produce 4-ethylphenols [105]. It is worthwhile mentioning that recently a 4-week randomized double-blind placebo-controlled study demonstrated some beneficial effects in ASD of *Lactobacillus plantarum* PS128 [106]. These findings may contrast a pathological role of 4-ethylphenol and the associated host metabolite 4EPS in ASD. This calls for further research into the role of 4-ethylphenol and 4EPS and the bacteria involved in their production in ASD.

To conclude, there are various candidates for bacterial *p*-cresol and 4-ethylphenol production, some of which have previously been associated with ASD in animal models or in humans [19, 59]. The most promising genus for association with *p*-cresol in ASD is *Clostridioides*, as these bacteria are known for their prominent *p*-cresol production and have continuously been hypothesized to play a role in ASD. For the production of 4-ethylphenol and 4EPS, the specific bacteria involved are less clear, but also here clostridial species are candidate bacteria. Future studies assessing both microbial dysregulation and metabolite levels in ASD patients could provide evidence for the existence of a connection between specific dysregulation and levels of *p*CS and 4EPS.

THE POTENTIAL EFFECT OF BACTERIAL METABOLITES ON BRAIN IN AUTISM SPECTRUM DISORDER

After being produced by the intestinal microflora, some bacterial metabolites are thought to be able to exert neurotoxic effects on the brain when they are present at high concentrations. An example is the proposed role for tetanus neurotoxin (TeNT) produced by *Clostridia* species in ASD development [25]. In a similar way, *p*CS and 4EPS may travel to the central nervous system and possibly affect neuroglial cells and thereby processes like neuroinflammation, phagocytosis and affect synaptic pruning in the CNS. Neuroinflammation is a key finding in autistic patients and is characterized by marked activation of microglial cells and concomitant increased levels of inflammatory cytokines and chemokines in the CSF [10, 11, 107]. Microglial cells are a specialized population of macrophages in the CNS. They play a role in innate immune function through release of inflammatory mediators and recognition and subsequent phagocytosis of microorganisms or damaged or infected cells [108]. Additionally, microglial cells are crucial for neuronal development as they are involved in synaptogenesis and responsible for the pruning of redundant synaptic connections during childhood, a process that is necessary for the development of functional neural circuits [109].

As a result of neuroinflammation, there may be changes in the cytokines and chemokines secreted by microglial cells, which may in turn disrupt synapse maintenance. The resulting changes in dendritic branching and spine density possibly contribute to under- and hyper-connectivity in various brain regions frequently observed in autistic patients [110]. Dysregulations in the process of synaptic pruning can disrupt the excitatory/inhibitory balance of synapses, which may contribute to neurodevelopmental disorders such as ASD [111].

The Potential Effects on Blood-Brain-Barrier Integrity

The metabolites discussed here are postulated to have neurotoxic effects [15], but before assessing whether the metabolites *p*CS and 4EPS affect processes like neuroinflammation in ASD, their ability to reach the brain needs to be established, as transport to the CNS is required for the exertion of such direct effects. In order to reach the brain, the metabolites have to pass the blood-brain-barrier (BBB), the border between systemic circulation and the CNS. Results from one *in vivo* study show that *p*CS can indeed reach the brain, as *p*CS was shown to accumulate in the brain in mice with renal failure [112]. This indicates that *p*CS can cross the BBB in mice with renal failure, where the microbiota is dysregulated and serum levels of *p*CS and other uremic toxins are high, seemingly similar to in individuals with ASD [113]. To this point, ability to reach the CNS has not been demonstrated directly for 4EPS. However, Hsiao et al. (2013) found that systemic administration of 4EPS led to changes in behavior in mice (e.g. inducing anxiety-like behavior), an effect that can be hypothesized to be caused by 4EPS directly affecting the brain [19].

There is limited data on *p*CS and 4EPS reaching the brain and the mechanisms facilitating this. Different mechanisms of moving from systemic circulation into the CNS can therefore be proposed. One factor likely to be involved is the impaired integrity and thus increased permeability of the BBB that is associated with ASD [114]. Abnormalities in endothelial BBB permeability can parallel the ASD-associated abnormalities in the gut microbiota. The microbiota has been identified as a regulator of BBB integrity, as germ-free mice have increased BBB permeability and reduced expression of tight junction proteins that regulate barrier function. These effects are reversed when germ-free animals are colonized with conventional gut microbiota [115]. Impaired BBB integrity has also been reported for an animal model of chronic kidney disease [116]. This may be mediated by urea and other uremic toxins, as brain endothelial barrier function is impaired upon exposure to urea or serum from uremic patients in cell culture studies [116]. While more data is needed, these findings do suggest a role for (microbiota-derived) uremic toxins in BBB permeability. Due to the similarities in uremic toxins that have been found in uremic patients and individuals with ASD, it could be possible that similar mechanisms are involved in the disrupted BBB integrity in ASD. In turn, this disrupted integrity could facilitate entry of microbiota -derived metabolites into the CNS (**Figure 2A**).

In general, uptake of uremic toxins into tissues is thought to involve transport though organic anion transporters (OATs) [112]. OATs are expressed at barrier epithelia in various tissues in both facilitate the transport of a variety of drugs, dietary compounds and urinary toxins, usually after the products have been modified in some way (e.g. hydroxylated, sulfonated or glucuronidated) [117]. For transport and clearance of certain microbiota-derived molecules, the expression of OAT1 and OAT3 in the kidneys is especially important, demonstrated by the accumulation of microbiota-derived uremic toxins in OAT1 and OAT3 knockout studies. The accumulating molecules include *p*CS, 4-HPA and indoxyl sulfate (IS), a product from tryptophan metabolism that is similar to *p*CS [117]. OATs, presumably preferentially OAT3, are responsible for the uptake of *p*CS in rat kidneys [118]. OAT3 is also expressed in the brain of rats, where it is suggested to be involved in molecular transport across the BBB [119]. IS was found to be transported across the BBB via OAT3 in rats [120]. This transport by OAT3 however, is effluent, from the brain/CSF to the blood, and thus forms a mechanism of eliminating toxins such as IS from the brain, which is necessary for protection of the brain [120]. These findings allow for speculations on whether OATs could provide a way across the BBB for the metabolites, *p*CS and 4EPS, as well, and perhaps facilitate influx and/or efflux of these compounds in the CNS (**Figure 2A**).

Effects on Microglial Cells

The importance of the host microbiota in regulating microglial homeostasis is indicated by the finding that germ-free mice have defective microglia and subsequently impaired innate immune responses in the CNS, both of which can be restored by colonization with conventional microbiota [121]. Microbiota-derived short chain fatty acids seem to be the regulators of microglial homeostasis [121], but additional microbiota-derived molecules can be involved too.

Assuming that *p*CS and 4EPS can indeed reach the CNS, it can be hypothesized that both metabolites either increase microglial activation and effector functions or suppress them. Effects of low concentrations of *p*CS on macrophages are pro-inflammatory, shown by increased phagocytosis and reactive oxygen species production, whereas higher concentrations of *p*CS have immunosuppressive effects, resulting in the inability to initiate a proper immune response [78, 79]. Thus, based on the limited experimental knowledge available, both options appear to be possible. Increased microglial activation and a subsequent state of neuroinflammation is associated with ASD [10], however, the same has been hypothesized for defective microglia with a decrease in microglia-mediated synaptic pruning [111]. The mechanisms underlying both of these ASD-associated states is not fully understood, and possibly, the ASD-associated metabolites discussed here could play a role.

Effects on Microglial Phagocytosis

One study investigating microglial density and morphology in postmortem ASD samples, found significantly increased somal volume in microglia from ASD patients compared to control [107]. Additional morphological alterations included a reduction in the number of processes extending from the cell bodies as well as a shortening and thickening of these processes. This more amoeboid morphology is characteristic of microglial reactivity and phagocytic activity [108]. A phagocytic morphology is required in early life for the process of synaptic pruning, however, if such a morphology persists, it can be an indicator of a state of chronic activation. A hypothesis fitting with this scenario is that the bacterial metabolites could play a role in the increased inflammatory activity of microglial cells in which an upregulation of microglial phagocytosis could be expected.

An alternative possibility is that of inducing neuroinflammatory processes, the bacterial metabolites dysregulate or suppress the immune system. Rather than an anti-inflammatory effect, this points toward a dysregulated immune response against toxins such as LPS, which are supposed to initiate a proper immune response in order to protect the brain from damage. An immunosuppressive role is supported by studies finding that *p*CS affected the immune system's ability to initiate a normal immune response against LPS [78, 80]. These immunosuppressive effects of *p*CS and 4EPS may extend to phagocytotic capacity of microglia in the CNS. Impaired leukocyte phagocytosis has been observed in uremic patients undergoing dialysis, again strengthening the possibility of a connection between uremic toxins and immune suppression [122]. Inhibition of microglial autophagy has been shown to impair degradation of synapses and debris, indicating disturbed synaptic pruning [123]. Microglial autophagy and phagocytosis are both mechanisms by which the cell can degrade extracellular materials such as pathogens, damaged cells and surplus synapses. The inhibition of autophagy, and thus synaptic pruning, results in ASD-like social and behavioral defects as well as repetitive behaviors in mice [123]. Thus, it could also be possible that the bacterial metabolites contribute to ASD pathophysiology through impaired microglial activity and subsequent impaired synaptic pruning. Alternatively, deficient microglia may be damaging to the brain as protection against invading pathogens is no longer guaranteed. This can be especially

problematic in combination with impaired BBB integrity, allowing pathogens and toxins to enter the CNS, where a proper immune response cannot be initiated, allowing invading pathogens and toxins to exert their damage on the brain.

Whether and how precisely *p*CS and 4EPS affect microglial phagocytosis and whether this contributes to the pathogenesis of ASD is currently unknown (**Figure 2B**), and presents an opportunity for future research, as described in the next section.

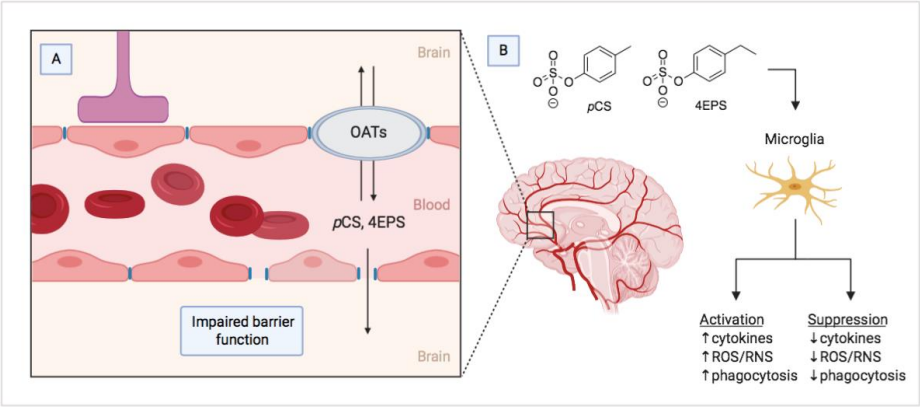

Figure 2. A. Two proposed routes for *p*CS and 4EPS to move from the bloodstream into the brain. The first is through the impaired blood-brain-barrier associated with ASD and the second through organic anion transporters that may be expressed in the blood-brain-barrier. **B.** If *p*CS and 4EPS pass the blood- brain-barrier and enter the central nervous system, these bacteria-derived metabolites might affect microglial cells, either by activating or suppressing them. OATs, organic anion transporters; *p*CS, *p*-cresyl sulfate; 4EPS, 4-ethylphenyl sulfate; ROS/RNS, reactive oxygen/nitrogen species. Created by BioRender.

DISCUSSION

While a causal role of microbial dysregulation and microbiota-derived metabolites such as *p*CS and 4EPS in ASD has not been confirmed, it is still possible to think about possible therapeutic strategies based upon such a connection between the microbiota, metabolites and the brain. The idea that probiotics may be beneficial in the context of ASD has been explored by a few studies but study populations are small, heterogeneous and the interventions differ among studies, making it difficult to draw conclusions about the effects of probiotic interventions [97]. Additionally, studies taking specific metabolites of bacterial AAA fermentation into account are scarce. One preclinical study has shown a beneficial effect of *B. fragilis* treatment in lowering serum levels of 4EPS in a mouse model of autism, but whether this can be translated to humans remains to be studied [19].

Further research can contribute to the design of a probiotic therapy aimed at normalizing microbial dysregulation associated with altered metabolite levels in ASD patients. For example, if an increase in clostridial species in the gut of autistic patients is found to be directly associated with increased levels of *p*CS and 4EPS, a therapy targeted against clostridial bacteria can be beneficial by lowering metabolite levels and thereby their possibly detrimental effects on the brain and behavior. Possibly, probiotic administration of other species can be used to normalize or prevent dysregulated microbiota. The idea of using probiotic therapies to relieve both intestinal and behavioral abnormalities associated with neurodevelopmental

disorders is supported by various studies, for example by the research performed by Buffington et al. (2016). They found a single commensal strain, *Lactobacillus reuteri*, to restore intestinal and social behavior abnormalities associated with Maternal High-Fat Diet offspring [124]. Additionally, there is evidence that probiotics are connected to lower risks of *Clostridioides difficile* infections [125]. With clostridial species implied as troublemakers in ASD, such a method to prevent infection and keep specific bacteria under control might also be beneficial in autism. Additionally, probiotic treatment to restore epithelial integrity in the gut may prevent metabolites from entering systemic circulation, which may prevent some complications. There are already indications that probiotics can indeed restore disrupted intestinal barrier integrity [19, 126].

Another interesting approach is targeting the elevated levels of the metabolites directly, instead of the bacteria producing them. An oral sorbent, AST-120, is available for treatment of chronic kidney disease, in which it adsorbs uremic toxins and their precursors within the intestinal tract [127]. By adsorbing *p*-cresol and 4-ethylphenol in the intestine, the accumulation *p*CS and 4EPS in serum and urine is prevented [81]. Moreover, one preclinical study showed that *p*CS accumulation in the brain of uremic mice can be reduced to normal by AST-120 [112]. While this has not been studied in relation to ASD, and it may not be a sustainable method, it does present a way of targeting the metabolites directly. In addition, Pascucci et al. have shown *p*-cresol administration exacerbates ASD-like behaviors in BTBR mice, which is associated with enhanced dopamine and its metabolites DOPAC and HVA levels in specific brain areas [43]. Previously, it has been shown with in-vitro studies that *p*-cresol inactivates Dopamine β-Hydroxylase as an alternative substrate of this enzyme. In this way, *p*-cresol binds to this enzyme competitively and ends up with the inhibition of metabolism dopamine to norepinephrine, resulting in that less dopamine is converted to norepinephrine [128-130].

While the ideas described above are currently merely hypothetical, the idea of such novel targeted therapies is intriguing, especially in light of the current absence of therapies targeting underlying mechanisms of ASD. Even so, in spite of new ideas and promising findings from preclinical studies, the mechanistic and translational knowledge is still lacking. Whether we can connect the different aspects to form a complete story that really contributes to the understanding and possibly treatment of ASD depends on future research.

CONCLUSION

The notion that the microbiota is in some way involved in neurodevelopmental disorders such as ASD continues to acquire evidence. As outlined in this review, specific microbiota-derived metabolites may form a link between the microbiota and the brain, and future studies on the effects of such metabolites may provide insights into the pathophysiology and possibly, etiology of ASD. The metabolites highlighted in this report, *p*CS and 4EPS have been associated with ASD in multiple studies, but strong evidence for causal involvement is still missing. These metabolites are produced from fermentation of AAA by a range of intestinal bacteria, most notably bacteria from the *Clostridioides* genus. These bacteria are among the dysregulated bacteria often observed in ASD patients. Future research may elucidate whether these and other metabolite-producing bacteria are indeed involved in ASD. Both *p*CS and 4EPS have been singled out as promising biomarkers and along with the microbiota, as possible targets for novel therapies, either through pharmacological or nutritional interventions targeting the microbiota -gut-brain axis.

AUTHOR CONTRIBUTIONS

YZ and MB: literature research and drafting the manuscript. AK and PP: conceptualization and refining the manuscript. NP, LP, and JG: critical reading of the manuscript. All authors have read and approved the manuscript.

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Chapter 3

The gut-brain axis in Autism Spectrum Disorder: A focus on the metalloproteases ADAM10 and ADAM17

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Abstract: Autism Spectrum Disorder (ASD) is a spectrum of disorders that are characterized by problems in social interaction and repetitive behavior. The disease is thought to develop from changes in brain development at an early age, although the exact mechanisms are not known yet. In addition, a significant number of people with ASD develop problems in the intestinal tract. A Disintegrin And Metalloproteases (ADAMs) include a group of enzymes that are able to cleave membrane-bound proteins. ADAM10 and ADAM17 are two members of this family that are able to cleave protein substrates involved in ASD pathogenesis, such as specific proteins important for synapse formation, axon signaling and neuroinflammation. All these pathological mechanisms that are involved in ASD. Besides the brain, ADAM10 and ADAM17 are also highly expressed in intestines. ADAM10 and ADAM17 have implications in pathways that regulate gut permeability, homeostasis and inflammation. These metalloproteases might be involved in microbiota-gut-brain axis interaction in ASD through the regulation of immune and inflammatory responses in the intestinal tract. In this review, the potential roles of ADAM10 and ADAM17 in the pathology of ASD and as targets for new therapies will be discussed with the major focus is on the gut-brain axis.

Keywords: Autism Spectrum Disorder (ASD); Gut-Brain Axis; Neuroinflammation; Metalloproteases; A Disintegrin And Metalloprotease (ADAM); ADAM10; ADAM17; Ectodomain Shedding

1. INTRODUCTION

Autism Spectrum Disorder (ASD) is a spectrum of neurodevelopmental disorders that is generally diagnosed early in life and will might persist across the whole lifespan. It is mainly characterized by deficiency in social interaction and communication and the presence of specific stereotyped behavior [1]. The prevalence of ASD is approximately 1.5% in developed countries, as determined in 2012 by the World Health Organization [2] and predominantly affects males [3]. Globally, the incidence of ASD has shown a 35-fold increase (1 in 59 children in the USA and 1 in 89 in the EU are affected) compared to the 60s and 70s (Centers for disease control and prevention data & statistics on autism spectrum disorder, 2019; ASDEU consortium autism spectrum disorders in the European union, 2018). A diagnosis is performed using behavioral assessment due to the absence of reliable biomarkers.

Although ASD pathogenesis remains elusive, it is thought that it results from early altered brain development and neural reorganization [4,5]. Some clinical aspects are found in large groups of people diagnosed with ASD, such as altered neuronal connectivity, increased synaptic density [6], neuroinflammation [7], microbiota dysbiosis [8-10], dysregulated immune responses [11-15] and gastrointestinal abnormalities [16-18]. In addition, the most replicated neuroanatomical finding in infants and early childhood of people diagnosed with ASD is an enlarged brain volume, called macrocephaly or megalocephaly [19,20]. Genetically, it is estimated that 400-1000 genes are involved in ASD susceptibility and it is thought that there are more that have and will be discovered in the near future [21]. The estimated heritability of ASD was 83% in a recent population-based cohort of children born in Sweden [22] and Bailey et al. reported a similar result [23]. Among these genetic predispositions, the membrane-bound synaptic genes for Amyloid Precursor Protein (APP), Neural glial-related Cell Adhesion Molecule (NrCAM), Neuroligins (NLGNs), Neurexins (NRXNs) and Protocadherins (PCDHs) were widely identified as candidate genes of ASD [24-28], as their deficiency in mice led to ASD-like behavior, such as deficits in spatial memory and learning, increased repetitive and stereotype grooming behavior and compromised social interaction [29-31]. Interestingly, the amount of these ASD-related proteins in membrane is controlled by the metalloproteases ADAM10 and ADAM17 through proteolytically cleaving these transmembrane proteins by which ADAM10 and ADAM17 might be involved in ASD pathogenesis [32-36].

A Disintegrin And Metalloproteases (ADAMs) are a subgroup of the metzincin family of metalloproteases, which also consists of Matrix Metalloproteases (MMPs), ADAMs with thrombospondin motifs (ADAMTSs) and Snake Venom Metalloproteases (SVMPs) [37,38]. ADAMs are ubiquitously expressed and are able to regulate sperm-egg interactions, cell proliferation, differentiation, migration and cell fate determination [39]. ADAM10 and ADAM17, are the two most investigated members of the ADAM family. Interestingly both ADAM10 and ADAM17 are highly expressed in the brain as well as intestines. In the Central Nervous System (CNS), ADAM10 and ADAM17 are able to regulate axon guidance and synaptic functions through controlling the cleavage of synaptic proteins, such as APP, NrCAM, NLGNs, NRXNs and PCDHs. Importantly, ADAM10 plays a vital role in synaptic pruning by cleaving the chemokine fractalkine (CX3CL1) that binds to its receptor CX3C chemokine receptor 1 (CX3CR1) to induce microglia-mediated synapse elimination [40]. ADAM17 also regulates neuroinflammation, attributing to its capacity of converting membrane-bound TNF-α into a soluble form that recognizes the TNF-α receptors I and II and consequently triggers inflammatory responses. In the intestinal tract, ADAM17 can regulate intestinal inflammation, intestinal barrier permeability and inflammatory responses by cleaving several cytokines, such as TNF-α and lymphotoxins [41-44]. Moreover, ADAM10 can control intestinal permeability by cleaving the transmembrane proteins Notch [45-47] and E-cadherin [48]. However, there

are only a few reports available that elucidate the roles and functions of ADAM10 and ADAM17 in gut-to-brain pathology of ASD.

Taken together, ADAM10 and ADAM17 regulate synaptic function, neuroinflammation and brain development, as well as intestinal barrier function, inflammation and immunity, which are involved in the pathogenesis of ASD. In this review, we aim to elaborate on the potential role of ADAM10 and ADAM17 in the pathogenesis of ASD with a major focus on the gut-brain axis.

2. STRUCTURE OF METALLOPROTEASES

The metzincin family of metalloproteases has four family members, ADAMs, MMPs, ADAMTSs and SVMPs [37,38]. They are called metzincins for the conserved Met residue at the active site and the use of a zinc ion in the enzymatic reaction. Collectively, the metalloproteases are able to degrade all extracellular structures. This family members have some corresponding protein domains (Figure 1). All members start with a signal peptide at their N-terminal that allows them to be located to the secretory pathway. Immediately following the signal peptide is a pro-domain. This ensures enzyme latency until cleaved by pro-protein convertases [49]. After the pro-domain, all members of the metzincin family contain a metalloprotease domain, which holds its catalytic activity. After this, some major structure differences are found between members. For ADAMs, SVMPs and ADAMTSs, the metalloprotease domain is followed by a Disintegrin domain and then a cysteine-rich domain. For the ADAMTS, the cysteine-rich domain follows the Thrombospondin region. The ADAMs contain an Epidermal growth factor (EGF)-like domain, followed by a transmembrane and cytoplasmic domain. SVMP and ADAMTS are soluble proteins. Membrane-type MMP and MMP contain a Hemapexin domain after their metalloprotease domain, necessary for substrate selectivity and for binding with Tissue Inhibitors of Metalloproteinases (TIMPs), the main MMP inhibitor [37] and the MT-MMP contains a transmembrane region with a cytoplasmic tail [37,50,51].

In this review, the focus will be on two types of ADAMs, ADAM10 and ADAM17. The MMPs, MT-MMPs, ADAMTSs and SVMPs are beyond the scope of this review. Little is known about the possible role of MT-MMPs, ADAMTSs and SVMPs in ASD, however, it should be noted that we do not rule out that MMPs, such as MMP9, might also play a role in the pathogenesis ASD [52,53].

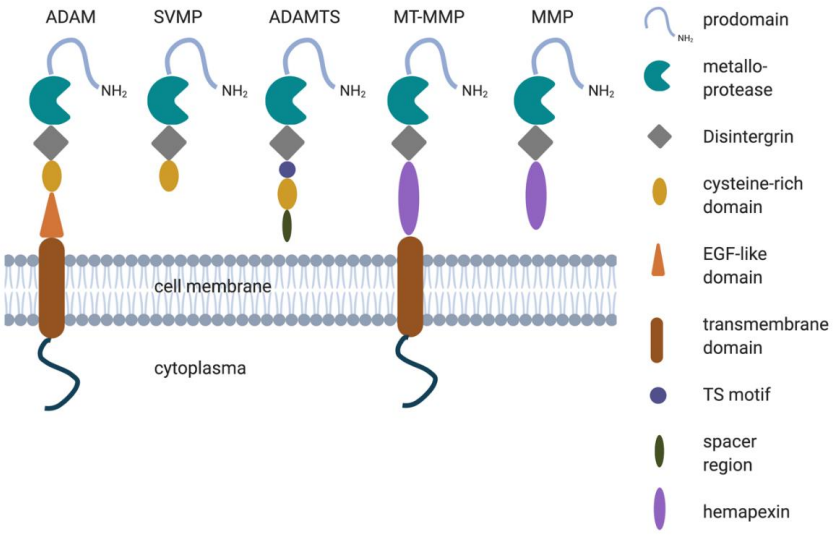

Figure 1. Protein structure of the members of the metzincin family of metalloproteases: A Disintegrin And Metalloprotease (ADAM), Snake Venom Metalloprotease (SVMP), A Disintegrin And Metalloprotease Thrombospondin motif (ADAMTS), membrane-type matrix metalloproteinases (MT-MMP) and MMP.

ADAMs

ADAMs are a family of type I transmembrane proteins characterized mainly by their ability to cleave membrane-bound proteins at their extracellular domain. The cleavage generates a soluble protein fraction in the extracellular space, a process called 'ectodomain shedding'. This will influence signaling pathways of other cells by decreasing the amount of membrane-bound receptors or by increasing the amount of soluble ligands [50]. ADAMs are proteins of approximately 750 amino acids that contain several structurally conserved domains, which determine its biological function. The metalloprotease domain can contain a catalytic site with a zinc-binding motif, which is mediated by three histamine residues (HEXGHXXGXXHD) [54]. Around 24 ADAMs have been identified in humans, of which only 12 contain the metalloprotease domain with the active zinc-binding site [55,56]. The biological function of these proteolytically active ADAMs (ADAM8, 9, 10, 12, 15, 17, 19, 20, 21, 28, 30 and 33) is determined by their substrates and includes sperm-egg interactions, cell migration, axon guidance, inflammation and cell fate determination [39]. In addition, ADAMs have been implicated in different pathologies, such as cancer [57], inflammation [58] and Alzheimer's Disease (AD) [59,60].

3. ADAM10 IN THE CENTRAL AND ENETRIC NERVOUS SYSTEMS

The most extensively studied member of the ADAMs family is ADAM10. There are more than a hundred substrates cleaved by ADAM10 in the CNS [35] and its expression, maturation and substrates selectivity are regulated by the TspanC8 subfamily of tetraspanin, consisting of Tspan5, 10, 14,15,17 and 33 [61-63], as different Tspan-ADAM10 complexes might adopt different conformation and space to their substrate [62,64,65]. ADAM10 is ubiquitously expressed in the brain [66], where it is located at the synapse and in synaptic vesicles and functions as a sheddase of other synaptic proteins [67], which makes ADAM10 to control CNS

processes, such as development, synaptogenesis and axon targeting. In addition, ADAM10 is expressed in the intestinal tract [68]. In Section 5 the role of ADAM10 in the intestines is discussed. Table 1 lists the major ADAM10 substrates in the CNS that will be discussed below.

Table 1. The reduction in shedding of ADAM10 substrates after the conditional deletion of ADAM10 in embryonic primary cortical neuron cultures [35]. Abbreviations: Fractalkine (Cx3cl1), Neuroligin-1 (NLGN-1), Protocadherin-9 (PCDH9), Neural glial-related Cell Adhesion Molecule (NrCAM), Neuroligin-3 (NLGN-3) and Amyloid-β Precursor Protein (APP).

ADAM10 substrates	Shedding reduction in ADAM10$^{-/-}$ neurons
Cx3cl1	91%
NLGN-1	83%
PCDH9	71%
NrCAM	66%
NLGN-3	62%
APP	20%

3.1. Amyloid-β Precursor Protein (APP)

APP is a transmembrane protein involved in cell adhesion and neurite pruning [69,70]. It consists of an extracellular N-terminus domain, a transmembrane region and a C-terminus, intracellular domain [71]. APP can be proteolytically cleaved by a group of secretases, α-, β- and γ-secretases (Figure 2). β-site APP cleaving enzyme 1 (BACE1) and γ-secretases induce the amyloidogenic pathway, where APP is cleaved extracellularly to create a soluble fraction, sAPPβ, the main component of AD plaques, Amyloid β-peptide (Aβ) and Amyloid Precursor Intracellular Domain (AICD) [72]. α-Secretases activate the non-amyloidogenic pathway by creating the soluble fraction sAPPα, P3 and AICD. ADAM10 is the main α-secretase of APP in the nervous system [32]. ADAM10 cleaves APP in the Aβ domain, which inhibits the formation of the pathological plaques that cause AD, and consequently creates sAPPα instead. Therefore, the non-amyloidogenic pathway of APP is thought to serve a neuroprotective function at this point [72]. Interestingly, studies show that while ADAM10 is the constitutive secretase, ADAM17 is the stimulatory secretase of APP [32].

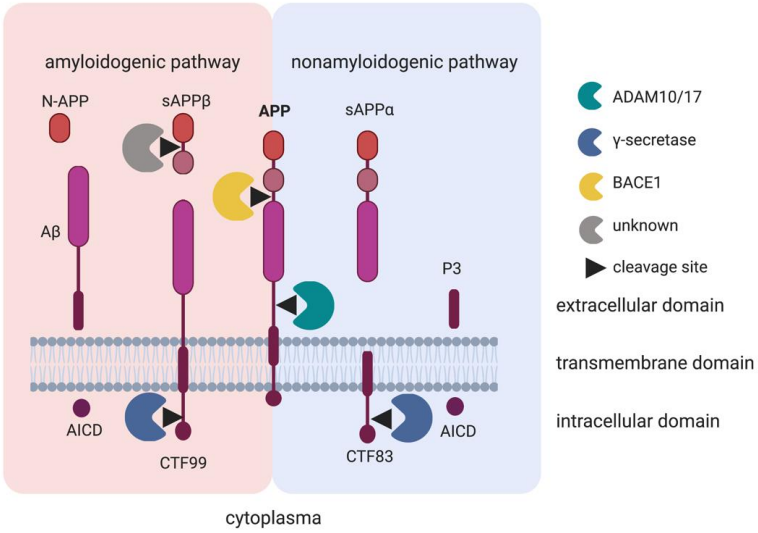

Figure 2. Overview of cleavage products of APP by different secretases. Cleavage of APP by BACE1 initiates the amyloidogenic pathway, where sAPPβ and C-Terminal Fragment 99 (CTF99) are created. γ-secretases further process the CTF99 to create the neurotoxic Aβ protein and the Amyloid Precursor Intracellular Domain (AICD). Meantime, sAPPβ is also cleaved by additional secretases at an unknown site to generate N-terminal of APP (N-APP). Cleavage of APP by either ADAM10 or ADAM17 initiates the non-amyloidogenic pathway which creates sAPPα and C-Terminal Fragment 83 (CTF83). γ-secretases then cleave CTF83 to create P3 and AICD.

Recent studies have started to investigate the roles of ADAM10 and ADAM17 in neurodevelopmental diseases. Of all ADAM10 substrates, APP is the most described in the context of ASD. It has been shown that there is an increase of sAPPα levels in the plasma of children diagnosed with severe ASD aged between 5-17 years [28] [73]. In the fragile X mental retardation 1 knockout (Fmr1 KO) mouse model of Fragile X Syndrome, significantly increased levels of both sAPPα and ADAM10 are found at postnatal day 21 [74], but not in adulthood. These findings are in line with the concept that ASD is a result of early altered brain development as the prenatal and perinatal period is most critical for synaptogenesis. In parallel with these main findings, Westmark et al. found that genetically decreasing APP and Aβ levels are able to ameliorate the autistic phenotype in adult Fmr1 KO mice [75]. Additionally, Lahiri et al. hypothesized that increased sAPPα levels can activate neuroprotective pathways and microglia, which result in neuronal overgrowth and neuroinflammation, leading to an increased brain volume that is also observed in ASD [19,20,76].

Importantly, the N-terminal of APP (N-APP) is a ligand of Death receptor 6 (DR6), which is highly expressed in oligodendrocytes and neurons. N-APP is a cleavage product of sAPPβ by a still undetermined mechanism and the specific cleavage site is unknown. The binding of N-APP to DR6 triggers neuronal death via caspase pathways *in vitro* and *in vivo* and consequently a role for N-APP/DR6 in neurodegeneration has been proposed [70], Furthermore, DR6 negatively regulates oligodendrocyte survival, maturation and myelination, which is related

to microglia activation, phagocytosis and neuroinflammation [77-79]. Colombo et al. demonstrated that DR6 of Schwann cells (SCs) negatively regulates the myelination of the Peripheral Nervous System (PNS) and that DR6 KO mice showed precocious myelination in the PNS [80]. SCs underlies the sheath of most of peripheral nerves and regulates the myelination of the nervus vagus in the PNS [81-83]. Vagal stimulation is recognizably involved in ASD development. Sgritta et al. demonstrated that Lactobacillus reuteri (L. reuteri) rescued social behavior in ASD mice (SH3 and multiple ankyrin repeated domains 3B KO mice) , but not in vagotomized mice. These findings indicate that L. reuteri might ameliorate ASD-like behavior in the ventral tegmental area of ASD mice in a vagus nerve-dependent manner [84,85]. In addition, Jin et al. proposed transcutaneous vagus nerve stimulation is a promising treatment for ASD, but the exact mechanism is not clear [86]. Recently, DR6 cleavage is decreased by 50% in ADAM10-deficient murine neurons and consequently it is a substrate of ADAM10 [80]. However, the potential role of ADAM10-mediated cleavages of APP and its receptor DR6 in the pathology and treatment of ASD is barely investigated.

Overall, ADAM10 and possibly ADAM17 are able to regulate APP shedding and create sAPPα fractions at the expense of sAPPβ. As elevated sAPPα levels are found in the plasma of children with severe ASD behavior, it is probable that ADAM10/17-mediated APP shedding contributes to the development of disturbed brain development in ASD. More studies are needed to elucidate the specific mechanisms of APP shedding in ASD.

3.2. Neuroligins (NLGNs) and neurexins (NRXNs)

NLGNs are synaptogenic adhesion proteins located at the post synapse which trans-synaptically binds to the presynaptic partner NRXNs to form a NRXN/NLGN complex, necessary for efficient neurotransmission. These two proteins recruit key synaptic proteins, such as scaffolding proteins and neurotransmitter receptors after the initial contact of an axon with its target cell. Therefore, they are essential for synaptic formation, maturation and differentiation [87-89]. There are five types of NLGNs (NLGN1, NLGN2, NLGN3, NLGN4 and NLGN4Y) and three NRXNs (NRXN1, NRXN2 and NRXN3) in the human genome. They are involved in ASD pathogenesis. Among the NLGNs, NLGN3 is the strongest candidate, followed by NLGN1 and of the NRXNs, NRXN1 is the strongest candidate [90]. Loss-of-function variants of NRXN1 have been found in ASD patients [24,26,27]. Interestingly, variants of the other two types, NRXN2 and NRXN3, are much rarer. Furthermore, NLGN-1 KO mice display deficits in spatial memory and learning and an increased repetitive, stereotypical grooming behavior, which is accompanied by a reduced ratio of NMDA to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) at the corticostriatal synapses [29]. NLGN-1 is shed by ADAM10 for 83% and NLGN-3 for 62%, as described in ADAM10-deficient neurons (Table 1) [35]. As the other types of NLGNs are not cleaved by ADAM10, we will not discuss them further. NLGN-1 is found exclusively on glutamatergic neurons whereas NLGN-3 is found on both glutamatergic and GABAergic synapses [91,92]. The proteolytic cleavage by ADAM10 of membrane-bound NLGN-1 increases by either N-methyl-D-aspartate (NMDA) receptor activation or by binding to the secreted form of NRXNs [92]. Interestingly, the secreted form of NRXN2 and NRXN3 may also be generated by ADAM10 or ADAM17 [33,34]. A recent study discovered that NRXN1 is primarily cleaved by ADAM10 in hippocampal neurons [93].

Only limited reports are available on the role of intestinal NLGN-1, NLGN-3 and NRXN. NLGN-3 is expressed in the enteric nervous system as well and gastrointestinal dysfunction is found in people and mice with a R451C missense mutation in this NLGN-3 that have an ASD phenotype [94]. Very recently, it has been shown that ASD-associated NLGN-3 mutations as well as NLGN-3 KO mice have more cecal Nitric Oxide (NO)-producing neurons and more activated enteric macrophages [95]. These phenomena might explain the presence of intestinal symptoms in these NLGN-3 deficiency mice [94,96] and ASD patients [97,98], such

as a disturbed intestinal transit and intestinal inflammation. Additionally, NLGN-1 and NRXN are shown to be important for the development of the enteric nervous system in rats [99]. It is rather speculative to link enhanced intestinal ADAM10/17 expression to loss of NLGN-1, NLGN-3 or NRXNs and ASD-related intestinal dysfunction.

Taken together, ADAM10 cleaves NLGN-1, NLGN-3 and possible NRXNs. Following this, it can be hypothesized that increased ADAM10 levels or activity will lead to an increased cleavage of NLGN-1, NLGN-3, NRXNs, therefore terminating their function at the synapse and disrupting neurotransmission. Disturbances in neurotransmission are also described in people diagnosed with ASD. It has been shown that a decreased glutamate concentration in the striatum correlates with the severity of social ASD symptoms, suggesting that glutamate/γ-aminobutyric acid (GABA) abnormalities in the corticostriatal circuitry may contribute to ASD development [100]. Moreover, it was shown that NLGN-1 shedding has a negative effect on NRXN1 stability [101], which also seems to be primarily cleaved by ADAM10 [93]. In conclusion, ADAM10 is a major regulator of synaptic functions of NLGN/NRXN complexes and the loss of NLGN-1 and NLGN-3 in mouse models induces autistic-like phenotypes [29,102,103], which highlights a potential connection between ASD and ADAM10-mediated cleavages of NLGN/NRXN complexes.

3.3. Protocadherins (PCDHs)

Cadherin is a family of calcium-dependent cell adhesion proteins consisting of three family members: classic cadherins, desmosomal cadherins and PCDHs [104]. PCDHs are mainly expressed in the developing CNS [105]. One member of this family that is an ADAM10 substrate is PCDH9 [35]. The conditional deletion of ADAM10 in primary neuron cultures resulted in a 71% reduced shedding of PCDH9 [35], which has been proposed to play a role in synaptogenesis [106]. It remains unclear what the effect of ADAM10 shedding is on PCDH9 function. In PCDH9-deficient mice, long-term social and object recognition deficits were determined [30], but without any changes in perception, sociability or fear memory. Furthermore, the PCDH9 KO mice showed impairments in sensorimotor development and structural changes in layers of sensory cortices [30]. The behavioral dysfunction in PCDH9 KO mice are also presented in ASD. Moreover, Copy Number Variation (CNV), including deletion, duplication, translocation and inversion, of the PCDH9 gene have been found in patients with ASD [24]. Additionally, another member of PCDHs, PCDH8, has also been identified as a substrate of ADAM10 [35] and regarded as a candidate ASD gene in Caucasian females [107]. Furthermore, Breuillard et al. demonstrated, although with limited sample size, that PCDH19 genetic defects frequently emerged in female ASD children with epilepsy and intellectual disability [108]. Obviously, PCDHs play an important role in ASD pathogenesis, as it seems that ADAM10 is the main sheddase of PCDH8 and PCDH9 [108]. Indeed, the role of ADAM in the loss of function of PCDHs is not yet investigated in ASD. To elucidate this connection further, more research will be necessary.

3.4. Neural glial-related cell adhesion molecule (NrCAM)

NrCAM is part of the L1 family of cell adhesion molecules of immunoglobulin superfamily (I gCAMs) and a cell adhesion molecule [109]. NrCAM is involved in brain development where it controls dendritic spine densities, axon guidance and targeting and neurite outgrowth [110-112]. NrCAM was shown to be an ADAM10 substrate where deletion of ADAM10 in primary neurons reduces NrCAM shedding by 66% [35]. In addition, Brummer et al. recently showed that ADAM17 deletion in primary neurons did not alter NrCAM proteolysis and that ADAM10 controls NrCAM cell surface expression levels and NrCAM-dependent neurite outgrowth *in vitro* [36].Furthermore, mice with a conditional ADAM10 knock-out in neurons showed

increased cell surface expression levels of NrCAM [35,36] and a reduced number of dendritic spines[113], whereas NrCAM knock-out mice show increased dendritic spines densities. As NrCAM is a member of the Sema3F complex which mediates spine retraction [114], increased NrCAM surface levels would be expected to decrease spine density. A potential role for soluble NrCAM in axon signaling becomes apparent in NrCAM-deficient [115] and ADAM10-deficient mice [35]. Both mouse models present with axonal targeting deficits within their olfactory bulbs with axons overshooting their mark. This would suggest a functional role for the soluble fraction of NrCAM generated by ADAM10 cleavage, although more research is needed to obtain more information about this role. Currently, no studies report on a possible peripheral/intestinal role of NrCAM in relation to ASD.

