Neuroimmunomodulation of autistic-like behavior: targeting mTOR via dietary interventions

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Neuroimmunomodulation of autistic-like behavior: targeting mTOR via dietary interventions

Neuroimmunomodulatie van autistisch-achtig gedrag: mTOR als een target via dieet interventies

(met een samenvatting in het Nederlands)

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Contents

CHAPTER ONE

General Introduction

Autism spectrum disorder (ASD) is a spectrum of neurological disorders which are characterized by, among others, impaired social behavior, communication, and/or stereotyped behaviors[1]. In recent years, the prevalence of ASDs seems to increase[1]. This can be attributed to a change in diagnostic criteria, an increased awareness, and diagnosis at a younger age[2,3]. The prevalence rate of ASD estimated from different studies in different countries ranges from less than 0,1% to 2.65%[3]. A meta-analysis by Fombonne (2009) indicates that the best estimate of ASD is between 0.6 and 0.7%. The prevalence of ASDs differs between males and females, the ratio is estimated to be 4.2:1[2].

In addition to behavioral deficits, autistic individuals often present with comorbidities like sleep disorders and gastrointestinal disorders[4]. Recent studies have pointed out that immune disturbance may importantly contribute to ASDs[5]. Multiple studies have indicated a link between immune disturbances and ASDs[5–7]. However, there is no definitive answer yet as to whether and how exactly immune disturbances can lead to ASD. The research into the relation between immune disturbances and ASD is still very controversial and in its early phases[5]. Many different branches of the immune system seem to be involved. Studies found, among others, deregulated activation of microglia and astroglia (supporting brain cells)[8,9], abnormal levels of pro-inflammatory cytokines[9], autoimmunity[10], increased T cell activation[11], deregulated innate immune functioning[5] and mutations in genes controlling the functioning of cells that are involved in the immune response[5,12–14]

One of the immune disturbances often reported in ASD is allergy, especially food allergy. A food allergy is an unfavorable immune reaction that can occur repeatedly on exposure to specific food components, in particular to proteins[15]. Food allergies are a prevailing type of allergy in the general population. It has been difficult to estimate the exact amount of people suffering from food allergies, but it has been estimated to be between 2 and 10% of the general population[16].

Food allergies can be either IgE-mediated or non-IgE-mediated. The exact mechanism behind non-IgE mediated food allergy is poorly understood and symptoms are mostly long-lasting and of gastrointestinal nature[17]. The mechanism of the type 1, IgE mediated allergic (hypersensitivity) reaction, on the other hand, are very well understood (Fig. 1). In the first phase of type 1 hypersensitivity reactions sensitization to the food allergen takes place (sensitization phase). During this phase allergens cross the mucosal barrier and are presented via MHC-II to naïve T-cells that develop into T helper cell type 2 (Th2). Th2 cells induce the production of IgE antibodies by B-cells. IgE binds to the FcεRI receptor on mast cells. In the challenge phase, upon a second exposure to the same allergen cross-

linking of cell-bound IgE occurs, leading to mast cell degranulation, which releases several mediators including histamine and tryptases. These mediators lead to most of the visible effects of an allergic reaction[17], which often include a combination of several symptoms. The most common symptoms are skin reactions (the appearance of hives or swelling of the skin), respiratory symptoms like rhinitis, ocular symptoms (lacrimation and red or itchy eyes), and gastrointestinal symptoms like nausea, vomiting, cramps and diarrhea[17]. It has been shown from parental reports that food allergies are observed more often in autistic individuals than in the general population[18,19]. The number of autistic children with food allergies may be even higher since impaired communication in these individuals could lead to underdiagnoses[18]. In addition, Lucarelli et al. found an increase in hallmark behaviors for ASDs after a challenge with cow's milk. Moreover, Lucarelli et al. showed that autistic children had higher antibody counts against several proteins found in cow's milk than the general population[20].

Another indication that allergies are common in people with ASD comes from the observation that levels of Th2 cells in individuals with ASDs are elevated[11,21,22]. Furthermore, higher levels of cytokines secreted by Th2 cells, IL-4, -5 and -13 have been observed in peripheral blood mononuclear cells of children with ASD as compared to controls[11]. Cells expressing the anti-inflammatory cytokine IL-10, on the other hand were found in lower amounts[23]. Cells secreting this cytokine are mostly regulatory T (Treg) cells, which are important for suppression of allergic responses and for suppression of the immune system in general[23]. In addition, levels of transforming growth factor-ß (TGF-ß), another cytokine expressed by Treg cells, were significantly decreased in children with ASD as compared to controls of the same age[24,25]. A recent report by Hashim, H and his coworkers also found this decrease in plasma levels of TGF-ß in autistic patients. The lower level of Tregs in ASD was correlated to the severity of autism, suggesting that there may be a potential causal relationship[26]. TGF-ß was found to play an important role in brain development, in particular in regulating the survival and the differentiation of neurons, and in regulating the growth of synapses, as observed in invertebrate models[27,28]. Therefore, a lack of TGF-ß could contribute to a disturbed brain functioning and be somehow involved in the development of the autistic phenotypes in autistic patients[25].

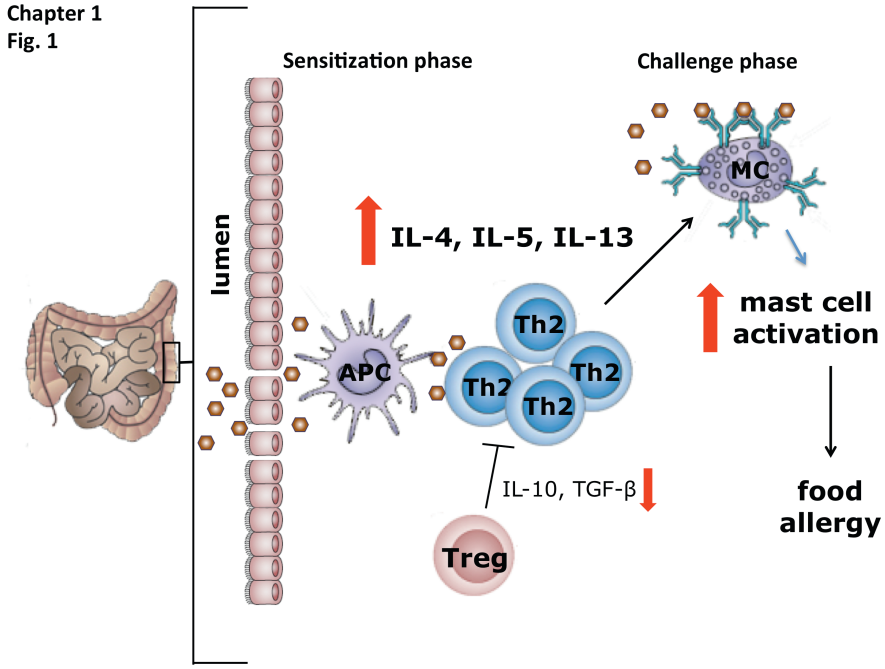

Fig. 1. Schematic overview of IgE-mediated food allergic reactions. During sensitization phase, allergens cross the mucosal barrier and are presented by antigen presenting cells (APC) to naïve T cells that develop into Th2 cells at the expense of regulatory T cells (Treg). Cytokines such as IL-4, IL-5, IL-13 produced by Th2 cells induced the proliferation and maturation of antigen-specific IgE producing B cells/plasma cells. IgE binds to the FcεRI receptor on mast cells (MC). During challenge phase, upon a second exposure to the same allergen IgE antibodies bound on mast cells are cross-linked, leading to mast cell degranulation and eventually food allergic symptoms.

Mast cell activation has also been implicated in the development of the autistic phenotypes[7,29]. Mast cells can directly interact with neurons both in the brain[30] and in the gut[31,32], which can signal to the central nervous system, and could in this way influence behavior. A study by Theoharides et al. investigated mastocytosis in children with ASD. Mastocytosis encompasses several disorders which are characterized by proliferating and hyperactivated mast cells in several organs, amongst which the skin and intestines. This study, comprising 41 participants, indicated that the incidence of mastocytosis is ten times higher in ASD patients than in the general population[33]. The increased gut permeability could be caused or severed by mast cell hyper-activation[33]. In addition, 15% of the people suffering from mastocytosis show aberrant behaviors, like concentration problems, decreased attention span, memory impairment, irritation and distraction[33], which are similar to the ones encountered in ASD, indicating that mast cells can influence behavior.

ASD is thought to be a neurodevelopmental disorder culminating from environmental and multiple genetic factors. The genes involved in ASD are variable and diverse, but mutations related to the mammalian target of rapamycin (mTOR) pathway, including NF1[34], PTEN[35], TSC1[36], TSC2[37], eIF4E[38], FMRP[39], are among the genes most highly associated with ASD (Fig. 2). NF1, PTEN, and TSC1/TSC2 act as negative regulators of mTOR complex 1 (mTORC1), of which single gene mutations lead to enhanced mTOR activity in the brain in mouse models[35,37,40]. The activity of mTOR enhances the phosphorylation of mTORC1 downstream proteins such as S6K and 4E-BPs, promoting cap-dependent protein translation[41–43]. The increased translation of neuroligins results in an increased synaptic excitation/inhibition ratio, which may eventually induce the development of ASD phenotypes[43].

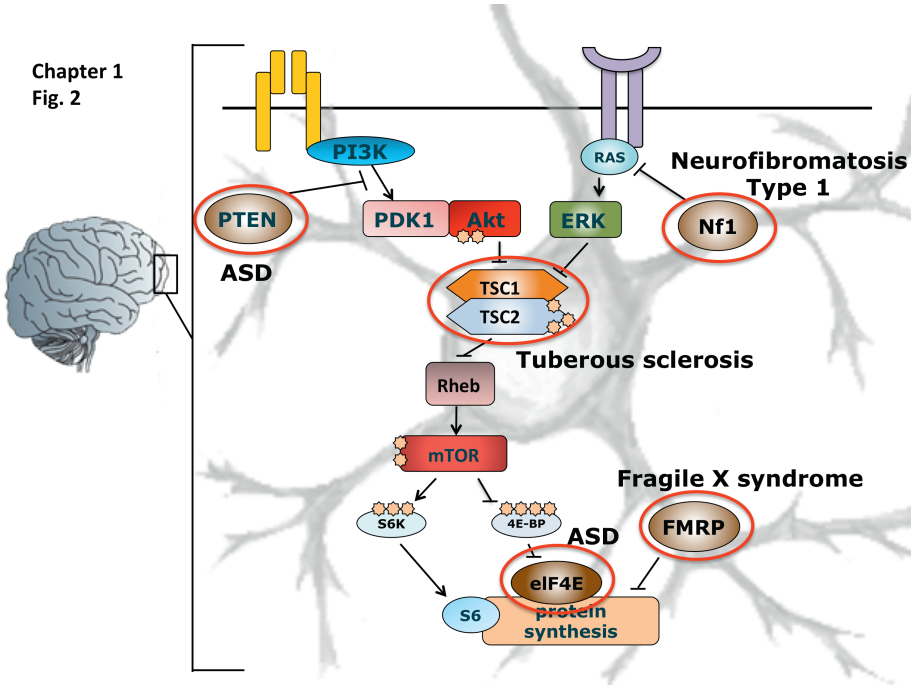

Fig. 2. Schematic representation of the mTOR signaling pathway in ASD. Various mutations on the mTOR signaling pathway (indicated in red circles) such as PTEN, Nf1, TSC, eIF4E, FMRP affect the protein synthesis machinery, leading to the development of ASD. Adapted from Ehninger, et al[37,40].

Apart from the important role in neurological disorders, the mTOR signaling pathway is also involved in directing immune responses (Fig. 3). mTORC1 activity is required for T helper cell differentiation and mTORC2 signaling is required for Th2 differentiation[44]. mTOR deficient T cells develop into Treg cells[45]. In addition, the mTORC1 pathway was

shown to play a critical role in function and activation of mast cells[46]. For these reasons, hyperactivity of mTOR in the intestinal tract might lead to a disturbance of effector and regulatory T cells in the direction of an inflammatory profile possibly associated with allergy. Taken together, the findings reveal that the mTOR signaling pathway could be disturbed both at the level of the immune system, the gastro-intestinal tract and the brain in ASD, and as such be considered as a 'linking pin' at the level of the gut-immune-brain axis. Manipulation of the mTOR signaling pathway may therefore serve as a therapeutic target for inflammation-associated neurodevelopmental disorders including ASD.

Chapter 1
Fig. 3

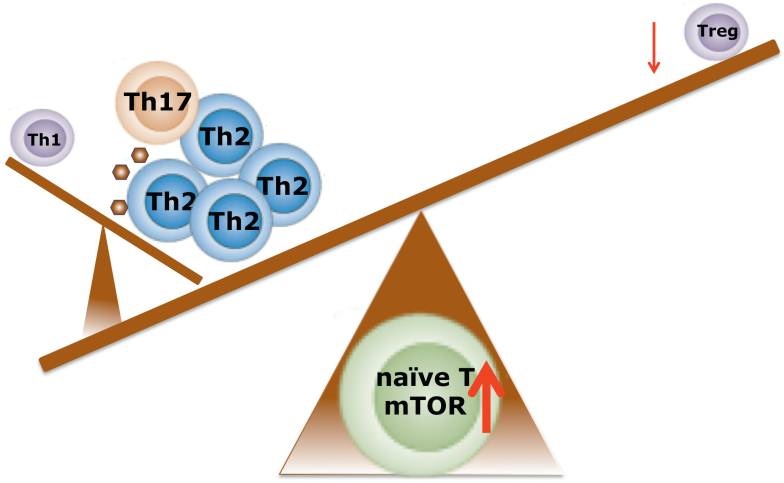

Fig. 3. Schematic representation regarding the possible role of mTOR activity in the balance of T cell profiling in food allergy. Enhanced mTOR activity is required for Th2 and Th17 cell differentiation. Suppression of the mTOR activity induces the differentiation into regulatory T cells (Treg). Adapted from Delgoffe, et al[44]. and Kim, et al[46].

Aim and outline of this thesis

This thesis aims at understanding the role of the brain-immune axis in ASD-like behaviors, in particular concentrating on the mTOR signaling pathway that plays a central role in both immune system, intestinal tract and brain.
Two models are employed, a mouse model of cow's milk allergy (CMA) and a genetic autistic mouse model. The major aims of the thesis are as follows:

1. to investigate the involvement of the mTOR signaling pathway in development of ASD-like behaviors and immune disturbances in a mouse model of cow's milk allergy.

2. to investigate the effect of individual amino acids or amino acid combinations on activation of the mTOR signaling pathway in antigen-IgE-activated BMMCs.

3. to investigate the effect of an mTOR-targeting amino acid diet and a multi-nutrient supplementation diet containing neuroprotective and anti-inflammatory ingredients on behavioral and immunological deficits in a CMA mouse model and BTBR autistic mouse model.

Chapter 1 provides a general introduction describing the scientific background of the thesis and highlighting the importance of the mTOR signaling pathway in development of ASD-related behavioral deficits and regulation of immune responses, in particular allergic responses. In **Chapter 2** we summarize the current findings regarding the neuro-immune interactions in diverse preclinical and clinical studies and point out that targeting intestinal disturbances may be beneficial for the treatment of ASD. **Chapter 3** demonstrates that a food allergic reaction induces reduced social interaction, increased repetitive behavior and impaired spontaneous alternation in a mouse model of cow's milk allergy. We demonstrated that neuronal activation is increased in the prefrontal cortex and reduced in the paraventricular nucleus of CMA mice. Dopaminergic activity is decreased in the prefrontal cortex and increased in the amygdala of CMA mice. CMA mice displayed increased levels of 5-HT and number of 5-HT positive cells in the intestinal tract. **Chapter 4** demonstrates that enhanced mTOR signaling in the specific brain regions and ileum is centrally involved in behavioral and immunological deficits of CMA mice. Phosphorylation of mTORC1 downstream proteins p70 S6K and 4E-BP1 is enhanced in the prefrontal cortex and amygdala and ileum of CMA mice. Rapamycin inhibited the enhanced activity of p70 S6K and 4E-BP1 in the specific brain regions and

ileum and normalized behavior. Rapamycin also increased the expression of regulatory T cell-associated transcriptional marker in the ileum of CMA mice. **Chapter 5** shows that the individual amino acids of leucine, isoleucine, valine or amino acid combination of leucine, isoleucine, and valine enhance the mTORC1 pathway in antigen-IgE-activated mast cells. The individual amino acids of histidine, lysine, and threonine or amino acid combination of histidine, lysine, threonine inhibit the mTORC1 pathway in antigen-IgE-activated mast cells. The results of this study led to the design of an mTOR targeting amino acid diet. **Chapter 6** demonstrates that dietary intervention with the mTOR-targeting amino acid diet or a multi-nutrient supplementation diet containing neuroprotective and anti-inflammatory ingredients improved autistic-like behavior of CMA mice. The amino acid diet and multi-nutrient supplementation diet were also shown to inhibit the enhanced mTOR signaling pathway in the prefrontal cortex and amygdala of CMA mice. Furthermore, dietary intervention with the amino acid diet improved the repetitive self-grooming behavior of BTBR mice and inhibited the mTOR signaling in the prefrontal cortex and somatosensory cortex of BTBR mice, while the multi-nutrient supplementation diet had no effect on autistic behavior of BTBR mice and on the mTOR signaling in the brain of BTBR mice. **Chapter 7** further demonstrates the effects of the amino acid diet and the multi-nutrient supplementation diet containing neuroprotective and anti-inflammatory ingredients on immunological responses in CMA mice, to further investigate the role of the gut-immune–brain axis in ASD-like behavior. Finally, the main findings are summarized and discussed with perspectives for future research in **Chapter 8**.

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CHAPTER TWO

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

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Abstract

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

1. Introduction

Autism spectrum disorders (ASDs) comprise autism, pervasive developmental disorder not otherwise specified (PDD-NOS) and Asperger's disorder. These pervasive neurodevelopmental disorders are characterized by impairments in social interaction and communication and the presence of limited, repetitive and stereotyped interests and behaviors (Johnson and Myers, 2007 and Vandereycken, 2003). So far, no biomarkers for ASD have been identified. Therefore, diagnosis of ASD is entirely dependent on behavioral observations, according to the DSM-IV criteria (American-Psychiatric-Association, 2000). Literature suggests that the prevalence of ASD has increased 20 times, from a rate around 1:2500 in the mid-1980s to a rate of 9:1000 at present (Genuis, 2009 and MMWR, 2009). Nowadays, early diagnostic tools are available (Luyster et al., 2009) and diagnostic stability has been established (Zwaigenbaum et al., 2009). Although many believe that the ASD escalation is a consequence of better and earlier diagnosis, improved awareness and expanding criteria to fulfill the diagnosis, some believe that these changes do not adequately account for the rapid rise (Hertz-Picciotto and Delwiche, 2009).