In NrCAM-deficient male mice, the loss of NrCAM leads to ASD-related behavioral alterations in sociability, acquisition of a spatial task and reversal learning [31]. Marui et al. identified that seven single nucleotide polymorphisms (SNPs) within the NrCAM gene are associated with ASD in Japanese children [25]. Bonora et al. also detected in 48 unrelated individuals with ASD several polymorphisms in the promoter and untranslated region of NrCAM and suggested that reduction in expression of this gene might be involved in ASD susceptibility [116]. In contrast, Hutcheson et al. showed that there is no association between ASD susceptibility and NrCAM gene in subsets of chromosome 7-linked families [117]. In order to further explore the relation between ASD and NrCAM, and the possible role of ADAM10-induced cleavage of NrCAM more studies are needed.

3.5. Fractakine (CX3CL1)

CX3CL1 is the only member of the δ subfamily of chemokines that is constitutively and abundantly expressed in the brain-specifically, in glial cells and neurons [118]. CX3CL1 recognizes its receptor CX3CR1, which is exclusively expressed in microglia [119,120]. CX3CL1-CX3CR1 signaling is necessary for immune response, neuroinflammation, synaptic pruning and brain development through maintaining the phagocytic function of microglia [40,119]. Moreover, CX3CL1 was identified as a substrate of ADAM10 and cleaved by ADAM10 to generate soluble CX3CL1 that acts as a ligand of CX3CR1 in the brain [35,121,122]. Currently, there is little known about the link between ADAM10-mediated cleavage of CX3CL1, microglial phagocytosis and ASD. However, it has been shown that CX3CR1 KO mice exhibits a deficiency in microglia engulfment and consequently show an increased density of dendritic spines and immature synapses as well as a synaptic pruning deficiency [119]. Furthermore, there is a defect in synaptic elimination in both CX3CL1 KO mice and CX3CR1 KO mice, which was phenocopied after ADAM10 inhibition in wild type mice [40]. Insufficient synaptic elimination is a cause of hyperconnectivity in the brain, which is related to the macrocephaly found in ASD patients. Additionally, Rogers et al. demonstrated that CX3CR1 KO mice show impairments in synaptic plasticity and cognitive function [123], which are symptoms of neurodevelopmental diseases, including ASD.

There are limited reports available that discuss the contribution of ADAM10-mediated cleavage of CX3CL1 to the intestinal disturbances found in ASD. Interestingly, the CX3CL1-CX3CR1 levels are critical for the sex difference in high-fat-food induced obesity. For instance, females are much more resistant to diet-induced obesity due to the higher expression levels of CX3CL1 than in males. Strengthening this point, female CX3CR1 KO mice phenocopied ‘male-like' microglial activation and increased susceptibility to diet-induced obesity. Conversely, increasing the CX3CL1 levels in the male mice brain converted them to a ‘female-like' metabolic phenotype with a decrease of microglial activation and weight gain [124]. These sex differences may be involved with gender difference in ASD. However, more research is necessary.

Taken together, the important role that CX3CL1-CX3CR1 signaling plays in the phagocytic function of microglia and consequently synaptic pruning, emphasizes the potential of this ADAM10 substrate to contribute to ASD pathology. Importantly, more research will be needed to further explore alterations in CX3CL1 expression in people diagnosed with ASD and its contribution to the disease.

4. ADAM17 IN THE CENTRAL NERVOUS SYSTEM

ADAM17 is one of most extensively studied member of the ADAMs family and is ubiquitously expressed in all tissues and cell types. Similar to the regulation of ADAM10 by TspanC8, the selectivity or specificity of substrates in ADAM17 is regulated by the iRhoms subfamily that is one of the rhomboid superfamilies of intramembrane proteases and consists of iRhom 1 and iRhom2 [125,126]. In addition, iRhoms also regulate ADAM17 enzymatic maturation from its exit from the endoplasmic reticulum to the membrane [125,127]. ADAM17 was first discovered as the enzyme responsible for the proteolytic cleavage of TNF-α and therefore, ADAM17 was originally called TNF-α Converting Enzyme [128]. Currently, it is known that ADAM17 is responsible for the shedding of over 90 substrates. Some substrates of ADAM17, such as TNF-α, Tumor necrosis factor-α receptor, Interleukin-6 receptor (IL6-R) and Triggering receptor expressed in myeloid cells-2 (TREM2), are involved in the onset of immune responses and neuroinflammation [129]. The influence of neuroinflammation in ASD has been recently reviewed [7]. Additionally, it was shown that ADAM17 expression levels increased with age in juvenile people diagnosed with ASD [130], although this was reported in relation to the elevation of sAPPα and not with respect to neuroinflammation, inflammation and immune responses. We will discuss the proteolytic cleavages of these substrates focusing on neuroinflammation and immunity in the CNS and their role in ASD below. More information involving ADAM17 in the intestinal tract will be provided in Section 5.

4.1. Tumor necrosis factor-α (TNF-α)

ADAM17 is the main protease of TNFα, a pro-inflammatory cytokine that can elicit its pro-inflammatory potential only after being proteolytically released from the cell surface [128,131]. Although deletion of the ADAM17 gene abolishes TNF-α shedding by 90% in ADAM17$^{-/-}$ macrophages and neutrophils, it seems that there are other proteases, amongst others MMP7 and proteinase 3, that are also able of proteolytically cleaving TNF-α [131]. In addition, ADAM10 was identified as major sheddase when ADAM17 is deficient in fibroblasts, indicating that there is a compensation between ADAM10 and ADAM17 [132]. These reports demonstrated that these two metalloproteases are important in TNF-α cleavage. It was shown that increased levels of TNF-α have been found in cerebellum and hippocampus in a murine model for ASD induced by Valproic Acid (VPA) [133] and in brain cortex of people diagnosed with ASD [134].

Together, there is an increase in pro-inflammatory cytokines in the brain of people diagnosed with ASD and it seems to be that decreasing the levels of these specific cytokines has a beneficial effect on disturbed sociability. As ADAM17 has been shown to be the main protease that controls TNF-α shedding from the cell membrane, an interesting connection between ADAM17-mediated TNF-α cleavage, neuroinflammation and ASD remains to be elucidated.

4.2. Interleukin-6 receptor (IL6-R)

IL6-R is a known substrate of ADAM17. The cytokine IL-6 has both pro- and anti-inflammatory properties, which is determined by the receptor signaling type [129,135]. Signaling via the membrane-bound IL-6R is called classic signaling. This type of signaling can

only occur on cell types that express IL-6R on their surface and is thus limited to hepatocytes and several leukocyte subsets and results in an anti-inflammatory response [136,137]. Signaling via soluble forms of the IL-6R, which is called *trans*-signaling, can occur on all cell types, because the IL-6/soluble IL-6R (sIL-6R) complex can directly bind to and activate the ubiquitously expressed glycoprotein 130 (gp130). The trans-signaling accounts mainly for the pro-inflammatory properties of IL-6 [137,138]. Interestingly, research in transgenic mice showed that proteolytic cleavage of IL-6R to create the soluble form is carried out by both ADAM17 and ADAM10 [139] and that approximately 85% of sIL6-R is a result of a proteolytic cleavage *in vivo* [140]. Increased levels of IL-6 in the brain have been found in a murine model for ASD [133] and in brain cortex of ASD patients [134]. Moreover, it has been shown that overexpressing IL-6 in the mouse brain mediates neuronal circuitry imbalances and induces ASD-like behavior [141]. Furthermore, one study showed that blocking the trans-signaling pathway of IL-6 led to improved social behavior in a murine ASD model by continuously infusing IL-6 trans-signaling blocker sgp130Fc protein [142]. As ADAM17 and ADAM10 are capable of proteolytically cleaving IL6-R to create the soluble IL6/sIL6-R complex, it will be of interest to investigate whether reducing the levels of these metalloproteases and consequently the level of sIL6-R will lead to reduced ASD-like behavior [142].

4.3. Triggering receptor expressed in myeloid cells-2 (TREM2)

ADAM17 is the main protease shedding TREM2 under steady state conditions [143]. Whether ADAM10 has a similar effect on TREM2 is still under debate [143]. TREM2 is a type I transmembrane protein and exclusively expressed by microglia [144,145]. TREM2 deficiency in mice decreases the number of microglia and activated microglia in hippocampus and increases synaptic and spine density [145]. This may be involved with increased expression of ligand of TREM2 induced by apoptotic neurons [146]. Furthermore, it is demonstrated that TREM2 is essential for initiating microglia-dependent synaptic pruning during early brain development [145]. Synaptic pruning is essential to remove synapses and keep normal brain connectivity during brain development. Shedding of TREM2 of microglial cells by ADAM17 might result in reduced synaptic pruning and associated neuronal overgrowth. The shedding of TREM2 results in a soluble fraction, sTREM2. Recently, Zhong et al. demonstrated that sTREM2 is able to activate microglia and increases neuroinflammatory responses both *in vitro* and *in vivo* [147], however, by which mechanisms remains to be elucidated.

Reports on the connection between TREM2 and ASD are currently scarce. Trem2-deficient mice display increased synaptic density, enhanced excitatory neurotransmission and reduced social and repetitive behavior [145]. Also, in post-mortem brain tissue of ASD patients, reduced TREM2 levels were found in the age group of 5-23 [145]. Furthermore, TREM2 protein levels of brain tissue were inversely correlated to Autism-Diagnostic Interview-revised score [145]. However, the levels of sTREM2 were not determined in this study. In general, reduced TREM2 could result in an altered control of microglial pruning and consequently this would lead to increased synaptic density. In ASD patients, synaptic density is increased on apical dendrites of pyramidal neurons from cortical layer 2 in frontal, temporal and parietal lobes and layer 5 only in the temporal lobe [6]. Therefore, the relationship between lower TREM2 levels and increased synaptic density in ASD patients seems plausible.

Taken together, it is difficult to determine the contribution of ADAM17 or ADAM10 mediated shedding of TREM2 in people diagnosed with ASD. The decreased levels of TREM2 in juvenile ASD could be related to either lower baseline TREM2 protein levels or to increased shedding of the protein by proteases. This can be determined by measuring sTREM2 levels in the same post-mortem tissue.

5. ADAM10 AND ADAM17 IN THE GUT-IMMUNE-BRAIN AXIS

The contribution of the intestines to ASD pathogenesis remains a field of active research. 46.8% of people diagnosed with ASD present with at least one intestinal symptom [148], such as constipation [149] and diarrhea [16]. Furthermore, a meta-analysis revealed that children with ASD show four times more intestinal symptoms than controls [150]. The gut-brain axis refers to the bidirectional interaction between these organs [151]. Alterations in this pathway could lead to increased permeability of both the intestinal and brain barriers. Research emphasizing the role of the intestine shows that in post-mortem duodenal tissue of people diagnosed with ASD elevated levels of pore-forming proteins and decreased levels of barrier-forming proteins were found in the tight junction of the intestinal epithelium [53]. These findings suggest a 'leaky gut', which could lead to more circulating bacterial metabolites in the blood of people diagnosed with ASD and activation of the immune system associated with an enhanced cytokine response [152-154]. Entering the brain by circulating cytokines and bacterial metabolites via blood is regulated by the Blood Brain Barrier (BBB). The BBB allows the selective entrance of compounds to the brain through the expression of receptors and transporters, which are necessary for maintaining brain homeostasis [155,156]. Disruption of the BBB, for example by chronic systemic inflammation, will increase the permeability and allow cytokines and bacterial metabolites to enter the brain directly. In turn this could result in neuroinflammation and neuronal dysfunction [156,157]. There are limited studies investigating the effect of ADAM10 and ADAM17 on the immune system, the intestinal homeostasis, intestinal microbiota, in intestinal inflammation and epithelial and endothelial (BBB) barrier function. These studies are discussed below.

5.1. ADAM10 and ADAM17 and blood-brain-barrier permeability

There is little known regarding the effect of ADAM10 or ADAM17 on the BBB permeability. Schulz et al. demonstrated ADAM10 increases endothelial permeability by specifically cleaving Vascular Endothelial Cadherin (VE-cadherin) in human umbilical vein endothelial cells [158]. The low density lipoprotein receptor-related protein 1 (LRP1) prevents endocytic transport of Aβ [159,160]. LRP1 is located at the abluminal surface of the brain endothelium by which Aβ is then released into the systematic circulation. ADAM10 KO and ADAM10 inhibition facilitates clearance of Aβ in the brain through decreasing proteolytic cleavage of LRP1 by ADAM10 in mice and in human brain microvessel endothelial cells [161]. It seems plausible that ADAM10 plays an important role in changing BBB permeability through proteolytic cleavage of junction and transporter proteins in the BBB.

5.2. ADAM10 in the intestinal tract

ADAM10 is widely expressed in intestinal epithelial cells [68] and involved in modulating intestinal permeability by targeting its substrates Notch and E-cadherin [48]. Research in conditional ADAM10-deficient mice points out that when ADAM10 is deleted in intestinal cells, there is early lethality caused by altered intestinal morphology and changes in cell differentiation [45]. Furthermore, it has been shown that intestinal morphology changes were due to the loss of Notch receptor signaling caused by shedding of ADAM10 [45]. The Notch receptor is a recognized substrate of ADAM10 that is ubiquitously expressed in all epithelial cell types. Notch receptor determines intestinal stem cell fate and controls intestinal homeostasis [162]. An increase of cleaved Notch-1 decreases transepithelial electrical resistance, indicative for reduced intestinal barrier function, and tight junction protein Claudin-5 expression in Caco-2 cells. In addition, levels of cleaved Notch-1 are increased in colonic epithelium of patients with Crohn's disease [47]. E-cadherin is one of the most important junction molecules involved in maintenance of intestinal epithelial integrity. It was demonstrated that E-cadherin is specifically cleaved by ADAM10 in mouse embryonical fibroblasts and by the absence of soluble E-cadherin in ADAM10-deficient mice [48]. Taken

together, these findings suggest that ADAM10-mediated shedding of Notch receptor and E-cadherin downregulates epithelial cell migration and adhesion and influences intestinal barrier function.

Finally, there might be role for ADAM10 (possibly also ADAM17) in cleaving APP in relation to weight gain as children diagnosed with ASD have a higher risk for developing overweight [163]. More recently, it has been demonstrated that APP mediates diet-dependent weight gain probably through enhanced TNF-α and IL-6 secretion by macrophages and potentiation of cholesterol uptake by colonic epithelial cells [164-166]. Moreover, high fat diet-induced APP production in white adipose tissue leads to mitochondrial dysfunction [167]. However, the role of ADAM10 in cleaving APP in relation to weight gain has not been studied. Taken together, it can be hypothesized that possibly, ADAM10-induced dysregulated APP can be involved in the development of obesity in ASD.

In conclusion, ADAM10 has an important role in maintaining intestinal homeostasis. However, there are no studies conducted about the role of enhanced ADAM10 on intestinal functioning in ASD and this will be crucial to deepen our understanding of this involvement.

5.3. ADAM17 in the intestinal tract

ADAM17 is ubiquitously expressed in all intestinal epithelial cells. In a murine model of decreased ADAM17 expression, where its activity is significantly reduced, normal intestinal epithelial cell proliferation is not compromised. However, there is a less effective response of the intestines to inflammation [168]. ADAM17 seems to be an essential component in regulating intestinal inflammation. The pro-inflammatory cytokines TNF-α and IL-6 [8] can disrupt the tight junction structure in the intestine and contribute to inflammation [169,170]. As ADAM17 cleaves TNF-α [171] and IL-6R [139], it seems that ADAM17 activity is tightly connected to the intestinal barrier integrity via a proinflammatory route. Recently, research pointed out that the polyphenol, resveratrol, is able to ameliorate social deficits in the VPA mouse model of ASD, probably attributing to its anti-inflammatory properties [172]. Resveratrol also reduced pro-inflammatory cytokine levels such as IL-6 and TNF-α in the BTBR T+tf/J mouse model of ASD, which indicates that inhibition of inflammation may be promising to a treatment of ASD [173]. Additionally, with the observed increased levels of TNF-α, sIL6R/IL6 complexes [134,174] and ADAM17 [130] in ASD patients, it is tempting to assume that this metalloprotease could be crucial in the development of ASD by alteration of the gut-brain axis.

5.4. ADAMs and intestinal microbiota

Another important aspect of the gut-brain axis is the intestinal microbiome. Intestinal microbiota consist of cohabitating microorganisms involved in regulating host immunity and inflammation [175-177]. There is bidirectional communication between the intestinal microbiota and the brain [178-180]. Although there are limited reports on the contribution of ADAM10 or ADAM17 to intestinal microbiota dysbiosis, it is possible to connect several studies and hypothesize.

The composition of the intestinal microbiome is altered in ASD children compared to normal healthy individuals [8,9,84,151]. Their microbial composition contains a higher proportion of Gram-negative bacteria [181], which increases the expression of lipopolysaccharides (LPS) [182]. LPS can activate the innate immune system through the activation of Toll-Like Receptor 4 (TLR4) [183,184]. Activation of TLR4 stimulates the production of pro-inflammatory cytokines in the intestinal and causes neuroinflammation in the brain by activation of microglia [185,186]. Strikingly, activation of TLR4 stimulates the ADAM17-dependent shedding of TNF-α [187]. Furthermore, pro-inflammatory cytokines and

LPS treatments are both able of increasing active ADAM10 levels *in vitro* [188]. It can be suggested that the altered microbiome in people diagnosed with ASD might be able to activate ADAM17 and ADAM10 through increased LPS and proinflammatory cytokines production.

A higher incidence of *Clostridium perfringens* in fecal samples of ASD children has been described compared to healthy children [189,190]. In the intestines, several species of *Clostridium perfringens* generate potent toxins that are the causatives of fatal intestinal and CNS diseases in animals [191]. Delta-toxin is one of these that perturbs intestinal epithelial barrier function in human intestinal epithelial Caco-2 cells through enhancing ADAM10 activity in a dose- and time-dependent way, which is blocked in the presence of ADAM10 inhibitor [192,193]. Therefore, the altered microbiome and related metabolites in ASD patients seem to be able to activate ADAM17 and ADAM10 by increasing the production of LPS and Delta-toxin. However, there is little known about the effects of other bacterial-generated metabolites on ADAM10 and ADAM17, such as *p*-cresol, its derivative *p*-cresyl sulfate (*p*CS) and 4-ethylphenylsulfate (4EPS). Levels of *p*-cresol and its conjugated derivative *p*CS are increased in urine and fecal samples in ASD children [97,194,195]. Urinary p-cresol has been suggested as a biomarker for ASD in small children because of its significant elevation [195]. 4EPS treatment induced ASD-like behavior in mice [8]. Mishra et al. showed that there was no difference in cecal bacterial microbiota composition and load between ADAM17 conditional KO mice and control mice, but the conditional knockout of ADAM17 decreased the peritoneal spread of bacteria following sepsis induction compared to control mice [196], which might be involved with reduced cleavages of TNF-α shedding and other proinflammatory cytokines by ADAM17.

Additionally, some substrates of ADAM17 or ADAM10 can regulate microbiota composition. Angiotensin-converting enzyme 2 (ACE2), a substrate of ADAM17 [197,198], plays an emerging role in the pathogenesis of cardiovascular and lung diseases through changing the composition of intestinal microbiota, such as increasing the ratio of the *Firmicutes* to *Bacteroidetes* and decreasing *Bifidobacterium* genus, which raises the potential relation of ADAM17-mediated ACE2 shedding and intestinal dysbiosis [199-201]. Meantime, it is demonstrated that enteric infection coupled with chronic Notch receptor pathway inhibition in mice is associated with bacterial dysbiosis compared to control mice, indicated as a significant decrease in Bacteroidetes phyla with concomitant increases in Firmicutes, Proteobacteria and Verrucomicrobia phyla [46]. It seems that Notch receptor inhibition changed the microbiota composition in enteric infected mice, which implies that ADAM10 overexpression may have a similar effect on microbiota composition through cleavage of the Notch receptor. Taken together, intestinal microbes can regulate ADAM10 or ADAM17 activity by producing bacterial metabolites and, in turn, ADAM10 or ADAM17 can also change intestinal microbiome composition. However, these connections remain unclear and more research is needed.

5.5. ADAMs and the immune system

The development and function of the immune system is highly dependent on intestinal microbiota, as demonstrated by the limited immune activity in germ free mice [202]. Intestinal bacterial fermentation produce a wide range of metabolites on the basis of tryptophan, tyrosine and phenylalanine from our daily diet, such as serotonin, short-chain fatty acids (SCFAs), indole-containing metabolites and *p*-cresol [203-205]. The role of SCFAs and other not mentioned bacterial metabolites in ASD have been extensively reviewed [177]. These metabolites can regulate immune responses and inflammatory responses by recognizing their receptor on epithelial cells or entering into the systemic circulation or the brain. SCFAs promote the number, function and differentiation of colonic Treg cells in mice [203,206]. In

addition, SCFA-specifically, butyrate-being fuel for epithelial cells promote intestinal barrier integrity [207,208]. In the brain, SCFAs also increases microglia maturation and function in mice [204]. However, the exact mechanisms how these metabolites affect host immune and brain functions remains to be investigated. In recent years, alterations in the gut-brain axis have been presented as possible pathological causes of ASD and targeting the intestinal microbes has been recognized as a promising treatment for ASD [8,16,178,209]. For example, maternal immune activation (MIA) induced by polyinosinic:polycytidylic acid (polyI:C) injection led to intestinal dysbiosis in the male offspring associated with in defects in communicative, stereotypic, anxiety-like and sensorimotor behaviors. Oral administration of *Bacteroides fragilis* restored these ASD-like symptoms [8]. The roles of ADAM10 and ADAM17 in the effects of bacterial metabolites in the immune system in ASD has not been researched well, however, it is possible to speculate about its potential involvement.

Transforming growth factor β1 (TGFβ1) is one member of TGFβ family and, generally, regulates T lymphocytes and antigen-presenting cells as an immunosuppressor [210]. Some studies showed that there is a significant decrease of TGFβ1 level in plasma or serum of ASD children [211,212]. Moreover, one study showed that TGF-β1 might be considered as a biomarker of ASD severity. Increasing TGFβ1 level in plasma of ASD children consequently improved behavioral rating score [213]. TGFβ1 also is essential to microglial development, phenotypes and functions *in vitro* and *in vivo*, which is connected with ASD pathogenesis [214-216]. Besides, TGFβ1 plays a vital role in modulating social interaction and repetitive behavior in mice hippocampus. It was demonstrated that adult hippocampal TGFβ1 overexpression increases social interaction and decreases self-grooming and depression-related behaviors and early hippocampal TGFβ1 overexpression reversely decreases those behaviors [217]. Kawasaki et al. illustrated that TGFβ1 signaling is dependent on ADAM 17 activity, and thus, modifying ADAM17 genetic variants enhances TGFβ1 signaling activity through cleaving less type 1 TGF-beta receptor (TGF-βR1) in mice and human [218]. It is demonstrated that TGFβR1 is a substrate of ADAM17 and its cleavage by ADAM17 downregulates TGFβ1 signaling through decreasing cell surface TGF-βR1 [218,219]. In addition, Vasorin is a type1 transmembrane protein and it is cleaved by ADAM17 to generate soluble Vasorin that binds to TGFβ1 as a suppressor [220,221]. To our knowledge, there is little known about the ADAM17-mediated TGFβ1 signaling in ASD pathogenesis.

T-helper 17 lymphocytes (TH17) cells and their effector cytokines interleukin 17 (IL-17) are necessary for immune responses against extracellular bacteria and fungi, and their dysregulation is thought to underlie numerous inflammatory and autoimmune diseases, such as inflammatory bowel disease and multiple sclerosis [222]. Choi et al. demonstrated that maternal immune activation induced ASD-like behavioral phenotypes in offspring and compromised their cortical brain development in an IL-17a-dependent manner. IL17a KO or IL-17a blockage with an antibody rescued ASD-like phenotypes [223]. The pro-inflammatory cytokine IL-6 inhibits TH17 cell differentiation from CD4+ T-cell subset as an upstream regulator of IL-17a [224,225]. Therefore, IL-6 is also necessary for maternal immune activation-induced ASD-like phenotypes in the offspring [226]. It is demonstrated that ADAM17 regulates IL-6 signaling through controlling IL-6 receptor (IL-6R) *in vitro* and *in vivo* [227]. Horiuchi et al. reported that conditional ADAM17 KO mice have increased serum levels of IL-17 compared with control littermates, indicating that decreased ADAM17 activity is associated with a downregulation of IL-17 secretion *in vivo* [187], but the role of IL-17 receptor in this process is not investigated and the definite mechanism remains to be elucidated. It is possible that the increased IL-17 level in ADAM17 KO mice is a result from the increased cleavage of IL-17 receptor by ADAM17 [228], but it is barely studied. These findings make it promising to investigate the potential roles of ADAM10 and ADAM17 in the pathogenesis of ASD from an immunological perspective.

6. METALLOPROTEASES ADAM10 AND ADAM17 AS THERAPEUTIC TARGETS FOR AUTISM SPECTRUM DISORDERS

The metalloproteinases ADAM10 and ADAM17 are or might be involved in different aspects of ASD pathogenesis. Strikingly, as can be concluded from Table 2, it seems that the enhanced expression and activity of ADAM10 or ADAM17 might contribute to several aspects of ASD. Therefore, the reduction or inhibition of these targets could be interesting as therapeutic strategies for ASD. Although not much attention has been given in research to targeting metalloproteases, some possible therapeutic options will be discussed below.

6.1. TIMPs

The natural inhibitors of the metalloproteases are the Tissue Inhibitors of Metalloproteases (TIMPs). There are four members of the TIMP family [229,230]. In general, the TIMPs can inhibit all MMPs, but the strength of MMP inhibition differs between TIMP. Interestingly, TIMPs inhibit ADAMs with higher specificity. For example, ADAM10 is specifically inhibited by TIMP-1 and TIMP-3 [231]. Moreover, TIMP-3 also inhibits ADAM17 [232]. The main limitation of metalloprotease inhibitors is their lack of selectivity. Therefore, an inhibitor can affect other enzymes as well, which could lead to undesirable side effects. For instance, increasing TIMP-3 levels can be an interesting therapeutic target, as this can possibly decrease both ADAM10 and ADAM17 activity in ASD patients, however, TIMP-3 also has an inhibitory effect on most MMPs [233,234]. Thus, the search for selective inhibitors is of critical importance in order to be used as a therapeutic drug.

6.2. ADAM inhibitors

In search for molecules with a great selectivity for ADAM10 and ADAM17, GI254023X compound has been identified as a potent and selective inhibitor of ADAM10 with 100-fold higher selectivity than ADAM17 [235,236]. In addition, Mahasenan et al recently synthesized and tested the compound (1*R*,3*S*,4*S*)-3-(hydroxycarbamoyl)-4-(4-phenylpiperidine-1-carbonyl) cyclohexyl pyrrolidine-1-carboxylate that showed high potency of inhibiting ADAM10 [237]. This compound can also cross the BBB [237]. Furthermore, Hirata et al. showed that the inhibitor KP-457 has over 50 times higher selectivity for ADAM17 than ADAM10 or any other MMPs [238].

TspanC8 members regulate ADAM10 maturation and substrate selectivity. Six TspanC8 members can form six different Tspan-ADAM10 complexes, which preferentially cleave different substrates as six scissors [36,62,65,125]. Therefore, the development of inhibitors targeting these complexes is beneficial compared to the side effects of broad ADAM inhibitors. Taken together, these studies showed that effective inhibitors are available and might be beneficial for ASD treatment.

6.3. Probiotics, bacterial metabolites and prebiotics?

As discussed above, intestinal dysbiosis has been frequently described in children suffering from ASD. Pro-, prebiotic and even microbiota transfer therapy (MTT) interventions have been proposed as a promising treatment for ASD children [16,239-242]. Of interest are the bacterial metabolites 4EPS, as well bacterial toxin LPS, that induces ASD-like behavior in mice by unknown mechanisms [8,243]. Furthermore, bacterial LPS-induced increase of ADAM10 expression is important for proinflammatory immune cell responses [244]. Other important bacterial metabolites are SCFA. Increase of enteric SCFAs levels in ASD-mice [245], as well as in ASD children, are demonstrated [246,247]. The precise mechanism of action of SCFA in relation to ASD-like behavior is not known, but effects on mitochondrial function or

epigenetic alterations in the brain may be involved [248]. Given the important roles of ADAM10 and ADAM17 in gut function, immunity and brain, it will be interesting to study whether and how intestinal microbiota-derived metabolites, such as 4EPS, LPS, p-cresol and SCFA, affect ADAM10/17 activity in the intestinal tract and in the brain related to ASD. When, indeed, an important role of these bacterial metabolites on ADAM10/17 activity is established, then targeting the intestinal microbiota with pre- and probiotics, as well as MTT, may be useful in ASD.

6.4. Targeting ADAM10 and ADAM17 in ASD: some considerations

The strategy of targeting ADAM 10 and/or ADAM17 as future treatments in ASD raises several issues. First, the processes in which ADAMs are involved are critical for cell, tissue and organ functioning, therefore ADAM10 and ADAM17 inhibitors might have serious side effects. Both proteases contribute to developmental and regenerative processes; for example, disruption of ADAM17 in mice leads to death, and studies in KO mice show that ADAM10 is vital for early development [125]. Specific targeting of ADAM10 or ADAM17 at the right time and in the right location might be the way to go. Secondly, it should be investigated at which location ADAM10 and ADAM17 should be targeted: in the intestines or the brain. For the latter compounds that are able to pass the BBB are essential. Indirect targeting through manipulation of the intestinal microbiota with pre-, pro or postbiotics might be a safer way to inhibit the enhanced ADAM10 and ADAM 17 activity in ASD.

Table 2. The effect of ADAM10 and ADAM17 on different substrates and their involvement in Autism Spectrum Disorder (ASD) patients. ↓: downregulated; ↑: upregulated

Protein Name	Gene Symbol	ADAM10 shedding	ADAM17 shedding	ASD patients
Amyloid Precursor Protein	APP	↑sAPPα [32]	↑sAPPα [32]	↑sAPPα [28]
Neuroligin-1	NLGN-1	↓Synaptogenic activity [92]		Common variants [249,250]
Neuroligin-3	NLGN-3	↓Synaptogenic activity [92]		R415C transition[251,252] Common variants[250]
Neurexin-1	NRXN-1	↓Synaptogenic activity[93]		Loss-of-function variants[24,26,27]
Neural glial-related Cell Adhesion Molecule	NrCAM	↑ Axon targeting activity [35]		SNPs & Common variants[25]
Protocadherin9	PCDH9	No data available		Copy Number Variants[24]
Fractalkine	CX3CL1	↑ Synaptic pruning [35,121,122]		No data available
Tumor Necrosis Factor-α	TNF-α		↑pro-inflammatory activity [131]	↑in blood and brain [134]
Interleukin-6 Receptor	IL-6R	↑pro-inflammatory pathways [139]	↑pro-inflammatory pathways [139]	↑IL-6 in blood and brain [134]
Triggering Receptor Expressed in Myeloid cells-2	TREM2		↓TREM2 membrane receptor levels [143]	↓in post-mortem brain tissue age 5-23 [145]

7. Outlook and conclusions

ASD is a highly heterogeneous disorder that includes multiple affected genes, altered synaptic density, neuroinflammation, low-grade systemic immune activation and an intestinal phenotype, including 'leaky gut'. Therefore, it is challenging to pinpoint what the exact underlying cause of this neurodevelopmental disease is. The ASD-associated enhanced expression and/or activity of the metalloproteases ADAM10 and ADAM17 provide an overarching hypothesis that affects many different aspects that seem to be involved at least in part in ASD pathology. ADAM10 is responsible for the proteolytic cleavage of several key proteins involved in synapse formation, axon signaling and cell adhesion and for regulating intestinal permeability. Furthermore, ADAM17 has a pivotal role in the shedding of proteins that regulate the onset of (neuro)inflammation and immune responses. Additionally, the

effect of ADAM10 and ADAM17 on intestinal microbiota composition and the effects of bacterial metabolites on ADAM10 and ADAM17 expression and activity remains to be investigated. Taken together, these two metalloproteases seem responsible for activating key pathways that seem to be altered in ASD pathogenesis. Figure 3 provides an overview of the pathways where ADAM10 and ADAM17 are possibly involved in the pathogenesis of ASD. In conclusion, enhanced expression and/or activity of ADAM10 or ADAM17 could possibly be involved in the induction and maintenance of ASD-like phenotypes in the brain as well as systemically and in the intestinal tract. Consequently, this hypothesis suggests that reducing the levels or activity of ADAM10 or ADAM17 could be a potential therapeutic target in ASD patients.

In order to provide more evidence to support this hypothesis, it is necessary to further determine if there is any altered ADAM10 and ADAM17 expression and/or activity in ASD-associated mouse models or in ASD patients. Moreover, more studies need to be conducted to investigate the role and molecular mechanisms of ADAM10 and ADAM17 which will shed light on the molecular pathogenesis and possible targets for treatment of ASD. Additionally, it will be interesting to screen metalloproteases specific inhibitor and then test specific inhibitors of ADAM10 and ADAM17 in ASD animal models.

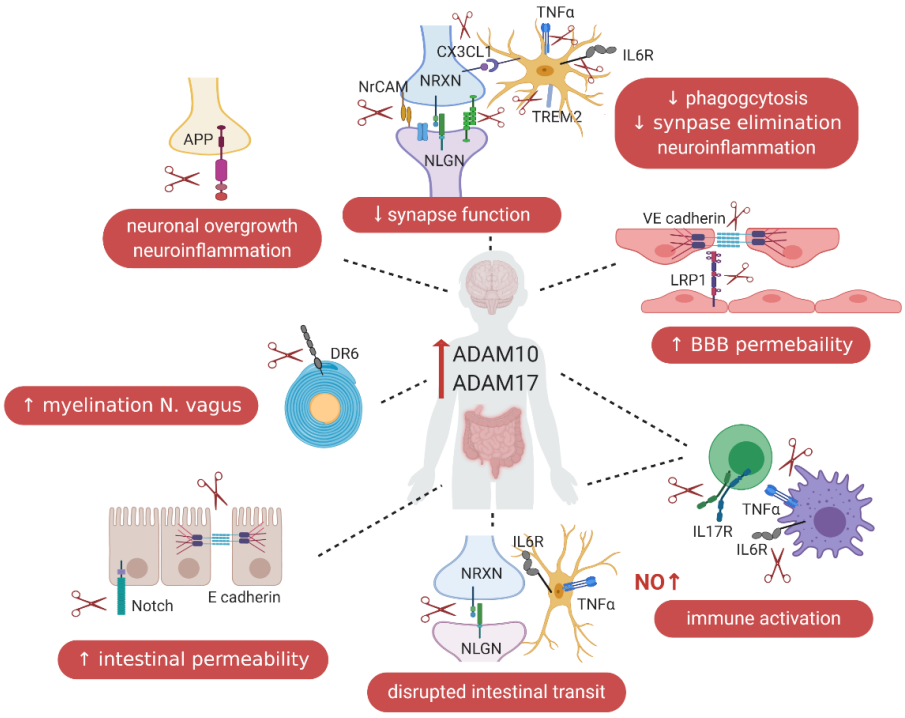

Figure 3. Overview of the proposed role of ADAM10 and ADAM17 in ASD pathology, with a focus on gut-immune-brain axis. In intestine, the increased activity or levels of ADAM10 and ADAM17 will lead to increased intestinal permeability by cleaving more E-cadherin and Notch, increased intestinal inflammation by cleaving more IL-6R and TNF-α, disrupted intestinal transit by cleaving more NLGN3 and increased myelination of nervus vagus by cleaving more DR6 in Enteric Nervous System; these contribute to intestinal dysfunctions. In the brain, the elevated activity of ADAM10 and ADAM17 will result in increased neuronal growth, synaptic density, dendritic spines densities and larger brain volume by cleaving more synaptic molecules, such as NLGN, NRXN, NrCAM, and APP. Furthermore, it will result in decreased synaptic elimination and microglial phagocytosis by cleaving more CX3CL1 and TREM2, increased neuroinflammation by cleaving more IL-6R, TNF-α and TREM2, and increased BBB permeability by cleaving more VE-cadherin and LRP1. These processes will lead to an altered brain development and functions. Additionally, increased ADAM10 and ADAM17 activity will lead to immune activation by cleaving more IL-17R, IL-6R and TNF-α. All of these can participate ASD development and involve in ASD pathogenesis.

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Chapter 4

The autism spectrum disorder-associated bacterial metabolite *p*-cresol derails the neuroimmune response of microglial cells partially via reduction of ADAM17 and ADAM10

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Abstract: The bacterial metabolite 4-methylphenol (*para*-cresol or *p*-cresol) and its derivative *p*-cresyl sulfate (*p*CS) are elevated in the urine and feces of children with autism spectrum disorder (ASD). It has been shown that *p*-cresol administration induces social behavior deficits and repetitive behavior in mice. However, the mechanisms of *p*-cresol, specifically its metabolite *p*CS that can reach the brain, in ASD remain to be investigated. The *p*CS has been shown to inhibit LPS-stimulated inflammatory response. A Disintegrin And Metalloprotease 10 (ADAM10) and A Disintegrin And Metalloprotease 17 (ADAM17) are thought to regulate microglial immune response by cleaving membrane-bound proteins. In the present study, a neuroinflammation model of LPS-activated BV2 microglia has been used to unveil the potential molecular mechanism of *p*CS in ASD pathogenesis. In microglial cells *p*CS treatment decreases the expression or maturation of ADAM10 and ADAM17. In addition, *p*CS treatment attenuates TNF-α and IL-6 releases as well as phagocytosis activity of microglia. In *in vitro* ADAM10/17 inhibition experiments, either ADAM10 or ADAM17 inhibition reduces constitutive and LPS-activated release of TNF-α, TNFR-1 and IL-6R by microglial cells, while it increases constitutive and LPS-activated microglial phagocytotic activity. The *in vivo* results further confirm the involvement of ADAM10 and ADAM17 in ASD pathogenesis. In *in utero* VPA-exposed male mice, elevated concentration in serum of *p*-cresol-associated metabolites *p*CS and *p*-cresyl glucuronide (*p*CG) is associated with a VPA-induced increased ADAM10 maturation and a decreased ADAM17 maturation that is related with attenuated levels of soluble TNF-α and TGF-β1 in the mice brain. Overall, the present study demonstrates a partial role of ADAM10 and ADAM17 in the derailed innate immune response of microglial cells associated with *p*CS-induced ASD pathogenesis.

Keywords: Autism spectrum disorder; Valproic acid; *p*-cresol; *p*-cresyl sulfate; ADAM10; ADAM17; neuroimmune response

1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in social communication and interaction, impairments in memory and learning activity, as well as the presence of repetitive behaviors that can have substantial impact on the quality of life [1]. Besides the behavioral and cognitive deficits, often autistic individuals suffer from gastrointestinal problems associated with gut microbiota dysbiosis [2, 3]. In recent years, reports point to a possible role of the gut-brain axis in ASD. Recently a thorough epidemiological review shows that the current worldwide ASD prevalence is 1%, which makes ASD one of the most frequently occurring neurodevelopmental disorders in childhood [4]. Additionally, ASD is diagnosed more in males than females with an approximate gender-bias of 4.1:1 [4-6]. At present, due to lacking clinically sensitive biomarkers, ASD diagnosis relies on behavioral evaluations and there are no effective treatments targeting the ASD core symptoms. Therefore, identifying therapeutical molecular targets for treatment are getting increasingly urgent [7-9]. Although the pathophysiology of ASD remains elusive, ASD is known to be caused by an interplay between genetic and environmental factors. A wide range of susceptibility genes have been identified accounting for 10–20% of ASD cases [10, 11]. Possible prenatal and postnatal environmental factors associated with an enhanced risk of ASD include maternal exposure to toxins or certain medications (including antibiotics, anti-epileptic medicine and selective serotonin reuptake inhibitors), infections and specific epigenetic factors [11-13]. Prenatally exposure to the anti-epileptic medicine valproic acid (VPA) at gestational day 10-13.5 in mice can induce substantial and permanent core symptoms of human ASD, including social impairments, repetitive behavior, and cognitive rigidity, as well as an intestinal phenotype [14, 15]. The *in utero* VPA-induced mice model is a widely recognized ASD model [14-17]. We and others have reported that *in utero* exposure to VPA during pregnancy present with social impairments and repetitive behavior, specifically in the male offspring , reflecting the sex-difference of ASD prevalence in human [14, 18].

In the brain dysregulated immunomodulation of microglia leads to immune abnormalities and synapse dysfunction in context of ASD pathogenesis [19]. Microglia is important resident immune cells in the brain, and regulate inflammatory responses and synaptic pruning, which orchestrates brain development, connectivity and homeostasis [19-21]. ASD patients show activated microglia in multiple brain regions [22, 23]. Enhanced microglia density also occurs in brain regions of *in utero* VPA-induced murine models [24, 25]. In recent years, it has been suggested that gut microbiota affects microglial maturation and function through producing bacterial metabolites [19, 26, 27]. 4-methylphenol (*para*-cresol or *p*-cresol) is a known uremic toxin and a metabolite produced by several bacterial families of the gut microbiota, including Clostridiaceae, Lachnospiraceae and Ruminococcaceae [28-30]. Once produced by bacterial fermentation of tyrosine and phenylalanine in intestine, 95% of *p*-cresol is metabolized by the host into 4-methylphenyl sulfate (*p*-cresyl sulfate, *p*CS) via O-sulfonation, a process that occurs primarily in the liver, and to a smaller extent in colonic epithelial cells [31-33]. The bacterial *p*-cresol and its derivative *p*CS are elevated in the urine and feces of ASD children [3, 34, 35]. In addition, *p*CS has been detected in mice brain tissues, suggesting that *p*CS can enter the brain and might further affect microglial function in brain [36, 37]. Furthermore, *p*-cresol and *p*CS have been proposed as potential biomarkers for ASD clinical diagnosis [35, 38]. Pascucci et al. have shown that *p*-cresol (intravenously delivered by tail vein injection) exacerbates ASD-like behaviors in BTBR mice [12]. Very recently, Bermudez-Martin et al. have described that *p*-cresol administration in drinking water induces social behavior deficits and repetitive behavior in mice by remodeling the gut microbiota [39]. However, the exact molecular mechanism of how *p*-cresol and *p*CS contribute to the pathogenesis of ASD still remains elusive.

ADAM10 and ADAM17 are two members of A Disintegrin And Metalloproteases (ADAMs) family that includes a group of enzymes that are able to cleave membrane-bound proteins. ADAM10 and ADAM17 cleave important protein substrates regulating neuronal networks and immune responses, respectively, such as synaptic molecules Neural Glial-Related Cell Adhesion Molecules (NrCAM) and neuro-inflammatory cytokine Tumor Necrosis Factor α (TNF-α), which are involved or derailed in ASD [40]. ADAM10 plays a key role in the modulation of the molecular mechanisms responsible for dendritic spine formation, maturation and stabilization and in the regulation of the molecular organization of the glutamatergic synapse [41, 42]. Moreover, ADAM10 maturation is increased in a mouse model of Fragile X syndrome (FXS) that is used as a murine ASD model [42]. It is feasible to conclude that the upregulation of ADAM10, resulting in enhanced cleavage of synaptic substrates, might be important in the synaptopathies of ASD. The involvement of ADAM10 in ASD needs to be further studied. In addition to cleaving TNF-α to trigger microglial immune response, ADAM17 regulates microglial phagocytosis capacity by cleaving Triggering receptor expressed in myeloid cells (TREM2) [43, 44]. Ray et al. have shown a significantly elevated soluble ADAM17 level in brain tissue of patients with ASD [45], but its role in ASD pathogenesis still remains to be investigated.

Several *in vitro* studies have shown *p*-cresol and *p*CS treatment decreases the LPS-induced inflammation and suppresses LPS-activated immune response in murine macrophages [46-48], but it is unknown whether *p*CS has similar effects on microglial functions in the brain. The aim of the present study is to investigate the connection between *p*CS, and ADAM10 and ADAM17, in the derailed immune response of microglial cells associated with ASD pathogenesis.

The present study investigated the direct effects of *p*CS on innate immune response and phagocytosis activity of microglial cells in the absence and presence of LPS stimulation to examine the connection between *p*CS and ADAM10/ADAM17 in the derailed immune response of microglial cells *in vitro*. In order to demonstrate whether ADAM10 and ADAM17 is affected in *in utero* VPA-exposed male mice, the expression and maturation of ADAM10 and ADAM17 as well as their downstream neuro-inflammatory cytokines soluble TNF-α and TGF-β1 in the mice brain tissues were measured (Supplementary materials & results)

2. Results

2.1. Effect of pCS on the expression of ADAM10 and ADAM17 of microglial cells constitutively and during LPS-induced inflammation.

The *p*-cresol host metabolites *p*CS and *p*CG levels were significantly increased in *in utero* VPA-exposed male mice compared to control mice (supplementary Figure S1). Decreased imADAM10, increased mADAM10 and ADAM10 maturation efficiency were observed in the hippocampus and other brain regions of *in utero* VPA-exposed male mice compared to control mice (supplementary Figure S2A-H). In addition, *in utero* exposure to VPA significantly decreased both mADAM17 content and ADAM17 maturation efficiency in the hippocampus compared to control mice (supplementary Figure S3A, D). ADAM17, also called TNF-α Converting Enzyme (TACE) [49], cleaves membrane bound TNF-α to soluble TNF-α (sTNF-α) and is an important regulator of TGF-β1 [50, 51], the VPA-reduced ADAM17 was associated with decreased expression of sTNF-α and TGF-β1 levels in the hippocampus (supplementary Figure S3E, F). These results show that enhanced systemic *p*CS is connected with dysregulated ADAM10/ADAM17 and ADAM17-associated cytokines in brain, which is involved with the pathogenesis of *in utero* VPA-induced ASD.

Because *p*CS can enter the brain [36, 37], we therefore examined the connection between *p*CS and ADAM10/ADAM17 in microglial cells. Using two cell viability assays (MTT and

LDH), it was demonstrated that 24 h exposure of BV2 cells up to a concentration of 500 µM *p*CS did not affect the viability of BV2 cells (supplementary Figure S4).

Exposure of BV2 cells to LPS showed a decrease in mADAM10, but showed no significant effect on imADAM10 and ADAM10 maturation efficiency (mADAM10/ imADAM10) compared to vehicle control (Figure 1A-H). The expression of imADAM10 and mADAM10 was barely affected by exposure of LPS-stimulated BV2 with *p*CS low (0.1, 0.5, 1 and 5 µM, Figure 1A-C) and high (5, 10, 50 and 150 µM, Figure 1E-G) concentrations compared to LPS control, this was also reflected by no significant change of ADAM10 maturation efficiency (Figure 1D, H). In contrast, *p*CS alone at the low concentrations reduced mADAM10 without affecting imADAM10 expression and ADAM10 maturation efficiency (Figure 1A-D). Furthermore, *p*CS alone at the high concentrations reduced the expression of imADAM10 and mADAM10 without affecting ADAM10 maturation efficiency (Figure 1E-H).

The 24 h incubation with LPS induced upregulation of ADAM17 expression in BV2 cells (Figure 1J, L). Co-incubation of LPS-stimulated BV2 cells with *p*CS at high concentrations (5, 10, 50 and 150 µM) demonstrated a reduced expression of ADAM17 (Figure 1L). Moreover, these concentrations of *p*CS reduced the constitutive ADAM17 expression in BV2 cells (Figure 1J, L).

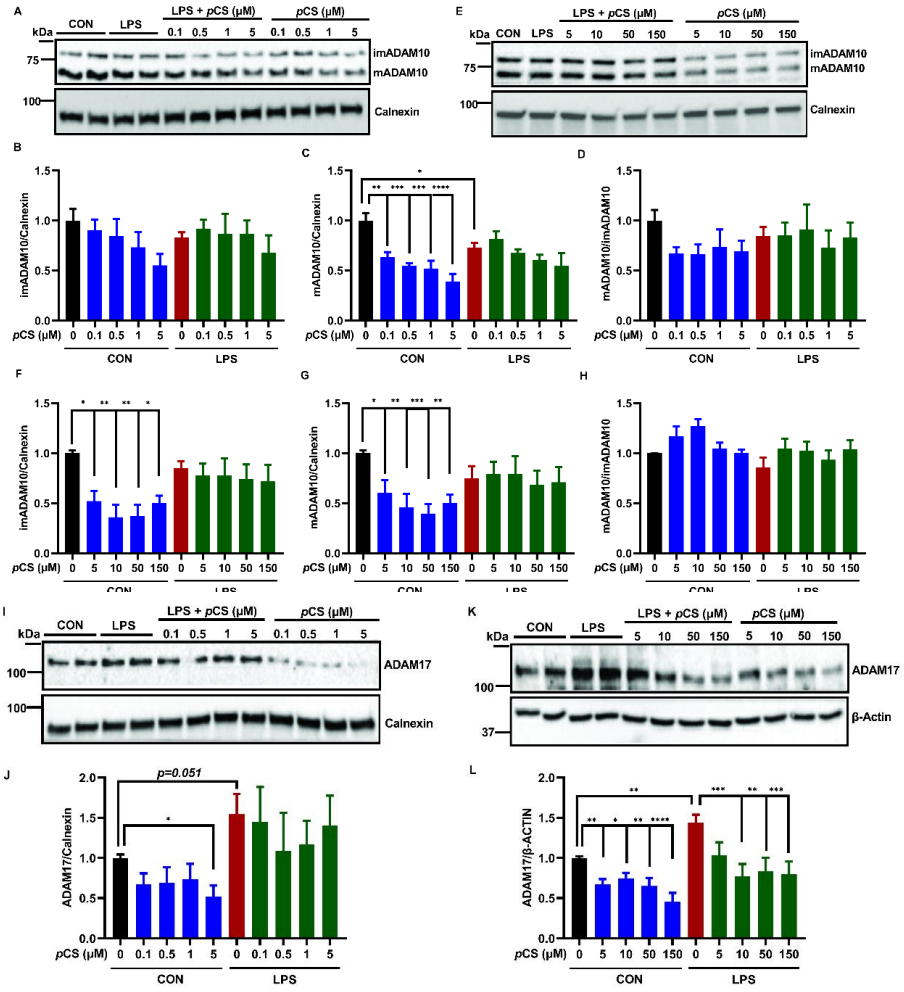

Figure 1. Effect of *p*CS on ADAM10 and ADAM17 expression of constitutive and LPS-activated BV2 microglial cells. BV2 microglia were incubated with *p*CS for 24 h in the absence or presence of 1000 ng/mL LPS. Cell lysate was collected for WB analysis. (A) The representative immunoblots of ADAM10 protein expression with low concentration range of *p*CS. (B-D) The quantification results of immature ADAM10 (imADAM10), and mature ADAM10 (mADAM10) and maturation efficiency (ratio mADAM10/imADAM10) of BV2 cells exposed to low concentration range of *p*CS (n=4 independent experiments). (E) The representative immunoblots of ADAM10 protein expression with high concentration range of *p*CS. (F-H) The quantification results of ADAM10 protein expression of BV2 cells exposed to high concentration range of *p*CS treatment (n=5 independent experiments). (I) The representative immunoblots of ADAM17 protein expression with low concentration range of *p*CS. (J) The quantification results of ADAM17 protein expression with low concentration range of *p*CS (n=4 independent experiments). (K) The representative immunoblots of ADAM17 protein expression with high concentration range of *p*CS. (L) The quantification results of ADAM17 protein expression with high concentration range of *p*CS (n=6 independent experiments). Calnexin or β-Actin were used as a loading controls. Black: constitutive protein expression set at 1; red: LPS-induced protein

expression; blue: effect of *p*CS on constitutive protein expression; green: effect of *p*CS on LPS-induced protein expression. Results were expressed as mean ± SEM. **P* < 0.05, ***P < 0.01*, ****P < 0.001, ****P < 0.0001*.

These results indicate that *p*CS might negatively affect the innate immune response of BV2 microglia through inhibiting ADAM17 and possibly through ADAM10. Given that ADAM10 and ADAM17 cleave TNF-α and other cytokine-related substrates, such as IL-6 receptor (IL-6R) and Tumor necrosis factor receptor 1 (TNFR-1) [52-54], we further investigated the potential role of ADAM10 and ADAM17 in innate immune and phagocytotic responses of BV2 microglial cells constitutively and after LPS stimulation.

2.2. ADAM10 and ADAM17 cleave TNF-α, TNFR-1 and IL-6R from BV2 microglia.

The role of ADAM10 and ADAM17 is not known in the release of TNF-α and IL-6 as wells as the cleavages of TNFR-1 and IL-6R from BV2 microglial cells during an innate immune response. To this end, we employed the effects of GI254023X, a selective and potent inhibitor of ADAM10 activity [55, 56], and TAPI-1, a compound preferential inhibiting ADAM17, followed by other metalloproteinases [57, 58] on constitutive and LPS-activated BV2 cells. A 24 h incubation with LPS resulted in a significant release of TNF-α, IL-6 and TNFR-1 compared to vehicle treated BV2 cells, whereas LPS did not affect sIL-6R levels (Figure 2A-D). Both ADAM10 inhibitor, GI254023X (5 μM), and ADAM17 inhibitor, TAPI-1 (25 μM) significantly decreased the constitutive and LPS-induced release of TNF-α and soluble TNFR-1 (sTNFR-1), indicating either ADAM10 or ADAM17 (and possibly other metalloproteases) regulate the cleavages of TNF-α and TNFR-1 in BV2 microglia (Figure 2A, B). GI254023X did not affect the LPS-induced release of IL-6 from BV2 cells, and TAPI-1 significantly enhanced the release of IL-6 (Figure 2C). In contrast, GI254023X and TAPI-1 did inhibit the release of soluble IL-6R (sIL-6R), indicating ADAM10 and ADAM17 control the cleavage of IL-6R (Figure 2D).

In the cell lysates we further investigated the effect of two inhibitors on the expression of ADAM10 or ADAM17 in BV2 microglial cells constitutively and under inflammation. Figure 2F shows imADAM10 was not affected by all treatments. LPS induced a reduced ADAM10 maturation efficiency (Figure 2H) confirming the findings presented in Figure 1. GI254023X and TAPI-1 significantly decreased constitutively and under inflammation mADAM10 resulting in reduced ADAM10 maturation efficiency (Figure 2G, H). Figure 2J demonstrates that LPS stimulation significantly increased ADAM17 expression compared to vehicle treated BV2 cells, as also demonstrated in Figure 1. Both inhibitors GI254023X and TAPI-1 did not decrease ADAM17 expression of BV2 cells constitutively or under inflammation (Figure 2J). Furthermore, GI254023X on its own significantly increased ADAM17 expression compared to vehicle treated BV2 cells (Figure 2J).

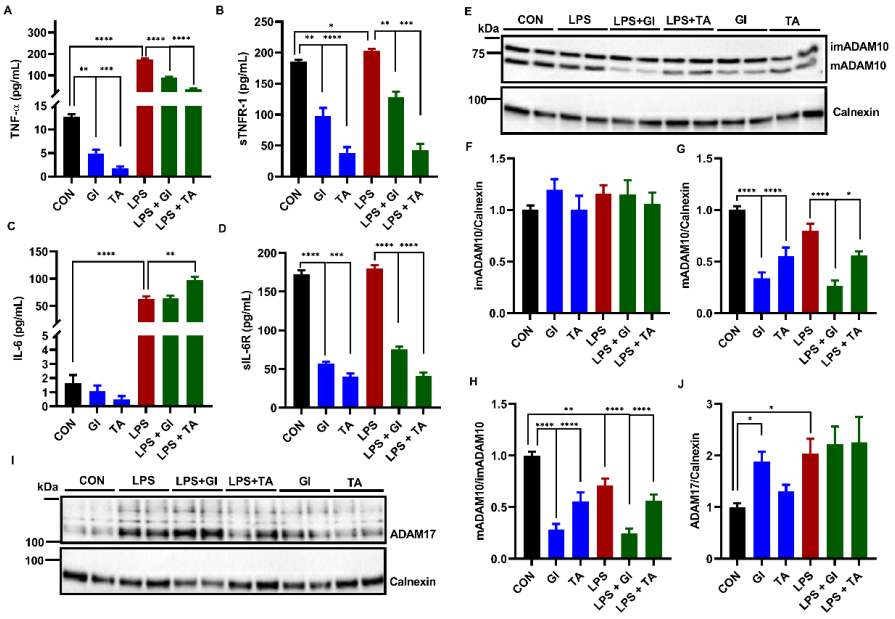

Figure 2. The effect of ADAM10 inhibitor (GI254023X) or ADAM17 inhibitor (TAPI-1) on the release of TNF-α, TNFR-1 and IL-6R by constitutively and under inflammation BV2 microglia. BV2 microglia were incubated with GI254023X (5 μM) or TAPI-1 (25 μM) for 24 h in the absence or presence of 1000 ng/mL LPS. The medium was collected for ELISA measurements and cell lysates were lysed for WB analysis. The concentration of TNF-α (A), sTNFR-1 (B), IL-6 (C) and sIL-6R (D) released in medium (n=6 from 6 independent experiments). (E) The representative immunoblots of ADAM10 protein expression in BV2 cell lysate. (F-H) The quantification of ADAM10 expression in BV2 cell lysate (n=10 from 5 independent experiments). (I) The representative immunoblots of ADAM17 expression in BV2 cell lysate. (J) The quantification results of ADAM17 in BV2 cell lysate (n=8 from 4 independent experiments). Calnexin was used as a loading control. Black: constitutive cytokine release or protein expression set at 1; red: LPS-induced cytokine release or protein expression; blue: effect of ADAMs inhibitor on constitutive cytokine release or protein expression; green: effect of ADAMs inhibitor on LPS-induced cytokine release or protein expression. Results were expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$ ***$P < 0.001$, ****$P < 0.0001$.

2.3. The effect of ADAM10 and ADAM17 inhibition on constitutive and LPS-induced phagocytosis by BV2 cells

As shown in Figure 3, 24 h LPS stimulation significantly increased phagocytosis by BV2 cells. GI254023X significantly potentiated LPS-induced phagocytosis compared to LPS (Figure 3A). In addition, TAPI-1 also significantly potentiated LPS-induced phagocytotic activity (Figure 3B). Furthermore, we demonstrated a small but significant increased constitutive microglial phagocytosis by GI254023X compared to vehicle control (Figure 3A). However, TAPI-1 treatment seemed not to significantly increase constitutive microglial phagocytosis (Figure 3B).

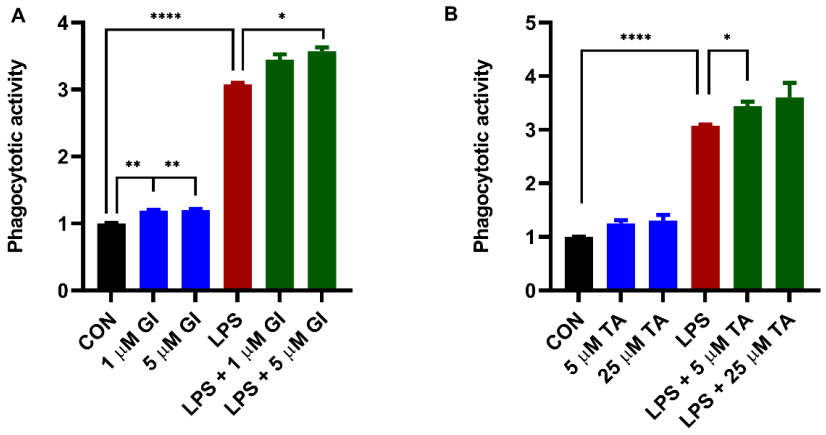

Figure 3. The effect of ADAM10 inhibitor (GI254023X) or ADAM17 inhibitor (TAPI-1) on constitutively and LPS-induced BV2 microglial phagocytosis activity. BV2 microglial cells were incubated with GI254023X (1 and 5 μM) or TAPI-1 (5 and 25 μM) for 24 h in the absence or presence of 1000 ng/mL LPS. (A) The effect of GI254023X treatment on BV2 microglial phagocytosis activity. (B) The effect of TAPI-1 treatment on BV2 microglial phagocytosis activity. Black: constitutive phagocytes set on 1; red: LPS-induced phagocytosis; blue: effect of ADAMs inhibitor on constitutive phagocytosis; green: effect of ADAMs inhibitor on LPS-induced phagocytosis. Results were expressed as mean ± SEM. n=4 from 4 independent experiments. **P* < 0.05, ***P < 0.01*, *****P < 0.0001*.

2.4. Effect of pCS exposure on innate immune response of microglial cells

We investigated the effect of *p*CS (0.1, 0,5, 1, 5, 10, 50 and 150 μM) on constitutive and LPS-induced TNF-α and IL-6 releases by BV2 microglia. Figure 4A, B demonstrated that LPS stimulation significantly increased the releases of TNF-α and IL-6 from BV2 microglia. Furthermore, Figure 4A showed that *p*CS from 1 μM to 50 μM significantly decreased LPS-induced TNF-α production by BV2 cells. In parallel, *p*CS from 5 μM to 50 μM significantly decreased constitutive TNF-α concentration compared to vehicle control. In addition, 5 to 150 μM *p*CS decreased LPS-induced IL-6 release by BV2 cells (Figure 4B). Meanwhile, *p*CS at concentrations of 1, 5, 10 and 50 μM decreased the basal IL-6 production compared to vehicle control.

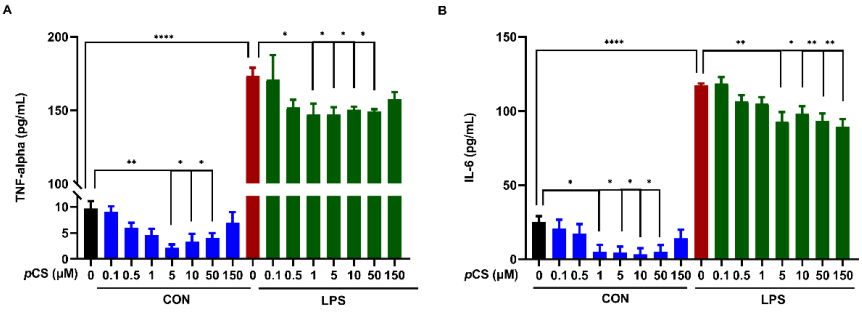

Figure 4. The effect of *p*CS on TNF-α and IL-6 releases by BV2 microglia. The BV2 microglial cells (5.000 per well) were incubated with *p*CS for 24 h with or without LPS (1000 ng/mL). (A)

The concentration of TNF-α in culture medium: n=3 from 3 independent experiments. (B) The concentration of IL-6 in culture medium: n=4 from 4 independent experiments. Black: constitutive cytokine release; red: LPS-induced cytokine release; blue: effect of *p*CS on constitutive cytokine release; green: effect of *p*CS on LPS-induced cytokine release. Results are expressed as mean ± SEM. **P* < 0.05, ***P < 0.01, ****P < 0.0001*.

2.5. Effect of pCS on phagocytosis response of microglial cells

24 h LPS stimulation of BV2 cells significantly increased phagocytosis by BV2 cells (Figure 5). Co-incubation of BV-2 cells with *p*CS and LPS did not result in a significantly different phagocytotic response compared to LPS control, demonstrating that *p*CS cannot affect the LPS-induced phagocytotic response (Figure 5). In contrast, Figure 5B shows that *p*CS (10, 50 and 150 µM) significantly decreased constitutive phagocytotic activity of BV2 cells compared to vehicle control.

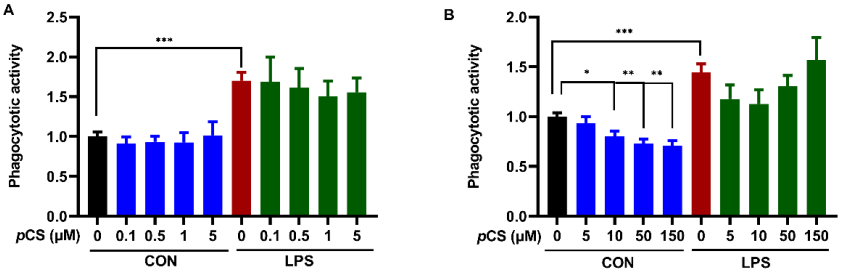

Figure 5. The effect of *p*CS on constitutive and LPS-induced (1000 ng/mL) phagocytosis response of BV2 microglia. (A) The effect of *p*CS (0.1, 0,5, 1, 5 µM) on phagocytosis response of BV2 microglia (n=4 independent experiments). (B) The effect of *p*CS (5, 10, 50 and 150 µM) on phagocytosis response of BV2 microglia (n=6 independent experiments). Black: constitutive phagocytes set on 1; red: LPS-induced phagocytosis; blue: effect of *p*CS on constitutive phagocytosis; green: effect of *p*CS on LPS-induced phagocytosis. Results were expressed as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P < 0.001*.

3. Discussion

The present study investigated the effects of *p*CS on innate immune response and phagocytosis activity of microglial cells, which is partially linked to ADAM10 and 17 functions that are dysregulated in the brain tissues of a murine model of ASD. Previous findings showed that *p*CS or *p*-cresol decreases LPS-activated immune response of murine macrophage [46-48]. This study showed that *p*CS inhibited constitutive and LPS-induced releases of TNF-α and IL-6 from microglial cells. To date this is the very first report showing that the *p*CS-induced derailed immune response of microglial cells is associated with reduced expression or maturation of ADAM17 and to a lesser extend of ADAM10. To further explore the role of ADAM10 and ADAM17 in immune responses of BV2 microglial cells, the effects of respective inhibitors GI254023X and TAPI-1 on LPS-stimulated microglial cells has been studied as well. ADAM10 and ADAM17 collectively cleaved TNF-α, TNFR-1 and IL-6R of constitutive and LPS-activated microglial cells. This finding is in line with previous findings that ADAM17 has been confirmed as the primary sheddase cleaving TNF-α with concurrent involvement of ADAM10 in mouse macrophages [52]. The sTNF-α forms homotrimers to recognize TNFR to regulate immune response [59, 60]. TNFR has also been identified as a substrate for ADAM10 [54], and the current study shows that ADAM17 cleaved TNFR-1 as well of microglial cells. IL-6 binds to IL-6R to trigger an immune response, during this process IL-6 signaling needs the involvement of

membrane-bound IL-6R to form the IL-6/IL-6R/gp130 complex [61]. ADAM17 mainly controls these through cleavage of membrane bound IL-6R to soluble IL-6R [53, 62], and indeed both ADAM17 and ADAM10 seemed to play a role in the cleavage of IL-6R from microglial cells. Unexpectedly, the TAPI-1 increased LPS-stimulated IL-6 concentration in BV2 cells, which is consistent with previous finding, showed that TAPI-1 increases LPS-stimulated IL-6 level in monocytes [63]. In the present study, TAPI-1 induces a decrease sIL-6R release under LPS stimulation. This will result in more membrane-bound IL6-R expression. Therefore, we suggest that the TAPI-1-induced upregulation of IL-6 release by LPS exposed BV2 cells can be explained by the activation of membrane bound IL6-R, resulting in more IL-6 release. Previously it has been demonstrated that IL-6 can induce IL-6 secretion by epithelial cells [64]. In addition, LPS treatment increased ADAM17 expression of microglial cells, indicating the involvement of ADAM17 in LPS-induced neuroinflammation of microglial cells. In contrast, LPS stimulation triggered a significant decrease in ADAM10 maturation in microglial cells, which is consistent with findings in macrophages [52]. TAPI-1 and GI254023X inhibited ADAM10 activity via attenuating its maturation in constitutive and activated microglial cells, which is in line with recent findings showing that these two ADAM inhibitors reduced ADAM10 maturation in monocytes, and that GI254023X treatment inhibits ADAM10 maturation *in vitro* and *in vivo* [65]. GI254023X significantly increased ADAM17 expression in microglial cells, which is in line with previous finding that has shown an overcompensation of ADAM17 in ADAM10 deficiency lymph nodes [66]. In addition, ADAM10 functions as the major TNF-α sheddase in ADAM17-deficient fibroblasts [67]. The decreased mADAM10 triggered by GI254023X observed in the present study might increase ADAM17 expression reversely. As TAPI-1 might also decrease mADAM10, it also might trigger a lower increase in ADAM17 expression at this point. In summary, ADAM10 and ADAM17 collectively cleave TNF-α, TNFR-1 and IL-6R in constitutive and activated microglial cells, indicating that ADAM10 and ADAM17 might regulate immune responses of microglial cells collectively. Regarding TNF-α, the effect of *p*CS on microglial cells is comparable to that of ADAM17 and ADAM10 inhibitors, suggesting that via these ADAMs *p*CS can derail the innate immune response of microglia observed in ASD [46, 68].

Besides innate immune responses microglial cells have phagocytotic capacities as well which are important during brain development through regulation of synaptic pruning. In the current study, *p*CS only inhibited constitutive microglial phagocytosis without affecting LPS-induced phagocytosis. Similar results have been reported for *p*CS and phagocytosis of macrophages [46]. Impaired synaptic function due to a reduction in microglia is found to be associated with impaired connectivity and ASD-like behaviors [69]. ADAM10 and ADAM17 seemed not to play a role in the *p*CS-induced reduction of phagocytosis, since both ADAM10 and ADAM17 inhibitors enhanced the LPS-induced phagocytosis of microglial cells. ADAM proteases inhibition using the nonspecific ADAM inhibitor GM6001 increases microglial phagocytosis [44]. Moreover, recently it has been demonstrated in mice that postnatal enhanced phagocytotic activity of microglial cells is associated with ASD-like behavior [70]. These findings pave the way for our findings suggesting both ADAM10 and ADAM17 play a crucial role in regulating microglial phagocytosis, which is not affected by *p*CS.

Ample studies have suggested that the microbiota-gut-immune-brain axis plays an important role in ASD pathogenesis. The supplementary *in vivo* results in this report further supported the link between bacterial metabolite derived *p*CS and ADAM10/17 in VPA-induced ASD model in mice. It is reported that various species within the Firmicutes family of Clostridiaceae are involved in *p*-cresol production from tyrosine and phenylalanine via fermentation [71-74]. Elevated levels of Clostridiaceae species in fecal samples from ASD patients have been reported [75, 76], and several studies have shown that *p*-cresol metabolites, *p*CS or *p*CG, are elevated in urine and feces of autistic children associated with altered gut microbiota composition [34, 35, 77]. *P*-cresol alters dopamine metabolism, enhances glutamine, and decreases

γ-aminobutyric acid in autistic children [12, 78, 79]. It has been shown that ADAM10 activity plays an important role in regulating glutamatergic synapses [41, 80]. Excessive ADAM10 activity hampers spine maturation and impairs synaptic plasticity through cleaving more APP into sAPPα to promote Glutamate Receptor 5 Signaling in a mouse model of ASD [42]. Therefore, the increased mADAM10 might relate to the *p*-cresol-induced decreased glutamine in *in utero* VPA-exposed male mice. In addition, glutamate activates N-methyl-D-aspartate (NMDA) receptors of microglia to trigger morphological activation and release of inflammatory mediators [81-83], the decreased ADAM17 activity might associate with *p-cresol*-induced decrease in glutamine or increase in γ-aminobutyric acid, and might participate in these inflammatory processes in *in utero* VPA-exposed male mice. These remains to be investigated. Given the VPA-induced elevated *p*CS level in serum of male mice and the direct effects of *p*CS on ADAM10/ADAM17 in BV2 microglial cells, this points to an underlying mechanism of the intestinal bacterial metabolite *p*-cresol in the pathogenesis of ASD to be associated with ADAM10/ADAM17 in the brain.

ADAM10 is the main α-secretase shedding APP to generate soluble amyloid precursor protein-α (sAPPα) [84, 85]. sAPPα is upregulated during spine formation and plays a pivotal role in synaptogenesis leading to increased spine density [86, 87]. Alteration of spine number and morphology is believed to underlie many neurological disorders including ASD [88]. Moreover, increased mature ADAM10 has been shown to dysregulate APP cleavage to induce synaptic dysfunction in mice, deficient in the Fragile X mental retardation protein (FMRP) that leads to Fragile X syndrome and ASD-like behaviors [42], this supports the present findings that ADAM10 maturation was increased in *in utero* VPA-exposed male mice brain. The increased mADAM10 expression can trigger synaptic dysfunction in the brain through cleaving additional synaptic substrates, including NrCAM, Neuroligins, Neurexins and Protocadherins [84, 89, 90]. To further unravel the potential role of ADAM10 in ASD-associated changes in synaptic structures is beyond the scope of this study.

Regarding ADAM17, increased levels of soluble ADAM17 α-secretase in the brains of ASD patients has been reported [45]. ADAM17 can be cleaved by other ADAMs to be transformed into soluble ADAM17 [91, 92], and this can result in decreased membrane-bound ADAM17 level in the brain concurrently [45, 93, 94]. These findings are in line with the decreased mADAM17 expression in the brain of *in utero* VPA-exposed male mice. ADAM17 is important for the cleavage of membrane-bound cytokines [95]. The VPA-induced decreased hippocampal mADAM17 level was associated with decreased hippocampal TGF-β1 levels, which is consistent with the attenuated TGF-β1 in the circulation of ASD children [96, 97]. In contrast to the present findings that soluble TNF-α was reduced in hippocampus of *in utero* VPA-exposed male mice, an increased expression of TNF-α mRNA in both cerebellum and hippocampus has been reported [98], as well as an increase in TNF-α in the brain cortex of ASD patients [99]. This contrasting result might be attributed to different measurements, in previous studies all TNF-α forms at gene or total protein level were measured, but this present study measured soluble TNF-α levels, excluding the membrane-associated form.

In conclusion, this present study demonstrates that in microglial cells *p*CS attenuated the expression or maturation of ADAM10/17 that control the cleavages of TNF-α, TNFR-1 and IL6R. Indeed *p*CS inhibited the release of TNF-α by microglial cells. Our *in vitro* results are confirmed by the decreased mADAM17 and the attenuated downstream cytokines sTNF-α and TGF-β1 in brain tissue of mice *in utero* exposure to VPA. In addition, *p*CS inhibited IL-6 release of constitutive and LPS-activated BV2 microglial cells and attenuated phagocytosis capacity of constitutive BV2 microglial cells. The *p*CS-induced decrease of ADAM10 in microglial cells does not reflect the increased ADAM10 maturation in the brain of *in utero* VPA-exposed male mice. This suggests that ADAM10 maturation might be enhanced in other brain cells, such as neurons, which makes sense regarding the important role of ADAM10 in synapse regulation [84,

89]. In addition, mice lacking ADAM10 also shows synaptic dysfunction, altered brain connectivity in the cortex and hippocampus [100, 101].

To date, this is the very first study that reports on the possible involvement of *p*CS-induced changes of ADAM10 and ADAM17 in the derailed neuroimmune response in context with ASD, which also sheds light on identifying ADAM10 and ADAM17 as potential targets for ASD treatment. Further studies are needed to examine the direct causal link between *p*-cresol, ADAMs activity and ASD development via the microbiota–gut–brain axis. In addition, targeting intestinal *p*-cresol producing bacteria with microbiome-based therapies might reduce its possible detrimental effect on ADAM10 and ADAM17 in the CNS, and might therefore reduce ASD symptoms.

4. Materials and methods

4.1. BV2 microglial cell culture and treatments

BV2 cells were cultured as described before [102] at 37°C and 5% CO2 in medium (Dulbecco's modified eagle medium (Gibco, NY, USA), 10% Fetal bovine serum (Gibco, NY, USA) and 1% penicillin/streptomycin (Gibco, NY, USA)). BV2 cells were used to investigate possible effects of *p*CS on cell viability, on ADAM10 and ADAM17 expression, on the inflammatory response as well as on phagocytosis.

4.2. BV2 microglial cell viability

To assess the effect of *p*CS on cell viability, 5,000 BV2 cells/well were seeded into 96-well plate (3599, Corning, NY, USA). On the following day the BV2 cells were incubated with *p*CS (concentration range: 5 -150 µM) for 24 h. After 24 h, 50 µL medium of each well was transferred into new 96-well plate and the content of lactate dehydrogenase (LDH) in medium was measured by Cytotoxicity Detection KitPLUS (LDH) (4744926001, Sigma) according to the manufacturer's instructions. In the meantime, the medium leftover was discarded and 100 µL DMEM containing 0.5 mg/mL MTT (M2128, Sigma) was added to the cells for 4 h incubation at 37°C under 5% CO2. The DMEM was removed and then 200 µL DMSO was added into each well. Finally, the OD values were measured at wavelength of 570 nm. In all viability experiments, 1 µM and 10 µM Rotenone dissolved in DMSO was employed as positive control [103-105].

4.3. BV2 microglial ADAM10 and ADAM17 expression and inflammatory response

To investigate the effect of *p*CS on the expression of ADAM10 and ADAM17 50,000 BV2 cells/well were seeded in a 12-well plate (3512, Corning, NY, USA). To assess the effect of *p*CS on the release of TNF-α and IL-6, 5,000 BV2 cells/well were seeded in a 96-well plate (3599, Corning, NY, USA). The cells were incubated with *p*CS (concentration range: 0.1 -150 µM) for 24 h in the presence or absence of 1000 ng/mL LPS stimulation (L3024, Sigma). The medium in 96-well plate was collected for measuring of TNF-α and IL-6 release by ELISA. The BV2 cells in 12-well plate were lysed for Western blotting analysis.

For ADAMs inhibition experiments, 50,000 BV2 cells/well were seeded into 12-well plate overnight and incubated with 5 µM GI254023X [106] (ADAM10 inhibitor SML0789, Sigma) or 25 µM TAPI-1 [107] (ADAM17 inhibitor B4686, APExBIO, USA) for 24 h with or without 1000 ng/mL LPS stimulation on the following day. The medium was collected for ELISA measurements and the BV2 cells lysed for Western blotting analysis.

All BV2 cell samples used for Western blotting analysis were lysed on ice for 30 min with RIPA buffer (20188, Sigma) containing 1% TritonX-100 detergent, Proteinase Inhibitor Cocktail (1:200), 5 µM GI254023X and 10 mM 1,10-Phenanthroline.

4.4. Phagocytosis activity assay

To assess the effect of *p*CS on phagocytosis, 50,000 BV2 cells/well were seeded into 96-well plate (3599, Corning, NY, USA), immediately followed by incubation with *p*CS (concentration range: 0.1 -150 μM) for 24 h in the presence or absence of 1000 ng/mL LPS stimulation. To study the effect of ADAM10 and ADAM17 inhibition on constitutive and LPS-stimulated BV2 cells, BV2 cells were incubated with GI254023X (1 μM or 5 μM) or TAPI-1 (5 μM or 25 μM), respectively, for 24 h with or without 1000 ng/mL LPS. BV2 microglial phagocytotic effect was measured with Vybrant™ Phagocytosis Assay Kit (V6694, Thermo Scientific) according to manufacturer's instructions. Briefly, the medium was discarded completely after 24 h incubation, and then incubated with K-12 strain bioParticles for 2 h at 37°C, followed by Trypan Blue solution incubation for 1 minute. Finally, the fluoresce intensity was measured using ~480 nm excitation, ~520 nm emission.

4.5. Cytokine & cytokine receptor ELISAs

(Soluble) TNF-α, TGF-β1, IL-6, IL-6R and TNFR-1 in the medium of BV2 cells or in the supernatants of brain tissue homogenates (see supplementary materials) were quantified using mouse TNF-α ELISA kit (430901, BioLegend, USA), human/mouse TGF-β ELISA kit (88-8350-88, Invitrogen, USA), mouse IL-6 ELISA kit (431301, BioLegend, USA), mouse IL-6R ELISA Kit (RAB0314, Sigma) and Mouse/Rat TNF R-1 Quantikine ELISA Kit (MRT10, R&D Systems) using manufacturer's instructions. Due to the strong inhibition of constitutive IL-6 release from BV2 microglial cell by *p*CS (1, 5,10 and 50 μM) treatment, IL-6 concentrations in medium after exposure to *p*CS (1, 5,10 and 50 μM) were under the detection limit that were set as '0'.

4.6. Immunoblotting brain tissue and BV2 microglial cells

As described previously [89, 108], the protein concentration in BV2 microglial cell supernatants and brain tissue (see supplementary materials) were quantified by Pierce™ BCA Protein Assay Kit (23225, Thermo Scientific, Vantaa, Finland). Then the supernatant was denatured at 95℃ for 5 min in 4X Laemmli Sample Buffer (1610747, Bio-Rad, USA) containing 50 mM Dithiothreitol (1610611, Bio-Rad, USA). Afterwards, 10 to 30 μg of protein was loaded and separated on 4–15% gradient precast polyacrylamide gels (5671084, Bio-Rad, USA). Next, proteins were transferred to PVDF membranes (1704157, Bio-Rad, USA) with Trans-Blot Turbo Transfer System. The membranes were blocked in 5% skim milk-PBST for 1 h and then incubated with the primary antibody overnight at 4°C. The membranes were washed with PBST buffer and incubated with anti-rabbit (1:3000, Dako, P0448, USA) or anti-mouse (1:3000, Dako, P0260, USA) secondary antibodies for 1 h at room temperature. After washing, the membranes were exposed to Clarity Western ECL Blotting Substrate (1705060, Bio-Rad Laboratories, Hercules, CA, USA), and were imaged using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) to detect bands. Image J software (version 1.52v, National Institutes of Health, USA) was used to quantify the density of the bands. The following antibodies were used: ADAM10 primary antibody (1:1000, ab124695, Abcam), TACE/ADAM17 primary antibody (NBP2-15281, 1:500, Novus Biologicals, used for BV2 microglia samples), ADAM17 primary antibody (AB19027, 1:1000, Sigma, used for brain tissue samples), β-Actin primary antibody (1:3000, MA5-15739, Invitrogen, USA) or Calnexin primary antibody (1:3500, PA5-34754, Invitrogen, USA) as loading control.

4.7. Statistics

All data analysis and statistics were performed using GraphPad Prism (version 9.1.1; GraphPad software, La Jolla, CA, USA). For multiple comparisons of the *in vitro* data, one-way ANOVA was used with Dunnett's multiple comparison test. The *in vivo* results were analyzed by two-tailed Student's test. The results were expressed as mean ± SEM. $P<0.05$ is considered to be statistically significant.