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

2. Gastrointestinal disturbances in ASD

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

3. Gastrointestinal pathology in ASD

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

A factor that might contribute to the gastrointestinal disturbances among autistic individuals is an abnormal composition of gut microbiota. Several groups have studied the intestinal microbiota of the autistic population and found a different composition of several microbial species compared to healthy controls. These ASD-related microbial species mainly comprised various Clostridium strains, Ruminococcus, Bacteroidetes, Bacteroides, Firmicutes and Desulfovibrio species (Finegold et al., 2002, Finegold et al., 2010, Parracho et al., 2005 and Song et al., 2004). A recent paper by Adams et al. (2011) demonstrated lower levels of Bifidobacterium and higher levels of Lactobacillus (all strains) in ASD, both considered to be beneficial bacteria (Adams et al., 2011). Colonization of Clostridum species to the expense of Bifidobacterium have been associated with higher risks of food allergy in children and with the development of (pediatric) inflammatory bowel diseases as well (Adlerberth et al., 2007, Schwiertz et al., 2010, Vanderploeg et al., 2010 and Willing et al., 2010). Interestingly, antibiotic

treatment of ASD children did not only lead to gastrointestinal improvements, but also improvements in cognitive skills (Sandler et al., 2000).

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

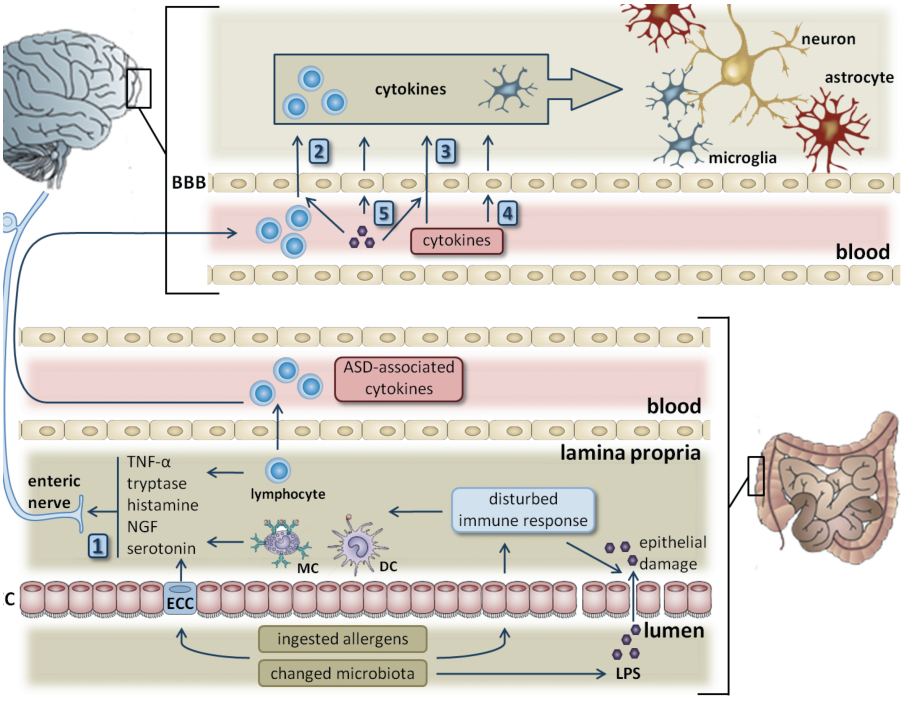

Fig. 1. Possible pathways involved in neuroimmune interactions in ASD. Upon immune disturbance in the gastrointestinal tract, intestinal epithelial cells (IECs) become more permeable and enterochromaffin cells (ECCs), lymphocytes, mast cells (MCs) and dendritic cells (DCs) secrete all kinds of neuroimmune factors that can stimulate enteric nerves (1). In addition, ASD-associated cytokines (IL-1ß, IL-4, IL-5, IL-6, IL-12, IL-13, IFN-γ, TNF-α) and lymphocytes are present in the circulation. Subsequently, lymphocytes can pass the blood-brain barrier (BBB)

(2), serum cytokines (IL-1ß, IL-6, IFN-γ, TNF-α) can pass the blood-brain barrier (3) and cytokines (IL-1ß, TNF-α) can bind to brain endothelial cells inducing an immune response at the brain side (4). LPS can increase the permeability of the blood-brain barrier, enhancing cytokine and lymphocyte infiltration, or bind to brain endothelial cells inducing an immune response at the brain side (5). The immune response in the brain can consist of an increased number of lymphocytes and cytokines (IL-1ß, IL-6, CXCL-8, IL-10, IFN-γ, TNF-α, CCL-2 and GM-CSF), also produced by neuroglia, resulting in changed neuronal homeostasis.

4. Neuroimmune interactions at the side of intestinal inflammation

4.1. Pathway of intestinal inflammation

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

4.2. Serotonin: neurotransmitter and mediator of inflammation

The serotonergic system has been implicated in the pathogenesis of ASD since

increased levels of blood serotonin (5-hydroxytryptamine; 5-HT) were first described in children with autism (Schain and Freedman, 1961). Subsequent studies demonstrated that about one-third of the patients with ASD has blood hyperserotonemia (Anderson et al., 1987 and Hanley et al., 1977). On the other hand, the capacity of 5-HT synthesis in the global brain was decreased in children with autism (Chugani et al., 1999), indicating a lower brain 5-HT availability. The cause of ASD-related hyperserotonemia is thought to arise from genetic (Coutinho et al., 2007), gastrointestinal (Minderaa et al., 1987 and Mulder et al., 2010) or immune (Burgess et al., 2006 and Warren et al., 1986) changes. Based on intestinal low-grade inflammation, blood hyperserotonemia and low 5-HT synthesis in the brain, we propose the following hypothesis. During an inflammatory response in the gut, 5-HT is produced and released by enterochromaffin cells and intestinal inflammatory cells such as mast cells and platelets, resulting in a faster moving gut and an increase in secretion, vasodilatation and vascular permeability. This, in turn, leads to problems in functional dysmotility, stool consistency (diarrhea or constipation) and infiltration of leukocytes in the intestinal wall. Because of the increased utilization of dietary tryptophan by the gut, there will be less tryptophan available for passage through the blood-brain barrier. As a result, brain 5-HT levels are reduced and this may lead to mood and cognitive dysfunctions found in ASD. Indeed, the availability of tryptophan was demonstrated to be important, since depletion of tryptophan from the diet increased autistic behavior in affected adults (McDougle et al., 1996). More research is required to establish whether 5-HT metabolism can be a therapeutic target in ASD, either by providing dietary tryptophan or by pharmaceutical treatments such as selective serotonin reuptake inhibitors (SSRIs). Recently, it was reported that there is no evidence for a beneficial effect of treatment with SSRIs in autistic children and only limited evidence exists for the effectiveness of SSRIs in adults suffering from ASD (Williams et al., 2010). Perhaps, targeting the ASD-associated low-grade intestinal inflammation might be more successful in restoring the availability of tryptophan for 5-HT synthesis in the brain.

4.3. Food Allergy in ASD

A disturbed intestinal immune reaction can be directed against food particles, initiating an allergic response. Food allergy has often been suggested to be present among autistic individuals. Parental reports indicate that food allergy is more common in the autistic population compared with healthy controls (Gurney et al., 2006 and Jyonouchi et al., 2008). It is important to take into account that ASD children are likely underdiagnosed for food allergies, because of their impaired ability to express their discomfort. Lucarelli

et al. (1995) observed that an oral challenge with cow's milk protein led to worsening of some of the behavioral symptoms specific for ASD. They also found significantly higher serum levels of IgA, IgG and IgM for casein and IgA for lactalbumin and ß-lactoglobulin in children with ASD compared with healthy controls (Lucarelli et al., 1995). Furthermore, the intake of milk protein was a significant predictor of constipation in the autistic population (Afzal et al., 2003). Therefore, patients with ASD often exclude gluten and milk protein from their diet, better known as a gluten-free, casein-free diet. Some publications on gastrointestinal disturbances in ASD compared ASD patients on a gluten and milk free diet with ASD patients on an unrestricted diet. For instance, eosinophil infiltration in intestinal biopsies of children with regressive autism and gastrointestinal disturbances was significantly less abundant in those on a gluten and milk free diet compared with those on an unrestricted diet (Ashwood et al., 2003). Moreover, the ASD patients that excluded gluten and milk proteins, showed a significant reduction in the enhanced intestinal permeability compared with ASD patients on a unrestricted diet (de Magistris et al., 2010). In addition to the beneficial effects on gastrointestinal disturbances, a gluten and milk free diet was claimed to improve autistic behavior as well. Indeed, parents reported improvements in social behavior and linguistic skills (Elder et al., 2006). Few studies have been performed on the efficacy of a gluten and casein elimination diet in autistic individuals, showing improvements in rituals, verbal communication, interpersonal relations and learning (Hsu et al., 2009, Knivsberg et al., 2002, Millward et al., 2008 and Whiteley et al., 2010). Unfortunately, these studies comprised either small cohort studies or case reports and could therefore not confirm the beneficial outcome of a gluten-free, casein-free diet. More research is necessary to strengthen these findings.