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Supplementary materials and methods

1. Isolation of brain tissues and blood collection of *in utero* valproic acid murine model of ASD

Specific pathogen-free BALB/cByJ breeding pairs from Charles River laboratories (Maastricht, the Netherlands) were housed under a 12 h light/dark cycle with free access to food and water. All animal procedures were conducted according to governmental guidelines and approved by the Ethical Committee of Animal Research of Utrecht University, Utrecht, the Netherlands (CCD number AVD108002017826). All females were mated until a vaginal plug was detected, indicated as gestational day 0 (G0). On G11, after neural tube closure, pregnant females were treated subcutaneously with 600 mg/kg valproic acid (VPA, Sigma, Zwijndrecht, the Netherlands, VPA: 100 mg/ml) or phosphate buffered saline (PBS) [1, 2]. The offspring were weaned on postnatal day 21 (P21). On postnatal day 50, male mice were euthanized by decapitation to collect blood and brain.

To assess systemic *p*CS levels, blood was collected in MiniCollect Tube (450533, Greiner BIO-ONE, the Netherlands), then it was centrifugated at 20,000 g, 10 min, 4℃. Next the serum was collected for *p*CS and *p*CG measurements by LC-MS/MS.

To assess ADAM10 and ADAM17 (see: material & methods 3) as well as neuroinflammation (see: materials & methods 5), the following brain regions were isolated and collected: hippocampus, prefrontal cortex, cerebellum and olfactory bulbs, and the rest of brain tissue was also collected. The different brain regions were homogenized at 4 ℃ using the STET buffer without detergents (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, Proteinase Inhibitor Cocktail (1:200, P8340, Sigma) 5 µM GI254023X (SML0789, Sigma), 10 mM 1,10-Phenanthroline (131377, Sigma)) [3]. After centrifugation at 12,000 g, 15 min, 4℃, the supernatants were collected for ELISA measurements. For immunoblotting, brain tissue was further lysed on ice for 30 min with RIPA buffer (20188, Sigma) containing 1% TritonX-100 detergent, Proteinase Inhibitor Cocktail (1:200), 5 µM GI254023X and 10 mM 1,10-Phenanthroline, next it was centrifugated again at 12,000 g, 15 min, 4℃ to collect the supernatant for Western blotting analysis, and tissue debris were discarded.

2. Assessment *p*-cresol metabolites in serum of *in utero* VPA murine model of ASD

*p*CS and *p*CG were purchased from AlsaChim (Illkirch-Graffenstaden, France). d7-*p*-cresyl sulfate (potassium salt) and d7-*p*-cresyl glucuronide were purchased from IsoSciences (Ambler, PA, USA) and Toronto Research Chemicals (North York, Ontario, Canada), respectively. Water (LC-MS grade) and acetonitrile (CAN; HPLC-S grade) were obtained from Biosolve (Valkenswaard, The Netherlands). LC-MS/MS equipment and software for controlling, data recording and processing (Xcalibur version 2.07) were supplied by Thermo Fischer Scientific (San Jose, CA, USA).

Serum samples were processed prior to LC-MS analysis. A 20-µL serum or surrogate matrix (for calibration and quality control) was added to 30 µL of cold (4 °C) ACN containing internal standards d7-*p*CS and d7-*p*CG, and subsequently vortexed and centrifuged for 5 minutes at 10000g. A 35-µL of the supernatant was collected and diluted with 200 µl of ultra-pure water before analysis with an Accela LC system (quaternary pump and autosampler), coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer with heated electrospray ionization (ESI). A Waters ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 µm particles) combined with an ACQUITY UPLC HSS T3 VanGuard pre-column (5 mm × 2.1 mm, 1.8 µm particles) was used and kept at a temperature of 40 °C.

Supplementary results

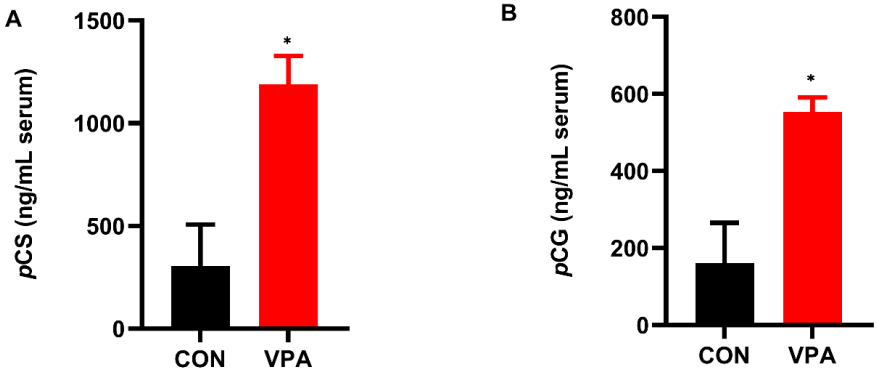

Figure S1. Effects of *in utero* exposure to valproic acid (VPA) on *p*-cresyl sulfate *(p*CS, A) and *p*-cresyl glucuronide (*p*CG, B) levels in serum of male offspring. Results are expressed as mean ± SEM; **P* < 0.05. n=3 *in utero* VPA-exposed mice; n=6 control mice.

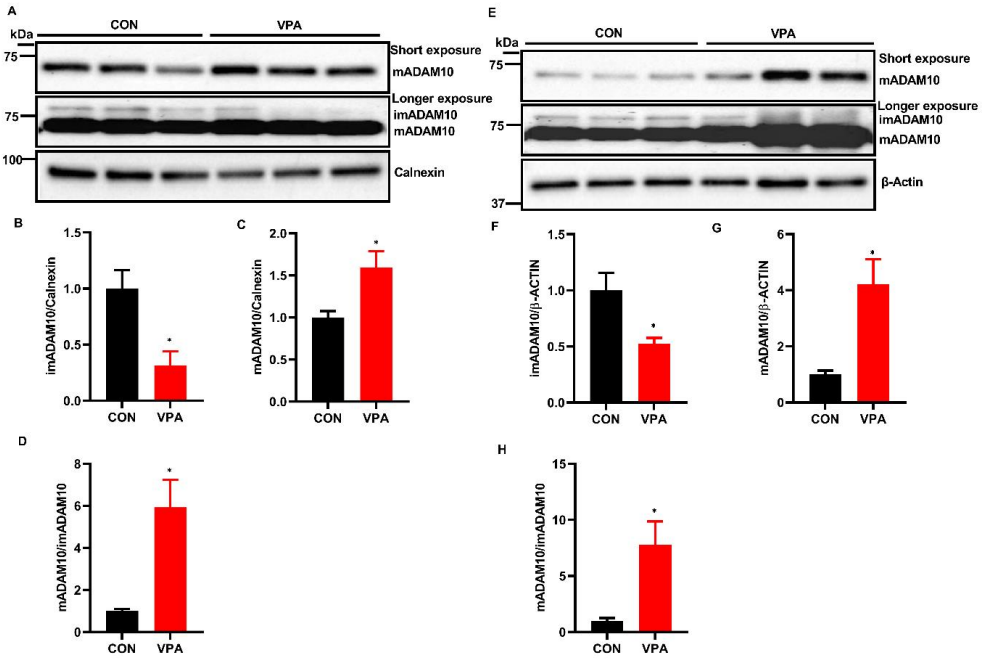

Figure S2. Effects of *in utero* exposure to valproic acid (VPA) on ADAM10 protein expression and maturation efficiency in the brain of male mice. (A) The immunoblots of hippocampal ADAM10. (B, C & D) The quantification results of hippocampal immature ADAM10 (imADAM10), mature ADAM10 (mADAM10) and maturation efficiency (ratio mADAM10/imADAM10). (E) The immunoblots of ADAM10 in other brain regions (excluding hippocampus, olfactory bulb, PFC and cerebellum). (F, G & H) The quantification results of imADAM10, mADAM10 and the maturation efficiency (ratio mDAM10/imADAM10) in other brain regions. Calnexin or β-Actin was used as a loading control. Results were expressed as mean ± SEM. *$P < 0.05$. n=3 VPA-exposed and control mice.

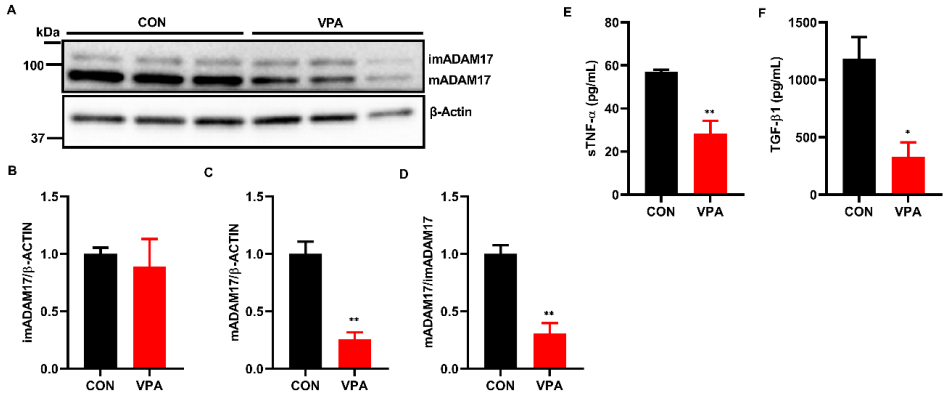

Figure S3. The effects of *in utero* exposure to valproic acid (VPA) on ADAM17 protein expression and levels of soluble TNF-α and TGF-β1 in the hippocampus of male mice. (A) Immunoblots of hippocampal ADAM17. (B, C & D) The quantification results of hippocampal immature ADAM17 (imADAM17), mature ADAM17 (mADAM17) and maturation efficiency (mADAM17/imADAM17 ratio). β-Actin was used as loading control for western blotting. (E & F) The hippocampal soluble TNF-α (sTNF-α) and TGF-β1 levels. Results were expressed as mean ± SEM, n=3. **P* < 0.05, ***P* < 0.01.

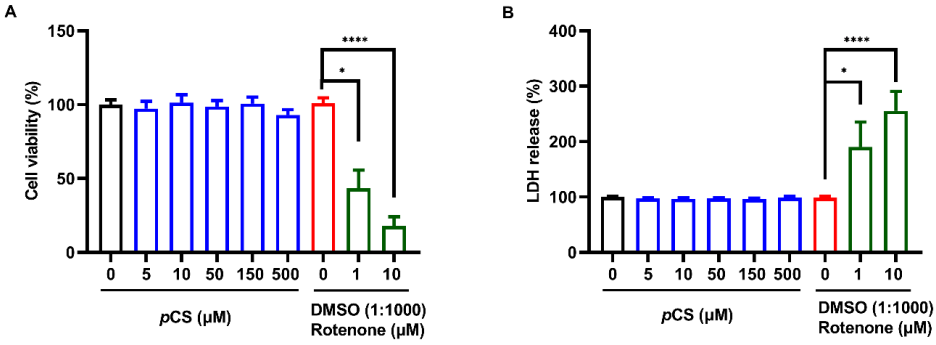

Figure S4. The cell viability measurements with *p*CS treatment in BV2 microglial cells. 5.000 BV2 microglia was seeded into 96-well plate each well overnight, next it was incubated with a concentration range of *p*CS treatments for 24 hours. (A) The cell viability percentage with *p*CS. (B) The LDH release in medium with *p*CS. n=4 from 4 independent experiments. Results were expressed as mean ± SEM. **P < 0.05, ****P < 0.0001*. Black: cell viability under control condition set at 100%; red: effect of vehicle (DMSO) on cell viability; blue: effect of *p*CS on cell viability; green: effect of rotenone on cell viability.

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Chapter 5

Inhibiting ADAM10 ameliorates the pathogenesis of autism spectrum disorder in a murine model through regulating the gut-brain axis: a preliminary study

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Abstract: Increased intestinal permeability and synaptic dysfunction are thought to be involved in Autism Spectrum Disorder (ASD). ADAM10 is a member of the A Disintegrin And Metalloproteases (ADAMs) family that is able to cleave membrane-bound proteins, including E-cadherin as well as Neuroligin-1 and NrCAM, which are associated with intestinal permeability and synaptic function, respectively. Little is known about the potential role of ADAM10 in ASD pathogenesis. In the present study, we use a valproic acid (VPA)-induced murine model for ASD to investigate the role of ADAM10 in ASD pathogenesis along the gut-brain axis. The results show that inhibiting ADAM10 by GI254023X decreased the *in utero* VPA-induced increase in E-cadherin cleavage and increased Claudin-3 expression in male mice colon. In addition, inhibiting ADAM10 decreased the *in utero* VPA-induced increase of maturation of ADAM10 in the prefrontal cortex, cerebellum and hippocampus in these mice. Furthermore, ADAM10 inhibition attenuated *in utero* VPA-induced increased cleavage of Neuroligin-1 in the prefrontal cortex. The behavioral results indicate that inhibiting ADAM10 improved *in utero* VPA-induced deficit in cognitive function which might be associated with the increased ADAM10-mediated Neuroligin-1 cleavage in the prefrontal cortex. The ADAM10 inhibitor did not affect *in utero* VPA-induced impairments in social interaction. In addition, neither *in utero* VPA-exposure nor inhibiting ADAM10 showed no effect on locomotor activity and anxiety-like behavior. Overall, these results suggest that ADAM10 inhibition ameliorates increased intestinal permeability and synaptic dysfunction in *in utero* VPA-exposed male mice possibly via the gut-brain axis.

Keywords: Autism spectrum disorder, Valproic acid, ADAM10, Neuroligin-1, NrCAM, Gut-brain axis

Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disease characterized by social interaction impairments and communication, deficits in learning and memory activity as well as the presence of stereotypical behavior [1]. It is one of the most frequently occurring neurodevelopmental disorders in childhood with 1% prevalence worldwide, which leads to increasingly large burdens on ASD children and their families [2]. However, there are no effective treatments ameliorating the detrimental ASD core symptoms so far [3, 4]. Therefore, the urgency for identifying therapeutical molecular targets for treatment of detrimental ASD symptoms is expanding. Although the mechanisms of ASD pathogenesis are not fully understood, it is now known that it is a complex disorder with multifactorial etiology that implicates both genetic and environmental factors [5]. Many ASD susceptibility genes, including Neuroligin-1 [6-8] and Neural Glial-Related Cell Adhesion Molecules (NrCAM) [9, 10], have been identified and are contributing to 10–20% of clinical ASD cases [5, 11]. Environmental factors include the prenatal exposure to the anti-epileptic medicine Valproic acid (VPA) [12, 13], which has been widely used as a murine model to mimic ASD core symptoms [14].

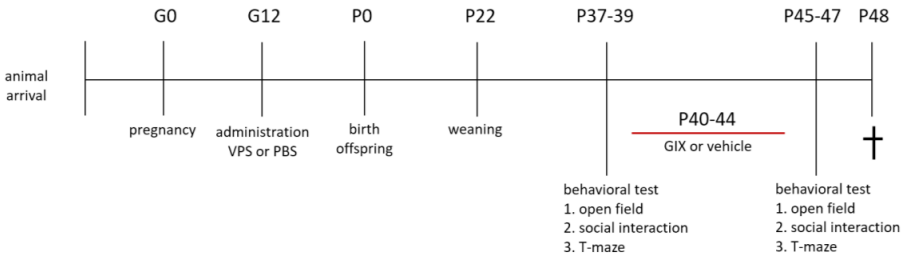
The gut-brain axis has been reported to play an important role in the pathogenesis of ASD and is therefore a potential target for improving detrimental core symptoms, including intestinal problems [15, 16]. ASD children show co-morbidities in the intestines, for instance, the leaky gut and intestinal inflammation associated with intestinal problems [17, 18]. E-cadherin is an adherens junction protein that regulates intestinal permeability in mice and epithelial permeability in colonic epithelial cell cultures [19, 20]. Moreover, synaptic dysfunction in the brain is observed in ASD [21]. As described above, the synaptic adhesion molecules Neuroligin-1 and NrCAM have been acknowledged as ASD susceptibility genes. Furthermore, both Neuroligin-1 deficient mice [8] and NrCAM-deficient mice [22] show impairments in sociability and cognitive function compared to wild-type mice. ADAM10 is one member of A Disintegrin And Metalloproteases (ADAMs) family that is able to cleave membrane-bound proteins [23]. ADAM10 can cleave E-cadherin as well as synaptic molecules Neuroligin-1 and NrCAM by which ADAM10 might control intestinal permeability and synaptic function [19, 20, 24-26].

The potential role of ADAM10 in the ASD gut-brain axis pathogenesis needs further research. In the present preliminary study, we aim to investigate the role of ADAM10 in ASD pathogenesis and its potential as a therapeutic target in an *in utero* VPA-induced ASD murine model. First, we tested the effect of an ADAM10 inhibitor (GI254023X) on ASD-like behaviors such as social interaction, locomotor activity and cognitive function. In addition, the effect of ADAM10 inhibition on the E-cadherin cleavage in colon associated with intestinal permeability was investigated in *in utero* VPA-exposed male mice. Next, the effect of *in utero* VPA-exposure and ADAM10 inhibition on the cleavages of two synaptic molecules, Neuroligin-1 and NrCAM, in several brain regions of mice were measured. Taken together, our results show that ADAM10 inhibition might be a potential target for ASD treatment by improving the intestinal barrier function and reducing the dysregulated synaptic function in *in utero* VPA-exposed male mice.

Materials & methods

1. Animals and experimental design

Specific pathogen-free BALB/cByJ breeding pairs from Charles River laboratories (Maastricht, the Netherlands) were housed under a 12h light/dark cycle with free access to food and water. All animal procedures were conducted according to governmental guidelines and approved by the Ethical Committee of Animal Research of Utrecht University, Utrecht, the Netherlands (CCD number AVD108002017826). As shown in the following schematic, all females were mated until a vaginal plug was detected, indicated as gestational day 0 (G0). On G12, after neural tube closure, pregnant females were treated subcutaneously with 600 mg/kg valproic acid (Sigma, Zwijndrecht, the Netherlands, VPA: 100 mg/ml) or phosphate buffered saline (PBS). After birth, on postnatal day 22 (P22), the male pups were separated from their mothers and housed together in groups of 3-5 mice. On P37, the male pups were subjected to open-field (OF) test and social interaction (SI) test, and on P38 and P39 to three trials of T-maze tests each day. On P40-P44, they received an intraperitoneally administered dose of either vehicle (DMSO) or ADAM10 inhibitor GI254023X for five consecutive days [27]. The ADAM10 selective inhibitor GI254023X (GIX) (Okeanos BioTech.CO, LTD, China) was administered at a dose of 200 mg/kg, dissolved in 30 µL DMSO (D2650, Sigma-Aldrich), so a total volume of 30 µL per day per mouse was injected intraperitoneally. On P45-P47, OF, SI and T-maze tests were performed again. Finally, on P48, all mice were sacrificed by decapitation to collect intestinal tissues and brain tissues for further analysis. The male offspring was divided into four experimental groups, based upon the postnatal injection with an ADAM10 inhibitor (GIX) or vehicle (DMSO)) of vehicle (PBS) or VPA *in utero* exposed mice: 1. PBS-vehicle group: n = 6; 2. PBS-GIX group: n= 7; 3. VPA-vehicle group: n = 3; 4. VPA-GIX group: n = 3.



2. Tissue homogenization

The colon was homogenized with Precellys Control Device (5500 rpm, 2*20s). Samples were put in beads-containing homogenisation tubes (P000918-LYSK0-A, Precellys) with 1XRIPA lysis buffer (20188, Sigma) containing Proteinase inhibitor (1:200, P8340, Sigma), 5 µM GI254023X (SML0789, Sigma) and 10 mM 1,10-Phenanthroline (131377, Sigma) [28]. After 20 min lysing on ice, the homogenized solution was centrifuged (12000g, 4°C, 15 min). Then supernatant was collected for protein quantification using Pierce™ BCA Protein Assay Kit (23225, Thermo Scientific, Vantaa, Finland).

Brain tissues, specifically the cerebellum, prefrontal cortex (PFC), hippocampus, were homogenised using a 2 mL Tenbroeck Tissue Grinder (62400493, DWK Life Sciences) with hypotonic buffer (10 mM Tris, pH 7.4, 2 mM EDTA) containing 5 µM GI254023X and 10 mM 1,10-Phenanthroline and Proteinase inhibitor (1: 200) [28, 29]. Samples were centrifuged (20,817 rpm, 4°C, 1 hr) after 10 min incubation. Supernatants of soluble fraction were transferred into tubes. Next, pellets were thoroughly resuspended in 1XRIPA buffer containing 5 µM GI254023X and 10 mM 1,10-Phenanthroline and Proteinase inhibitor (1: 200), then incubated for 30 min on ice with shaking. After centrifugation (20817 rpm, 4°C, 10 min), supernatants of insoluble fraction were transferred into fresh tubes. Next, both supernatants were subjected to protein quantification using Pierce™ BCA Protein Assay Kit. Finally, samples

were denatured in 4x Laemmli buffer (161-0747, Bio-Rad, CA, USA) containing 50 mM Dithiothreitol (1610611, Bio-Rad, USA) for immunoblotting analysis.

3. Immunoblotting intestine and brain tissue

As described previously [28, 30], 10 to 30 µg of protein was loaded and separated on 4–15% gradient precast polyacrylamide gels (5671085, Bio-Rad, USA). Next, proteins were transferred to PVDF membranes (1704157, Bio-Rad, USA) with Trans-Blot Turbo Transfer System. The membranes were blocked in 5% skim milk-PBST for 1 hour and then incubated with the primary antibody overnight at 4°C. The membranes were washed with PBST buffer and incubated with anti-rabbit (1:3000, Dako, P0448, USA) or anti-mouse (1:3000, Dako, P0260, USA) secondary antibodies for 1 hour at room temperature. After washing, the membranes were exposed to Clarity Western ECL Blotting Substrate (1705060, Bio-Rad, Hercules, CA, USA) and were imaged using the ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA) to detect bands. Image J software (version 1.52v, National Institutes of Health, USA) was used to quantify the density of the bands. The following antibodies were used: ADAM10 primary antibody (1:1000, ab124695, Abcam), E-cadherin primary antibody (1:1000, 610182, BD Science), Claudin-3 primary antibody (1:1000, 341700, Invitrogen), Neuroligin-1 primary antibody (1:1000, 129111, Synaptic Systems), NrCAM primary antibody (1:1000, ab24344, Abcam), β-Actin primary antibody (1:3000 to 5000, MA5-15739, Invitrogen, USA) or Calnexin primary antibody (1:3500, PA5-34754, Invitrogen, USA) as loading control.

4. Social interaction test

SI was performed as previously described by [31] to assess social behavior. After habituation of the target mouse to the open field arena (45x45 cm, black walls and floor) with two small cylindrical cages (perforated Plexiglas, allowing visual, olfactory, minimal tactile interaction), an unfamiliar age- and gender-matched interaction mouse was introduced in one of the cylindrical cages for 5 minutes. The Plexiglas cages were cleaned with water and soap, the arena with 70% ethanol solution to eradicate any odors. The next round was started when the cages and arena were dry. The arenas were continually recorded on video by a camera placed above them, and later analyzed using EthoVision XT video tracking software (EthoVision XT version 15, Noldus, Wageningen, the Netherlands). Hence, the interaction zone (cage containing the interaction mouse) and reference empty zone (cage without a mouse) were set digitally. Time spent in interaction zone and reference zone; time spent on moving or not moving; mobility state and total distance moved were measured for the target mouse.

5. T-maze test

The T-maze spontaneous alternation test was performed to test for cognitive functioning [32-35]. A T-maze apparatus was used for this test, consisting of a base (45 cm) with two lateral arms (each 30 cm) that could be closed by using a wooden plate at the start. The mouse was put in at the base of the T-maze, nose towards the backwall, to explore the environment. Once the mouse walked into one of the lateral goal arms, closing the arm to confine mouse in there for 30 seconds. Then, the mouse was put back its home cage for 2 minutes, the entire apparatus was cleaned with 70% ethanol, and the mouse was put back at the base, allowing to choose an arm once again. A total of six trials was performed per mouse, divided over 2 consecutive days with each trial consisting of two runs, and around 90 minutes between each trial. The alternation rate (number of trials in which a mouse alternated divided by the total number of trials) was determined for each mouse.

6. Open field test

The OF test was adapted from a previously described protocol to test for anxiety-like behavior [36]. The open-field arena was digitally divided in an outer zone and center zone, allowing

measurements of time spent in the outer and center zone. This test was performed at two points in time for each mouse, before and after receiving the 5-day GIX- or vehicle treatment, P37 and P45, respectively.

7. Statistics
The data after GIX injection were analyzed by two-way analysis of variance (ANOVA), followed by Tukey post-hoc test. The behavioral data before GIX injection were analyzed using two-tailed Student's test. The correlation between sNeuroligin-1 and sociability & cognitive function in the prefrontal cortex of mice were performed by Two-tailed Pearson correlation analysis. All data analysis and statistics were performed using GraphPad Prism (version 9.1.1; GraphPad software, La Jolla, CA, USA). $p<0.05$ is considered to be statistically significant.

Results

1. The effects of *in utero* VPA-exposure and ADAM10 inhibition induced by GIX on E-cadherin cleavage in the colon.

As shown in figure 1A and B, *in utero* exposure to VPA seemed to increase mature ADAM10 (mADAM10) in the colon of *in utero* VPA-exposed mice compared to *in utero* PBS-exposed mice, but no significant difference was found. Surprisingly, GIX treatment also seemed to increase mADAM10 without clear significance in the colon of *in utero* VPA- or PBS-exposed mice (figure 1A and B). The expression of adhesive junctional molecule E-cadherin is negatively associated with intestinal permeability [19, 20]. In addition, E-cadherin has been identified as a substrate of ADAM10. ADAM10 cleaves the full-length E-cadherin (FL E-cadherin), generating a C-terminal fragment of E-cadherin (CT E-cadherin), resulting in a decrease in FL E-cadherin as well as a concurrent increase in CT E-cadherin fragment [20, 25]. Figure 1C and D show that *in utero* VPA-exposure significantly decreased membrane-bound FL E-cadherin in colon compared to *in utero* PBS-exposed mice, which can be rescued by GIX treatment. Moreover, *in utero* exposure to VPA increased CT E-cadherin in the colon ($p=0.069$ in figure 1E). GIX treatment inhibited the VPA-induced increased colonic CT E-cadherin as well as CT/FL cadherin ratio ($p=0.23$ in figure 1E and $p< 0.05$ in figure 1F, respectively). In *in utero* PBS-exposed male mice, GIX treatment resulted in significant increase of colonic CT E-cadherin (figure 1E) and in ratio of CT E-cadherin/FL E-cadherin (figure 1F) compared to vehicle-treated mice, respectively. Figure 1H shows that *in utero* VPA-exposure induced a trend of increase in Claudin-3 expression ($p=0.051$) in the colon of male mice compared to PBS-exposed mice. GIX treatment significantly increased the colonic Claudin-3 expression in *in utero* PBS-exposed male mice compared to vehicle-treated mice (figure 1H), but did not affect *in utero* VPA-exposure-induced colonic Claudin-3 expression.

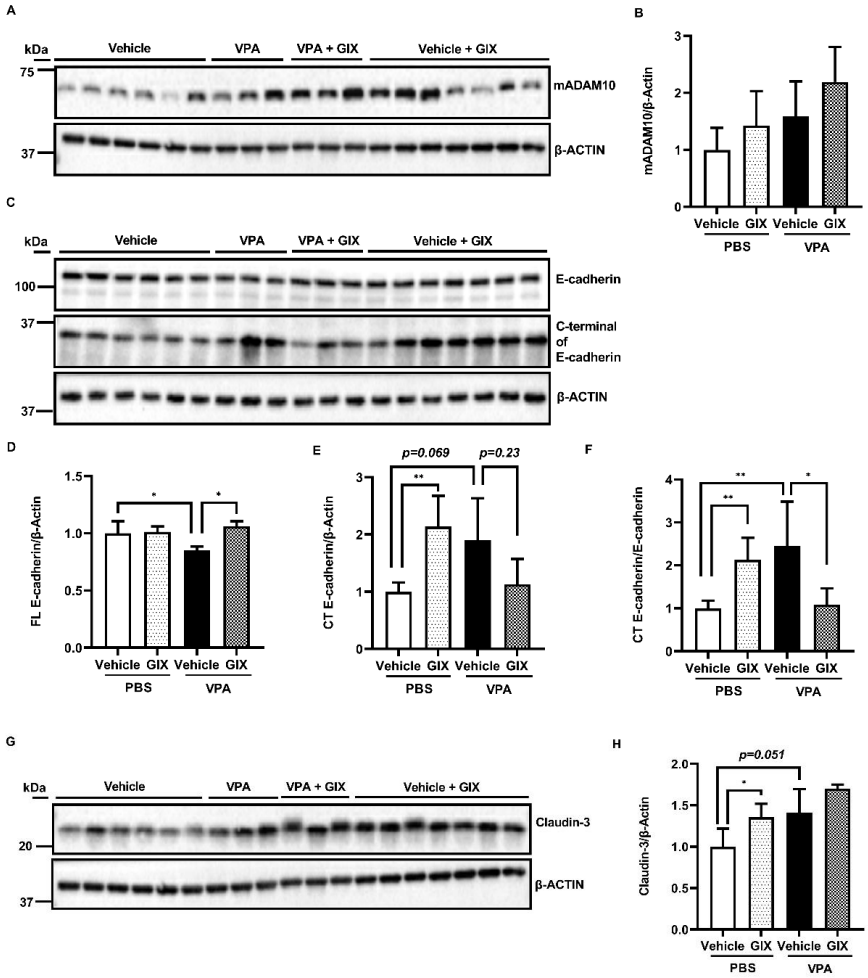


Figure 1: Effect of *in utero* VPA-exposure and postnatal GIX-induced ADAM10 inhibition on E-cadherin and Claudin-3 in mice colon. (A) The immunoblots of mature ADAM10 (mADAM10). (B) The quantification results of mADAM10. (C) The immunoblots of E-cadherin and CT E-cadherin. The quantification results of E-cadherin (D) and CT E-cadherin fragment (E). (F) The ratio of CT E-cadherin to FL E-cadherin. (G) The immunoblots of Claudin-3. (H) The quantification results of Claudin-3. β-Actin was used as a loading control. Data are expressed as mean ± SD. **P < 0.05*, ***P < 0.01*. n = 6 PBS-vehicle mice; n= 7 PBS-GIX mice; n = 3 VPA-vehicle mice; n = 3 VPA-GIX mice.

2. The effects of *in utero* exposure to VPA and ADAM10 inhibition induced by GIX on Neuroligin-1 and NrCAM cleavage in the brain.

Our previous study has shown an upregulation of mADAM10 in brain tissue of mice *in utero* exposed to VPA (Chapter 4, manuscript submitted), but it remains unclear of how increased mADAM10 may contribute to ASD pathogenesis in brain. ADAM10 can cleave synaptic molecules Neuroligin-1 and NrCAM by which ADAM10 regulates synaptic function in brain [26, 37]. We measured the cleavage of these two substrates in prefrontal cortex (PFC, figure 2),

cerebellum (figure 3), and hippocampus (figure 4) by assessing protein levels of neuroligin and soluble neuroligin (sNeuroligin-1) as well as NrCAM and soluble NrCAM (sNrCAM). Figures 2A & B, 3A & B and 4A & B show that *in utero* VPA-exposure increased mADAM10 expression in the PFC, cerebellum and hippocampus compared to PBS-exposed mice, respectively. The VPA-induced increased expression of mADAM10 was significantly reduced by GIX treatment in the PFC, cerebellum and hippocampus compared to vehicle-treated *in utero* VPA-exposed mice. The increased mADAM10 expression in the different brain regions induced by *in utero* VPA-exposure is associated with cleavage of Neuroligin-1, leading to decreased membrane-associated Neuroligin-1 in PFC and hippocampus, but not cerebellum, when compared to PBS-exposed mice (figure 2D, figures 3D and 4D, respectively). In all investigated brain regions, *in utero* VPA-exposure increased sNeuroligin-1 as well as the ratio of sNeuroligin-1/Neuroigin-1 compared to *in utero* PBS-exposed mice (figures 2E & F, 3E & F and 4E &F). GIX treatment did not affect Neuroligin-1 expression of any of the investigated brain regions of *in utero* VPA-exposed mice (Figures 2D, 3D & 4D). However, the VPA-induced increased sNeuroligin-1 (figure 2E) and sNeuroligin-1/Neuroigin-1-ratio (figure 2F) found in PFC were significantly decreased by GIX treatment compared to vehicle-treated *in utero* VPA-exposed mice. GIX treatment did not reduce the VPA-induced trend of increase in sNeuroligin-1 or sNeuroligin-1/Neuroigin-1 ratios in cerebellum or hippocampus (Figures 3E & F and 4E & F, respectively). In contrast, GIX treatment seemed to upregulate sNeuroligin-1 in the cerebellum of *in utero* either VPA- or PBS-exposed mice and in the hippocampus of PBS-exposed mice, which is also reflected by an increased sNeuroligin-1/Neuroligin-1 ratio especially in PBS-exposed mice (Figures 3E & F and 4E & F, respectively).

As for the cleavage of NrCAM in PFC, cerebellum and hippocampus, neither *in utero* VPA-exposure nor GIX treatment affected levels of membrane-bound NrCAM (figures 2H, 3H and 4H, respectively), soluble NrCAM (sNrCAM) (figures 2I, 3I and 4I, respectively) and the ratio of sNrCAM/NrCAM (figures 2J, 3J and 4J, respectively) in *in utero* VPA-exposed mice. In contrast, in *in utero* PBS-exposed mice, GIX treatment significantly increased NrCAM expression in the PFC, (figure 2H), did not affect the sNrCAM level *(*figure 2I), and significantly decreased sNrCAM/NrCAM-ratio (figure 2J) compared to vehicle-treated mice. In cerebellum and hippocampus, none of the descripted effects were found (figures 3H-J and 4H-J, respectively).

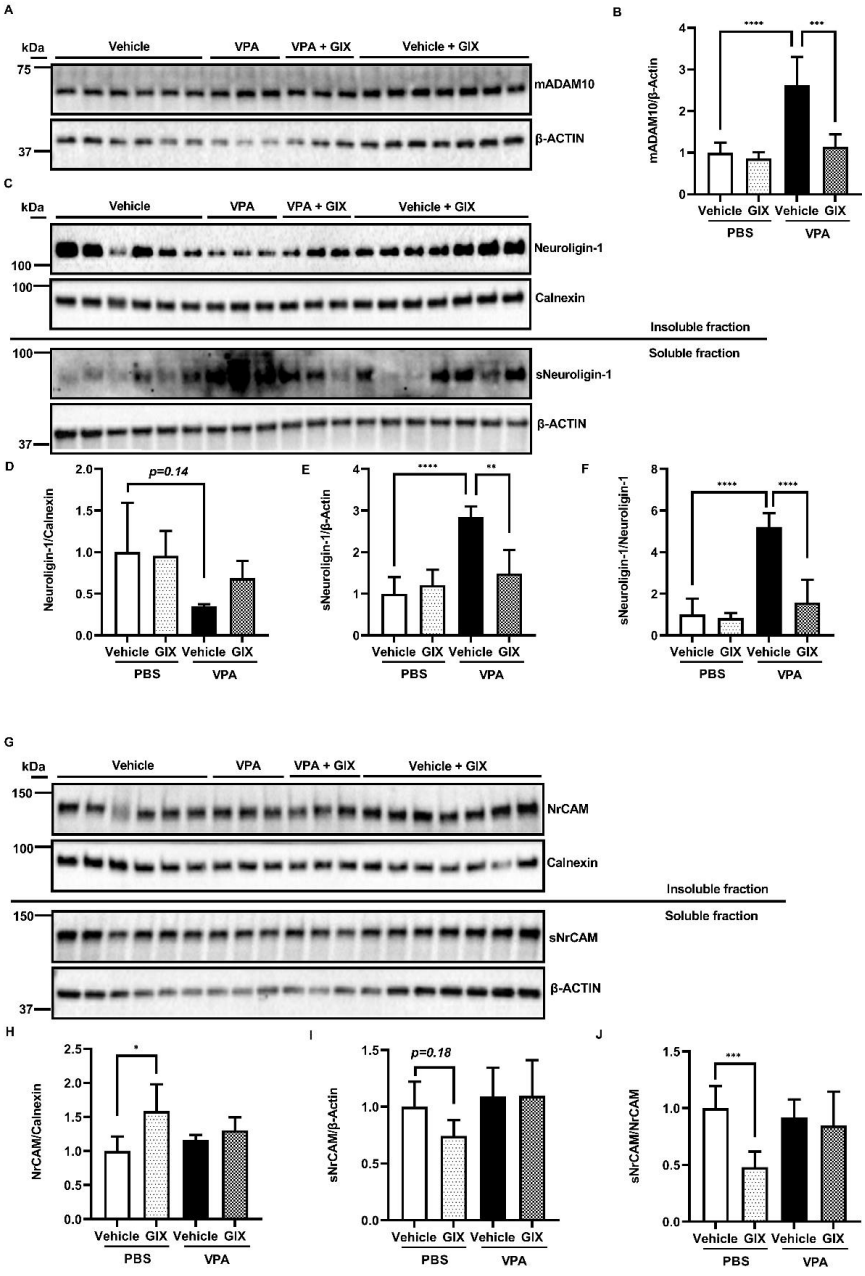


Figure 2: Effect of *in utero* VPA-exposure and postnatal GIX-induced ADAM10 inhibition on cleavages of Neuroligin-1 and NrCAM in PFC. (A) The immunoblots of mADAM10. (B) The quantification results of mADAM10. (C) The immunoblots of Neuroligin-1 and sNeuroligin-1. (D) The quantification results of Neuroligin-1 (E) and sNeuroligin-1. (F) The ratio of sNeuroligin-1 to Neuroligin-1. (G) The immunoblots of NrCAM and sNrCAM. The quantification results of NrCAM (H) and sNrCAM (I). (J) The ratio of sNrCAM and NrCAM. β-Actin or Calnexin was used as loading control. Data are expressed as mean ± SD. **P < 0.05, **P < 0.01, ***P <*

*0.001, ****P < 0.0001.* n = 6 PBS-vehicle mice; n= 7 PBS-GIX mice; n = 3 VPA-vehicle mice; n = 3 VPA-GIX mice.

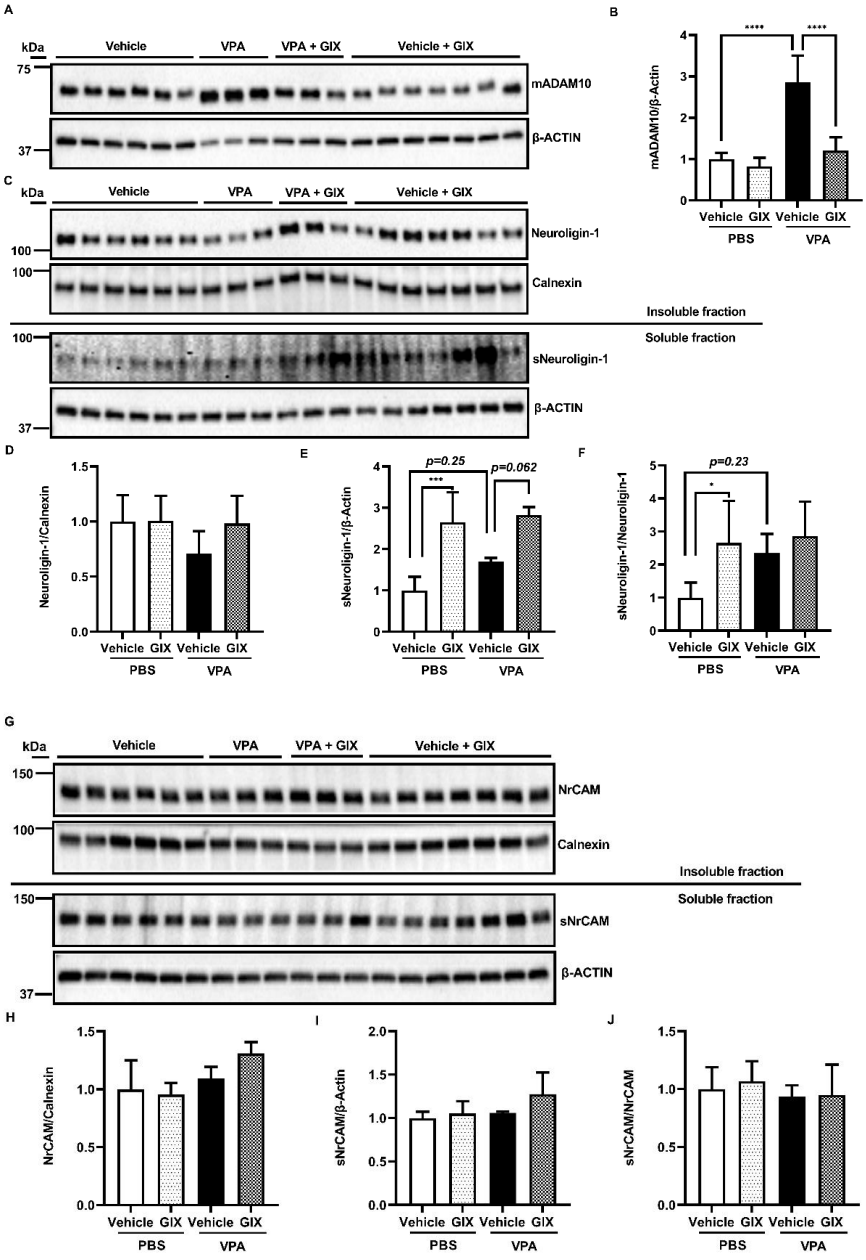


Figure 3: Effect of *in utero* VPA-exposure and postnatal GIX-induced ADAM10 inhibition on cleavages of Neuroligin-1 and NrCAM in cerebellum. (A) The immunoblots of mADAM10. (B) The quantification results of mADAM10. (C) The immunoblots of Neuroligin-1 and sNeuroligin-1. The quantification results of Neuroligin-1 (D) and sNeuroligin-1 (E). (F) The ratio of sNeuroligin-1 to Neuroligin-1. (G) The immunoblots of NrCAM and sNrCAM. (H) The

quantification results of NrCAM and sNrCAM (I). (J) The ratio of sNrCAM and NrCAM. β-Actin or Calnexin was used as loading control. Data are expressed as mean ± SD. **P < 0.05, ***P < 0.001, ****P < 0.0001.* n = 6 PBS-vehicle mice; n= 7 PBS-GIX mice; n = 3 VPA-vehicle mice; n = 3 VPA-GIX mice.

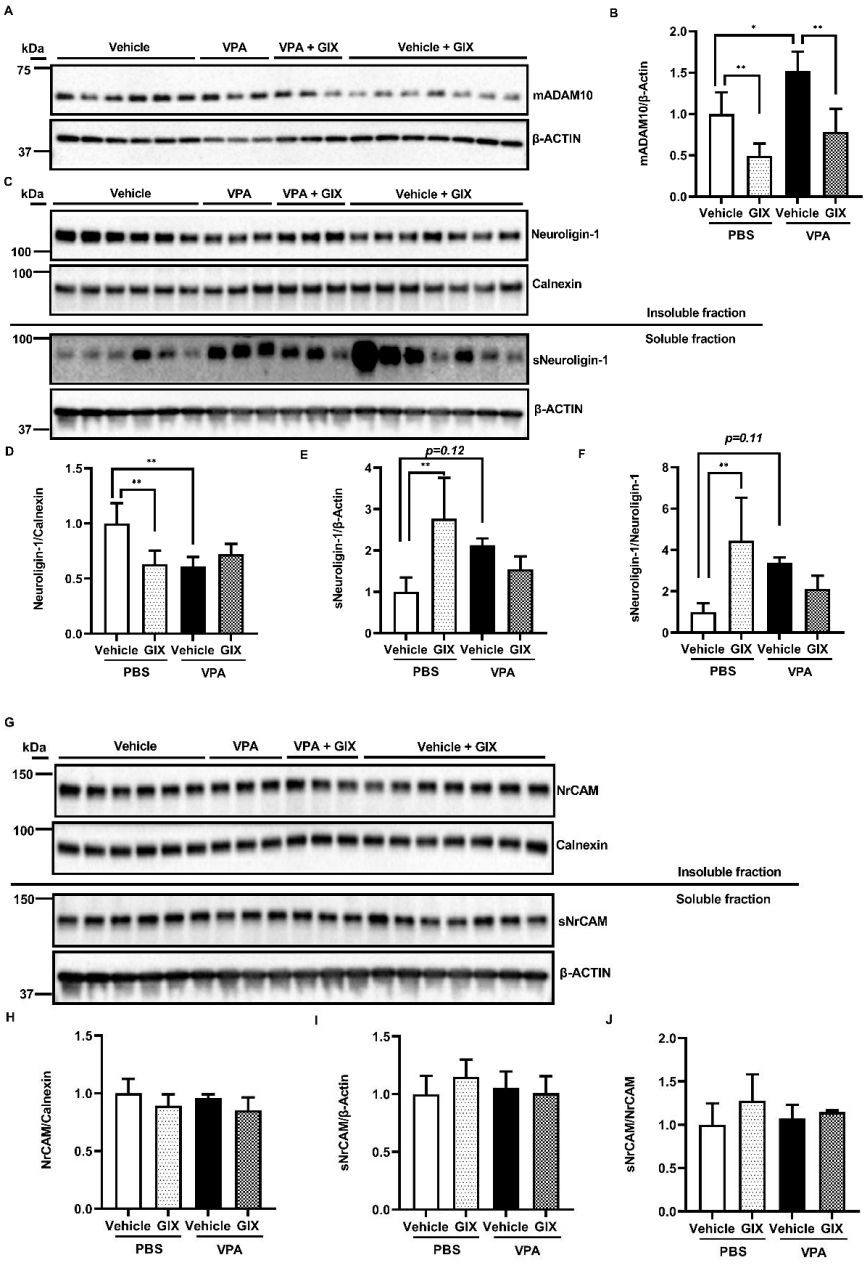


Figure 4: Effect of *in utero* VPA-exposure and postnatal GIX-induced ADAM10 inhibition on cleavages of Neuroligin-1 and NrCAM in hippocampus. (A) The immunoblots of mADAM10. (B) The quantification results of mADAM10. (C) The immunoblots of Neuroligin-1 and sNeuroligin-

1. The quantification results of Neuroligin-1 (D) and sNeuroligin-1 (E). (F) The ratio of sNeuroligin-1 to Neuroligin-1. (G). The immunoblots of NrCAM and sNrCAM. The quantification results of NrCAM (H) and sNrCAM (I). (J) The ratio of sNrCAM and NrCAM. β-Actin or Calnexin was used as loading control. Data are expressed as mean ± SD. **P < 0.05, **P < 0.01.* n = 6 PBS-vehicle mice; n= 7 PBS-GIX mice; n = 3 VPA-vehicle mice; n = 3 VPA-GIX mice.

3. The effects of *in utero* VPA-exposure and GIX-induced ADAM10 inhibition on ASD-like behaviors.

The effects of *in utero* VPA-exposure without or with GIX-induced ADAM10 inhibition on social interaction and locomotor activity, cognitive functioning as well as anxiety-like behavior after 5 days GIX- or vehicle-treatment were assessed on days P45-47 by social interaction test, T-maze test, and open-field test, respectively. Figure 5A shows a schematic representation of the social interaction test set-up, illustrating the interaction zone (zone 1, with a mouse) and the other zone as reference (zone 2, without a mouse). *In utero* VPA-exposed mice showed a decreased trend of interaction time with other mice (zone 1) and an increased time spent in zone 2 compared to *in utero* PBS-exposed male mice, indicating that *in utero* exposure to VPA induced disturbed social interaction, which was not affected by GIX treatment (figure 5B & C). In addition, in *in utero* PBS-exposed male mice, GIX treatment seemed to reduce mice interaction time spent in zone 1 compared to vehicle-treated control mice (figure 5B). Figures 5D-G show that both *in utero* VPA-exposure and GIX treatment had no clear effect on mice locomotor activity, indicated by the unaffected time moving (figure 5D) or not moving (figure 5E), distance moved (figure 5F) and mobile state (figure 5G).

Figure 6A shows that for *in utero* VPA-exposed mice, the spontaneous alteration rate seemed to decrease by 20% compared to *in utero* PBS-exposed mice. GIX-treatment seemed to rescue the VPA-induced reduced cognitive function. Given that in the PFC, *in utero* VPA-exposure increased sNeuroligin-1 fragment and GIX-treatment further rescued the VPA-induced sNeuroligin-1 increase, the correlations between sNeuroligin-1 fragment in PFC, and mice sociability and cognitive function were analyzed. Figure 6B shows there was no significant correlation between sNeuroligin-1 fragment in PFC and mice social interaction time. Figure 6C shows a clear negatively regulatory relationship between sNeuroligin-1 fragment level in PFC and mice alteration rate.

Besides these autistic behaviors, the anxiety-like behavior was assessed as well. As shown in figure 7A, both *in utero* VPA-exposure and GIX treatment did not clearly affect the time mice spent in the center.

Additionally, the effects of *in utero* VPA-exposure on social interaction and locomotor activity (supplement data figure S1), cognitive functioning (figure S2) and anxiety-like behavior (figure S3) before GIX treatment was also assessed on P37-39. The *in utero* VPA-exposed mice did not show clear difference in the time spent in zone 1 and zone 2 (figure S1B), in the time moving or not moving (figure S1C), in the distance moved (figure S1D), and in the mobile state (figure S1E) compared to *in utero* PBS-exposed mice, respectively. Figure S2A shows that *in utero* VPA-exposure did not change spontaneous alteration rate compared to *in utero* PBS-exposed mice. Figure S3A shows that *In utero* VPA-exposure did not affect the time spent in the center compared to the mice *in utero* exposure to PBS.

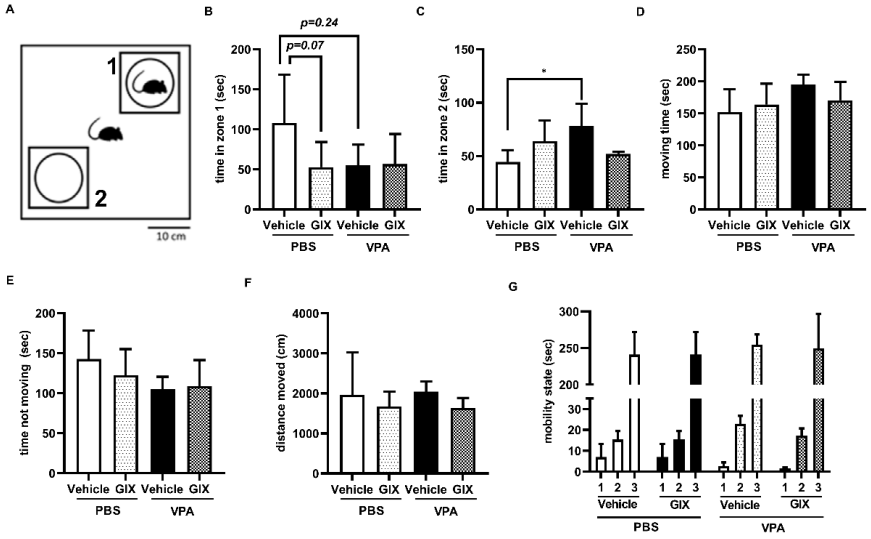


Figure 5: Effects of *in utero* VPA-exposure and postnatal GIX-induced ADAM10 inhibition on mice social interaction time and mice locomotor activity at day P45. (A) The schematic representation of the social interaction test in an open-field arena, zone 1 is the interactive zone interacting with the other mice and zone 2 is the empty zone. (B) The interaction time of mice spent in zone 1 (B) and zone 2 (C) after 5 days GIX or vehicle treatment. The time moving (D) or not moving (E) spent in open-field arena. (F) The distance moved in open-field arena. (G) The mice mobility state in open-field arena. 1 represents highly mobile, 2 represents mobile and 3 represents immobile. Data shown represent mean ± SD. $P<0.05$ is considered to be statistically significant. n = 6 PBS-vehicle mice; n= 6 PBS-GIX mice; n = 3 VPA-vehicle mice; n = 3 VPA-GIX mice**.**

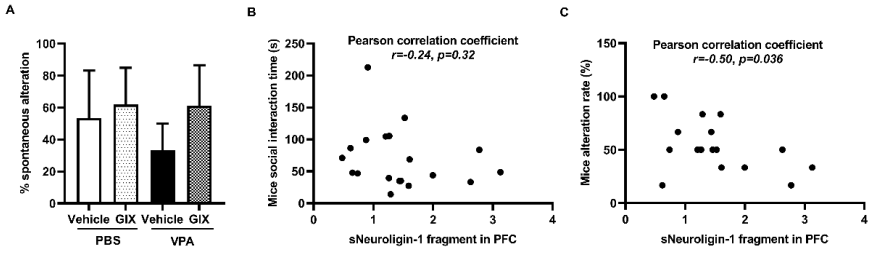


Figure 6: Effects of *in utero* VPA-exposure and postnatal GIX-induced ADAM10 inhibition on cognitive function. (A) At day P46-47 mice spontaneous alteration rate in T-maze test after 5 days GIX or vehicle treatment. (B) The correlation analysis of sNeuroligin-1 fragment in PFC and mice social interaction time spent in zone 1. (C) The correlation analysis of sNeuroligin-1 fragment in the PFC and mice alteration rate. Data shown represent mean ± SD. $P<0.05$ is considered to be statistically significant. n = 6 PBS-vehicle mice; n= 6 PBS-GIX mice; n = 3 VPA-vehicle mice; n = 3 VPA-GIX mice**.**

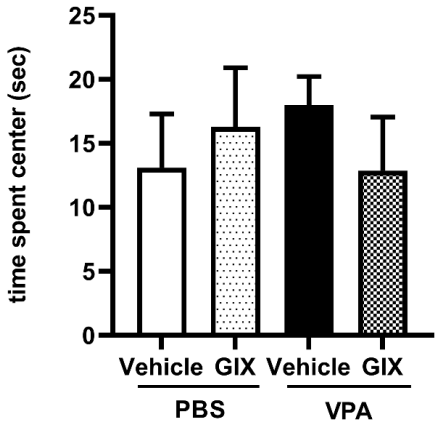


Figure 7: Effects of *in utero* VPA-exposure and postnatal GIX-induced ADAM10 inhibition on anxiety-like behavior. The time mice spent in center at day P45 after GIX injection. Data shown represent mean ± SD. n = 6 PBS-vehicle mice; n= 7 PBS-GIX mice; n = 3 VPA-vehicle mice; n = 3 VPA-GIX mice**.**

Discussion

In recent years, the gut-brain axis has been proposed to be involved in the pathogenesis of ASD and therefore might represent a therapeutic target [38, 39]. The metalloprotease ADAM10 might be a linking pin, as ADAM10 can regulate intestinal permeability by cleaving adherens junction protein E-cadherin and synaptic function by cleaving synaptic molecules Neuroligin-1 and NrCAM [23]. In the present preliminary study, we report on the possible role of ADAM10 in the gut-brain axis in a VPA-induced murine model for ASD.

As a murine model of ASD, *In utero* exposure to VPA in mice mimics core ASD symptoms, including social and cognitive impairments and the presence of repetitive behavior [14]. It has been reported that *in utero* exposure to VPA in mice causes up to 25% abortion rate and 36.3% fetal resorption rate [40, 41]. The abortion rate is as high as 44.4% in monkeys gestational exposure to VPA [42]. Clinically, an abortion rate as high as 25% occurs in pregnant women who receive VPA as an anti-epilepsy therapy [43]. The high abortion rate also occurred in the present preliminary study. In addition, the low breeding rate and low gender ratio of male offspring to female offspring happened together to the present study. These factors led to a small sample size of *in utero* VPA-exposed male mice. Therefore, the investigations with these mice are a preliminary study, paving way for future research.

In the colon of *in utero* PBS- or VPA-exposed male mice, ADAM10 inhibitor GIX did not clearly affect ADAM10 maturation, indicating that GIX does not inhibit ADAM10's activity through attenuating ADAM10 maturation. In recent years, phosphatidylserine exposure to the cleavage site of substrates was found to be necessary to maintain ADAM10's and ADAM17's cleavage activity [44, 45]. Furthermore, their inhibitors can block the binding sites of phosphatidylserine to substrates to attenuate their activity [44, 45]. Since E-cadherin is a substrate of ADAM10, the decreased E-cadherin cleavage by GIX treatment demonstrated the reduced ADAM10 activity by GIX treatment in colon of *in utero* VPA-exposed mice. In addition, we observed reduced levels of membrane-associated E-cadherin in colonic tissue of *in utero* VPA-exposed mice, which could be ameliorated by inhibiting ADAM10 with GIX. Membrane-bound E-cadherin can be cleaved by ADAM10 to generate soluble fragments both *in vivo* and *in vitro* [25]. GIX treatment attenuates E-cadherin cleavage by ADAM10 and enhances intestinal epithelial integrity in a Delta-toxin-exposed epithelial cell model [20]. Furthermore, the same authors have also shown that GIX treatment improves villi damage and intestinal permeability induced by Delta-toxin in mouse ileal tissue [19]. Taken together, these and our results indicate that intestinal permeability might be increased resulting from less membrane-bound E-cadherin triggered by an increase in ADAM10-associated E-cadherin cleavage in the colon of *in utero* VPA-exposed mice, which can be reduced by GIX treatment. In addition, *in utero* VPA-exposure in male mice seemed to upregulate the tight junction protein Claudin-3 expression, which was not affected by ADAM10 inhibition. GIX treatment also increased Claudin-3 expression in the colon of *in utero* PBS-exposed mice. Therefore, it can be concluded that the *in utero* VPA-induced upregulation in Claudin-3 expression might be the result of a feedback mechanism associated with the VPA-induced reduction in adhesion junction protein E-cadherin to control the dysregulated epithelial barrier and is independent of ADAM10.

In murine brain tissues, we found that *in utero* exposure to VPA increased mADAM10 levels compared to *in utero* PBS-exposed mice, which is consistent with our previous results (Chapter 4). Furthermore, we found that GIX treatment attenuated VPA-induced mADAM10 expression in these brain regions (PFC, cerebellum, and hippocampus), which is supported by the finding that GIX treatment inhibits ADAM10 activity by attenuating its maturation *in vivo* and *in vitro* [27]. Neuroligin-1 and NrCAM are cleaved by ADAM10 as demonstrated in conditional ADAM10 knockout mice brains and ADAM10 deficient primary neuron [26, 37]. In the prefrontal cortex of *in utero* VPA-exposed mice GIX treatment decreased VPA-induced

increase in Neurpligin-1 cleavage, indicating that inhibiting ADAM10 with GIX can improve synaptic dysfunction associated with Neurologin-1 through attenuating VPA-induced increase in mADAM10 in PFC. Although our results show an increased trend of Neuroligin-1 cleavage in the cerebellum and hippocampus of *in utero* VPA-exposed mice, we found that GIX treatment strikingly triggered an increase of this cleavage in these two brain regions of *in utero* PBS-exposed mice. Neuroligin-1 is a post-synaptic molecule that trans-synaptically binds to its presynaptic ligand neurexin-1 to control synapse development and function [37, 46, 47]. Neurexin-1 can be collectively cleaved by ADAM10, ADAM17 and other metalloproteases in mouse hippocampus and primary neurons [37, 47]. Furthermore, the soluble fragment of neurexin-1 or other neurexins after cleavage by metalloproteinases can increase cleavage of Neuroligin-1 by ADAM10 [37]. In addition, increased mADAM10 enhances NrCAM cleavage while decreasing Amyloid precursor protein (APP) cleavage in cells overexpressing NrCAM and APP. Moreover, increased mADAM10 triggered by GIX treatment can reversely decrease NrCAM cleavage in primary neurons [24, 28]. GIX treatment can prevent ADAM10 maturation from immature ADAM10, but, in parallel, it can also attenuate mADAM10 degradation in mice or primary neurons [27, 28]. Additionally, our previous results showed a decrease in ADAM17 maturation in hippocampus of *in utero* VPA-exposed mice (Chapter 4). Taken together, these findings might explain the seemingly contradictive effect of GIX treatment on Neuroligin-1 cleavage in the hippocampus and cerebellum of mice.

We also measured NrCAM cleavage in the PFC, cerebellum, and hippocampus. *In utero* VPA exposure did not affect NrCAM and sNrCAM protein expression in all three brain regions compared to *in utero* PBS-exposed mice. The GIX treatment only inhibited NrCAM cleavage in PFC of *in utero* PBS-exposed mice while having no effects on NrCAM cleavage in mice hippocampus and cerebellum, indicating that ADAM10 cleaves its substrates in a brain region-specific way. Six members of TspanC8 family (Tspan5, Tspan10, Tspan14, Tspan15, Tspan17, and Tspan33) can differentially regulate ADAM10 maturation, activity and substrate selectivity through forming six different scissor complexes [48-50]. Tspan5/ADAM10 and Tspan14/ADAM10 scissor complex are preferential to Notch signaling [51, 52], and Tspan15/ADAM10 scissor complex is important to Cadherins [53, 54]. At present, Tspan15 has been shown to be able to regulate ADAM10 maturation and substrate selectivity in mouse brain [53], although the role of other members of TspanC8 family in ADAM10 maturation, activity, and substrate selectivity is not clear. To date, the molecular mechanisms of how *in utero* exposure to VPA increases ADAM10 maturation also remain to be elucidated. Investigating upstream regulators of ADAM10 would pave way for identifying more ADAM10 substrates associated with ASD pathogenesis in a tissue- or cell-specific way, such as synaptic molecules Protocadherins and Neurexins, Adherens junction vascular endothelial cadherin (VE-cadherin), as well as cytokine receptors Interleukin-6 receptor and Tumor necrosis factor receptor.

Prenatal exposure to VPA in mice shows impairments in sociability and cognitive function [34, 55, 56]. In the present study, *in utero* VPA-exposed mice seemed to show potential impairments in these two ASD-like behaviors. Our preliminary data show that ADAM10 inhibition seemed not to influence the *in utero* VPA-induced reduced social interaction. In contrast, ADAM10 inhibition seemed to improve the cognitive function of *in utero* VPA-exposed mice. In addition, the cognitive function significantly correlated with sNeuroligin-1 fragment level in the PFC. sNeuroligin-1 originates from membrane-bound synaptic molecule Neuroligin-1 by means of ADAM10-mediated cleavage [26, 37]. Neurologin-1 deficient mice show impaired cognition function [8]. Moreover, *in utero* VPA exposure into mice shows an decrease in neuronal Neuroligin-1 expression that delays neuronal maturation [57]. Taken together, these results indicate that the increased sNeuroligin-1 fragment in the PFC is connected to compromised cognitive function in *in utero* VPA-exposed male mice, suggesting

that an increased ADAM10-mediated cleavage of Neuroligin-1 in the PFC might be a potential mechanism of VPA-induced ASD.

In summary, the present preliminary study found that the ADAM10 inhibitor GIX decreased *in utero* VPA-induced E-cadherin cleavage. In mouse brain, ADAM10 inhibition decreased *in utero* VPA-induced maturation of ADAM10 in the PFC, the cerebellum, and the hippocampus. Furthermore, ADAM10 inhibition attenuated *in utero* VPA-induced increased cleavage of Neuroligin-1 in the PFC. Taken together, these results suggest that ADAM10 inhibition might ameliorate increased intestinal permeability associated with increased E-cadherin cleavage and synaptic dysfunction in relation to increased Neuroligin-1 cleavage in *in utero* VPA-exposed mice. Future studies can further investigate the effect of ADAM10 inhibition on ASD-like behaviors, such as sociability and cognitive function, in *in utero* VPA-exposed mice and other ASD models. In addition, investigating the molecular mechanisms of how *in utero* VPA-exposure increases ADAM10 maturation associated with ASD pathogenesis will shed light on further identifying ADAM10 as a potential target for ASD treatment.

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Conflicts of interest

JG is a part time employee at Danone Nutricia Research, Utrecht, Netherlands. All other authors report no potential conflicts of interest.

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Supplementary results

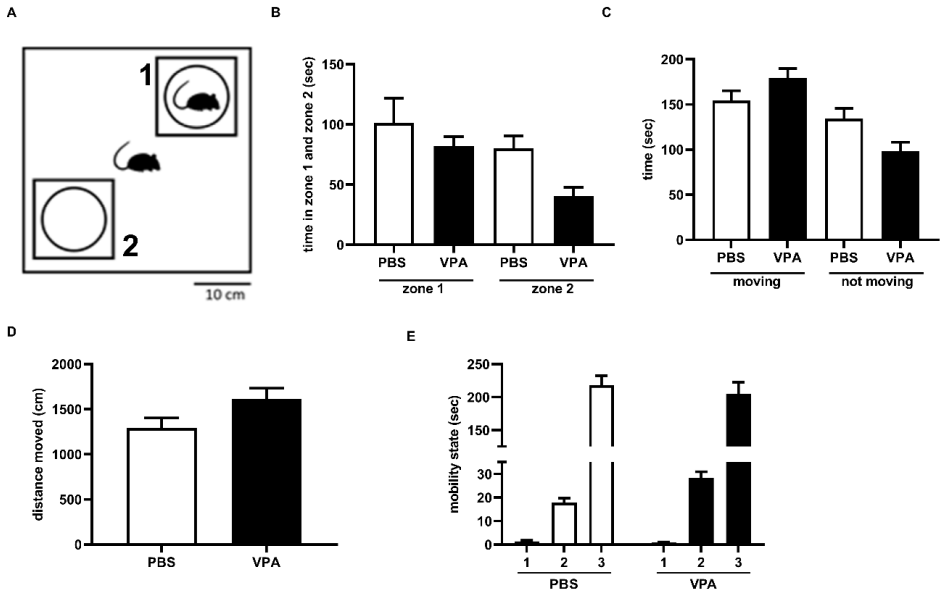

Figure S1: Effects of *in utero* VPA-exposure before GIX treatment on mice social interaction and mice locomotor activity at day P37. (A) The schematic representation of the social interaction test in an open-field arena, zone 1 represents the interactive zone interacting with the other mice and zone 2 represents the empty zone. (B) The time spent in zone 1 and zone 2 before GIX injection. (C) The time moving or not moving in open-field arena. (D) The distance moved in open-field arena. (E) The mice mobility state in open-field arena. 1 represents highly mobile, 2 represents mobile and 3 represents immobile. Data shown represent mean ± SEM. n = 12 PBS-injected mice; n= 6 VPA-injected mice**.**

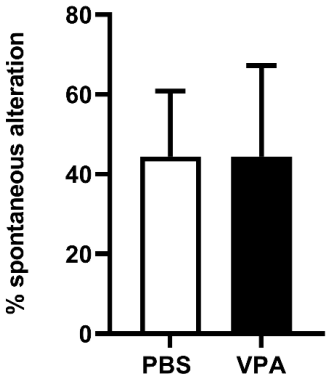

Figure S2: Effects of *in utero* VPA-exposure before GIX treatment on cognitive function at days P38-39. Mice spontaneous alteration rate in T-maze test. Data shown represent mean ± SD. n = 12 PBS-injected mice; n= 6 VPA-injected mice**.**

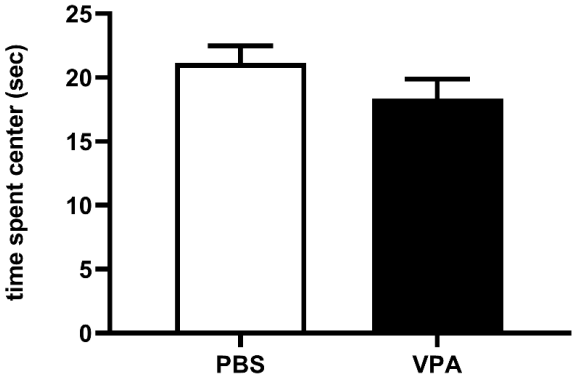

Figure S3: Effects of *in utero* VPA-exposure before GIX treatment on anxiety-like behavior. The time mice spent in center at day P37 before GIX injection. Data shown represent mean ± SEM. n = 13 PBS-injected mice; n= 6 VPA-injected mice**.**

Chapter 6

The interaction between intestinal bacterial metabolites and Phosphatase and tensin homolog in autism spectrum disorder

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Abstract Intestinal bacteria-associated *para*-cresyl sulfate (*p*CS) and 4-ethylphenyl sulfate (4EPS) are elevated in autism spectrum disorder (ASD). Both metabolites can induce ASD-like behaviors in mice, but the molecular mechanisms are not known. Phosphatase and tensin homolog (PTEN) is a susceptibility gene for ASD. The present study investigated the relation between *p*CS and 4EPS and PTEN in ASD in a valproic acid (VPA)-induced murine ASD model and an *in vitro* LPS-activated microglial model. The VPA-induced intestinal inflammation and compromised permeability in the distal ileum was not associated with changes of PTEN expression and phosphorylation. In contrast, VPA reduced PTEN expression in the hippocampus of mice. *In vitro* results show that *p*CS and 4EPS reduced PTEN expression and derailed innate immune response of BV2 microglial cells. The PTEN inhibitor VO-OHpic did not affect innate immune response of microglial cells. In conclusion, PTEN does not play a role in intestinal inflammation and compromised permeability in VPA-induced murine model for ASD. Although *p*CS and 4EPS reduced PTEN expression in microglial cells, PTEN is not involved in the *p*CS and 4EPS-induced derailed innate immune response of microglial cells. Further studies are needed to investigate the possible involvement of reduced PTEN expression in the ASD brain regarding synapse function and neuronal connectivity.

Keywords: ASD, PTEN, VPA, *p*CS, 4EPS, LPS, neuroimmune response.

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disease that characterizes with the core symptoms of impairments in social interaction and communication, deficits in cognitive function as well as the presence of stereotyped behavior [1]. Recent epidemiological surveys show the worldwide prevalence of ASD is about 1% [2]. At present, there is no effective treatment for targeting ASD detrimental core symptoms [3]. Although the ASD pathogenesis is not clear, the abnormal changes along microbiota-gut-brain axis have been reported to be involved in the pathogenesis of ASD [4]. The involvement of the gut in ASD is also supported by frequently reported gastrointestinal comorbidities, including intestinal inflammation and compromised intestinal barrier [5-8].

Children with ASD show changes in gut microbiota and microbiota-associated metabolites [9-11]. *Para*-cresyl sulfate (*p*CS) and 4-ethylphenyl sulfate (4EPS) are host metabolites derived from two bacterial metabolites, *para*-cresol (*p*-cresol) and 4-ethylphenol, originating from intestinal bacterial fermentation of tyrosine and phenylalanine [12]. Both *p*CS and 4EPS have been shown to be elevated in urine, feces or serum of children with ASD [9, 10, 13, 14]. We have demonstrated elevated *p*CS in the serum of the valproic acid (VPA)-induced mouse model for ASD [15]. Furthermore, *p*-cresol administration in drinking water causes social behavior deficits and repetitive behavior in mice through remodeling of the gut microbiota [16]. In addition, intraperitoneal injection of 4EPS into mice induces anxiety-like behavior, a common co-morbidity that may contribute to core ASD symptoms [17, 18]. Moreover, a clinical trial has recently shown that an oral gastrointestinal (GI)-restricted adsorbent AB-2004 decreases urine *p*CS and 4EPS levels and ameliorates ASD-like behaviors of ASD children without serious side-effects [19]. However, it is unclear yet how these two bacteria-derived metabolites are mechanistically linked with ASD.

Phosphatase and tensin homolog (PTEN) is a well-recognized ASD susceptibility gene and a germline mutation in PTEN has been identified in up to 20% of children diagnosed with ASD with macrocephaly [20, 21]. Studies in mouse models demonstrate that deletion of PTEN in the cerebral cortex and hippocampus results in increased rates of macrocephaly and abnormal social interactions [22]. Moreover, PTEN expression in brain is decreased in *in utero* valproic acid (VPA)-exposed male mice [23]. PTEN inhibition or knockdown attenuates neuroinflammation in mice [24, 25]. In contrast, loss-of-function mutation of PTEN in mice upregulates neuroinflammation and enhances microglial phagocytosis [26, 27]. Gut-derived *p*CS and 4EPS are regarded as an important protein-bound uremic toxins associated with chronic kidney disease in human and rodent models [28, 29]. Of interest is the recent finding that in uremic mice a downregulated PTEN expression is associated with peripheral inflammation, indicating the *p*CS can affect PTEN expression [30]. Our previous study has demonstrated that *p*CS derailed the innate immune response as well as phagocytosis of BV2 microglial cells [15]. However, little is known about the interaction between bacteria-derived *p*CS and 4EPS and PTEN in ASD-associated intestinal inflammation and derailed neuroimmune responses in the brain.

The current study aims to investigate the potential relation between PTEN and *p*CS and 4EPS, using *in vivo* and *in vitro* models to unravel the molecular mechanisms involved of how *p*CS and 4EPS contribute to ASD. First, in tissues obtained from *in utero* VPA-exposed male mice the intestinal and brain PTEN phosphorylation and/or expression was assessed. Subsequently BV2 microglial cells were used to investigate whether *p*CS or 4EPS has a direct effect on PTEN expression. Next, the role of PTEN in microglial neuroimmune response and phagocytosis associated with *p*CS and 4EPS was investigated.

Materials and methods

1. Mice

As previously described [31], specific pathogen-free BALB/cByJ breeding pairs from Charles River laboratories (Maastricht, the Netherlands) were housed under a 12 h light/dark cycle with free access to food and water. All animal procedures were conducted according to governmental guidelines and approved by the Ethical Committee of Animal Research of Utrecht University, Utrecht, the Netherlands (CCD number AVD108002017826). All females were mated until a vaginal plug was detected, indicated as gestational day 0 (G0). On G11, after neural tube closure, pregnant females were treated subcutaneously with 600 mg/kg VPA (Sigma, Zwijndrecht, the Netherlands, VPA: 100 mg/ml) or phosphate buffered saline (PBS). The offspring was weaned on postnatal day 21 (P21). On postnatal day 50, male mice were euthanized by decapitation to collect intestinal tissue and brain tissue (*in utero* PBS-exposed male mice: n=3; *In utero* VPA-exposed male mice: n=3).

2. Tissue homogenization

Mice hippocampus, prefrontal cortex, cerebellum and olfactory bulbs, and the rest of brain tissue was also isolated. These brain regions were homogenized at 4 ℃ using STET buffer without detergents (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, Proteinase Inhibitor Cocktail (1:200, P8340, Sigma), 5 µM GI254023X (SML0789, Sigma), 10 mM 1,10-Phenanthroline (131377, Sigma)) [32]. After centrifugation at 12,000 g, 15 min, 4℃, brain tissue lysate was further lysed on ice for 30 min with RIPA buffer (20188, Sigma) containing 1% TritonX-100 detergent, Proteinase Inhibitor Cocktail (1:200), 5 µM GI254023X and 10 mM 1,10-Phenanthroline, next it was centrifugated again at 12,000 g, 15 min, 4℃ to collect the supernatant for Western blotting (WB) analysis after protein quantification with Pierce™ BCA Protein Assay Kit (23225, Thermo Scientific, Vantaa, Finland).

For intestinal tissues, the distal ileum was homogenised with Precellys Control Device (5,500 rpm, 2*20s), then samples were put in beads-containing homogenisation tubes (P000918-LYSK0-A, Precellys) with RIPA lysis buffer containing Proteinase inhibitor (1:200), phosphatase inhibitor (1:50, ab 201112, Abcam), 5 µM GI254023X and 10 mM 1,10-Phenanthroline. After 20 min lysing on ice, the homogenised solution was centrifuged (12,000g, 4°C, 15 min). Next, supernatant was used for protein quantification using Pierce™ BCA Protein Assay Kit. Then all supernatants were denatured at 95℃ for 5 min in 4X Laemmli Sample Buffer (1610747, Bio-Rad, USA) containing 50 mM Dithiothreitol (1610611, Bio-Rad, USA).

3. Cell culture and treatments

As previously described [33], BV2 cells were cultured at 37°C and 5% CO2 in the medium (Dulbecco's modified eagle medium (DMEM) (Gibco, NY, USA), 10% Fetal bovine serum (Gibco, NY, USA) and 1% penicillin/streptomycin (Gibco, NY, USA)). BV2 cells were used to investigate possible effects of *p*CS (A8895, APExBIO, USA) or 4EPS (TRC-E925865, Hölzel Diagnostika, the Netherlands) on cell viability, on PTEN, Inducible nitric oxide synthase (iNOS) and Cyclooxygenase-2 (COX-2) expression, on the inflammatory cytokines TNF-α and IL-6 as well as on microglial phagocytosis.

4. BV2 microglial cell viability

To investigate the effect of 4EPS on cell viability, 5,000 BV2 cells/well were incubated with 4EPS (concentration range: 0.1-400 µM) for 24 hours. After 24 hours, 50 µL medium of each well was transferred into new 96-well plate and the content of lactate dehydrogenase (LDH) in medium was measured by Cytotoxicity Detection KitPLUS (LDH) (4744926001, Sigma) according to the manufacturer's instructions. Meanwhile, the medium left was removed and 100 µL DMEM containing 0.5 mg/mL MTT (M2128, Sigma) was added to the cells for 4 hours of incubation at 37°C under 5% CO_2. 200 µL Dimethyl sulfoxide (DMSO) was added into each

well after the DMEM was removed. Finally, optical density values were measured at wavelength of 570nm. In all viability experiments, 1 μM and 10 μM Rotenone dissolved in DMSO was used as positive control [34, 35].

5. PTEN expression and inflammatory response in BV2 microglial cells

To study the effect of *p*CS and 4EPS on the release of TNF-α and IL-6 or on the expression of PTEN, COX-2 and iNOS in cells, 5,000 BV2 cells/well were seeded in a 96-well plate (3599, Corning, NY, USA) or 50,000 BV2 cells/well in a 12-well plate (3512, Corning, NY, USA), respectively. On the following day, the cells were incubated with *p*CS and 4EPS concentrations in the presence or absence of 1,000 ng/mL LPS stimulation (L3024, Sigma). After 24 hours of incubation, the medium in 96-well plate was collected for measurements of TNF-α and IL-6 by enzyme-linked immunosorbent assay (ELISA). The BV2 cells in 12-well plate were lysed in RIPA lysis buffer containing Proteinase inhibitor (1:200), 5 μM GI254023X and 10 mM 1,10-Phenanthroline for WB analysis. For experiments to study the effect of PTEN inhibition on the inflammatory response of microglial cells, 5,000 BV2 cells/well were seeded into 96-well plate overnight. On the following day, BV2 cells were incubated with a potent PTEN inhibitor VO-OHpic trihydrate (V8639, Sigma) [36] for 24 hours in the absence or presence of 1,000 ng/mL LPS stimulation. Finally, the medium was used for measurements of inflammatory cytokines TNF-α and IL-6 using ELISA.

6. Phagocytosis activity assay

50,000 BV2 cells/well were seeded into 96-well plate (3599, Corning, NY, USA). To assess the effect of 4EPS on phagocytosis, BV2 cells were immediately incubated with 4EPS concentrations (0.1, 1, 10 and 100 (μM)) for 24 hours in the presence or absence of 1,000 ng/mL LPS stimulation. To study the effect of PTEN inhibition on constitutive and LPS-stimulated microglial phagocytosis, BV2 cells were immediately incubated with VO-OHpic trihydrate concentrations (50, 100, 200 and 400 (nM)) for 24 hours with or without 1,000 ng/mL LPS. Next the phagocytic effect was measured with Vybrant™ Phagocytosis Assay Kit (V6694, Thermo Scientific) according to manufacturer's instructions. Briefly, the medium was discarded completely after 24 hours incubation, and cells were incubated with K-12 strain bioParticles for 2 hours at 37°C, followed by Trypan Blue solution incubation for 1 minute. Finally, the fluoresce intensity of BV2 cells was measured using ~480 nm excitation, ~520 nm emission.

7. ELISA

TNF-α and IL-6 concentrations after *p*CS treatment (5, 10, 50 and 150 (μM)) in the medium of BV2 cells were measured by mouse TNF-α ELISA kit (88-7324-88, Invitrogen, USA) and mouse IL-6 ELISA kit (88-7064-88, Invitrogen, USA) according to manufacturer's instructions. TNF-α and IL-6 concentrations with 4EPS (0.1, 1, 10 and 100 (μM)) or VO-OHpic trihydrate (50, 100, 200 and 400 (nM)) treatment in the medium of BV2 cells were measured by mouse TNF-α ELISA kit (430901, BioLegend, USA) and mouse IL-6 ELISA kit (431301, BioLegend, USA) according to manufacturer's instructions. IL-6 concentrations in medium after exposure to 4EPS treatment (0.1, 1 and 10 μM) were under the detection limit that were set as '0'.

8. Immunoblotting mouse tissue and BV2 microglial cells

As described previously [32, 37], 10-30 μg of protein was loaded and separated on 4–15% gradient precast polyacrylamide gels (5671084, Bio-Rad, USA). Next, proteins were transferred to PVDF membranes (1704157, Bio-Rad, USA) with Trans-Blot Turbo Transfer System. The membranes were blocked in 5% skim milk-PBST for 1 hour and then incubated with the primary antibody overnight at 4°C. The membranes were washed with PBST buffer and incubated with anti-rabbit (1:3,000, Dako, P0448, USA) or anti-mouse (1:3,000, Dako,

P0260, USA) secondary antibodies for 1 hour at room temperature. After washing, the membranes were exposed to Clarity Western ECL Blotting Substrate (1705060, Bio-Rad Laboratories, Hercules, CA, USA), and were imaged using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) to detect bands. Image J software (version 1.52v, National Institutes of Health, USA) was employed to quantify the density of the bands. The following primary antibodies were used: PTEN primary antibody (1:1,000, 9556S, CST), phosphorylated(p)-PTEN (Ser380/Thr382/383) primary antibody (1:1,000, 9549S, CST), COX-2 primary antibody (1:1,000, 12282S, CST), iNOS primary antibody (1:500, MAB9502, Novus Biologicals), E-cadherin primary antibody (1:1,000, 610182, BD Science), and β-ACTIN primary antibody (1:3000, MA5-15739, Invitrogen, USA) or Calnexin primary antibody (1:3,500, PA5-34754, Invitrogen, USA) as loading control.