The majority of allergies is characterized by a T helper (Th) 2-type immune reaction. Th2 effector cells produce Th2 cytokines (interleukin (IL)-4, IL-5 and IL-13) and can activate memory B cells to secrete immunoglobulins (Valenta, 2002). Supporting the suspected role of allergy in ASD, there seems to be an imbalance in Th1 and Th2 cytokines in these patients. Indeed, peripheral blood mononuclear cells (PBMCs) of children with ASD produced significantly higher levels of IL-4, IL-5 and IL-13 than their matched controls (Molloy et al., 2006). In blood of ASD children, interferon (IFN)-γ and IL-2 positive helper and cytotoxic T cells were less abundant than in blood of healthy controls. In contrast, IL-4 positive helper and cytotoxic T cell numbers were enhanced (Gupta et al., 1998). In addition to these data on a disturbed Th1/Th2 balance, a lower IFN-γ/IL-10 ratio was observed in male rats prenatally exposed to valproic acid, a well-characterized animal

model for autism (Schneider et al., 2008). In response to cow's milk protein, PBMCs from ASD children with and without gastrointestinal disturbances produced more tumor necrosis factor (TNF)-α and IL-12 than those from control subjects (Jyonouchi et al., 2005). Furthermore, there were less IL-10 positive T cells present in both the periphery and the gut mucosa of ASD children with gastrointestinal symptoms, compared with non-inflamed controls and children with Crohn's disease (Ashwood and Wakefield, 2006). T cells that produce the anti-inflammatory cytokine IL-10 are mainly inducible T regulatory cells. Allergen-specific T regulatory cells are predominantly present in healthy individuals to suppress an allergic response. Less IL-10 positive T cells are therefore associated with enhanced Th2 responses. Plasma levels of another T regulatory cytokine transforming growth factor (TGF)-ß, were decreased as well, as observed by two groups (Ashwood et al., 2008 and Okada et al., 2007). Low TGF-ß levels were inversely correlated with behavioral scores (Ashwood et al., 2008). This indicates that regulatory T cell responses are decreased in individuals with ASD and that the lack of suppressive capabilities of the immune system could be involved in the expression of autistic behavior.

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

4.4. Association between ASD and maternal allergic diseases

Cumulating to the importance of allergy in the pathophysiology of ASD is the finding that mothers, diagnosed for asthma or allergies (such as atopic eczema and rhinitis) during the second trimester of their pregnancy, had a greater than two fold elevated risk for ASD in their offspring (Croen et al., 2005). In addition, there was an enhanced association observed between allergic conditions and autism in families with more than one ASD-affected child. This observation suggests that genes underlying atopy may be related to the etiology of ASD. (Croen et al., 2005). Recently, King (2011) hypothesized that epigenetic disruption of brain development is caused by gestational exposure to allergy-associated inflammatory mediators (for example IL-6 and histamine) (King, 2011). These mediators promote retinoic acid and estradiol gene transcription, resulting in overexposure of the fetus to retonic acid and estradiol. Retinoic acid (a vitamin A metabolite) is required for growth and development. An excess in vitamin A or retinoic acid is associated with brain abnormalities reminiscent of those present in ASD, such as cerebellar malformations, cranial nerve abnormalities and abnormalities of the dopaminergic system (London, 2000). Estradiol is known to defeminize the fetal brain, playing an important role in sexual differentiation. Overexposure to estrogen affects a wide range of cognitive functions, which are characteristic for autistic individuals such as anxiety, motor deficits, stereotype and repetitive movements, hyperactivity and attention deficits (King, 2011 and McEwen et al., 1999).

4.5. Other immune processes in ASD

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

with higher aberrant behavior scores (Ashwood et al., 2011b). The heterogeneity of autistic disorders may be the reason behind these conflicting data.

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

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

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

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

The endothelial cell layer of the blood-brain barrier is surrounded by a basal lamina, which is in direct contact with pericytes and astrocytes, with microglia in close attendance. Physiological changes in neuroglial cells can influence the blood-brain

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

5.2. Cytokines enter the brain and influence neurons via neuroglia

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

In postmortem brains of autistic patients, enhanced activation of astrocytes and microglia was observed (Vargas et al., 2005). Astrocyte activation was identified in the

subcortical white matter of the midfrontal gyrus and the anterior cingulate gyrus and in the granular cell layer, the Purkinje cell layer and the white matter of the cerebellum. In addition, enhanced astrocyte activation was observed in the striatum, hippocampus and cerebral cortex of mice with fragile X syndrome (highly related to autism) (Yuskaitis et al., 2010). Microglia activation was predominantly observed in granular cell layer and white matter of the cerebellum of ASD brains (Vargas et al., 2005). It is unclear when or how neuroglia become activated in the brain of autistic patients. To investigate this, Vargas et al. (2005) additionally characterized cytokine and chemokines profiles in the midfrontal gyrus, the anterior cingulate gyrus and the cerebellum of ASD brains and cerebrospinal fluid. Enhanced levels of IL-6, IFN-γ, CCL2, CCL4, CXCL8 and CXCL10 were found in the cerebrospinal fluid of autistic children. In postmortem brains of autistic individuals, TGF-ß was increased in all three brain regions (midfrontal gyrus, anterior cingulate gyrus and cerebellum) and pro-inflammatory chemokines CCL2 and thymus and activation-regulated chemokine (TARC) were increased in the anterior cingulate gyrus and the cerebellum. Furthermore, the anterior cingulate gyrus showed increased levels of a wide range of pro-inflammatory cytokines and chemokines, including IL-6, IL-10, CCL7, CCL22, CCL23, CXCL9, and CXCL13 (Vargas et al., 2005). Another group measured cytokine profiles in the frontal cerebral cortex of ASD brains and observed enhanced levels of pro-inflammatory cytokines IL-6, TNFα and granulocyte macrophage colony stimulating factor (GM-CSF), Th1 cytokine IFN-γ and chemokine CXCL8 (Li et al., 2009). Because no enhanced lymphocyte infiltration was observed in the brains of autistic individuals, it may be more likely that neuroglia become activated upon stimulation by infiltrated cytokines. The activated neuroglia can produce immune factors, as described above, consequently adapt neuronal homeostasis and functioning, leading to alterations in behavior.

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

Brain endothelial cells function as a barrier between blood and brain and regulate the infiltration of immune factors. In addition to this barrier function, brain endothelial cells are known to be activated by cytokines and to produce cytokines themselves (Fig. 1: pathway 4). IL-1ß (Cao et al., 1996) and TNF-α (Bugno et al., 1999), two cytokines that are also relevant in ASD, can bind to brain endothelial cells and induce an immune response (Stanimirovic and Satoh, 2000). In turn, brain endothelial cells are important sources of pro-inflammatory mediators, such as prostaglandins, leukotrienes, cytokines and chemokines (Stanimirovic and Satoh, 2000, Vadeboncoeur et al., 2003 and Verma et al., 2006). The factors that they produce, including cytokines such as IL-6, GM-CSF

and TNF-α and chemokines like CCL2 and CXCL8 (Verma et al., 2006 and Zhang et al., 1999), can be released both at the side of the brain and the blood vessel. When brain endothelial cells secrete immune factors at the brain side, astrocytes and microglia become activated and consequently influence neuronal functioning and thereby behavior.

5.4. LPS influences neurons via the blood-brain barrier

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

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

The mammalian target of rapamycin (mTOR) is a highly conserved, intracellular serine/threonine kinase that regulates cell growth and metabolism in response to a wide variety of signals, including growth factors, nutrients, energy and inflammatory factors (Hay and Sonenberg, 2004, Sengupta et al., 2010 and Wullschleger et al., 2006). mTOR belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and serves as the catalytic subunit of two structurally and functionally distinct multi-protein complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Fig. 2 depicts a schematic illustration of the major upstream and downstream mTOR signaling

pathways. Rapamycin, which is a macrolide produced by soil bacterium Streptomyces hygroscopicus (Vezina et al., 1975), disrupts mTORC1 complex formation (Kim et al., 2002). mTORC2 shares several proteins with mTORC1 and was originally described as a rapamycin-insensitive complex, as acute rapamycin treatment is unable to inhibit mTORC2 (Sarbassov et al., 2005). However, subsequent studies have shown that, in some cell types, prolonged rapamycin treatment inhibits the assembly of mTORC2 (Sarbassov et al., 2006).

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

In patients with ASD, several mutations in genes are found that are strongly linked to the mTOR signaling pathway. Tuberous sclerosis is a genetic disorder caused by heterozygous mutations in the mTOR pathway related Tuberous sclerosis complex (Tsc)1 or Tsc2 genes and is commonly associated with the autistic phenotype. Mice with a heterozygous mutation in the Tsc2 gene ($Tsc2^{+/-}$ mice) demonstrate enhanced mTOR signaling in hippocampus, which contributes to learning and memory impairments in $Tsc2^{+/-}$ mice. Treatment of adult $Tsc2^{+/-}$ mice with rapamycin reversed the learning and memory impairments (Ehninger et al., 2008). In addition, $Tsc1^{+/-}$ mice also displayed reduced levels of social behavior and cognitive function (Goorden et al., 2007). PTEN (phosphatase and tensin homolog deleted on chromosome ten) acts as a phosphatase that dephosphorylates one of the upstream TSC/mTOR-associated signal transduction molecules, resulting in enhanced activity of mTOR. Mutations in PTEN are associated with a wide variety of human neurological disorders, including ASD (Rosner et al., 2008). PTEN gene mutation analysis has been suggested for patients with macrocephaly, a condition that is observed in 20% of patients with ASD (Butler et al., 2005). Pten knock-out mice with deletion of Pten in neurons in the cortex and hippocampus develop autistic phenotypes such as macrocephaly and reduced social behavior. Moreover,

changes in cell morphology have been observed, including neuronal hypertrophy and loss of neuronal polarity, which means that the establishment of axons and dendrites in these neurons is disrupted. Treatment with rapamycin in Pten knock-out mice reversed neuronal hypertrophy and macrocephaly and ameliorated ASD-related, abnormal behaviors (Zhou et al., 2009). Furthermore, mTOR is involved in protein synthesis-dependent synaptogenesis. Activation of mTOR pathway can increase the production of synaptic signaling proteins and the formation of new spine synapses in the prefrontal cortex of rats. mTOR inhibition with rapamycin blocked synaptic protein synthesis and antidepressant behavioral responses in rats (Li et al., 2010).