9. Statistics

All data analyses and statistics were performed using GraphPad Prism (version 9.1.1; GraphPad software, La Jolla, CA, USA). The *in vivo* results were analyzed by two-tailed Student's test. Two-tailed Pearson correlation was used for correlation analysis of E-cadherin and iNOS expression in the distal ileum of mice. For multiple comparisons of the *in vitro* data, one-way ANOVA was used, followed by Dunnett's multiple comparisons as post-hoc test. All data are shown as mean ± SEM or mean ± SD; $P<0.05$ is considered to be statistically significant.

Results

Valproic acid-induced murine model for ASD

1. PTEN expression and PTEN phosphorylation did not change in the distal ileum of male mice after *in utero* exposure to VPA.

Previous studies have shown intestinal inflammation in VPA-induced murine model of ASD [31]. COX-2 expression was significantly increased to six folds in the distal ileum of *in utero* VPA-exposed mice compared to control mice, indicating a VPA-induced intestinal inflammatory response (figure 1A & B). Furthermore, membrane-bound E-cadherin was significantly decreased in the distal ileum of *in utero* VPA-exposed mice compared to control mice (figure 1C & D). The decreased membrane-bound E-cadherin leads to increased C-terminal fragment of E-cadherin levels (CT E-cadherin). Indeed, we found a trend of increased CT E-cadherin (figure 1E *P=0.083*) and significantly increased ratio of CT E-cadherin to membrane-bound E-cadherin (figure 1F). It has been shown that iNOS co-localizes with adhesive protein E-cadherin, indicating E-cadherin might regulate iNOS expression or vice versa [38-40]. The decreased E-cadherin expression was associated with a decreased iNOS expression in the distal ileum of *in utero* VPA-exposed mice compared to control mice (figure 1G & H (*P=0.056*). Furthermore, the Pearson correlation analysis shows a strong correlation between E-cadherin expression and iNOS expression (r=0.87, P=0.024, figure 1I). Next, we assessed whether PTEN expression or PTEN phosphorylation is changed in the inflamed distal ileum of *in utero* VPA-exposed male mice. As shown in figure 1K & M, both PTEN and phosphorylated PTEN expression show no differences between *in utero* VPA-exposed and control mice.

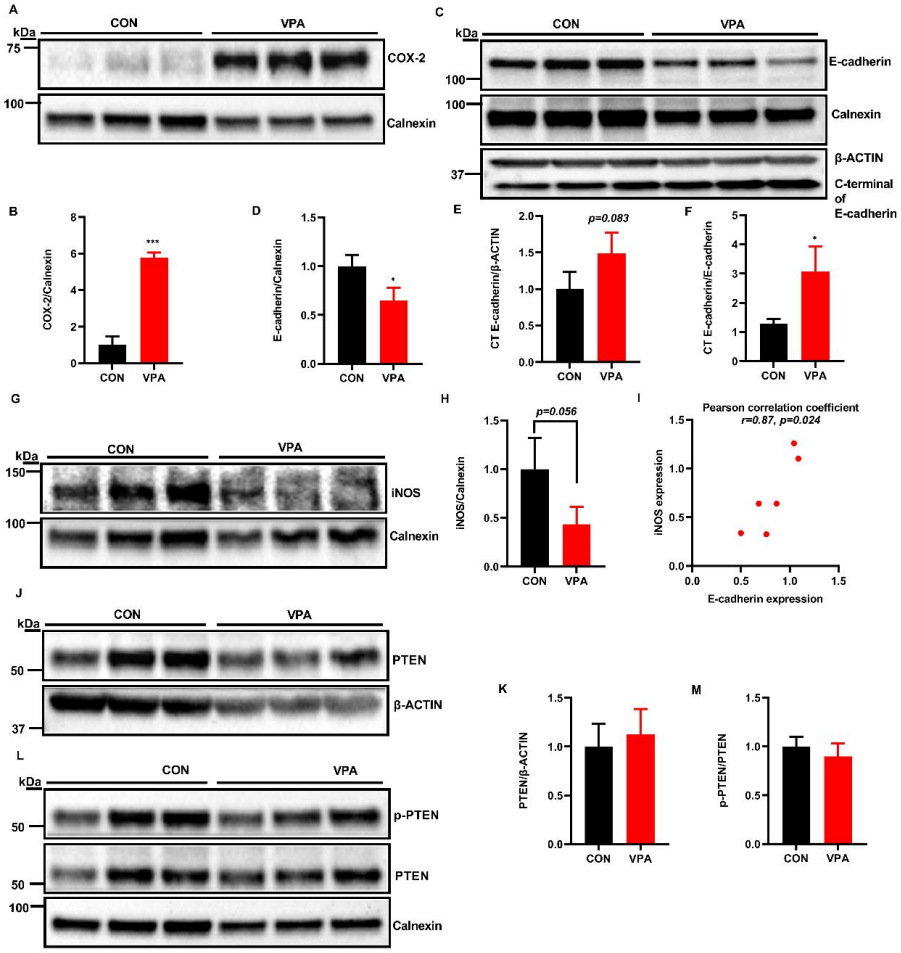

Figure 1. he effects of *in utero* VPA-exposure on COX-2, iNOS, E-cadherin, as well as PTEN expression and PTEN phosphorylation in the distal ileum of male mice. (A) Immunoblots of COX-2. (B) The quantification of COX-2 expression. (C) The immunoblots of membrane-associated E-cadherin and C-terminal (CT) fragment of E-cadherin. (D, E) The quantification of E-cadherin and CT E-cadherin expression. (F) The ratio of CT E-cadherin to E-cadherin. (G) The immunoblots of iNOS. (H) The quantification of iNOS expression. (I) Pearson correlation analysis of E-cadherin and iNOS. (J) The immunoblots of PTEN. (K) The quantification of PTEN expression. (L)The immunoblots of phosphorylated PTEN (p-PTEN). (M) The quantification of p-PTEN expression. Calnexin or β-ACTIN were used as loading control. n=3 for *in utero* PBS- or VPA-exposed male mice. Results are shown as mean ± SD. **P < 0.05, ***P < 0.001.*

2. *In utero* exposure to VPA attenuated PTEN expression in hippocampus, without effects in other brain regions in male mice.

PTEN expression significantly decreased in the hippocampus of *in utero* VPA-exposed male mice compared to control mice (figure 2A & B). In contrast, PTEN expression of *in utero* VPA-exposed mice was not significantly changed compared to control mice in the prefrontal cortex (supplementary figure 1A & B), cerebellum (supplementary figure 1C & D), olfactory bulb

(supplementary figure 1E & F), and in the rest of other mice brain regions (supplementary figure 1G & H).

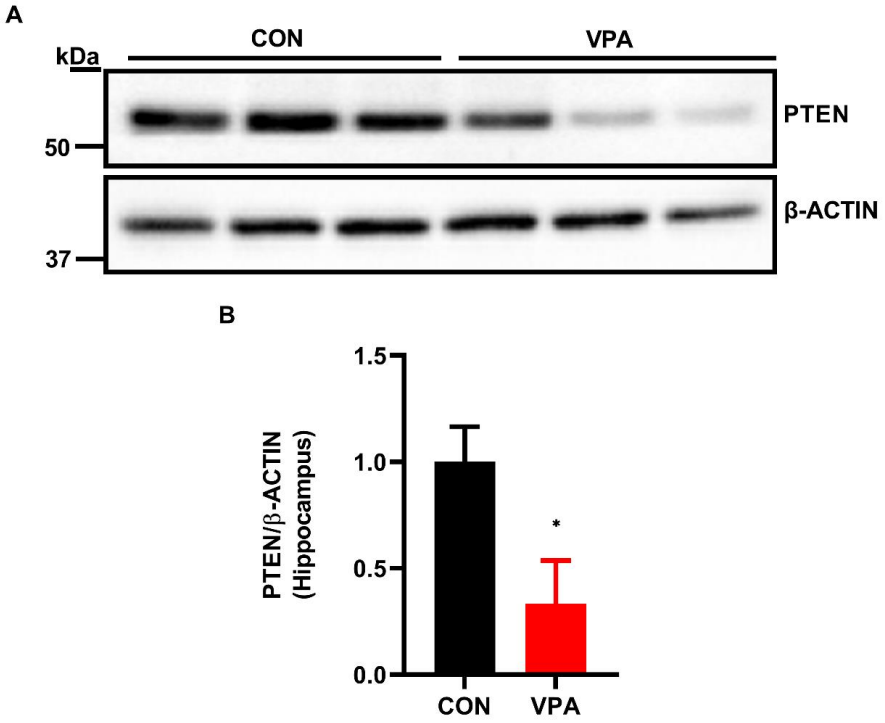

Figure 2. *In utero* VPA-exposure to male mice demonstrated a decreased PTEN expression in hippocampus. (A) The immunoblots of hippocampal PTEN. (B) The quantification of hippocampal PTEN expression. β-ACTIN was used as loading control. n=3 for *in utero* PBS- or VPA-exposed male mice. Results are expressed as mean ± SD. *$P < 0.05$.

These *in vivo* results indicate that *in utero* exposure to VPA down regulates PTEN expression in the hippocampus but not affect PTEN expression in the distal ileum in male mice. Elevated levels of the intestinal bacteria-derived metabolite *p*CS in serum of *in utero* VPA-exposed male mice has been shown in previous studies [15]. In the present study, we hypothesize that the intestinal bacteria-derived *p*CS and 4EPS induce ASD-like behaviors via affecting PTEN expression of microglial cells in the brain in this murine model for ASD. Therefore, the direct effect of *p*CS and 4EPS on PTEN expression in BV2 microglial cells and the possible association with neuro-immune responses and microglial phagocytosis function are investigated.

Inflammatory response of microglial cells

1. *p*CS and 4EPS attenuated constitutive and LPS-activated PTEN expression in BV2 microglial cells.

Previously we demonstrated that 24-hour exposure of BV2 microglial cells up to a concentration of 500 µM *p*CS did not affect their viability [15]. In the present study, we show that 4EPS up to a concentration of 400 µM did also not have toxic effects on BV2 microglial

cells after 24 hours incubation using two cell viability assays (MTT and LDH) (supplementary figure 2).

The direct effects of *p*CS and 4EPS on PTEN expression in BV2 microglial cells were assessed. As shown in figure 3A-D, 5 to 150 µM *p*CS, and 0.1 to 10 µM 4EPS significantly decreased constitutive PTEN expression dose-dependently compared to vehicle-incubated cells. In parallel, LPS stimulation significantly increased PTEN expression compared to control cells. Furthermore, *p*CS (at a concentration of 5 and 150 µM) and 4EPS (at a concentration of 1, 10 and 100 µM) significantly attenuated the LPS-induced increase of PTEN expression in BV2 microglial cells (figure 3A & B and figure 3C & D, respectively). These results suggest that these two bacteria-derived metabolites can directly reduce PTEN expression of microglial cells constitutively and under inflammation.

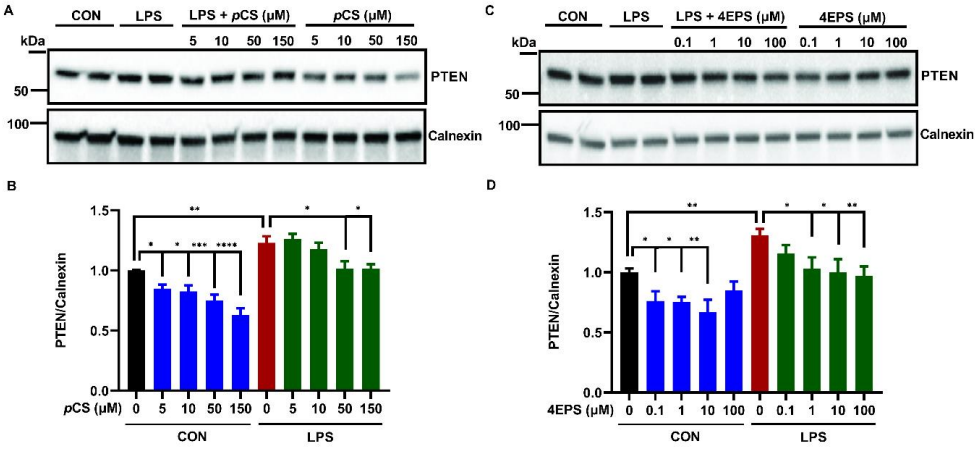

Figure 3. The effects of *p*CS or 4EPS on constitutive and LPS-activated PTEN expression in BV2 microglial cells. BV2 microglial cells were incubated with *p*CS or 4EPS for 24 hours in the absence or presence of 1,000 ng/mL LPS. Cell lysate was collected for WB analysis. (A) The representative immunoblots of PTEN with *p*CS treatment. (B) The quantification of PTEN expression of microglial cells exposed to *p*CS (n=4 from 4 independent experiments). (C) The representative immunoblots of PTEN with 4EPS treatment. (D) The quantification of PTEN expression of microglial cells exposed to 4EPS treatment (n=6 from 6 independent experiments). Calnexin was used as loading control. Black: control; red: control LPS-activated microglial cells; blue: constitutive active microglial cells; green: LPS-activated microglial cells. Results are shown as mean ± SEM. **P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.*

2. *p*CS and 4EPS attenuated LPS-induced TNF-α and IL-6 release by BV2 microglia cells, which was not regulated by PTEN.

As shown previously [15, 41, 42], LPS stimulation significantly increased TNF-α and IL-6 release from microglial cells (figure 4A-F). Confirming previous results, *p*CS inhibited the LPS-induced TNF-α and IL-6 release from microglial cells (figure 4A & B). Similar inhibitory effects were found for 4EPS (figure 4C & D). In addition, like *p*CS [15], 4EPS treatment reduced constitutive release of TNF-α and IL-6 from microglia (figure 4C & D).

Next, the effect of a PTEN inhibitor VO-OHpic (50, 100, 200 and 400 nM) on the constitutive and LPS-induced release of TNF-α and IL-6 by microglial cells was investigated [36]. As figure

4E & F show, VO-OHpic neither affected the constitutive nor the LPS-induced releases of TNF-α and IL-6 by microglial cells, indicating that PTEN is not involved in the constitutive or LPS-induced release of TNF-α and IL-6 by microglial cells. In summary, these results indicate that the *p*CS and 4EPS-induced inhibition of PTEN expression is not involved in the *p*CS and 4EPS-induced effects on constitutive and LPS-induced release of TNF-α and IL-6 of microglial cells.

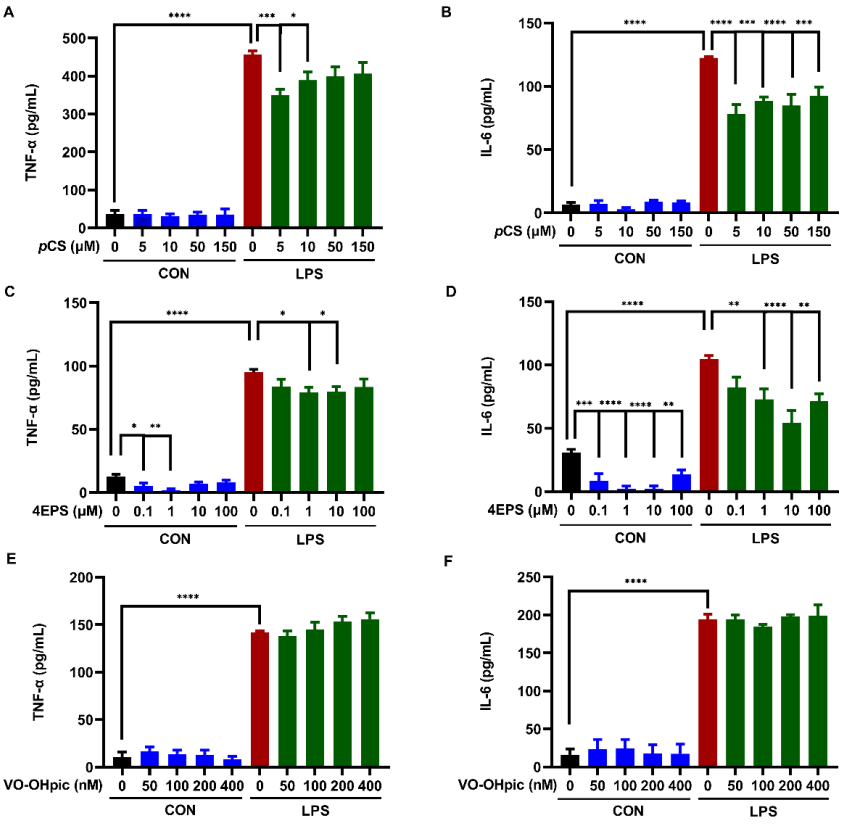

Figure 4. The effects of *p*CS, 4EPS and PTEN inhibitor VO-OHpic on TNF-α and IL-6 release from BV2 microglial cells. BV2 microglial cells were seeded with a density of 5,000 each well into 96-well plate, then exposed to *p*CS, 4EPS, and VO-OHpic for 24 hours in the absence or presence of 1,000 ng/mL LPS, respectively, culture medium was collected for ELISA measurements. (A, B) The TNF-α and IL-6 concentration in medium after *p*CS treatment. (n=6 and 4 from 6 and 4 independent experiments respectively). (C, D) The TNF-α and IL-6 concentration in medium after 4EPS treatment. (n=3 and 4 from 3 and 4 independent experiments respectively). (E, F) The TNF-α and IL-6 concentration in medium after VO-OHpic treatment. (n=4 and 3 from 4 and 3 independent experiments respectively). Black: control; red: control LPS-activated microglial cells; blue: constitutive active microglial cells; green: LPS-activated microglial cells. Results are shown as mean ± SEM. **P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001*.

In addition to the inflammatory cytokines TNF-α and IL-6, iNOS and COX-2 play important role in neuroinflammatory response in brain [43, 44]. It has been shown previously that LPS stimulation upregulates the protein expressions of these two inflammatory targets in BV2

microglial cells [41, 45], but there is little known about the effect of *p*CS and 4EPS on the expression of iNOS and COX-2. Therefore, the same *in vitro* model of BV2 microglial cells was further used to investigate the effect of *p*CS and 4EPS on iNOS and COX-2 expression in BV2 microglia.

3. *p*CS and 4EPS inhibited LPS-activated iNOS expression in BV2 microglial cells.

iNOS is an inflammation-induced protein and is barely constitutively expressed in microglial cells [46, 47]. As shown in figure 5A-D, LPS stimulation strongly increased iNOS expression in microglial cells compared to control, which can be attenuated by co-incubation with *p*CS (50 µM) and 4EPS (10 and 100 µM), respectively. These results further support that *p*CS and 4EPS can attenuate LPS-induced neuroinflammation in microglial cells.

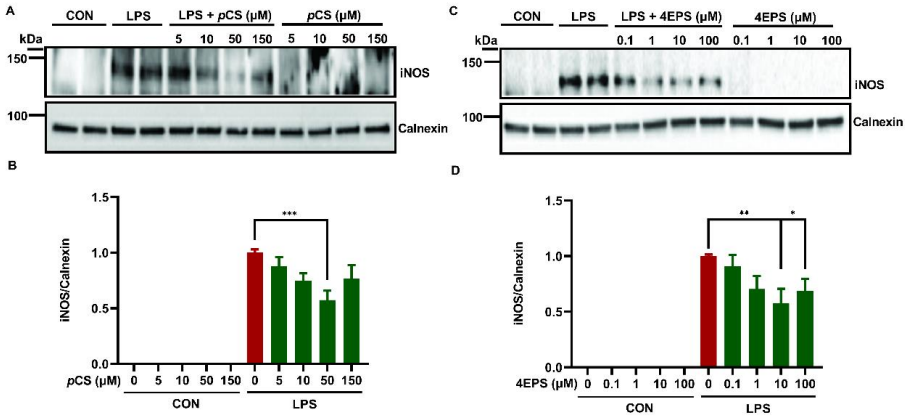

Figure 5. The effects of *p*CS or 4EPS on iNOS expression in BV2 microglial cells. BV2 microglial cells were seeded with a density of 50,000 per well into 12-well plate and then incubated with *p*CS or 4EPS for 24 hours in the absence or presence of 1,000 ng/mL LPS. Cell lysate was collected for WB analysis. (A) The representative immunoblots of iNOS with *p*CS treatment. (B) The quantification of iNOS expression after *p*CS treatment (n=4 from 4 independent experiments). (C) The representative immunoblots of iNOS with 4EPS treatment. (D) The quantification of iNOS expression after 4EPS treatment (n=4 from 4 independent experiments). Calnexin was used as loading control. Red: LPS-control; green: LPS-activated microglial cells. Results are shown as mean ± SEM. **P < 0.05, **P < 0.01, ***P < 0.001*.

4. *p*CS and 4EPS were unable to affect LPS-activated COX-2 expression. 4EPS alone increased constitutive COX-2 expression in BV2 microglial cells.

LPS stimulation significantly increased COX-2 expression in microglial cells (figure 6A-D). Neither *p*CS nor 4EPS treatment affected LPS-induced COX-2 expression. To better detect COX-2 bands without LPS stimulation, microglial cell lysates after *p*CS or 4EPS exposures were exposed longer separately. As shown in figure 6E & F, no clear difference was observed in relation to the constitutive COX-2 expression in microglial cells exposed to 5, 10, 50 or 150 µM *p*CS compared to control. In contrast, 1, 10 and 100 µM 4EPS significantly increased COX-2 expression in microglial cells compared to control without LPS stimulation (figure 6G & H). These results indicate that the bacterial metabolites *p*CS and 4EPS do not affect LPS-activated COX-2 expression, but 4EPS can increase constitutive COX-2 expression of microglial cells.

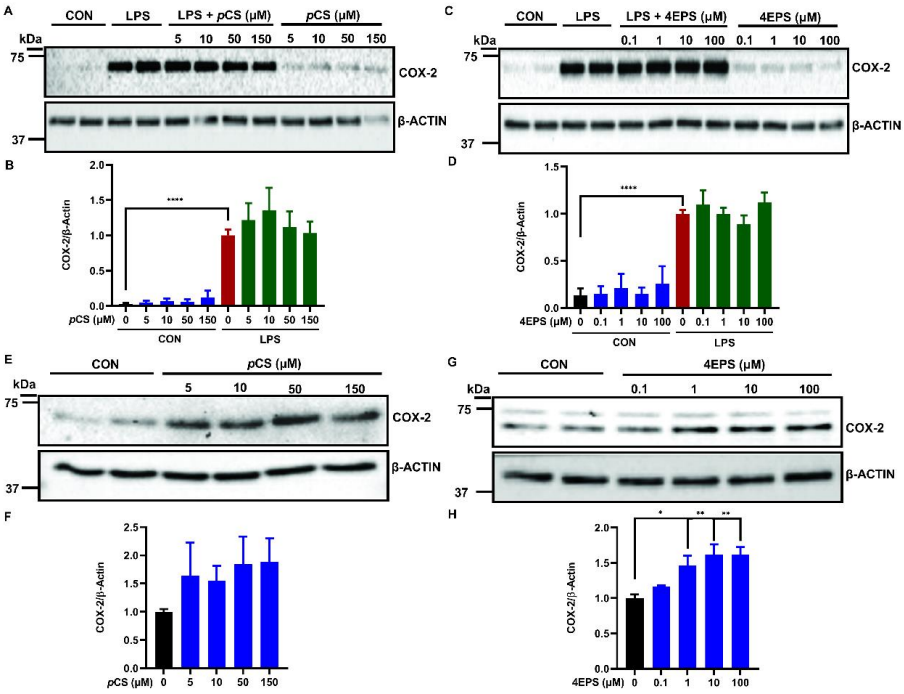

Figure 6. The effects of *p*CS or 4EPS on COX-2 expression in BV2 microglial cells. BV2 microglial cells were seeded with a density of 50,000 per well into 12-well plate and then incubated with *p*CS or 4EPS for 24 hours in the absence or presence of 1,000 ng/mL LPS. Next cell lysate was collected for WB analysis. (A) The representative immunoblots of COX-2 with *p*CS treatment. (B) The quantification of COX-2 expresison after *p*CS treatment (n=4 from 4 independent experiments). (C) The representative immunoblots of COX-2 with 4EPS treatment. (D) The quantification expression of COX-2 after 4EPS treatment (n=4 from 4 independent experiments). (E) The representative immunoblots of COX-2 with *p*CS treatment in the absence of LPS stimulation. (F) The quantification of COX-2 expression after *p*CS treatment in the absence of LPS stimulation. (n=4 from 4 independent experiments). (G) The representative immunoblots of COX-2 with 4EPS treatment in the absence of LPS stimulation. (H) The quantification of COX-2 expression after 4EPS treatment in the absence of LPS stimulation. (n=3 from 3 independent experiments). β-ACTIN was used as loading control. Black: control; red: control LPS-activated microglial cells; blue: constitutive active microglial cells; green: LPS-activated microglial cells. Results are shown as mean ± SEM. **P < 0.05, **P < 0.01, ****P < 0.0001.*

5. 4EPS or PTEN inhibitor VO-OHpic did not affect constitutive and LPS-activated microglial phagocytosis activity.

Previous results have shown that *p*CS treatment attenuated constitutive phagocytosis activity in microglial cells without affecting LPS-induced microglial enhanced phagocytosis activity [15]. In the present study, the effects of 4EPS or PTEN inhibitor VO-OHpic on microglial phagocytosis were studied. Confirming the previous finding, LPS stimulation significantly increases microglial phagocytosis activity (figure 7A & B). As shown in figure 7A, 4EPS treatments from 0.1μM to 100 μM did not affect constitutive or LPS-induced microglial phagocytosis. In addition, 50 nM to 400 nM VO-OHpic also did not affect microglial phagocytic

activity with or without LPS stimulation (figure 7B), indicating that 4EPS and PTEN do not play a role in microglial phagocytosis.

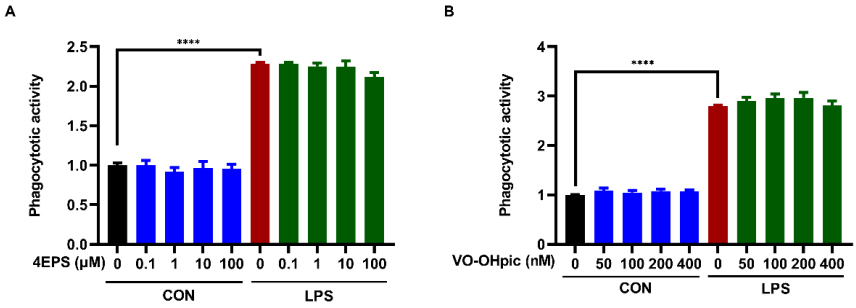

Figure 7. The effects of 4EPS and PTEN inhibitor on microglial phagocytosis function in BV2 cells. BV2 cells were incubated with 4EPS or VO-OHpic for 24 hours in the absence or presence of 1,000 ng/mL LPS respectively. (A). The effect of 4EPS treatment on BV2 microglial phagocytosis activity (n=4 from 4 independent experiments). (B). The effect of VO-OHpic treatment on BV2 microglial phagocytosis activity (n=5 from 5 independent experiments). Black: control; red: control LPS-activated microglial cells; blue: constitutive active microglial cells; green: LPS-activated microglial cells. Results are shown as mean ± SEM. *****P < 0.0001*.

Discussion

The present study investigated the relation between two bacteria-derived metabolites, *p*CS and 4EPS, and PTEN in the pathogenesis of ASD along the gut-brain axis using an *in utero* VPA-induced murine model of ASD and an *in vitro* LPS-activated microglial neuroinflammation model. In this current study, it was demonstrated that PTEN expression was not changed in the distal ileum of *in utero* VPA-exposed male mice, which is associated with enhanced levels of serum *p*CS. The observation that PTEN expression is reduced in the brain of *in utero* VPA-exposed male mice directed us to investigate the role of PTEN regarding the effects of *p*CS and 4EPS on microglial cell function *in vitro*. The decreased PTEN expression in the brain in the murine model for ASD is mirrored by *p*CS and 4EPS-induced decrease in PTEN in microglial cells. However, this *p*CS and 4EPS-induced reduction in PTEN expression does not appear to play a role in the bacteria-derived metabolite-induced derailed innate immune and phagocytotic response of microglial cells.

Increased intestinal permeability and intestinal inflammation frequently occur in children with ASD as well as in rodent models for ASD [5, 7, 8, 48]. Seike et al. have previously shown that *Clostridioides perfringens* Delta-toxin causes increased intestinal permeability or epithilial permeability through decreaseing E-cadherin expression in mouse ileal loop or epithelial Caco-2 cells, respectively [49, 50]. In the present study, it was demonstrated that *in utero* exposure to VPA decreased E-cadherin expression in distal ileum, indicative of increased intestinal permeability, which is also supported by the impaired intestinal permeability reported *in utero* VPA-exposed rats [51]. Glynne *et al.* have shown that E-cadherin co-localizes with epithelial iNOS [38]. In addition, decreased iNOS activity or expression increases intestinal permeability through attenuating the expression of tight junctional proteins in mouse ileal tissue [52]. These previous studies support the decreased iNOS expression that was observed in the distal ileum of *in utero* VPA-exposed male mice in the current study. In addition, it was demonstrated that the distal ileum of *in utero* VPA-exposed male mice was inflamed as indicated by enhanced COX-2 protein expression, which is supported by previous

study showing the presence of inflammation in small intestine of in *in utero* VPA exposed mice [31]. Next, PTEN and p-PTEN expression in the same ileal tissue were examined. We found that *in utero* exposure to VPA did not to affect (p-)PTEN expression in ileum. These data suggest that PTEN is not relevant for the compromised barrier and intestinal inflammation in the ileum of *in utero* VPA-exposed male mice.

Both *p*CS and 4EPS are found to be elevated in urine, feces or serum of children diagnosed with ASD and can induce ASD-like behaviors in mice [9, 10, 13, 14, 16-18, 53]. Furthermore, our previous study has shown an increase in serum *p*CS levels of in *in utero* VPA-exposed male mice [15]. *p*CS has been detected in mouse brain tissue [54, 55] and cerebrospinal fluid of parients with Parkinson's disease [56]. In addition, 4EPS has also been demonstrated to be able to reach the brain in mice [17]. In *in utero* VPA-exposed mice, the present study shows that hippocampal PTEN expression is decreased, which is in line with the previous finding that PTEN expression is decreased in hippocampus and cortex of in *in utero* VPA-exposed male mice [23]. Decreased PTEN expression in brain might induce ASD-like behaviors, including impaired social interaction and increased repetitive behavior, which has also been demonstrated previously with PTEN-deficient mice [23, 57]. However, it is not clear whether *p*CS or 4EPS derailes the neuroimmune responses of microglial cells via the induction of decreased PTEN in the hippocampus of *in utero* VPA-exposed male mice. This study showed that both *p*CS and 4EPS attenuated PTEN expression in BV2 micorglial cells constitutively and during LPS stimulation, indicating that these two ASD-associated bacterial derivates might be able to decrease PTEN expression directly in brain.

Next, we studied whether PTEN plays a role in the bacteria-derived metabolite-induced dysfunction of microglial cells *in vitro*. Both *p*CS and 4EPS attenuate constitutive and LPS-induced TNF-α and IL-6 release by BV2 microglial cells, which is supported by the finding that *p*CS attenuates LPS-induced inflammation in macrophages [58, 59]. However, PTEN inhibition did not affect the release of these cytokines in microglial cells, indicating that the *p*CS- and 4EPS-induced decreased PTEN expression does not play a role in the inhibitory effects of *p*CS and 4EPS on TNF-α and IL-6 release of by microglial cells. Interestingly, the present study showed that 4EPS, but not *p*CS, significantly increased constitutive COX-2 expression in microglial cells indicative for prostaglandin-associated inflammatory response. In contrast, COX-2 deficient mice show ASD pathogenesis and ASD-related behaviors [60-62]. The possible role of increased COX-2 expression triggered by 4EPS in microglial cells in the context of ASD remains to be investigated. Microglial phagocytosis is necessary to control synaptic function and brain development via regulating synaptic pruning and neuro-immune response [63, 64]. In the present study, we found that neither 4EPS nor PTEN inhibition affected BV2 microglial phagocytosis activity. PTEN deficient mice show upregulated neuroinflammation and microglial phagocytosis [26, 27]. In contrast, it has also been reported that PTEN inhibition or knockdown attenuates neuroinflammation in mice [24, 25]. This study reports that PTEN did not play a role in ASD-associated bacterial metabolite-induced derailed immune and phagocytosis response of microglial cells. Additionally, PTEN is essential to maintain neuronal growth, synaptic function and brain connectivity [65, 66]. Therefore, although beyond the scope of the present study, the role of the reduced PTEN expression observed in the brain of *in utero* VPA-exposed male mice, in the derailed neuronal network development and function remains to be further investigated.

In summary, this study demonstrated that both *p*CS and 4EPS decreased PTEN expression directly in microglial cells, which mirrors the decreased PTEN expression observed in the hippocampus of *in utero* VPA-exposed male mice. The current findings indicate that *p*CS and 4EPS-induced down-regulated PTEN does not play a role in ASD-associated neuroinflammation in the brain. Future studies are warranted in order to investigate how these two bacteria-derived metabolites affect neuronal synaptic function *in vitro* primary

neurons and in *in vivo* ASD murine models with a focus on PTEN.

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CRediT authorship contribution statement

Conceptualization—YZ, PPP, ADK; supervision—PPP, ADK, JG; investigation and data collection—YZ, NP, CVH; data analysis—YZ; writing—original draft preparation: YZ; writing—review and editing: YZ, NP, CVH, PPP, JG, ADK. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

JG is a part time employee at Danone Nutricia Research, Utrecht, Netherlands. All other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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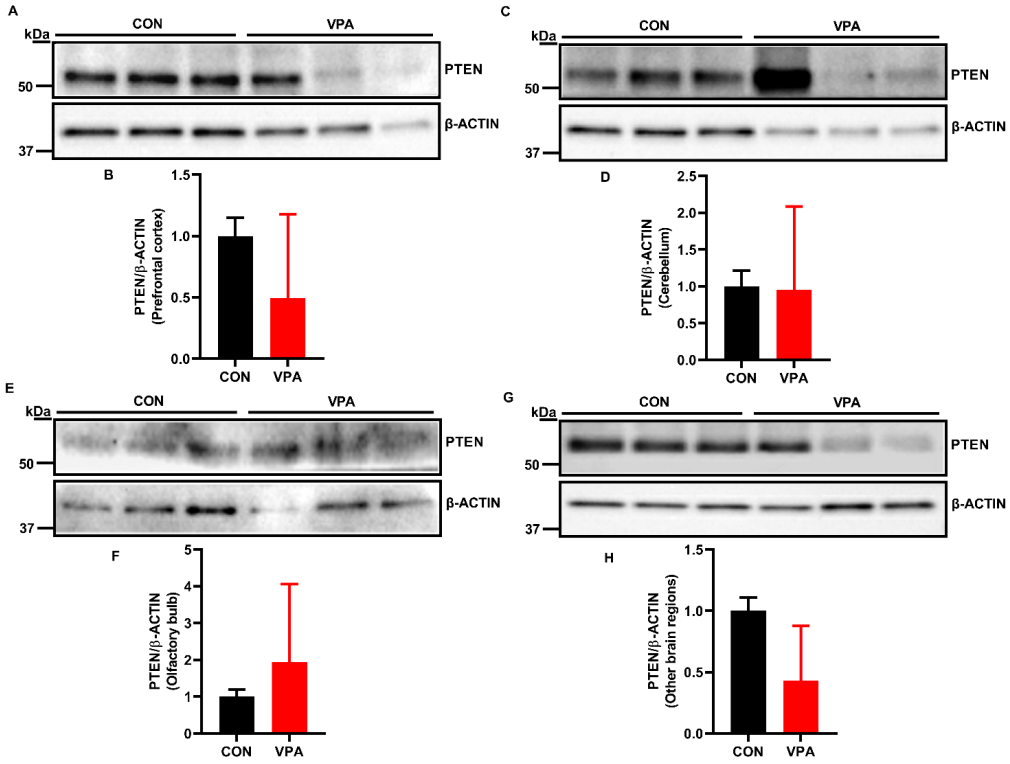
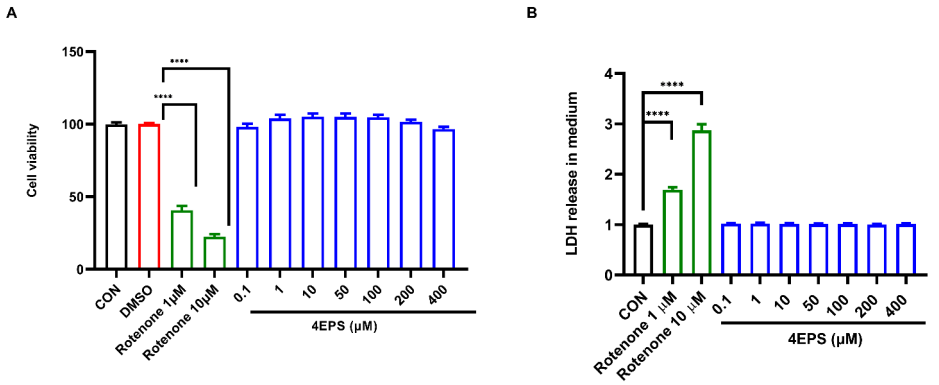
Supplementary results



Supplementary Figure 1. *In utero* VPA-exposure to male mice had no significant effects on PTEN expression in the prefrontal cortex, cerebellum, olfactory bulb, and the rest of other brain regions. (A) The immunoblots of PTEN in prefrontal cortex. (B) The quantification of PTEN expression in prefrontal cortex. (C) The immunoblots of PTEN in cerebellum. (D) The quantification of PTEN expression in cerebellum. (E) The immunoblots of PTEN in olfactory bulb. (F) The quantification of PTEN expression in olfactory bulb. (G) The immunoblots of PTEN in the rest of other brain regions. (H) The quantification of PTEN expression in the rest of other brain regions. β-ACTIN was used as loading control. n=3 for *in utero* PBS- or VPA-exposed male mice. Results are expressed as mean ± SD.



Supplementary Figure 2. The cell viability measurements with 4EPS treatment in BV2 microglial cells. 5,000 BV2 microglial cells were seeded per well into 96-well plate overnight, and were subsequently incubated with a concentration range of 4EPS treatments for 24 hours. (A) Cell viability in the presence of 4EPS. (B). LDH content in medium with 4EPS. n=3 independent experiments. Results are shown as mean ± SEM. **** $P < 0.0001$.

Chapter 7

Summary of key findings, general discussion and future perspectives

Major findings of this thesis

1. ADAM17 and ADAM10 plays an important role in microglial immune responses, demonstrated by the decreased TNF-α, TNFR-1 and IL-6R releases as well as the increased phagocytosis capacity after treatment with ADAM17 inhibitor, TAPI-1, or ADAM10 inhibitor, GI254023X.
2. PTEN does not play a role in microglial immune responses because the PTEN inhibitor did not affect TNF-α and IL-6 releases and/or the phagocytosis capacity.
3. The bacteria-derived metabolites, *p*CS and *p*CG, are elevated in the blood of *in utero* VPA-exposed male mice.
4. *p*CS and 4EPS decreased ADAM17 expression, ADAM10 expression and maturation as well as PTEN expression in microglial cells.
5. *p*CS and 4EPS attenuated TNF-α and IL-6 release of constitutive and LPS-activated microglial cells, which was associated with the *p*CS- and 4EPS-induced decreased ADAM17 and ADAM10 expression
6. *p*CS attenuated constitutive phagocytosis capacity of microglial cells, which seem not to be regulated by a reduced expression of ADAM17, ADAM10 or PTEN.
7. ADAM17, ADAM10 and PTEN are involved in ASD pathogenesis, indicated by decreased ADAM17 maturation, increased ADAM10 maturation and downregulated PTEN expression in the brain of *in utero* VPA-exposed mice.
8. PTEN is not playing a role in intestinal inflammation and enhanced permeability, because no differences in PTEN expression and phosphorylation were found in the intestine of autistic mice.
9. The inhibitor ADAM10, GI254023X, ameliorated the *in utero* VPA-induced compromised intestinal barrier and altered synaptic function in the brain of VPA-induced autistic mice.
10. GI254023X did not affect *in utero* VPA-induced social disturbance, but it seems to improve *in utero* VPA-induced impairment in cognitive function which was correlated with sNeuroligin-1 fragment level in mice brain.

Autism spectrum disorder (ASD) is a neurodevelopmental disorder usually diagnosed at early age and characterized by impairments in social interaction and communication, deficits in learning and memory and the presence of repetitive behavior [1]. In recent years, abnormalities along the gut-brain axis have been suggested to be associated with ASD pathogenesis. Children diagnosed with ASD frequently show to have a dysbiosis of gut microbiota that is composed of trillions of bacteria residing in the gastrointestinal tract [2, 3], causing changes in several bacterial metabolites production. Two bacterial of these metabolites, *p*-cresol sulfate (*p*CS) and 4-ethylphenyl sulfate (4EPS) are reported to be enhanced in blood, feces and urine of people diagnosed with ASD [4, 5]. Moreover, *p*CS and 4EPS treatment induces ASD-like behavior in mice [6-8], but the mechanisms behind it remain to be investigated. The targets A Disintegrin And Metalloprotease 10 (ADAM10), A Disintegrin And Metalloprotease 17 (ADAM17) and Phosphatase and tensin homolog (PTEN) play important roles in intestinal permeability, neuroimmune responses and synaptic function in brain that are causalities of ASD [9-13]. Investigating the possible connection between the two bacteria-derived metabolites *p*CS and 4EPS and these ASD-associated targets ADAM10, ADAM17 and PTEN will shed lights on explaining the reason why *p*CS and 4EPS might induce ASD. This thesis was focused on gaining insights into the possible molecular mechanisms of bacteria-derived metabolites in the pathogenesis of ASD to identify new targets for intervention. In this final chapter an overview and interpretation of the results is provided, and a future outlook is presented.