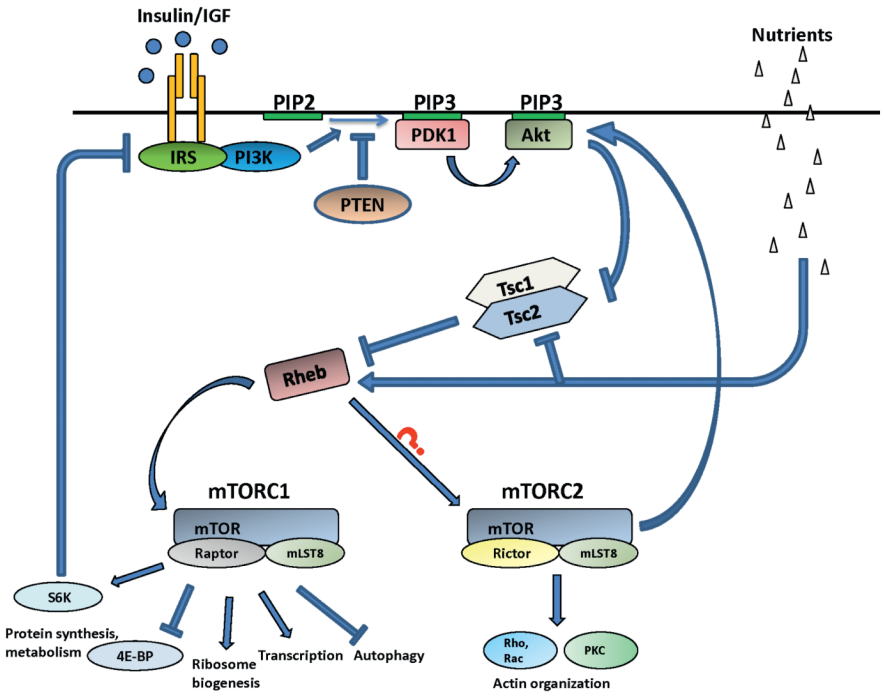

Fig. 2. Schematic illustration of mTOR signaling pathway. mTORC1 comprises four components apart from mTOR: regulatory associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8; also known as GßL), proline-rich AKT substrate 40 kDa (PRAS40), and DEP-domain-containing mTOR-interacting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

Currently, it is becoming more and more evident that mTOR also plays a central role in directing immune responses. A recent study suggests that Th1 and Th17 differentiation are specifically regulated by mTORC1 signaling. In contrast, Th2 differentiation is dependent on mTORC2 signaling, as T cells in which mTORC2 activity is eliminated failed to differentiate into Th2 cell both in vitro and in vivo but were able to differentiate into Th1 and Th17 cells (Li et al., 2010). Furthermore, it was shown that T cells differentiated into regulatory T cells in the presence of a conventional dose of rapamycin, which inhibits mTORC1 and mTORC2 (Li et al., 2010). Indeed, rapamycin-induced mTOR inhibition resulted in elevated Treg cells in tissue culture of nasal polyps obtained from patients suffering from chronic allergic rhinitis (Xu et al., 2009). Furthermore, mTORC1 activation in mast cells is associated with survival, differentiation, migration and cytokine production of the important 'allergic' cells (Kim et al., 2008). Finally, increased mTOR activity is shown to attenuate autophagy (Yu et al., 2010). This finding could explain the reduced clearance and maintenance of inflammatory cells at sites of allergic inflammation. In conclusion, because of its function in immune and neuronal pathways, mTOR may be a possible target for treatment in ASD.

7. Targeting the gastrointestinal tract in ASD

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

The use of Complementary and Alternative Medicine (CAM) practices for children with ASD is often reported (Golnik and Ireland, 2009, Hanson et al., 2007, Harrington et al., 2006, Levy et al., 2003, Levy and Hyman, 2003, Weber and Newmark, 2007 and Wong and Smith, 2006). Examples of such treatments include the use of vitamin and mineral supplements, secretin, melatonin and gluten-free, casein-free diets (Levy et al., 2003). At this moment, approximately 50% of parents with an ASD child have tried CAM (Levy and Hyman, 2003), and half of these are using a gluten-free, casein-free diet (Levy et al., 2003). Results from a recent study indicate that gluten and milk free diets improve behavior in children with ASD (Whiteley et al., 2010). This result suggests the presence of food hypersensitivity or allergy in the autistic population. The gluten and milk elimination diet can be supported by the use of a free amino acid composition to avoid dietary insufficiencies. In addition to the elimination diet, dietary ingredients such as omega-3 fatty acids and pre- and probiotics might be beneficial to the dietary management of autistic behavior and the associated gastrointestinal symptoms, because of their effects on CNS, immune system and/or on microbiota profile.

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

al., 2004), which may exacerbate the disease (Sandler et al., 2000), these patients may benefit from dietary supplementation with a prebiotic mixture.

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

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

Third, omega-3 fatty acids decrease the expression of adhesion molecules and prevent adherence of monocytes and macrophages (De Caterina and Libby, 1996 and Miles et al., 2000). Finally, omega-3 fatty acids have been shown to decrease the production of inflammatory cytokines (Calder, 2008). This means that supplementation of omega-3 PUFAs could be beneficial for patients with ASD, because omega-3 PUFAs can act either directly on neuronal responses or indirectly via the immune system and gastrointestinal tract.

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

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CHAPTER THREE

Autistic-like behavioural and neurochemical changes in a mouse model of food allergy

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Abstract

Food allergy has been suggested to contribute to the expression of psychological and psychiatric traits, including disturbed social behaviour and repetitive behaviour inherent in autism spectrum disorders (ASD). Most research in this field receives little attention, since fundamental evidence showing direct effects of food allergic immune responses on social behaviour is very limited. In the present study, we show that a food allergic reaction to cow's milk protein, induced shortly after weaning, reduced social behaviour and increased repetitive behaviour in mice. This food allergic reaction increased levels of serotonin (5-hydroxytryptamine; 5-HT) and the number of 5-HT positive cells, and decreased levels of 5-hydroxyindoleacetic acid (5-HIAA) in the intestine. Behavioural changes in food allergic mice were accompanied by reduced dopaminergic activity in the prefrontal cortex. Furthermore, neuronal activation (c-Fos expression) was increased in the prefrontal cortex and reduced in the paraventricular nucleus of the hypothalamus after exposure to a social target. We hypothesize that an intestinal allergic response regulates complex, but critical, neuroimmune interactions, thereby affecting brain circuits involved in social interaction, repetitive behaviour and cognition. Together with a genetic predisposition and multiple environmental factors, these effects of allergic immune activation may exacerbate behavioural abnormalities in patients with ASD.

1. Introduction

The intestinal tract continuously encounters foreign antigens and is therefore the most complex organ of the immune system. The majority of these antigens are harmless food antigens to which the body has formed a tolerogenic reaction. Genetic predisposition and environmental factors, however, can abrogate tolerance towards food allergens, leading to a Th2-directed immune response characterized by production of allergen-specific immunoglobulins during sensitization, and mast cell degranulation upon a second exposure to the allergen [1].

The intestinal tract is not only distinguished by its crucial immune function, but also exerts an important neurological function and is called 'the second brain' because of its abundant amount of enteric nerves. Evidence is emerging that intestinal immune disturbances can signal to the brain through various pathways, affecting behaviour and emotion [2]. Food allergy has been suggested to be one of the intestinal triggers that can contribute to the expression of various psychological and psychiatric traits, including anxiety, depression, migraine, schizophrenia, attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorders (ASD) [3], [4], [5] and [6]. Supporting the hypothesis that food allergy can affect mental disorders of psychosocial relevance, Meldrum et al. [7] recently observed social neurodevelopmental abnormalities in food allergic children at 18 months of age. Diagnosis of food allergy was associated with enhanced internalizing behaviour and a trend towards low social emotional scores. Intestinal problems are often reported in children with ASD [8] and [9] and milk intake was found to be a predictor of constipation [10]. Furthermore, a (gluten and) milk protein free diet is suggested to improve autistic behaviours [11], [12] and [13] and to restore the increased intestinal permeability observed in these children [14]. Preclinical studies on neurological effects of food allergy are limited. Mice immunized to ovalbumin (OVA) displayed increased anxiety 1 h after oral challenge with OVA [15]. Moreover, c-Fos staining of the paraventricular nucleus (PVN) of the hypothalamus and central nucleus of the amygdala was observed in these mice 90 min after OVA challenge, accompanied by increased serum levels of corticosterone.

Not only food allergy, but also other allergic diseases have been associated with neuropsychological sequelae [7], [16] and [17]. Symptoms of developmental and behavioural dysfunction were more frequent in children with asthma compared to control children and asthma severity was shown to correlate with greater behavioural difficulties [16] and [18]. In addition, ADHD was positively associated with eczema and asthma [19] and a preliminary report indicated that ASD was more prevalent among

children with mastocytosis [20], suggesting a role for mast cell activation in triggering neurological manifestations. Preclinical studies showed that OVA-immunized mice challenged via the airways displayed comparable brain activation as mice challenged via the oral route [15]. Furthermore, allergic rhinitis increased anxiety and reduced social interaction in rats and mice, one day after allergen challenge [21].