Two main challenges in ASD are the unclear pathogenetic mechanisms and the lack of effective intervention targeting ASD detrimental core symptoms [14, 15]. In recent years, the bacterial metabolite derivatives, *p*CS and 4EPS, have been shown to be elevated in blood, urine and feces of autistic individuals. Both metabolites have been proposed to be used as predictive biomarker for ASD diagnosis. In addition, preclinical studies have demonstrated that these two metabolites can induce ASD-like behavior in mice [5, 16, 17]. However, the underlying mechanisms indicating how the bacterial metabolites induce ASD remain still unclear. Using an *in utero* VPA-induced ASD murine model and an *in vitro* LPS-stimulated neuroinflammation model, this thesis illustrates that these two bacterial metabolites affect the expression and function of the metalloproteases, ADAM10, ADAM17, and the phosphatase, PTEN, that might be involved in the ASD-associated pathogenetic changes in neuroimmune response or synaptic function in the brain. In addition, this thesis further identifies that ADAM10 might be a target for ASD intervention either through direct pharmacological inhibition or via nutritional interventions targeting bacteria that are involved in *p*CS and 4EPS production to reduce ASD-associated detrimental symptoms.

1. ASD-associated bacteria-derived metabolites and their potential molecular leverage points in ASD

The levels of *p*-cresol derivatives, *p*CS and *p*CG, have shown to be elevated in the urine and feces of children diagnosed with ASD [4, 17]. In *in utero* VPA-exposed mice, a common murine model for ASD, increased intestinal Firmicutes at the expense of Bacteriodetes is reported previously, which is associated with *p*-cresol production in the intestinal tract [8, 18]. In Chapter 4 increased levels of both *p*CS and *p*CG in the serum of *in utero* VPA-exposed mice are presented confirming previous studies. In addition, *p*-cresol administration in mice induces social behavior deficits and repetitive behavior in mice [7], suggesting that the elevated *p*CS might be involved in the ASD-like symptoms in *in utero* VPA-exposed male mice. Moreover, the bacterial metabolite 4EPS, which is structurally similar to *p*CS, is significantly increased in blood and urine of ASD children and in a maternal immune activation murine model for ASD [5, 8]. Unfortunately, due to technical limitations we were not able to assess the levels of 4EPS in blood *in utero* VPA-exposed mice. 4EPS treatment has shown to cause some ASD-like behavior in mice as well as aberrant brain connectivity, which is associated with enhanced Notch signaling pathway in brain [6]. Notch signaling is important for brain development and function [19, 20]. Notch signaling depends on ADAM10 and possibly ADAM17, which are ASD-associated metalloproteases [21-23]. These metalloproteases cleave membrane-associated Notch, resulting in release of the intracellular domain that can modify gene expression in the cell. Therefore, elevated Notch signaling might be the result of enhanced ADAM10 activity in the 4EPS-treated mice brains. In addition, it has been demonstrated that Notch signaling represses PTEN expression *in vitro* and *in vivo* [24, 25]. However, it is unclear whether the 4EPS-meadiated enhanced Notch signaling involves enhanced ADAM10 activity or affects PTEN expression. The findings by us and others directed us to investigate whether the enhanced intestinal bacteria-derived metabolites *in utero* VPA-exposed mice are associated with changes in expression and/or activity of ADAM10, ADAM17 and PTEN in the brain and intestinal tract. Then we further investigated whether *p*CS and 4EPS can directly affect their expression or activity in microglial cells, important neuroimmune cells in the brain.

2. Expression of ADAM17, ADAM10 and PTEN in VPA-induced murine model for ASD

2.1. ADAM17

We measured the expression and maturation of ADAM17 and ADAM10 (in chapter 4) as well as the expression of PTEN (in chapter 6) in brain tissues of *in utero* VPA-exposed mice. We demonstrated that *in utero* VPA-exposure in mice decreased mature ADAM17 in the hippocampus. ADAM17 can be cleaved by other ADAMs to produce the soluble ADAM17 at the expense of cell membrane bound ADAM17 [26, 27], which might result in less ADAM17 and more soluble ADAM17, concurrently. Ray et al. have shown increased soluble ADAM17 levels in the brain of ASD patients [10], supporting the finding of decreased mature membrane bound ADAM17 in *in utero* VPA-exposed mice. ADAM17 controls neuroimmune responses via cleavage of membrane-bound TNF-α releasing soluble TNF-α by and regulates the TGF-β1 signaling [28, 29]. Indeed, the decreased mature ADAM17 expression observed in the

hippocampus of *in utero* VPA-exposed male mice was associated with decreased levels of soluble TNF-α and TGF-β1 (chapter 4). The consequences of decreased soluble TNF-α and TGF-β1 in the hippocampus of *in utero* VPA-exposed mice remain to be investigated. Of interest is that our *in vitro* studies show that ADAM17 inhibition in microglial cells resulted in reduced release of TNF-α (chapter 4).

Besides the neuro-immune function of microglial cell, the phagocytotic capacity of these cell is important for synaptic pruning which is in turn crucial for a normal brain development [30, 31]. In ASD, 'overpruning' - enhanced phagocytosis by microglial cells - is hypothesized to be responsible for the brain hyperconnectivity observed in ASD [32]. Enhanced microglial phagocytosis activity has been shown to be associated with ASD-like behaviors in mice and the number of activated microglial cells in post mortem brain of people diagnosed with ASD are increased as well [33, 34]. Previous studies has demonstrated that ADAM17 can reduce microglial phagocytosis via the cleavage of Triggering Receptor Expressed on Myeloid Cells-2 (TREM2), a receptor that activates phagocytosis [35-37]. Our *in vitro* data show that ADAM17 inhibition potentiated the phagocytosis capacity of microglial cells (chapter 4).[35-37] Taken all results together, the decreased mature ADAM17 in the brain of *in utero* VPA-exposed mice (chapter 4) might increase microglial phagocytosis resulting in overpruning , however, this remains to be investigated in future studies.

2.2 ADAM10

In chapter 4 we demonstrate an increased mature ADAM10 in the brain tissues of *in utero* VPA-exposed mice, which was further confirmed in chapter 5. Amyloid precursor protein (APP) is mainly cleaved by ADAM10 to generate soluble amyloid precursor protein (sAPP) that increases dendritic spine density [38-40], which is associated with altered brain weights and synaptic function most commonly occurred in people diagnosed with ASD with impaired cognitive function [41, 42]. Previously, Ray et al. have shown that sAPPα and total sAPP are increased in brain and blood of ASD children [10, 43]. Recently, *in utero* VPA-exposed rats and 4EPS-treated mice have shown to develop ASD-like behavior and abnormalities in dendritic spine density and brain connectivity, which is believed to be triggered by enhanced Notch signaling in the brain tissues [6, 44]. Notch is primarily cleaved by ADAM10 [21]. Taken together, these findings suggests that ADAM10 maturation and activity are increased not only in *in utero* VPA-exposed mice, but probably also in other ASD preclinical models as well as in ASD subjects. Apart from cleaving APP and Notch, ADAM10 has been shown to regulate synaptogenesis and brain connectivity as well through cleaving many other transmembrane synaptic adhesion molecules, including Neuroligin-1 and NrCAM [9, 45]. Despite of the unchanged NrCAM cleavage in the brain tissues of *in utero* VPA-exposed mice in chapter 5, in this chapter we demonstrated that the increased mature ADAM10 is associated with a decreased Neuroligin-1 expression and an increased Neuroligin-1 fragment in the same brain tissues. These results further indicate that increased ADAM10 maturation participates in ASD pathogenetic changes in synapse function. As for how the increased mature ADAM10 affects other synaptic adhesion molecules associated with ASD pathogenesis in *in utero* VPA-exposed mice remains to be investigated for further understanding.

In the intestinal tract, we focused on adhesion junction protein E-cadherin since ADAM10

regulates intestinal permeability through cleaving it to generate soluble E-cadherin fragments [46, 47]. In chapter 5 and chapter 6 we demonstrated that *in utero* exposure to VPA decreased E-cadherin expression with a concurrent increase in C-terminal E-cadherin fragment in the colon and distal ileum of *in utero* VPA-exposed male mice, respectively, indicating that ADAM10-meadiated cleavage of E-cadherin is enhanced in these tissues. In chapter 5, we did not see a clear increase in mature ADAM10 in the colon of *in utero* VPA-exposed male mice. This might be due to the limitation in sample size. In addition, the brain tissues were extracted into soluble fraction and insoluble fraction, the latter was used for measuring mature ADAM10. In contrast to brain tissues, the total fractions of colon tissues were used for mature ADAM10 measurements. The total amount of mature ADAM10 cannot represent ADAM10's activity always since mature ADAM10 is distributed around the cell or tissue and only the cell surface-expressed mature ADAM10 has the ability to mediate proteolytic shedding of other surface molecules [48-50]. Our results indicate that *in utero* VPA-exposure might affect ADAM10's distribution inside the cell or throughout the intestinal tissue to increase membrane-anchored mature ADAM10. Taken together, in *in utero* VPA-exposed mice an increase in E-cadherin cleavage mediated by enhanced ADAM10 activity might be involved in ASD pathogenetic change in impaired intestinal barrier.

2.3 PTEN

In chapter 6 we demonstrated that PTEN expression is attenuated in the hippocampus of *in utero* VPA-exposed male mice. Confirming previous studies that showed that *in utero* VPA-exposed mice have a decreased PTEN expression in brain tissues and dendritic spine abnormalities, which is associated with impaired sociability and repetitive behavior [51]. PTEN deficient mice show aberrant synaptic plasticity in the hippocampus and ASD-like behaviors [52]. In addition, PTEN haploinsufficiency in mice show ASD-like behaviors and an overgrown brain that is a clinical symptom of ASD children before the age of 10 [42, 53]. These reports indicate that the decreased PTEN expression in the hippocampus of *in utero* VPA-exposed mice might trigger synaptic dysfunction that is a causality of ASD. Although this thesis did not further demonstrate the effect of decreased PTEN on synapse function and brain connectivity, previous research indicates the involvement of the decreased PTEN in ASD pathogenesis with respect to neuronal networks. In addition, PTEN loss-of-function in mice demonstrates neuroinflammation and increased microglial phagocytosis [13, 54]. In contrast, PTEN inhibition or knockdown attenuates neuroinflammation and microglial phagocytosis in mice [12, 55]. The role of decreased PTEN expression in the derailed neuroimmune responses and microglial phagocytosis in the VPA-induced ASD model in mice needs to be further confirmed. In addition, chapter 6 showed that *in utero* VPA-exposure induced intestinal inflammation and impaired intestinal barrier indicated by increased COX-2 expression and decreased E-cadherin in the distal ileum, respectively. Moreover, in chapter 6 we showed that PTEN expression and PTEN phosphorylation did not change in the distal ileum of *in utero* VPA-exposed male mice, indicating that PTEN is not involved in the ileal inflammation and the increased ileal permeability of *in utero* VPA-exposed male mice. The unchanged PTEN expression and PTEN phosphorylation might imply that it is ADAM10 that cleaves E-cadherin to regulate ileal permeability.

3. The role of ADAM17, ADAM10 and PTEN in neuroimmune response and phagocytosis by microglial cells

We further investigated whether or not ADAM10, ADAM17 and PTEN play a role in innate neuroimmune responses of microglia with focuses on Toll-like receptor 4 (TLR4)-induced inflammatory response and phagocytosis. To this end, GI254023X a selective inhibitor of ADAM10, TAPI-1 preferentially inhibiting ADAM17 and followed by other metalloproteinases, and VO-OHpic a potent PTEN inhibitor were added accordingly into microglial cells with or without LPS stimulation. Chapter 4 demonstrated that both ADAM10 and ADAM17 inhibition attenuated the release of soluble TNF-α, soluble TNFR-1 and soluble IL-6R by constitutive and LPS-activated microglial cells, indicating that both ADAM10 and ADAM17 cleave TNF-α, TNFR-1 and IL-6R. Although ADAM17 is identified as the sheddase of TNF-α in T lymphocyte and called TNF-α Converting Enzyme [29], ADAM10 also can partially cleave TNF-α in mouse macrophages [56]. Moreover, ADAM10 becomes the primary sheddase to cleave TNF-α when ADAM17 is deficient in fibroblasts [57]. The compensation effect of ADAM17 has been further confirmed in ADAM10 deficiency lymph nodes [58]. Our results and that of others indicate the overlapped function of ADAM17 and ADAM10 in cleaving substrates and might explain why either ADAM10 or ADAM17 cleaves TNF-α, TNFR-1 and IL-6R in microglial cells. In chapter 4 we also demonstrated that both ADAM10 and ADAM17 inhibition potentiated the phagocytic capacities of microglial cells constitutively and during LPS-activation. In addition to ADAM17 cleaving TREM2 and thereby suppressing phagocytosis, ADAM10 also has been suggested to be able to cleave TREM2 [37, 59]. In addition, ADAM10 cleaves fractalkine of neurons to generate the secreted fractalkine that recognizes the fractalkine receptor of microglia to activate microglial phagocytosis [60-62]. In conclusion, inhibiting ADAM17 or ADAM10 can potentiate constitutive and LPS-stimulated microglial phagocytosis. In chapter 6 we showed that PTEN inhibition with the inhibitor VO-OHpic did not affect the release of TNF-α and IL-6 as well as the phagocytosis of constitutive and LPS-activated microglial cells, indicating that PTEN does not play a role in neuroinflammatory response and microglial phagocytosis.

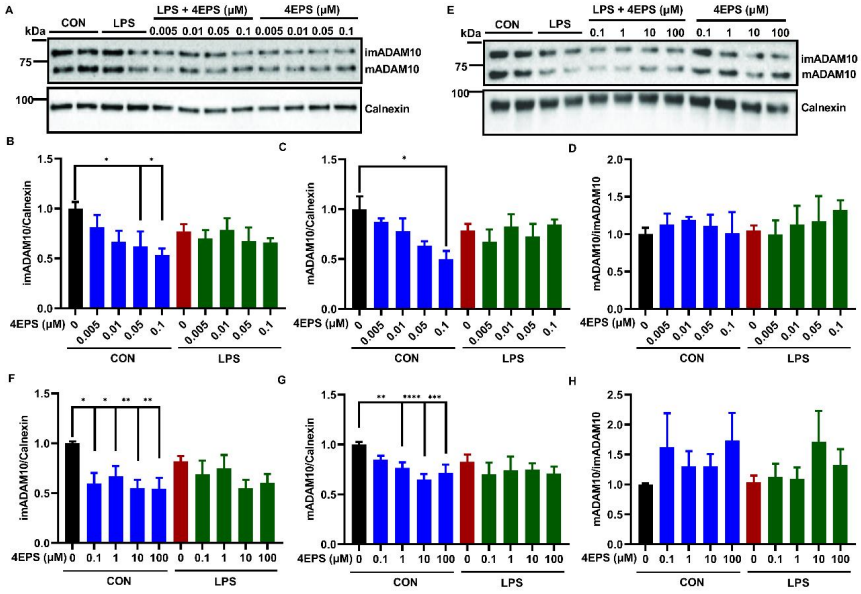
4. The role of ADAM17, ADAM10, PTEN as well as iNOS and COX-2 in ASD bacteria-derived metabolites, *p*CS or 4EPS, induced derailed immune responses of microglial cells

4.1 ADAM17

Previously it has been demonstrated that the ASD-associated bacterial metabolite *pCS* inhibits LPS-induced inflammation of mouse macrophages and Th1-type immune response in mice. In chapter 4 we demonstrated that *p*CS derailed the microglial immune response, indicated by the decreased TNF-α and IL-6 releases of constitutive and LPS-activated microglial cells, which was shown to be associated with *p*CS-induced downregulation of ADAM17 expression in microglial cells. Similar to *p*CS, in chapter 6 we demonstrated that another ASD-associated metabolite 4EPS decreased TNF-α and IL-6 releases of constitutive and LPS-activated microglial cells. In chapter 6 we did not present the effects of 4EPS on ADAM10 and ADAM17 expression of microglial cells, therefore we present the results here. Figure 1 shows that 4EPS attenuated ADAM17 expression in microglial cells constitutively and under inflammation, suggesting 4EPS-decreased TNF-α and IL-6 releases are attributed to the attenuated ADAM17

expression. As described before TNF-α is primarily cleaved by ADAM17 [29]. Our results indicate that potentially through the downregulation of ADAM17 both ASD-associated bacterial metabolites, *p*CS and 4EPS, attenuate TNF-α release in microglial cells. Our *in vitro* results are similar to the decreased ADAM17 maturation and associated reduced TNF-α observed in the hippocampus of *in utero* VPA-exposed mice (chapter 4). The combination of *in vitro* and *in vivo* data help to unravel the mechanism of how bacterial metabolites might affect the brain in ASD. Furthermore, chapter 4 demonstrated that ADAM17 can cleave TNFR-1 and IL-6R in microglial cells, the effects of *p*CS and 4EPS on these inflammatory cytokines receptors *in vitro* and *in vivo* remain to be studied.

In chapter 4 we showed that *p*CS attenuated constitutive phagocytosis of microglial cells while inhibiting ADAM17 with TAPI-1 oppositely potentiated the phagocytosis capacity. This striking observation might be caused by different inhibitory mechanisms of ADAM17 by *p*CS and TAPI-1. *p*CS inhibited ADAM17 expression directly while TAPI-1 inhibited ADAM17 activity without decreasing ADAM17 expression in microglial cells (chapter 4). ADAM17 inhibitors can inhibit ADAM17 activity through blocking the binding sites to phosphatidylserine exposure to prevent the cleavages of substrates [63]. In addition, it is possible that TAPI-1 inhibitor attenuates ADAM17 activity through changing the localization, cellular compartmentation, trafficking and maturation of ADAM17 [26, 64]. Taken together, we conclude that ADAM17 is not involved in the decreased microglial phagocytosis induced by *p*CS.



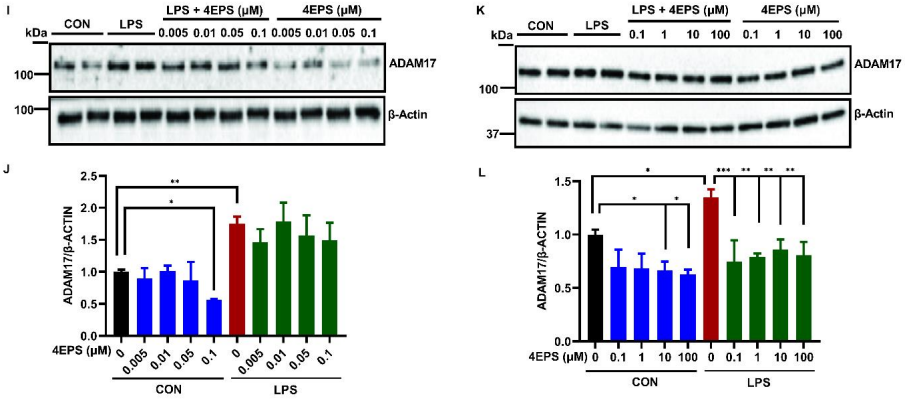


Figure 1. Effect of 4EPS on ADAM10 and ADAM17 expression of constitutive and LPS-stimulated BV2 microglial cells. BV2 microglia were incubated with 4EPS for 24 hours in the absence or presence of 1000 ng/mL LPS. Cell lysate was collected for WB analysis. (A) The representative immunoblots of ADAM10 with low concentration range of 4EPS. (B-D) The quantification results of immature ADAM10 (imADAM10), and mature ADAM10 (mADAM10) and maturation efficiency (ratio mADAM10/imADAM10) of BV2 cells exposed to low concentration range of 4EPS (n=3 independent experiments). (E) The representative immunoblots of ADAM10 with high concentration range of 4EPS. (F-H) The quantification results of imADAM10, mADAM10 and maturation efficiency of BV2 cells exposed to high concentration range of 4EPS treatment (n=6 independent experiments). (I) The representative immunoblots of ADAM17 protein expression with low concentration range of 4EPS. (J) The quantification results of ADAM17 protein expression with low concentration range of 4EPS (n=3 independent experiments). (K) The representative immunoblots of ADAM17 protein expression with high concentration range of 4EPS. (L) The quantification results of ADAM17 protein expression with high concentration range of 4EPS (n=5 independent experiments). Calnexin or β-Actin were used as a loading controls. Black: constitutive protein expression set at 1; red: LPS-induced protein expression; blue: effect of 4EPS on constitutive protein expression; green: effect of 4EPS on LPS-induced protein expression. Results were expressed as mean ± SEM. **P < 0.05*, ***P < 0.01*, ****P < 0.001*, *****P < 0.0001.*

4.2 ADAM10

Similar to ADAM17, ADAM10 is able to cleave TNF-α, TNFR-1 and IL-6R from microglial cells. Therefore, we examined the effect of ASD-associated bacteria-derived metabolites, *p*CS and 4EPS, on ADAM10 in microglial cells as well. *p*CS and 4EPS only attenuated ADAM10 expression and maturation of constitutive microglial cells (chapter 4 and figure 1). These findings indicating that *p*CS- and 4EPS-induced decreased of constitutive TNF-α and IL-6 release is regulated by ADAM10 inhibition. The LPS-induced TNF-α and IL-6 release was also inhibited by *p*CS and 4EPS, but here ADAM10 seems not to play a role (chapter 4 and 6). Both *p*CS or 4EPS-induced decrease of ADAM10 expression (immature ADAM10) mimics the decreased immature ADAM10 in the brain tissues of *in utero* VPA-exposed male mice (chapter

4). In contrast, the lack of effect by *p*CS and 4EPS on ADAM10 maturation on microglial cells fails to reflect the increased ADAM10 maturation in the brain tissues of *in utero* VPA-exposed mice (chapter 4 and chapter 6), which indicates the possibility that *p*CS and 4EPS increase ADAM10's maturation of other cells in the brain, like neurons. Future investigation on the effect of *p*CS or 4EPS on ADAM10 maturation as well as ADAM10-mediated cleavages of synaptic adhesion molecules in neurons can shed light on the mechanisms by which these two bacterial metabolites are involved in ASD.

4.3 PTEN

In chapter 6 we demonstrated *p*CS and 4EPS attenuated PTEN expression of constitutive and LPS-stimulated microglial cells, reflecting the decreased PTEN expression in the hippocampus of *in utero* VPA-exposed mice. Next, we investigated the involvement of PTEN in the *p*CS or 4EPS-derailed immune response of microglial cells. The PTEN inhibitor VO-OHpic did not affect the releases of TNF-α and IL-6 or phagocytosis capacity of microglial cells. It seems to be that PTEN does not play a role in microglial cell function. In addition, PTEN is not playing a role in the *p*CS- or 4EPS-induced attenuated immune response of microglia (chapter 6). Future studies should investigate the effects of *p*CS and 4EPS on PTEN expressions in neurons and on synaptic function, to elucidate a possible PTEN-downregulation in neurons of these ASD-associated bacterial metabolite.

4.4 iNOS and COX-2

iNOS expression in brain and intestine tract is connected to neuroinflammation and intestinal permeability, respectively [65-67]. A decreased iNOS expression was found in the distal ileum of *in utero* VPA-exposed male mice. Previously, it was reported that VPA-exposed mice develop an inflammation of the distal ileum [68]. iNOS expression co-localize with adhesion junction protein E-cadherin [65], and chapter 6 demonstrated a significant correlation between E-cadherin and iNOS expression in murine distal ileum. Together with our finding that *p*CS and 4EPS attenuated iNOS expression in microglial cells (chapter 6), these ASD-associated metabolites might affect intestinal permeability through downregulating iNOS-associated E-cadherin; importantly this hypothesis remains to be investigated in more detail. COX-2 in brain and intestine is associated with neuroinflammation and intestinal inflammation respectively [69, 70]. Chapter 6 showed that 4EPS increased COX-2 expression in microglial cells, in line with the increased COX-2 expression in the distal ileum of *in utero* VPA-exposed male mice. However, how important the role of bacteria-derived metabolites are for the ASD-associated enhanced intestinal COX-2 expression and the exact mechanism by which this inflammation-associated enzyme is activated remains to be further investigated.

5. Inhibiting ADAM10 is a potential target for ASD intervention

ADAM10 regulates intestinal permeability by cleaving adhesion tight junction protein E-cadherin into soluble fragments [46, 47, 71]. In brain ADAM10 regulates synaptogenesis by cleaving synaptic adhesion molecules Neuroligin-1 and NrCAM into relative fragments [9, 45]. We further studied the effects of ADAM10 inhibition by GI254023X in the VPA-induced murine model for ASD to investigate the role of ADAM10 in ASD pathogenesis along the gut-brain axis.

In chapter 5 we report preliminary findings that inhibiting ADAM10 by GI254023X decreased the *in utero* VPA-induced increase in E-cadherin cleavage and increased Claudin-3 expression in male mice colon. These data suggest that ADAM10 inhibition can improve the *in utero* VPA-induced impaired intestinal barrier in the colon of mice. In addition, GI254023X decreased the *in utero* VPA-induced increase of maturation of ADAM10 in the brain, which was associated with a reduction of cleavage of Neuroglin-1 the prefrontal cortex. This finding indicates that inhibiting ADAM10 might improve the VPA-induced synaptic dysfunction.

The behavioral test results indicate that ADAM10 inhibition improved *in utero* VPA-induced deficit in cognitive function. It has been shown that Neuroligin-1 deficient mice show compromised cognitive function [72]. These results indicate that the ADAM10-mediated cleavage of Neuroligin-1 plays an important role in the cognitive function of *in utero* VPA-exposed. Beside the cognitive function, we examined the social interaction of *in utero* VPA-exposed and PBS-exposed mice. ADAM10 inhibition did not affect the VPA-induced social interaction deficits in mice. In contrast, in control mice GI254023X induced impaired social interaction. ADAM10 cleaves more than 40 substrates that maintain a wide range of physiological function to develop and maintain brain function of mouse and human [45, 73, 74]. ADAM10 deficiency in mice shows synaptic dysfunction, altered brain connectivity in the cortex, hippocampus and olfactory bulb [45, 74, 75], which might lead to the disturbed social behavior and other altered behaviors. Therefore, balancing the level of ADAM10 in mouse brain would be a good option to reduce the detrimental effects of inhibiting ADAM10 for ASD treatment.

6. Summarizing conclusions

Using an *in utero* VPA-induced mouse model of ASD and an *in vitro* model of LPS-induced neuroinflammation of microglial cells, this thesis describes the involvement of ADAM17, ADAM10 and PTEN in ASD pathogenesis, and the potential connection with ASD-associated bacterial metabolites, *p*CS and 4EPS. The bacterial metabolites might be initial triggers by affecting the expression of these metalloproteases and phosphatase along the gut-brain axis. In addition, in this thesis preliminary data further suggest ADAM10 as a potential target for treatment of detrimental symptoms in ASD. Figure 2 gives a graphical overview of the potential mechanisms of action of bacterial-derived metabolites *p*CS and 4EPS in ASD along the gut-brain axis.

6.1 The interaction between bacterial metabolites and ADAM10, ADAM17 and PTEN in ASD pathogenesis

Our results demonstrate that ADAM10 and ADAM17 play an important role in the regulation of microglial immune response as well as the phagocytosis capacity. In addition, the *in vitro* studies demonstrate that *p*CS and 4EPS are able to reduce ADAM10 expression and maturation and ADAM17 expression, which is associated with reduced release of TNF-α and IL-6 as well as phagocytosis capacity of microglial cells. Moreover, elevated levels of *p*CS and *p*CG were detected in the blood of *in utero* VPA-exposed mice. ADAM10 maturation was increased in the brain of *in utero* VPA-exposed mice. In contrast, ADAM17 maturation was

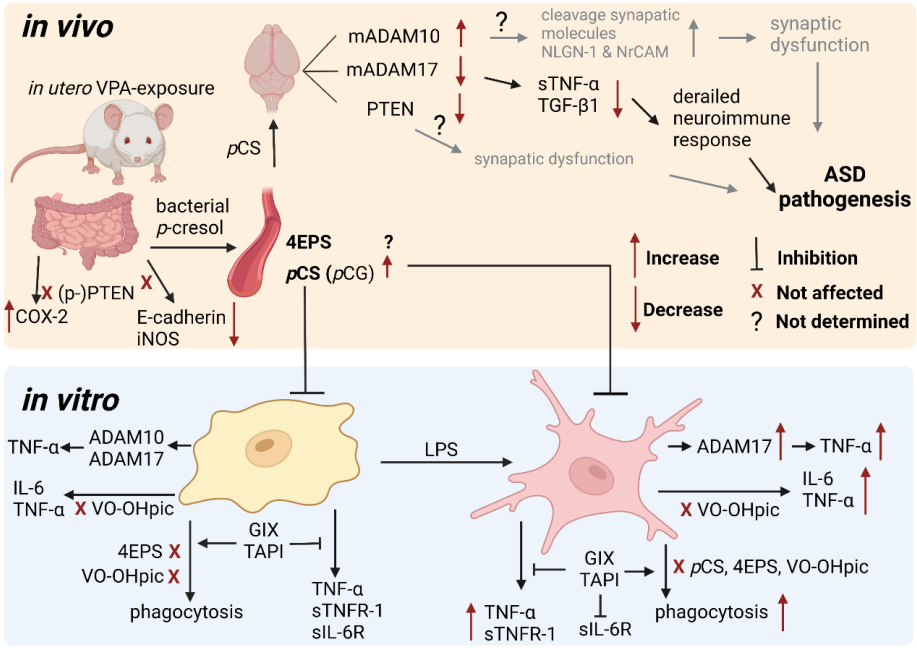
decreased in the brain of *in utero* VPA-exposed mice, which was associated with decreased levels of sTNF-α and TGF-β1. The *p*CS and 4EPS-induced decreased ADAM17 of microglial is in line with the decreased ADADM17 in the brain of *in utero* VPA-exposed mice. In conclusion, ADAM17 and ADAM10 might play a role in ASD pathogenesis, which could be associated with central effects of bacterial metabolites *p*CS and 4EPS.

We demonstrated a decrease in PTEN expression in the brain of *in utero* VPA-exposed mice. In the ileum of *in utero* VPA-exposed mice, increased ileal permeability and inflammation was not associated with PTEN expression or phosphorylation which was not changed. *In vitro* results demonstrated that *p*CS and 4EPS downregulated PTEN expression and attenuated the immune response of microglial cells. 4EPS had no effect on microglial phagocytosis activity. PETN inhibition with the VO-OHpic did not affect releases of TNF-α and IL-6 or the phagocytosis activity of microglial cells, which shows that PTEN does not play a role in the bacterial metabolites *p*CS or 4EPS-dysregulated immune and phagocytotic response of microglial cells. Given the vital role of PTEN in synaptic function, the *p*CS and 4EPS-induced decreased PTEN might dysregulate synaptic function in brain of *in utero* VPA-exposed male mice to induce ASD pathogenesis.

6.2 ADAM10 is a potential target for ASD intervention

We demonstrated that inhibiting ADAM10 with GI254023X reduced the maternal VPA-exposure-induced increased intestinal permeability *in utero* VPA-exposed mice. In addition, GI254023X attenuated the VPA-induced increased ADAM10 maturation in the brain, which was associated with a decreased cleavage of Neuroligin-1. Inhibiting ADAM10 did not affect the VPA-induced disturbed sociability and strikingly caused disturbed sociability in control mice. In contrast, GI254023X seems to improve cognitive function in *in utero* VPA-exposed mice. In addition, a negative correlation between sNeuroligin-1 fragment level in the prefrontal cortex and cognition scores. These data suggest that the improved cognitive function by inhibiting ADAM10 is associated with reduced Neuroligin-1 cleavage in the brain of autistic mice. Taken together, Inhibiting ADAM10 with the inhibitor GI254023X ameliorates the ASD pathogenesis and probably ASD-like behavior through regulating the gut-brain axis. In conclusion, our results suggest that ADAM10 might be a target for intervention to treat ASD-associated cognitive impairments. However, since the ADAM10 inhibitor induced impaired social behavior in control mice, caution is advised. Future studies should investigate targeting ADAM10 in other preclinical models for ASD before performing clinical trials.

A. The involvements of bacterial metabolites-associated targets ADAM17, ADAM10 and PTEN in ASD pathogenesis



B. The mechanisms of targeting ADAM10 for ASD interventions

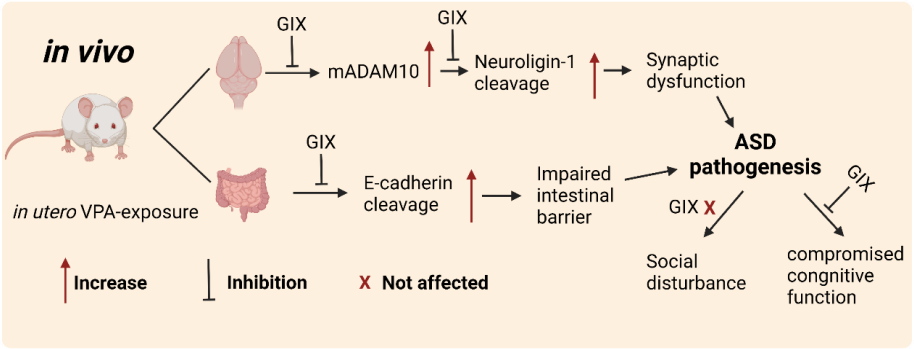


Figure 2. The relation between bacterial metabolites and ADAM17, ADAM10 and PTEN in ASD pathogenesis. (A) In the brain of autistic mice that shown increased bacterial metabolites *p*CS (possibly 4EPS), the expression of ADAM17 and PTEN10 is downregulated in contrast ADAM10 expression is enhanced. In microglial cells *p*CS and 4EPS attenuate the expression or maturation of ADAM17, ADAM10 and PTEN. In addition, *p*CS and 4EPS derail immune response of microglia via reducing ADAM17 or ADAM10 potentially contributing to ASD pathogenesis. The *p*CS or 4EPS-induced decreased PTEN is not associated with the dysregulated immune response of microglia, but the decreased PTEN might trigger ASD

pathogenesis by affecting synaptic function, which needs further investigation. (B) ADAM10 contributes in ASD pathogenesis through cleaving E-cadherin and Neuroligin-1 to dysregulate intestinal permeability and synaptic function in the brain, respectively. The ADAM10 inhibitor GIX ameliorates impaired intestinal barrier and synaptic function in autistic mice, and therefore ADAM10 inhibition might improve the compromised cognitive function. In contrast, social deficits in autistic mice were not affected by ADAM10 inhibition. GIX: ADAM10 inhibitor GI254023X; TAPI: ADAM17 inhibitor TAPI-1; VO-OHpic: PTEN inhibitor; LPS: lipopolysaccharide.

7. Future perspectives

This thesis shows the ASD-associated bacteria-derived metabolites *p*CS and 4EPS attenuated the expression or maturation of ADAM10 and ADAM17 and decreased PTEN expression. How these ASD-associated metabolites affect ADAM10, ADAM17 and PTEN expression remains to be elucidated in future studies. It might be possible that the bacterial metabolites affect transcription of genes encoding for ADAM10, ADAM17 and PTEN, which can be investigated by assessing mRNA expression. In addition, the maturation of ADAM10 and ADAM17 are regulated by TspanC8 subfamily of tetraspanin (Tspan5, 10, 14, 15, 17 and 33) and iRhoms subfamily (iRhom1 and iRhom2), respectively [9, 76]. Future research should investigate how *p*CS and 4EPS affect their upstream regulators to regulate the maturation of ADAM10 and ADAM17. ADAM17 and ADAM10 cleaves TNF-α, IL-6 receptor, TNF-α receptor, TREM2 and fractalkine that are essential to maintain a proper inflammatory response and phagocytotic capacity in microglia and astrocyte [3, 9, 37, 62, 77, 78]. It might be possible to further unravel the role of *p*CS and 4EPS in ASD pathogenesis through investigating the effects of their treatment on ADAM17 and ADAM17 in primary microglia and astrocyte. ADAM10 cleaves many synaptic molecules in neuron, such as APP, Neurexin-1 and Protocadherin 9, to maintain synaptogenesis, and neuronal network development and function [45]. In addition to ADAM10, PTEN is necessary for these processes by balancing excitatory (glutamatergic) and inhibitory (GABAergic) [79]. Therefore, investigating the effects of *p*CS and 4EPS treatment on ADAM10 and PTEN expression in neuronal cells and the consequences on network formation might also shed light on how these ASD-associated bacteria-derived metabolites damage synaptic function. 4EPS impairs oligodendrocyte differentiation and maturation in mice [6]. Oligodendrocytes highly expresses Death receptor 6 (DR6) that negatively regulates oligodendrocyte survival, maturation and myelination, which is associated with microglia activation, phagocytosis and neuroinflammation [80, 81]. In addition, the product of APP cleavage by ADAM10 binds to DR6 to trigger neuronal death [82]. Furthermore, DR6 has also been identified as a substrate of ADAM10 [83]. Investigating the effects of *p*CS and 4EPS treatment on DR6 expression in oligodendrocyte might unveil these metabolites-associated molecular mechanisms in ASD pathogenesis. In addition to playing a role in oligodendrocyte, DR6 regulates the myelination of the nervus vagus that plays an important role in the communication between the central nervous system and the enteric nervous system (ENS) [83-85]. Enteric neurons secrete neurotransmitters, such as serotonin and dopamine, that are associated with ASD pathogenesis [68, 86]. *p*-Cresol alters dopamine metabolism, enhances glutamine and decreases γ-aminobutyric acid in autistic children [87, 88]. It might be interesting to investigate the effects of 4-ethylphenol, 4EPS, p-cresol and pCS on dopamine

and serotonin production of ENS with a possible involvement of ADAM10-mediated DR6 cleavage.

Apart from the effects on (micro)glial cells and neurons, ADAM10 cleaves adhesions junction protein vascular endothelial cadherin (VE-cadherin) that controls permeability of blood brain barrier (BBB) [89]. Investigating the effects of *p*CS and 4EPS treatment on BBB and VE-cadherin might explain the mechanism why they are able to enter the brain and affect microglial and neuronal function. Like the cleavage of VE-cadherin in BBB, E-cadherin is also cleaved by ADAM10. E-cadherin regulates intestinal permeability in mice and epithelial permeability in colonic epithelial cells [46, 47], which opens the door to investigate how the bacterial metabolites affect epithelial permeability with a focus on E-cadherin in epithelial cells. In addition, ADAM17 regulates intestinal inflammation by cleaving TNF-α, IL-6 receptor and TNF-α receptor by which the bacterial metabolites might affect immunoregulatory function of epithelial cells in the intestinal tract, this also deserves to be investigated.

In order to further identify ADAM10 as a new target for ASD intervention, future studies need to confirm the preliminary results regarding the effect of ADAM10 inhibition in the VPA-induced and other rodent models for ASD, such as BTBR mice, SHANK knockout mice and the maternal immune activation murine model. Given that the decreased ADAM17 maturation and PTEN expression, it might be interesting to investigate what is the effect of ADAM17 and PTEN expression stimulating compounds in murine model for ASD. In addition, future development of targeting ADAM10 inhibitors to specific brain regions (such as PFC) can be promising to increase their bioavailability and reduce side-effects of the current inhibitor.

*p*CS and 4EPS are increased in ASD subjects and that preclinically it is demonstrated that *p*CS and 4EPS induce ASD-like symptoms [6, 7]. Future research should be focused on solutions to reduce the levels of *p*CS and 4EPS *in vivo* by using intestinal absorbent to eliminate the precursor bacterial metabolites like p-cresol and 4-ethylphenol. Additionally, microbiome-based therapies aim to engineer the intestinal ecosystem by means of probiotics or prebiotics, which would be promising to target the intestinal bacteria producing these bacterial metabolites. These interventions might decrease their possible detrimental effect on the targets ADAM10, ADAM17 and PTEN, and therefore improve ASD detrimental symptoms.

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Appendices

English laymen's summary

中文总结

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English laymen's summary

Autism spectrum disorder (ASD) is a neurodevelopmental disorder usually diagnosed at early age and characterized by impairments in social behavior and communication, the presence of repetitive behavior, and deficits in learning and memory. In recent years, abnormalities along the gut-brain axis have been suggested to be associated with ASD. Children diagnosed with ASD frequently suffer from intestinal problems associated with a different composition of gut bacteria. In the gut trillions of bacteria residing. Changes in the composition of the gut bacteria might lead to changes in bacterial metabolites production, that potentially can affect the host. Two of these bacterial metabolites, *p*-cresol sulfate (*p*CS) and 4-ethylphenyl sulfate (4EPS), are reported to be enhanced in feces, blood and urine of people diagnosed with ASD. Moreover, *p*CS and 4EPS exposure in mice induces ASD-like behavior, but the exact underlying mechanisms remain to be investigated.

Sheddases are cell membrane bound enzymes that cleave the extracellular part of proteins - like receptors, receptor ligands (for example inflammatory mediators) or (neural) adhesion molecules. Many sheddases are members of the so-called ADAM family and potentially play a role in the pathogenesis of disease. In addition, the phosphatase PTEN is an important enzyme that regulates neural cell growth and survival in the brain.

The enhanced levels of the sheddases, ADAM10 and ADAM17, and loss of function of the phosphatase PTEN are believed to play important roles in ASD via regulating intestinal permeability, immune responses and neuronal network function in the brain. Investigating the possible connection between the bacterial metabolites, *p*CS and 4EPS, and the ASD-associated targets ADAM10, ADAM17 and PTEN will shed light on potential mechanisms of the gut-brain axis in ASD.

This thesis is focused on gaining insights into the possible molecular mechanisms of bacterial metabolites in the pathogenesis of ASD to identify potential new targets for intervention and therapy. Using an ASD mouse model and a neuroinflammation cell model, this thesis illustrates that *p*CS and 4EPS affect the level and function of the sheddases, ADAM10 & ADAM17, and the phosphatase, PTEN, that might be involved in the ASD-associated changes in neuroimmune responses and/or neuronal network function in the brain. In addition, this thesis further identifies that ADAM10 might be a treatment-target for ASD either through direct pharmacological inhibition or via nutritional interventions targeting bacteria that are involved in *p*CS and 4EPS production aimed to reduce ASD-associated detrimental symptoms.

Association between bacterial metabolites and ADAM10 & 17 and PTEN expression in the brain

In this thesis we demonstrated increased levels of the bacterial metabolites, *p*CS and *p*CG, in blood of ASD mice confirming previous preclinical and human studies. Others have shown that *p*-cresol or 4EPS administration in mice induces social behavioural problems and repetitive behaviour in mice, suggesting that the elevated *p*CS might be involved in the ASD-like symptoms in our mouse model. Unfortunately, due to technical limitations we were not able to assess the levels of 4EPS in blood of ASD mice. Next, we measured the sheddases ADAM17 and ADAM10 as well as PTEN in brain tissues of ASD mice. We observed a decreased ADAM17 level, an increased ADAM10 level and downregulated PTEN expression in the brain of ASD mice. To further assess the role of the sheddases, ADAM10 & 17, and PTEN in derailed neuroimmune response in the brain of ASD, we used an *in vitro* cell model using

murine microglial cells. Microglial cells are a specialized population of immune cells, like macrophages, found in the brain. Microglial cells can release pro-inflammatory mediators and have the capacity of phagocytosis to remove damaged neurons and infections and are thus important for maintaining the health of the CNS.

The interaction between bacterial metabolites and ADAM10, ADAM17 and PTEN in ASD

The brain

Our results demonstrate that the sheddases, ADAM10 and ADAM17, play an important role in the regulation of immune response as well as the phagocytosis capacity of microglial cells. Inhibition of both sheddases compromised a proper immune response and enhanced phagocytosis of the microglial cells. In addition, our cell studies demonstrate that *p*CS is able to reduce ADAM10 and ADAM17 levels, which is associated with reduced release of inflammatory mediators as well as enhanced phagocytosis capacity of microglial cells. The other ASD-associated bacterial metabolite, 4EPS, showed similar effects on microglial cells, regarding ADAM10 and ADAM17 and the release of inflammatory mediators, however 4EPS did not affect the phagocytotic capacity.

The effects of the bacterial metabolite *p*CS on microglial cells mimic results found in the brain of ASD mice: the ADAM17 was found to be decreased which was associated with reduced inflammatory mediators. The pathological consequences of the decreased levels of ADAM17 and associated inflammatory mediators in the brain of ASD mice remain to be investigated.

In ASD, 'overpruning' - enhanced phagocytosis by microglial cells - is hypothesized to be responsible for the brain hyperconnectivity observed in ASD. Our cell studies show that ADAM17 inhibition potentiated the phagocytosis capacity of microglial cells. Taken all results together, the decreased ADAM17 in the brain of ASD mice might increase microglial phagocytosis resulting in overpruning, however, this remains to be investigated in future studies.

In contrast, ADAM10 was found to be increased in the brain of ASD mice not mirroring the results found in bacterial metabolite-exposed microglial cells. Microglial cells account about 7% of the cells found within the brain and the net increase of ADAM10 levels might be the result of increased levels in other cells, such as neurons.

In conclusion, ADAM17 and ADAM10 may play a role in ASD, which could be associated with central effects induced by the bacterial metabolites *p*CS and 4EPS.

Synaptic plasticity in the brain is the ability to strengthen or weaken the connection between neurons over time. PTEN deficient mice show abnormal synaptic plasticity in the brain and ASD-like behaviours. Indeed, our finding of the decreased PTEN level in the brain in our ASD mouse model might be involved in neuronal network dysfunction that is a causality of ASD. This thesis did not further demonstrate the effect of decreased PTEN on synapse function and brain connectivity, however, previous research indicates the involvement of the decreased PTEN in ASD pathogenesis with respect to neuronal networks. Our cell study showed that PTEN inhibition did not affect the release of inflammatory mediators as well as the phagocytosis, indicating that bacterial metabolite-induced reduction of PTEN does not play a role in the inflammatory response and phagocytosis of microglial cells. Given the vital role of PTEN in synaptic function, the *p*CS and 4EPS-induced decreased PTEN might dysregulate synaptic function in brain of ASD mice.

The gut

In addition to the brain, we also examined the expression of ADAMs and adhesion molecules in the gut. These are important regulators of intestinal integrity. In this thesis we demonstrate that in our mouse model for ASD decreased adhesion molecules in the intestinal tract was induced. This might result in enhanced intestinal permeability which is frequently found in people diagnosed with ASD. Our results indicate that ADAM10-mediated cleavage of adhesion molecules is enhanced in these tissues. However, we did not see a clear increase in ADAM10 in the gut of ASD mice. This might be due to the limitation in sample size, as well as technical limitations in measuring ADAM10 activity in intestinal tissues. Taken together, in ASD mice an increase in adhesion molecule cleavage mediated by enhanced ADAM10 activity might be involved in the impaired intestinal barrier in ASD.

In ASD mice intestinal inflammation was observed also as indicated by increased expression of the proinflammatory enzyme COX-2. In addition, we showed that PTEN levels did not change in the intestines of ASD mice, indicating that PTEN is not involved in the intestinal inflammation and the increased permeability in ASD.

ADAM10 is a potential target for ASD treatment

We demonstrate that inhibiting ADAM10 with the compound, GI254023X, reduced increased intestinal permeability ASD mice. In addition, GI254023X attenuated the increased ADAM10 in the brain of ASD mice, which was associated with a decreased cleavage of neuronal adhesion molecules. Inhibiting ADAM10 did not affect the disturbed social behaviour, but strikingly caused disturbed sociability in control mice. In contrast, GI254023X seems to improve cognition in ASD mice. In addition, a negative correlation existed between the level of cleaved neuronal adhesion molecules in the brain and cognition scores. These data suggest that the improved cognition by inhibiting ADAM10 is associated with reduced cleavage of neuronal adhesion molecules in the brain of autistic mice. Taken together, inhibiting ADAM10 with the inhibitor GI254023X ameliorates the ASD pathogenesis and probably ASD-like behaviour through or via regulating the gut-brain axis. In conclusion, our results suggest that ADAM10 might be a target for intervention to treat ASD-associated cognitive impairments. However, since the ADAM10 inhibitor induced impaired social behaviour in control mice, caution is advised. Future studies should investigate targeting ADAM10 in other preclinical models for ASD before performing clinical trials.

Future perspectives

This thesis shows the ASD-associated bacterial metabolites, *p*CS and to a lesser extend 4EPS, attenuated the levels of ADAM10 and ADAM17 and decreased PTEN expression in microglial cells. How these ASD-associated metabolites affect ADAM10, ADAM17 and PTEN expression remains to be elucidated in future studies. It might be possible that the bacterial metabolites affect transcription of genes encoding for ADAM10, ADAM17 and PTEN, which can be investigated by assessing mRNA expression. ADAM17 and ADAM10 cleave cell bound inflammatory mediators that are essential to maintain a proper inflammatory response and phagocytotic capacity in microglia and astrocyte. It might be possible to further unravel the role of *p*CS and 4EPS in ASD through investigating the effects of their treatment on ADAM17 and ADAM17 in primary microglia. ADAM10 cleaves many synaptic adhesion molecules to maintain neuronal network development and function. In addition to ADAM10, PTEN is necessary for these processes by balancing excitatory and inhibitory neurons. Therefore,

investigating the effects of *p*CS and 4EPS treatment on ADAM10 and PTEN expression in neuronal cells and the consequences on neuronal network formation and function might also shed light on how these ASD-associated bacterial metabolites damage the brain. In addition to the brain, it might be interesting to investigate the effects of ASD-associated bacterial metabolites on the function of the nervous system of the intestinal tract, that might be the gateway to the brain in ASD.

Apart from the effects on microglial cells and neurons, ADAM10 cleaves adhesion molecules that controls permeability of blood brain barrier (BBB). Investigating the effects of *p*CS and 4EPS and the potential role of ADAMs on BBB might explain the mechanism why they are able to enter the brain and affect microglial and neuronal function.

In order to further identify ADAM10 as a new target for ASD intervention, future studies need to confirm the preliminary results regarding the effect of ADAM10 inhibition in our and other rodent models for ASD. In addition, future development of targeting ADAM10 inhibitors to specific brain regions can be promising to increase their bioavailability and reduce side-effects of the current inhibitor. Given that a decreased ADAM17 and PTEN expression is found in our ASD mouse model, it might be interesting to investigate what is the effect of ADAM17 and PTEN expression stimulating compounds in preclinical models for ASD.

The bacterial metabolites, *p*CS and 4EPS, are increased in people diagnozed with ASD and that preclinically it is demonstrated that *p*CS and 4EPS induce ASD-like symptoms. Future research should be focused on solutions to reduce the levels of *p*CS and 4EPS by using oral absorbents to eliminate the precursor bacterial metabolites like *p*-cresol and 4-ethylphenol in the intestinal tract. Additionally, microbiome-based therapies that aim to engineer the intestinal ecosystem by means of probiotics or prebiotics, can be promising to target the intestinal bacteria producing these bacterial metabolites. These interventions might decrease their possible detrimental effect on the targets ADAM10, ADAM17 and PTEN, and therefore improve ASD detrimental symptoms.

中文总结

孤僻症是一种神经发育性疾病，通常在儿童时期确诊，主要特征是社交障碍，行为呆滞，学习记忆功能受损。近年来研究表明肠脑轴功能失调与孤僻症发病相关。孤僻症儿童通常伴有肠道功能障碍，这与肠道菌群的改变有关。在人体肠道内有数万亿菌群，它们的种类和含量发生改变时可引发其代谢产物发生变化，这可能反过来影响宿主。在这些改变的肠道菌群代谢产物中，对甲酚硫酸盐（*p*CS）和 4-乙基苯基硫酸盐（4EPS）最为备受关注。有研究表明 *p*CS 和 4EPS 在孤僻症患者的粪便，血液和尿液中浓度均升高，并且 *p*CS 和 4EPS 均可诱导小鼠孤僻症行为，但是其机制尚不明确。

脱落酶是跨膜蛋白酶，其可剪切跨膜蛋白样受体及其配体（如炎症介质）和神经黏附分子等。基质金属蛋白家族是脱落酶家族的成员，其可能参与疾病的发病进程。另外，人第 10 号染色体缺失的磷酸酶及张力蛋白同源的基因（PTEN）在调控脑内神经细胞的生长及功能中发挥重要作用。基质金属蛋白酶-10（ADAM10）和-17（ADAM17）活性改变及磷脂酶 PTEN 功能的缺失可通过调控肠道通透性，免疫反应和脑内神经网络的功能参与孤僻症的病理进程。因此，研究肠道菌群代谢产物 *p*CS 和 4EPS 与孤僻症相关的靶点 ADAM10/17 和 PTEN 之间的潜在关系将有助于阐明肠脑轴在孤僻症发病中的分子机制。

本论文聚焦于揭示肠道菌群代谢产物在孤僻症发病中的分子机制，并进一步为孤僻症的干预和治疗确立可能的分子靶点。本论文主要运用丙戊酸诱导的小鼠孤僻症模型和体外神经炎症模型阐明了 *p*CS 和 4EPS 可影响 ADAM10/17 和 PTEN 的功能及这些靶点相关的脑内神经免疫反应或神经网络功能参与孤僻症的发病。并且，本论文进一步阐明了 ADAM10 是孤僻症的潜在治疗或干预靶点，直接运用药理干预抑制 ADAM10 活性或运用营养学干预肠道菌群以减少 *p*CS 和 4EPS 的生成，这些都有望于减轻与孤僻症相关的不利症状。

_p_CS 和 4EPS 与 ADAM10/17 和 PTEN 在脑内的关系

本论文证明了 *p*CS 和 *p*CG 在孤僻症小鼠血液中升高，这与前期临床前和临床研究结果一致。研究表明 *p*CS 和 4EPS 处理可诱发小鼠社交障碍和刻板性行为，这表明在我们的小鼠模型中，血液中升高的 *p*CS 可能与孤僻症行为的出现有关。不幸的是由于技术限制，本论文未能测量孤僻症小鼠中 4EPS 的含量。然后，我们检测了孤僻症小鼠脑内脱落酶 ADAM10/17 和磷脂酶 PTEN 的水平，我们发现 ADAM17 水平降低，ADAM10 水平升高及 PTEN 表达下调。为了进一步评估 ADAM10/17 和 PTEN 在孤僻症小鼠脑内失调的神经免疫反应中的作用，我们运用了小鼠小胶质细胞。小胶质细胞于巨噬细胞类似，是脑内的免疫细胞。其能释放炎症介质，通过吞噬功能清除脑内受损的神经元和抵御病毒的侵害，因此，小胶质细胞对于维持健康的中枢神经系统非常重要。

在孤僻症中肠道菌群代谢产物 _p_CS 和 4EPS 与靶点 ADAM10/17 和 PTEN 的相互作用

脑内

我们的结果表明脱落酶 ADAM10/17 在小胶质细胞中的免疫反应及吞噬能力中起着重要作用。抑制这些脱落酶可使正常的免疫反应失调，小胶质细胞吞噬能力增加。另外，我们的细胞结果表明 *p*CS 能够减少 ADAM10/17 的水平，这可能与炎症介质减少和吞噬能力增加有关。另外一个孤僻症相关的细菌代谢产物 4EPS 在小胶质细胞中也展示出同样的效果。但是 4EPS 不影响小胶质细胞的吞噬能力。

*p*CS 对小胶质细胞的影响映证了孤僻症小鼠中的结果：小鼠脑内 ADAM17 活性降低，炎症介

质释放减少。在孤僻症小鼠脑内，由这些改变而引发的病理影响值得深入研究。在孤僻症中，由升高的小胶质细胞的吞噬能力而导致的'过度修剪'被认为与孤僻症中脑内的高度连接性有关。我们的细胞实验结果表明抑制 ADAM17 增强小胶质细胞的吞噬能力。综合考虑，孤僻症小鼠中减少的 ADAM17 可能增强小胶质细胞吞噬能力并导致'过度修建'。然而，机制仍不清楚，需要后续实验阐明。相反，ADAM10 水平在孤僻症小鼠脑内升高，这与小胶质细胞中的 *p*CS 和 4EPS 对 ADAM10 的实验结果不一致。小胶质细胞占脑内细胞的 7%，这表明小鼠脑内 ADAM10 水平升高可能是由于脑内其他细胞的 ADAM10 水平升高所致，比如神经元。总之，ADAM17/10 可能在孤僻症中起着重要作用，这与 *p*CS 和 4EPS 对 ADAM17/10 的作用有关。

突触可塑性是脑内神经元间连接增强或减弱的能力。PTEN 缺失小鼠表现出不正常的突触可塑性和孤僻症样行为。我们发现在我们的孤僻症小鼠中脑内 PTEN 水平减少，这可能改变神经元网络功能进而导致孤僻症。本论文没有进一步证明减少的 PTEN 水平对突触功能和大脑连接性的影响，但是前期研究已经表明减少的 PTEN 水平可通过影响神经元网络参与孤僻症的病理进程。我们的体外细胞实验结果证明抑制 PTEN 不能影响小胶质细胞炎症介质的释放及其吞噬能力，这表明由 *p*CS 和 4EPS 诱发的 PTEN 减少没有在小胶质细胞的炎症反应和吞噬能力中发挥重要作用。鉴于 PTEN 在突触功能中的重要作用，由 *p*CS 和 4EPS 诱发的 PTEN 减少可能在孤僻症小鼠脑内使突触功能失调。

肠内

除了脑内，我们还检测了肠内 ADAMs 和黏附分子的表达，这些是肠道通透性的重要调节因子。在本论文中，我们证明在我们的孤僻症小鼠模型中黏附分子表达减少，这可能导致肠道通透性增加，肠道通透性增加经常在孤僻症患者中发生。我们的结果表明 ADAM10 介导的黏附分子的剪切在这些肠道组织中升高。然而，我们并没有看到 ADAM10 水平明显增加，这可能是由于样本量少，或者由于技术限制没能检测肠道组织中 ADAM10 的活性。综上所述，孤僻症小鼠中由增加的 ADAM10 活性介导的黏附分子剪切的增加可能与孤僻症中受损的肠道通透性有关。在我们的孤僻症小鼠中，炎症蛋白环氧合酶-2 表达增加，表明小鼠肠道内存在炎症。另外，我们发现 PTEN 水平在孤僻症小鼠中没有发生明显改变，这表明 PTEN 与肠道炎症和增加的肠道通透性没有关系。

ADAM10 是干预治疗孤僻症的一个潜在靶点

我们证明用化合物 GI254023X（ADAM10 选择性抑制剂）抑制 ADAM10 改善了孤僻症小鼠中升高的肠道通透性。另外，GI254023X 减少孤僻症小鼠中脑内增加的 ADAM10 水平及其相关的神经黏附分子的剪切。抑制 ADAM10 并没有改善孤僻症小鼠的社交障碍，反而其在正常小鼠诱发了社交障碍，但是抑制 ADAM10 可能改善孤僻症小鼠的认知功能。另外，我们发现脑内神经黏附分子剪切与小鼠认知功能得分存在负相关性。这些数据表明由抑制 ADAM10 减少的神经黏附分子的剪切可能改善孤僻症小鼠的认知功能。综合考量，用 GI254023X 抑制 ADAM10 可通过调控肠脑轴减轻孤僻症小鼠的病理进程和孤僻症样行为。因此，我们的结果表明 ADAM10 可能是一个干预治疗孤僻症认知功能障碍的靶点，然而，鉴于 ADAM10 抑制剂诱发了正常小鼠的社交障碍，后续研究应该谨慎考量。将来的实验应该在实施临床研究前运用其他临床前孤僻症动物模型深入研究靶向 ADAM10 对孤僻症样行为的影响。

未来展望

本论文表明孤僻症相关的肠道菌群代谢产物 *p*CS 和 4EPS 减少了小胶质细胞 ADAM10/17 和 PTEN 的表达水平。关于 *p*CS 和 4EPS 如何影响这些靶点的表达水平仍需要后续研究阐明。

*p*CS 和 4EPS 可能在基因编码水平上影响这些靶点，这需要通过测量 mRNA 水平来证明。在胶质细胞中 ADAM10/17 可剪切跨膜炎症介质来维持其适当的免疫炎症反应和吞噬能力。后续研究可探讨这两个菌群代谢产物对原代小胶质细胞 ADAM10/17 的影响来阐明这两个代谢产物在孤僻症中的作用。ADAM10 可通过剪切很多突触黏附分子来维持神经元网络的发育和功能。另外，ADAM10 和 PTEN 对于维持兴奋性神经元和抑制性神经元的平衡是必需的。因此，探讨 *p*CS 和 4EPS 对神经元中 ADAM10 和 PTEN 及与其相关的神经网络的形成和功能的影响可能将有助于解释 *p*CS 和 4EPS 如何损害大脑。在孤僻症中，肠道神经系统是消化道通往大脑的门道，研究 *p*CS 和 4EPS 对肠道神经系统的影响或将有助于阐明 *p*CS 和 4EPS 诱发孤僻症样行为的机制。另外，ADAM10 可剪切黏附分子以调控肠道通透性和血脑屏障通透性，研究肠道菌群代谢产物和 ADAM10 对血脑屏障通透性的影响可能将有助于阐明为什么这些代谢产物能够进入大脑并影响小胶质细胞和神经元的功能。

为了进一步确立 ADAM10 可作为干预孤僻症的一个新靶点，后续研究需要在更多孤僻症动物模型中验证我们这些前期结果。另外，将 ADAM10 抑制剂靶向输送至指定脑区有望增加抑制剂的生物利用度和减少 GI254023X 抑制剂的副作用。鉴于 ADAM17 和 PTEN 水平在我们的孤僻症小鼠模型中减少，研究 ADAM17/PETN 激活剂对孤僻症动物模型中所产生的影响也显得有趣。

*p*CS 和 4EPS 水平在孤僻症患者中升高，临床前研究证明 *p*CS 和 4EPS 能够诱发孤僻症样行为，因此，未来研究应聚焦于研发利用口服吸收剂减少 *p*CS 和 4EPS 在肠道内产生的方案。此外，旨在通过益生菌或益生元改造肠道生态系统的基于微生物组的疗法有望靶向产生这些细菌代谢物的肠道细菌。这些干预措施有望降低它们对 ADAM10、ADAM17 和 PTEN 可能产生的不利影响，从而改善 ASD 的不利症状。

Nederlandse samenvatting

Autisme spectrum stoornissen (autisme) zijn een groep van neurologische ontwikkelingsstoornissen die meestal op jonge leeftijd wordt gediagnosticeerd en wordt gekenmerkt door stoornissen in sociaal gedrag en communicatie, de aanwezigheid van repetitief gedrag en problemen met leren en geheugen. In de afgelopen jaren is gesuggereerd dat afwijkingen van de darm-brein as mogelijk verband houden met autisme. Kinderen met de diagnose autisme hebben vaker last van darmproblemen en een andere samenstelling van darmbacteriën. In de darm leven biljoenen bacteriën. Veranderingen in de samenstelling van de darmbacteriën kunnen leiden tot veranderingen in de productie van bacteriële metabolieten, die mogelijk de gastheer kunnen beïnvloeden. Van twee van deze bacteriële metabolieten, *p*-cresolsulfaat (*p*CS) en 4-ethylfenylsulfaat (4EPS), is aangetoond dat ze verhoogd zijn in ontlasting, bloed en urine bij mensen met de diagnose autisme. Bovendien resulteert blootstelling aan *p*CS en 4EPS bij muizen in autisme-achtig gedrag, echter de exacte onderliggende mechanismen moeten nog worden onderzocht.

Zo genaamde sheddasen zijn aan celmembraan gebonden enzymen die het extracellulaire deel van eiwitten van de cel afsplitsen-zoals receptoren, receptorliganden (bijvoorbeeld ontstekingsmediatoren) of (neurale) adhesiemoleculen. Veel sheddasen zijn leden van de zogenaamde ADAM-familie en spelen mogelijk een rol bij een aantal ziekten. Bovendien is de fosfatase PTEN een belangrijk enzym dat de groei en overleving van cellen in de hersenen reguleert.

Aangenomen wordt dat de verhoogde niveaus van de sheddasen, ADAM10 en ADAM17, en het functieverlies van de fosfatase, PTEN, een belangrijke rol spelen bij autisme via het reguleren van de doorlaatbaarheid van de darm, afweerreacties en neuronale netwerkfuncties in het brein. Onderzoek naar het mogelijke verband tussen de bacteriële metabolieten, *p*CS en 4EPS, en ADAM10, ADAM17 en PTEN zal inzicht geven in mogelijke mechanismen van de darm-brein as bij autisme.

Dit proefschrift is gericht op het verkrijgen van inzicht in de mogelijke moleculaire mechanismen van bacteriële metabolieten in autisme om nieuwe aangrijpingspunten voor behandeling van nadelige symptomen te identificeren. Met behulp van een autisme-muismodel en een cel-model voor ontstekingsreacties in het brein laat dit proefschrift zien dat *p*CS en 4EPS de hoeveelheid en de functie beïnvloeden van de sheddasen, ADAM10 & ADAM17, en de fosfatase, PTEN. Bovendien tonen studies in dit proefschrift aan dat ADAM10 mogelijk een aangrijpingspunt voor behandeling van nadelige symptomen bij autisme zou kunnen zijn, hetzij door directe remming door een specifiek geneesmiddel, hetzij via voeding gericht op bacteriën die betrokken zijn bij de productie van *p*CS en 4EPS, met als doel de nadelige symptomen van autisme te verminderen.

Het verband tussen bacteriële metabolieten en ADAM10- & 17- en PTEN-activiteit in het brein

In dit proefschrift hebben we verhoogde niveaus van de bacteriële metabolieten, *p*CS en *p*CG, in het bloed van autisme-muizen aangetoond. Deze resultaten bevestigen eerdere proefdier studies maar ook enkele studies in de mens. Andere onderzoekers hebben aangetoond dat toediening van p-cresol of 4EPS bij muizen sociale gedragsproblemen en repetitief gedrag bij muizen veroorzaakt, wat

suggereert dat de verhoogde *p*CS mogelijk betrokken is bij de autisme-achtige symptomen in ons muismodel. Helaas konden we vanwege technische beperkingen de niveaus van 4EPS in het bloed van autisme-muizen niet meten. Vervolgens maten we de sheddasen ADAM17 en ADAM10, evenals PTEN in het brein van ASS-muizen. We vonden een verlaagd ADAM17, een verhoogd ADAM10 en verlaagd PTEN in het brein van autisme-muizen. Om de rol van de sheddasen, ADAM10 & 17, en PTEN in het brein van autisme verder te onderzoeken, gebruikten we een cellulair-model met microglia cellen. Microglia cellen zijn een gespecialiseerde populatie van afweercellen, die in het brein worden aangetroffen. Microglia cellen kunnen ontstekingsstofjes afgeven en hebben het vermogen tot fagocytose (zogenaamd opeten) om beschadigde neuronen en infecties te verwijderen en zijn om die redenen dus belangrijk voor het behoud van de gezondheid van het brein.

De interactie tussen bacteriële metabolieten en ADAM10, ADAM17 en PTEN bij autisme

Het brein

Onze resultaten tonen aan dat de sheddasen, ADAM10 en ADAM17, een belangrijke rol spelen bij de regulatie van de afweerreactie en de fagocytose van microglia cellen. Remming van beide sheddasen verstoorde een goede afweerreactie en versterkte fagocytose van de microglia cellen. Bovendien tonen deze studies aan dat *p*CS in staat is ADAM10 en ADAM17 te verlagen, wat gepaard gaat met een verminderde afgifte van ontstekingsstofjes en een verbeterde fagocytose van de microglia cellen. De andere autisme gerelateerde-bacteriële metaboliet, 4EPS, vertoonde vergelijkbare effecten op microglia cellen vergeleken met ADAM10 en ADAM17 met betrekking tot de ontstekingsstofjes echter 4EPS had geen invloed op de fagocytose.

De effecten van de bacteriële metaboliet pCS op microglia cellen bootsen resultaten na die in het brein van ASS-muizen zijn gevonden: de ADAM17 bleek te zijn verlaagd, wat geassocieerd was met verminderde ontstekingsstofjes. Echter de gevolgen van de verlaagd ADAM17 en ontstekingsstofjes in het brein van autisme-muizen moeten nog worden onderzocht en gevalideerd.

Bij autisme wordt verondersteld dat zogenaamde 'oversnoeien' - versterkte fagocytose door microglia cellen - verantwoordelijk is voor de verstoorde zenuwnetwerken die wordt waargenomen bij autisme. Onze cel-studies tonen aan dat remming ADAM17 van de fagocytose van microglia cellen versterkte. Alle resultaten bij elkaar genomen, zou de verminderde ADAM17 in het brein van autisme-muizen de fagocytose door microglia kunnen verhogen, wat resulteert in ‘oversnoei’, maar dit moet nog worden onderzocht in toekomstige studies.

Daarentegen bleek ADAM10 verhoogd te zijn in het brein van autisme-muizen, wat niet overeenkwam met de resultaten die werden gevonden in microglia cellen blootgesteld aan de bacteriële metabolieten. Microglia cellen zijn goed voor ongeveer 7% van de cellen die in het brein worden aangetroffen en de netto toename van ADAM10 kan het gevolg zijn van verhoogde niveaus in andere cellen, zoals zenuwen.

Samenvattend, ADAM17 en ADAM10 kunnen een rol spelen bij autisme, wat geassocieerd zou kunnen zijn met effecten in het brein veroorzaakt door de bacteriële metabolieten *p*CS en 4EPS.

Synaptische plasticiteit in het brein is het vermogen om de verbinding tussen zenuwen in de loop van de tijd te versterken of te verzwakken. Muizen die geen PTEN hebben, vertonen abnormale synaptische plasticiteit in het brein en autisme-achtig gedrag. Onze bevinding van verlaagd PTEN in het brein in ons autisme muismodel kan inderdaad betrokken zijn bij verstoorde zenuwnetwerken bij autisme. Dit proefschrift heeft het effect van verminderde PTEN op synapsfunctie en zenuwnetwerken niet verder onderzocht, maar eerder onderzoek wijst op de betrokkenheid van de verminderde PTEN bij autisme met betrekking tot zenuwnetwerken. Onze cel-studie toonde aan dat remmen van PTEN geen invloed had op de afgifte van ontstekingsstofjes en ook niet op de fagocytose, wat aangeeft dat door bacteriële metabolieten geïnduceerde vermindering van PTEN geen rol speelt bij de ontstekingsreactie en fagocytose van microglia cellen. Gezien de belangrijke rol van PTEN in de zenuw-zenuw contact, zou de *p*CS en 4EPS-geïnduceerde verminderde PTEN de functie van zenuwnetwerken in het brein van autisme-muizen kunnen ontregelen.

De darm

Naast het brein onderzochten we ook ADAM's en adhesiemoleculen in de darm. Zogenaamde adhesiemoleculen, moleculen die van belang zijn voor de verbinding tussen o.a. epiteelcellen, zijn belangrijk voor de regulatie van darmdoorlaatbaarheid. In dit proefschrift laten we zien dat in ons muismodel voor autisme minder adhesiemoleculen in het darmkanaal werden gemeten. Dit kan resulteren in een verhoogde darmdoorlaatbaarheid, wat vaak wordt aangetroffen bij mensen met de diagnose autisme. Onze resultaten suggereren dat ADAM10 mogelijk betrokken zou kunnen zijn bij afsplitsing van deze adhesiemoleculen in de darm. We zagen echter geen duidelijke toename van ADAM10 in de darm van autisme-muizen. Dit kan te wijten zijn aan de beperking van de steekproefomvang, evenals aan technische beperkingen bij het meten van ADAM10 in darmweefsel van de muis. Alles bij elkaar genomen, kan bij autisme-muizen een toename van de afsplitsing van adhesiemoleculen, mogelijke gemedieerd door ADAM10, betrokken zijn bij de zogenaamde lekkende darm bij autisme.

Bij autisme-muizen werd ook darmontsteking waargenomen, wat blijkt uit een verhoging van het ontstekingsenzym genaamd COX-2. Bovendien toonden we aan dat PTEN niet veranderd is in de darmen van autisme-muizen, wat aangeeft dat PTEN niet betrokken is bij de darmontsteking en de verhoogde doorlaatbaarheid bij autisme.

ADAM10 is een potentieel doelwit voor de behandeling van nadelige symptomen bij autisme

We tonen aan dat het remmen van ADAM10 met de stof, GI254023X, de verhoogde darmdoorlaatbaarheid van autisme-muizen verminderde. Bovendien verlaagde GI254023X de verhoogde ADAM10 in het brein van ASS-muizen, wat geassocieerd was met een verminderde afsplitsing van adhesiemoleculen tussen zenuwen. Het remmen van ADAM10 had geen invloed op het verstoorde sociale gedrag, maar veroorzaakte opvallend genoeg een verstoord gedrag bij de controle muizen. Daarentegen lijkt GI254023X het geheugen van autisme-muizen te verbeteren. Bovendien bestond er een negatief verband tussen het niveau van afgesplitste adhesiemoleculen in het brein en

geheugen-scores. Deze gegevens suggereren dat het verbeterd geheugen door remming van ADAM10 wordt geassocieerd met verminderde splitsing van adhesiemoleculen in het brein van autistische muizen. Alles bij elkaar genomen, verbetert het remmen van ADAM10 met de remmer GI254023X de darmproblemen en waarschijnlijk autisme-achtig gedrag door het reguleren van de darm-brein as. Onze resultaten suggereren dat ADAM10 een aangrijpingspunt zou kunnen zijn voor de behandeling van bijv. geheugenstoornissen bij autisme. Omdat de ADAM10-remmer echter een verminderd sociaal gedrag veroorzaakte bij controle muizen, is voorzichtigheid geboden. Toekomstige studies zouden zich kunnen richten op de rol van ADAM10 in andere muizen modellen voor autisme alvorens onderzoek in de mens uit te voeren.

Toekomstperspectieven

Dit proefschrift laat zien dat de autisme-geassocieerde bacteriële metabolieten, pCS en in mindere mate 4EPS, ADAM10, ADAM17 en PTEN verminderden in microglia cellen. Hoe deze autisme-geassocieerde metabolieten de ADAM10-, ADAM17- en PTEN-expressie beïnvloeden, moet nog worden opgehelderd in toekomstige studies. Het is mogelijk dat de bacteriële metabolieten het aflezen beïnvloeden van genen die coderen voor ADAM10, ADAM17 en PTEN, wat kan worden onderzocht door mRNA-expressie in microglia te meten. ADAM17 en ADAM10 splitsen cel-gebonden ontstekingsstofjes af die essentieel zijn voor het behoud van een goede afweerreactie en fagocytose van microglia. Het zou interessant zijn om de rol van pCS en 4EPS bij autisme verder te ontrafelen door de effecten van hun behandeling op ADAM17 en ADAM17 in primaire microglia van de mens te onderzoeken. ADAM10 splitst veel adhesiemoleculen in het brein om de ontwikkeling en functie van het zenuwnetwerk in goede banen te leiden. Naast ADAM10 is PTEN nodig voor deze processen door prikkelende en remmende zenuwen in evenwicht te brengen. Daarom zou het onderzoeken van de effecten van *p*CS en 4EPS op ADAM10- en PTEN in zenuwcellen en de gevolgen voor de vorming en functie van zenuwnetwerken ook licht kunnen werpen op hoe deze autisme-geassocieerde bacteriële metabolieten het brein beschadigen. Naast het brein kan het interessant zijn om de effecten van autisme-geassocieerde bacteriële metabolieten op de functie van het zenuwstelsel van het darmkanaal te onderzoeken. Dit zenuwstelsel zou de poort naar de hersenen kunnen zijn bij autisme.

Afgezien van de effecten op microglia cellen en zenuwen, splitst ADAM10 adhesiemoleculen die de doorlaatbaarheid van de bloed-hersenbarrière (BBB) regelen. Onderzoek naar de effecten van *p*CS en 4EPS en de mogelijke rol van ADAM's op BBB zou het mechanisme kunnen verklaren waarom ze de hersenen kunnen binnendringen en de functie van microglia en zenuwen kunnen beïnvloeden.

Om ADAM10 verder te onderzoeken als potentieel nieuw aangrijpingspunt voor de behandeling van de nadelige symptomen van autisme, moeten toekomstige studies de voorlopige resultaten met betrekking tot het effect van het remmen van ADAM10 in onze en andere proefdiermodellen voor autisme bevestigen. Bovendien kan de toekomstige ontwikkeling van ADAM10-remmers die op specifieke gebieden in het brein werken veelbelovend zijn om hun biologische beschikbaarheid te vergroten en bijwerkingen van de huidige remmer te verminderen. Gezien het feit dat er verminder ADAM17- en PTEN wordt gevonden in ons autisme-muismodel, kan het interessant zijn om te onderzoeken wat het effect is van ADAM17- en PTEN-stimulerende verbindingen in

proefdiermodellen voor autisme.

De bacteriële metabolieten, *p*CS en 4EPS, zijn verhoogd bij mensen met de diagnose autisme en in proefdieren is aangetoond dat *p*CS en 4EPS autisme-achtige symptomen veroorzaken. Toekomstig onderzoek moet gericht zijn op het verlagen van *p*CS en 4EPS door bijvoorbeeld orale absorptiemiddelen te gebruiken om de voorlopers van bacteriële metabolieten zoals p-cresol en 4-ethylfenol in het darmkanaal te elimineren. Bovendien kunnen op het microbioom gebaseerde therapieën zoals probiotica of prebiotica, veelbelovend zijn door het mogelijk verminderen van darmbacteriën die deze bacteriële metabolieten produceren. Deze behandeling zouden hierdoor de mogelijke nadelige effecten van *p*CS en 4EPS op de ADAM10, ADAM17 en PTEN kunnen verminderen en daardoor de nadelige symptomen van autisme kunnen verbeteren.

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Yuanpeng Zheng
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Curriculum vitae

Yuanpeng Zheng was born on May 27th, 1992, in Haifeng, Guangdong province, China. After graduation from the Renrong middle school at Haifeng in 2010, he started his bachelor program of (clinical) pharmacy in the School of Pharmaceutical Sciences at Guangdong Medical University in China. In July 2014, he obtained his bachelor's degree and continued his master program in Institute of Materia Medica, Chinese Academy of Medical Sciences & Peaking Union medical college & Tsinghua University in China. During his master period, he majored at pharmacology, and mainly worked on screening chemical compounds using *in vitro* and *in vivo* models to identify potential treatments for Parkinson's disease or Alzheimer's disease. Meantime, he contributed to unravelling the molecular targets of squamosamide derivative FLZ, a promising drug in clinic trials using for Parkinson's disease treatment. After he obtained his master's degree in 2017, he started his PhD project in Neuroproteomics, School of Medicine, Klinikum rechts der Isar, Technical University Munich, German Center for Neurodegenerative Diseases (DZNE), Munich, Germany. In Feb. 2019, he transferred his PhD position to the Department of Pharmacology of the Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands, under the supervision of Prof. dr. A.D. Kraneveld, Prof. dr. J. Garssen and Dr. P. Perez Pardo. His PhD project was focused on obtaining insights into the molecular mechanisms of bacterial metabolites in the pathogenesis of autism spectrum disorder (ASD). He found out several dysregulated targets by bacterial metabolites are associated with ASD pathogenesis, and identified one of them as a potential target for ASD intervention of detrimental symptoms. The results of this work have been described in this thesis.

List of publications

1. **Zheng, Y.**; Verhoeff, T. A.; Perez Pardo, P.; Garssen, J.; Kraneveld, A. D., The Gut-Brain Axis in Autism Spectrum Disorder: A Focus on the Metalloproteases ADAM10 and ADAM17. International journal of molecular sciences 2020, 22, (1).

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3. **Zheng, Y.**; Prince, N. Z.; Peralta Marzal, L. N.; Ahmed, S.; Garssen, J.; Perez Pardo, P.; Kraneveld, A. D., The Autism Spectrum Disorder-Associated Bacterial Metabolite p-Cresol Derails the Neuroimmune Response of Microglial Cells Partially via Reduction of ADAM17 and ADAM10. International Journal of Molecular Sciences, 2022. 23(19): p. 11013.

4. **Zheng, Y.**; Prince, N.Z.; van Hattem, C.; Garssen, J.; Perez Pardo, P.; Kraneveld, A. D., The interaction between intestinal bacterial metabolites and phosphatase and tensin homolog in autism spectrum disorder. Mol Cell Neurosci, 2022 Dec 30;103805.

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6. YAO XINSHENG (CN), YU YANG (CN), ZHANG DAN (CN), NI YANG (CN), BAO XIUQI (CN), LI LIN (CN), ZANG CAIXIA (CN), GAO HAO (CN), **ZHENG YUANPENG** (CN). Crocins Compounds and Uses Thereof. ZL201610284974.6, CN-105906672-A, CN-105906672-B, EP-3450444-A1, EP-3450444-A4, AU-2017255363-A1, AU-2017255363-B2, JP-2019514916-A, JP-6740372-B2, US-2019177354-A1,WO-2017185900-A1.

7. YAO XINSHENG, ZHANG DAN, YU YANG, BAO XIUQI, NI YANG, ZANG CAIXIA, GAO HAO, **ZHENG YUANPENG**. Composition, crocins active site, and uses thereof. ZL201610284549.7, CN-105935363-A, CN-105935363-B, JP-2019514915-A, JP-6782790-B2, EP-3449924-A1, EP-3449924-A4, EP-3449924-B1,AU-2017255362-A1,AU-2017255362-B2, US-10851129-B2, US-2019112326-A1,WO-2017185899-A1.

8. ZHANG DAN, WANG YUCHENG, LI ZIQIANG, BAO XIUQI, BAI XIAOGUANG, ZHANG GUONING, WANG JUXIAN, ZHOU LEI, LI QIANG, **ZHENG YUANPENG**,ZANG CAIXIA. Imidazolone-morphinan as well as preparation method and application thereof. ZL201610194651.8, CN-105712994-A, CN-105712994-B.

9. LI LI, WANG LIN, ZHANG DAN, BAO XIUQI, GAO QI, ZANG CAIXIA, **ZHENG YUANPENG**. Cumarin Mannich base and its purposes in neurodegenerative disease is prevented. CN-108203423-A.