Despite these clinical and preclinical indications, there is still much debate on the existence of food allergy-enhanced psychosocial disabilities and the question whether food allergy in mice affects social and repetitive behaviour has never been explored. Therefore, this study investigated the effects of a food allergic immune response on social interactions, repetitive behaviour and spontaneous alternation in mice and examined associated region-specific neuronal activation and monoamine levels.

2. Materials and methods

2.1. Cow's milk allergy mouse model

Three-week-old, specific pathogen free, male C3H/HeOuJ mice were purchased at Charles River Laboratories (L'Arbresle Cedex, France) and housed at the animal facility of the Utrecht University on a 12 h light–dark cycle with access to food and water ad libitum. Mice were bred and raised on a cow's milk protein-free diet (Special Diet Services, Witham, UK). All animal procedures were approved by and conducted in accordance with the guidelines of the Animal Ethics Committee of Utrecht University (approval number: DEC2009.I.12.112, DEC2011.I.08.082). After one-week habituation, mice were sensitized intragastrically (i.g.) with 20 mg whey/0.5 mL PBS containing 10 µg cholera toxin (CT, DMV International, Veghel, The Netherlands) as an adjuvant. Sham-sensitized control mice received CT alone. Mice were sensitized once a week for 5 consecutive weeks as previously described by Schouten et al. [22]. One week after the last sensitization, mice were challenged i.g. with 50 mg whey/0.5 mL PBS. The day after challenge, mice were exposed to a social behaviour test. To further exploit behaviour and avoid multiple behavioural testing on one day, self-grooming and T maze alternation was assessed one and two days after the last sensitization, respectively. An intestinal allergic response to the fifth sensitization is confirmed by elevated serum levels of mMCP-1 in whey-sensitized mice (data not shown).

2.2. Social interaction test

The behavioural assessment used was adapted from a previous description [23] and [24]. The morning after oral challenge, mice were exposed to a social interaction test (n = 10 per group). Mice were placed in a 45 × 45 cm open field, with a small perforated Plexiglas cage (10 cm diameter) located against one wall allowing visual, olfactory and minimal tactile interaction (Fig. 1a). Mice were habituated to the open field for 5 min and an age- and gender-matched unfamiliar target mouse was introduced in one of the cages for an additional 5 min. Open fields were cleaned with water followed by 70% ethanol after each test. By using video tracking software (EthoVision 3.1.16, Noldus, Wageningen, The Netherlands), an interaction zone around the cage was digitally determined. Time spent in the interaction zone, latency until first occurrence in the interaction zone and total distance moved was measured.

2.3. Self-grooming

The morning after the last sensitization, mice (n = 10 per group) were scored for spontaneous grooming behaviours as described earlier [25] and [26]. Each mouse was placed individually in an empty home cage (35 cm × 20 cm) without bedding and video recordings were used for behavioural scorings of frequency and cumulative time spent grooming all body regions. Open field was cleaned with water followed by 70% ethanol after each test. After a 5 min habituation period in the cage, each mouse was scored blindly for 5 min by two independent researchers. Inter-rater reliability was 97.8%.

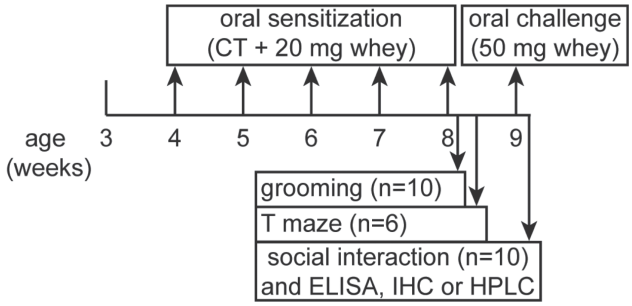

Fig. 1. A schematic overview of the sensitization and challenge protocol and the behavioural tests performed. Mice were exposed to one of the behavioural tests; self-grooming (one day after last sensitization), spontaneous alternation in the T maze (two days after last sensitization) or social interaction (one day after challenge). Serum, brain and intestinal tissues of mice that were exposed to the social interaction test were used for further analysis by ELISA, IHC and HPLC.

2.4. T maze spontaneous alternation

Two days after the last sensitization, spontaneous alternation was tested in a T maze set-up. The T-maze was (49 cm long × 10 cm wide × 19 cm high) and two lateral arms (32 cm long × 10 cm wide × 18 cm high). A trial consisted of 2 runs, with a time interval of 2 min. After the mouse (n = 6 per group) had been released from the start arm, the animal was free to choose between both goal arms. As soon as the animal had entered one goal arm, the other arm was closed and the animal was confined for 30 s in the goal arm by lowering the door. The animal was then returned to his home cage. After thoroughly cleaning the T-maze with 70% ethanol, the mouse was put back to the start arm and was free to choose one of the goal arms. Total of 5 trials was performed. The alternation ratio was defined as the amount of trials in which an animal alternated divided by the total amount of trials.

2.5. Measurements of serum mMCP-1 and whey-specific immunoglobulins

Blood was collected 16 h after oral challenge and centrifuged for 15 min at 14,000 rpm (n = 10 per group). Serum was stored at −70 °C until analysis. Concentration of mouse mast cell protease-1 (mMCP-1) in serum was determined using commercially available ELISA kits (Moredun Scientific Ltd., Penicuik, UK) according to the manufacturer's protocol. Serum concentrations of whey-specific IgE, IgG1 and IgG2a were measured by ELISA. Microlon plates (Greiner, Alphen aan de Rijn, The Netherlands) were coated with 20 μg/mL whey in carbonate/bicarbonate buffer (0.05 M, pH = 9.6; Sigma–Aldrich, Zwijndrecht, The Netherlands) overnight at 4 °C. Plates were blocked for 1 h in ELISA buffer (50 mM Tris, 137 mM NaCl, 2 mM EDTA, 0.05% Tween-20 and 0.5% BSA in PBS). Serum samples were incubated for 2 h. Plates were incubated with biotinylated rat anti-mouse IgE, IgG1 and IgG2a (1 μg/mL; BD Biosciences, Alphen aan de Rijn, The Netherlands) for 2 h. Plates were subsequently incubated with streptavidin–horse radish peroxidase (0.5 μg/mL; Sanquin, Amsterdam, The Netherlands) for 1 h and developed using o-phenylendiamine (Sigma–Aldrich, Zwijndrecht, The Netherlands). Reaction was stopped after 10 min with 4 M H_2SO_4 and absorbance was measured at 490 nm on a microplate reader (Bio-Rad, Veenendaal, The Netherlands). Results were expressed as arbitrary units (AU), composed using a titration curve of pooled sera from whey–alum i.p. immunized mice serving as an internal standard.

2.6. Immunohistochemistry for c-Fos

Since maximum c-Fos expression occurs between 1 and 3 h following exposure to a stimulus [27], mice were sacrificed 1.5 h after the social interaction test. Mice (n = 5

per group) were deeply anaesthetized with pentobarbital and perfused transcardially with PBS, followed by buffered 4% paraformaldehyde (pH 7.2). Brains were removed and post-fixed in the same fixative overnight, following cryoprotection with 30% sucrose (Sigma–Aldrich, Zwijndrecht, The Netherlands) in PBS. Coronal slices of 40 µm were sectioned using a cryostat (CM3050, Leica Microsystems, Rijswijk, The Netherlands), collecting 5 parallel series in PBS. Sections were incubated with 0.3% H_2O_2 in PBS for 30 min and blocked using PBS-BT (0.1% BSA and 0.3% Triton-X-100 in PBS) for 30 min. Sections were incubated overnight with rabbit anti-c-Fos (Santa Cruz Biotechnology, Heidelberg, Germany) 1:5000 in PBS-BT. Next day, sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch, Suffolk, UK) 1:1500 in PBS-BT for 90 min and subsequently incubated for 90 min with ABC–HRP complex (Vector Laboratories, Peterborough, UK) in PBS-BT. Reaction was visualized by incubation for 10 min in a chromogen solution consisting of 0.02% DAB (Sigma–Aldrich, Zwijndrecht, The Netherlands) and 0.03% ammonium-nickel-sulphate (Sigma–Aldrich, Zwijndrecht, The Netherlands) dissolved in 50 mM Tris buffer (pH 7.6) and subsequently 10 min in chromogen solution with H_2O_2. Sections were washed, mounted on adhesive microscope slides, dehydrated and coverslipped.

Initial screening of c-Fos patterns resulted in selection of 3 brain areas where potential changes were detected; prefrontal cortex (PFC), amygdala and hypothalamic paraventricular nucleus (PVN). Digital images were captured with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera, at a magnification of 10 or 20 times for PFC and PVN, respectively. Image J software allowed us to mark c-Fos immunoreactive nuclei. Numbers of c-Fos positive neurons were counted bilaterally from a single section and the sum was used as the outcome for a single animal.

2.7. HPLC for analysis of l-tryptophan, monoamines and metabolites in brain and intestine

After decapitation, brains (n = 8) were rapidly removed, frozen in isopentane (Sigma–Aldrich, Zwijndrecht, The Netherlands) and brain and intestinal tissue were stored at –70 °C. Bilateral regions of PFC, amygdala and PVN were dissected out with 500 µm coronal sections using a cryostat (Model 700, Laméris Instruments, Utrecht, The Netherlands). l-tryptophan (TRP), dopamine (DA), serotonin (5-hydroxytryptamine; 5-HT) and their metabolites (3,4-dihydroxyphenylacetic acid; DOPAC, 3-methoxytyramine; 3-MT, and homovanillic acid; HVA and 5-hydroxyindoleacetic acid; 5-HIAA, respectively) were measured simultaneously in brain and intestinal tissue by HPLC with electrochemical

detection using an Alexys 100 LC-EC system (Antec, Lelystad, The Netherlands) as previously described [28]. Tissue was sonicated in 50–100 µL ice-cold solution containing 5 µM clorgyline, 5 µg/mL glutathione and 0.6 µM N-methylserotonin (NMET, internal standard). To 50 µL homogenate, 12.5 µL 2 M $HClO_4$ was added and subsequently, 10 µl 2.5 M potassium acetate. After 15 min in ice water, the homogenates were centrifuged for 10 min at 15,000 × g (4 °C). The HPLC system consisted of a pump model P100, an autosampler model AS300 (both from Thermo Separation Products, Waltham, MA, USA), A ERC-3113 degasser (Erma CR. Inc., Tokyo, Japan), an ESA Coulochem II detector with 5011 analytical cell set at potential +450 mV (ESA Inc., Bedford, MA, USA), a data acquisition system (Atlas 2003, Thermo Electron Corporation, Brussels, Belgium) and a column (150 mm × 4.6 mm i.d.) packed with Hypersil BDS C18, 5 µm particle size (Alltech Associates, Deerfield, IL, USA). The mobile phase solution consisted of 50 mM citric acid, 50 mM phosphoric acid, 0.1 mM EDTA, 45 µl/L dibutylamine, 77 mg/L 1-octanesulfonic acid sodium salt, 10% methanol (pH = 3.4). Separation was performed at 45 °C using a flow rate of 0.7 mL/min. The concentration of each compound was calculated by comparison with both the internal and the external standards. The limit of detection (signal/noise ratio 3:1) was 0.3 nM. Turnovers of DA and 5-HT were calculated by dividing metabolite concentrations by monoamine concentrations ((DOPAC + 3-MT + HVA)/DA and 5-HIAA/5-HT, respectively).

2.8. Immunohistochemistry for 5-HT

Ileal and colonic tissue was removed, opened longitudinally, rolled and fixated with 10% formalin for at least 24 h. Formalin-fixed, paraffin-embedded tissue sections (5 µm) were incubated with 0.3% H_2O_2 in methanol for 30 min, rehydrated and incubated for 5 min with proteinase K solution (Dako, Enschede, The Netherlands). Non-specific background was blocked with 5% goat serum and sections were incubated overnight at 4 °C with rabbit anti-5-HT (1:8000 Sigma–Aldrich, Zwijndrecht, the Netherlands). Next day, sections were incubated with biotinylated goat anti-rabbit (1:200, Dako, Enschede, The Netherlands), followed by ABC–HRP complex (Vector Laboratories, Peterborough, UK). Staining was visualized using 0.05% DAB solution for 10 min and sections were counterstained with Mayer's haematoxylin (Merck Millipore, Amsterdam, The Netherlands). Digital images were captured with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera, at a magnification of 20 times. 5-HT positive cells in the epithelial layer of the intestinal mucosa were counted in 15 consecutive villi or crypts, for ileum and colon, respectively, at three different places

(proximal, middle and distal) in the intestinal swiss roll. Data were expressed as the number of 5-HT positive cells per 10 villi or crypts for ileum and colon, respectively.

2.9. Statistical analysis

Differences between groups were statistically determined with an unpaired two-tailed Student's t-test and experimental results are expressed as mean ± S.E.M. As data on the humoral response (Fig. 2) and latency until first occurrence in the interaction zone (Fig. 3C) were not normally distributed, differences between groups were statistically determined using a Mann–Whitney test and presented in a Box-and-Whisker Tukey plot. Results were considered statistically significant when $P < 0.05$. Analyses were performed using GraphPad Prism, version 5.03.

3. Results

3.1. Allergic response to oral whey challenge in whey-sensitized mice

The allergic response was assessed by measuring mMCP-1 levels as a marker for mucosal mast cell degranulation in serum collected 16 h after challenge. Serum mMCP-1 concentrations were increased in allergic whey-sensitized mice compared to control sham-sensitized mice ($P < 0.001$, Fig. 2A). Furthermore, levels of whey-specific IgE ($P < 0.001$, Fig. 2B), IgG1 ($P < 0.001$, Fig. 2C) and IgG2a ($P < 0.001$, Fig. 2D) were increased in serum of allergic mice compared to control mice.

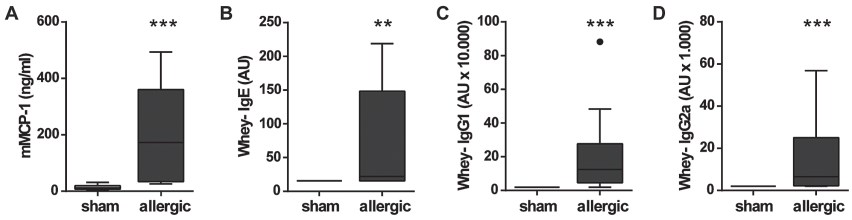

Fig. 2. Humoral response to oral whey challenge in whey-sensitized allergic mice and sham-sensitized control mice. Serum levels of mouse mast cell protease-1 (mMCP-1) (A) and arbitrary units (AU) of whey-specific immunoglobulin IgE (B), IgG1 (C) and IgG2a (D) were significantly increased in whey-sensitized allergic mice compared to sham-sensitized control mice. Mann–Whitney test was conducted and data are presented as Box-and-Whisker Tukey plots. $^{**}P < 0.01$, $^{***}P < 0.001$, n = 10 per group.

3.2. Food allergic mice display reduced social interaction and increased repetitive behaviour

To assess the effect of the allergic response on social behaviour, mice were exposed to a social interaction test, 14 h after challenge with the food allergen (Fig. 3A). Whey-sensitized allergic mice spent as much time in the interaction zone in absence of a social target, compared to sham-sensitized control mice ($P = 0.83$, Fig. 3B). However, in presence of a social target, allergic mice spent significantly less time in the interaction zone, compared to control mice ($P < 0.05$). Furthermore, latency of first approach to the social target was significantly increased in allergic mice compared to control mice ($P < 0.05$, Fig. 3C), while latency was not affected in absence of a social target ($P = 0.92$). Locomotor activity was not altered in allergic mice compared to control mice (no target: $P = 0.65$, target: $P = 0.98$, Fig. 3D). Moreover, reduced social interaction was not observed in a dextran sodium sulphate (DSS)-induced mouse model for colitis (Supplementary data), suggesting that reduced social interaction is not a general result of intestinal inflammation. DSS treatment, however, did reduce locomotor activity, compared to controls.

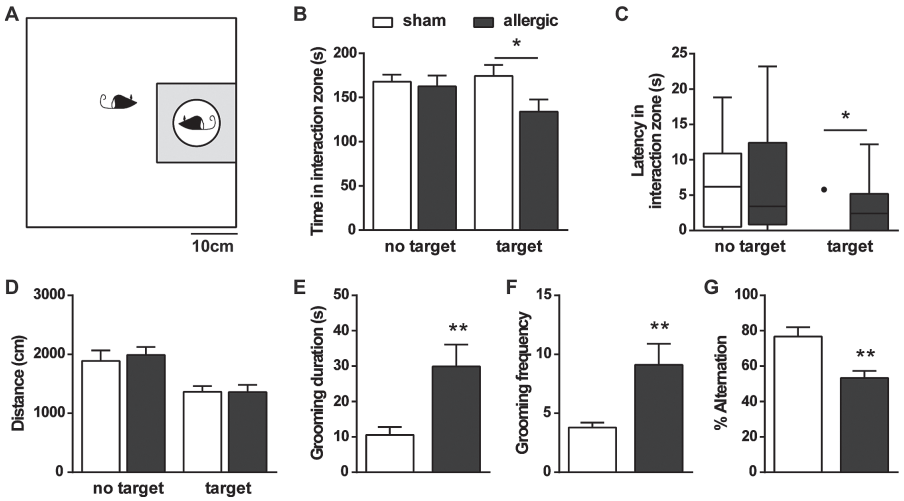

Fig. 3. Behaviour of food allergic mice compared to control mice. (A) Schematic representation of the social interaction test, illustrating location and size of the interaction zone (grey rectangle) and the cage (white circle) in which a gender- and age-matched unfamiliar mouse (target) was placed. Compared to sham-sensitized control mice, whey-sensitized allergic mice spent significantly less time in the interaction zone (B) and it took longer for them to approach the target mouse for the first time (C). One allergic animal was excluded from Fig. 2C as a significant outlier (Grubbs' test), because it took him 181 s before entering the interaction zone for the first time. Locomotor activity, measured by total distance moved, was similar in both groups (D). Cumulative time spent

grooming (E) and frequency of grooming (F), during a 5 min session in an empty home cage without bedding, were significantly increased in food allergic mice compared to controls. Spontaneous alternation ratio in the T maze, defined by the amount of trials in which an animal alternated divided by the total amount of trials, was significantly decreased in food allergic mice compared to controls (G). (B and D–G) Student's t-test was conducted and data are presented as mean ± SEM. (C) Mann–Whitney test was conducted and data are presented as Box-and-Whisker Tukey plots. *P < 0.05, **P < 0.01, (B–F) n = 10 per group, (G) n = 6 per group.

To further investigate behaviour of whey-sensitized allergic mice, self-grooming was scored as a measure of repetitive behaviour. Food allergic mice placed individually in a novel empty cage spent more time self-grooming than controls. Both cumulative time ($P < 0.01$, Fig. 3E) and frequency ($P < 0.01$, Fig. 3F) of grooming was significantly increased in allergic mice compared to controls.

Spontaneous alternation was tested in a T-maze set-up, as a measure for spatial memory [29] and/or to strengthen repetitive behaviour [30] and [31] in food allergic mice. In contrast to control mice that tend to choose the other arm after a successive introduction in the T-maze, food allergic mice alternated to a significantly lower rate ($P < 0.01$, Fig. 3G).

3.3. Neuronal activation is increased in the PFC and reduced in the PVN of allergic mice

Next, c-Fos expression was measured in the brain as a marker of neuronal activation, 2 h after social interaction. Compared to control mice, robust c-Fos induction was observed in the lateral and ventral orbital PFC (oPFC) of food allergic mice after exposure to a social target ($P < 0.05$, Fig. 4A and C). This induction was restricted to these orbital areas, since no change in neuronal activation was observed in the medial orbital (MO), prelimbic (PrL) and cingulate (Cg1) areas of the PFC. Furthermore, allergic mice showed a blunted c-Fos response in the PVN compared to control mice ($P < 0.05$ Fig. 4B and D). No differences in neuronal activation were observed in nuclei of the amygdala (data not shown).

3.4. Dopaminergic activity is decreased in the prefrontal cortex and increased in the amygdala of allergic mice

To determine the effect of the allergic immune response on the dopaminergic and serotonergic systems in the brain, monoamine and metabolite levels were measured in tissue homogenates of PFC, PVN and amygdala, 16 h after oral challenge. A significant decrease in levels of DA ($P < 0.05$, Fig. 5A) and its metabolites DOPAC, 3-MT and HVA ($P < 0.01$) were observed in the PFC of allergic mice compared to control mice. Although levels of 5-HT were not changed in the PFC ($P = 0.78$, Fig. 5B) the concentration of 5-HIAA

was significantly decreased in allergic mice compared to controls ($P < 0.05$). However, the proportion of change was marginal compared to the DA changes.

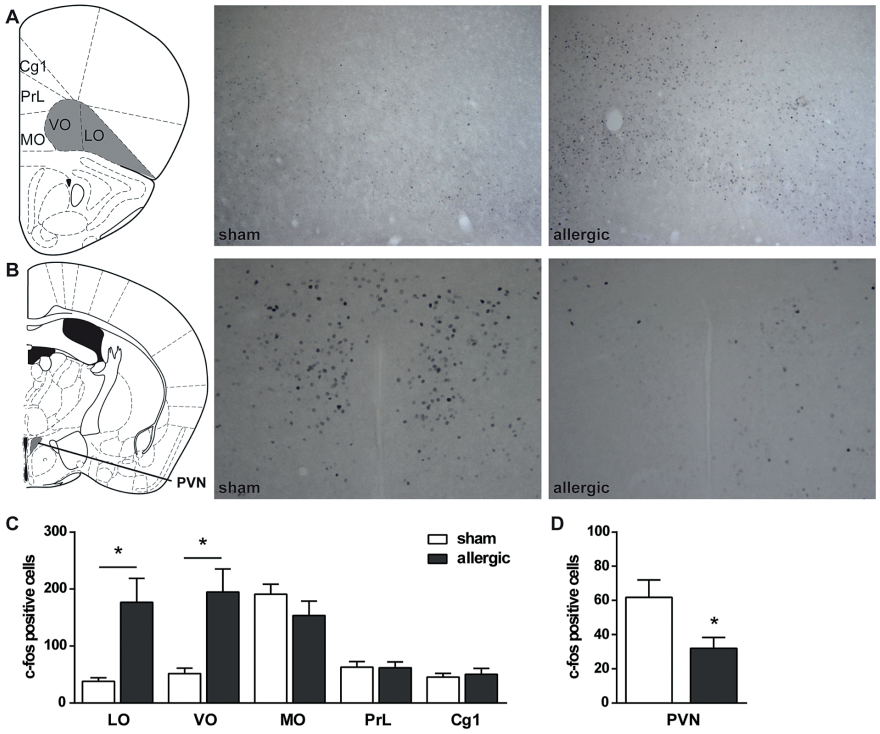

Fig. 4. Neuronal activation in the prefrontal cortex (PFC) and hypothalamic paraventricular nucleus (PVN) of food allergic mice, 2 h after the social interaction test. (A and C) Expression of c-Fos was significantly increased in whey-sensitized allergic mice compared to sham-sensitized control mice in the lateral (LO) and ventral (VO) orbital PFC, but not in the medial orbital (MO), prelimbic (PrL) and cingulate area 1 (Cg1) regions of the PFC. (B and D) Decreased c-Fos expression was observed in the PVN of allergic mice, compared to control mice. Student's t-test was conducted and data are presented as mean ± SEM. *$P < 0.05$, $n = 5$ per group.

Food allergy did not induce alterations in the dopaminergic (Fig. 5C) or the serotonergic (Fig. 5D) systems in the PVN. In the amygdala, a tendency towards an increase in DA levels ($P = 0.06$, Fig. 5E) and a significant increase in metabolite levels of DOPAC ($P < 0.05$), but not 3-MT ($P = 0.36$) and HVA ($P = 0.23$), were observed in allergic mice compared to control mice. Levels of 5-HT ($P < 0.05$, Fig. 5F), but not 5HIAA ($P = 0.35$), were increased in the amygdala of allergic mice compared to controls. Levels of tryptophan and turnover rates of 5-HT and DA in all three brain areas remained unaffected by a food allergic response in the intestine (data not shown).

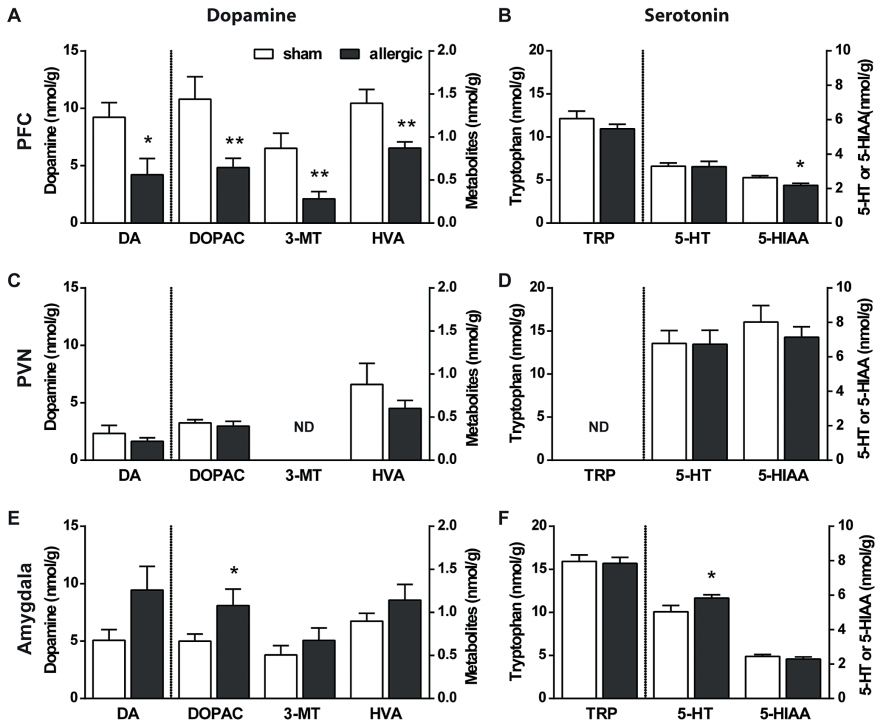

Fig. 5. Monoamine and metabolite levels in prefrontal cortex (PFC), hypothalamic paraventricular nucleus (PVN) and amygdala of food allergic mice. Concentrations of dopamine (DA), serotonin (5-HT; 5-hydroxytryptamine), their metabolites (3,4-dihydroxyphenylacetic acid; DOPAC, 3-methoxytyramine; 3-MT, and homovanillic acid; HVA and 5-hydroxyindoleacetic acid; 5-HIAA, respectively) and tryptophan (TRP) in brain tissue homogenates of the PFC (A and B), PVN (C and D) and amygdala (E and F) of whey-sensitized allergic mice compared to sham-sensitized control mice. Student's t-test was conducted and data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ND = not detectable, n = 8 per group.

3.5. Increased levels of 5-HT and number of 5-HT positive cells in the intestinal tract of food allergic mice

Considering the importance of the 5-HT system in intestinal neuroimmune interactions, levels of 5-HT and 5-HIAA were also measured in ileum and colon, 16 h after oral challenge. In the ileum of allergic mice, a significant increase in 5-HT levels was observed ($P < 0.05$, Fig. 6A), accompanied by decreased 5-HIAA levels ($P < 0.01$, Fig. 6B), leading to a reduced 5-HT turnover ($P < 0.01$, Fig. 6C). However, no significant changes were observed in 5-HT and 5-HIAA levels in colon of food allergic mice. Immunohistochemistry of 5-HT revealed that an increased number of 5-HT positive cells was present in the epithelial layer of both ileum ($P < 0.05$, Fig. 6D and E) and colon ($P < 0.05$, Fig. 6D and F) of allergic mice, compared to control mice. In contrast, number of 5-HT positive cells in the lamina

propria remained unaffected (data not shown), suggesting that there is an increase in EC cells, but not in other 5-HT positive cells.

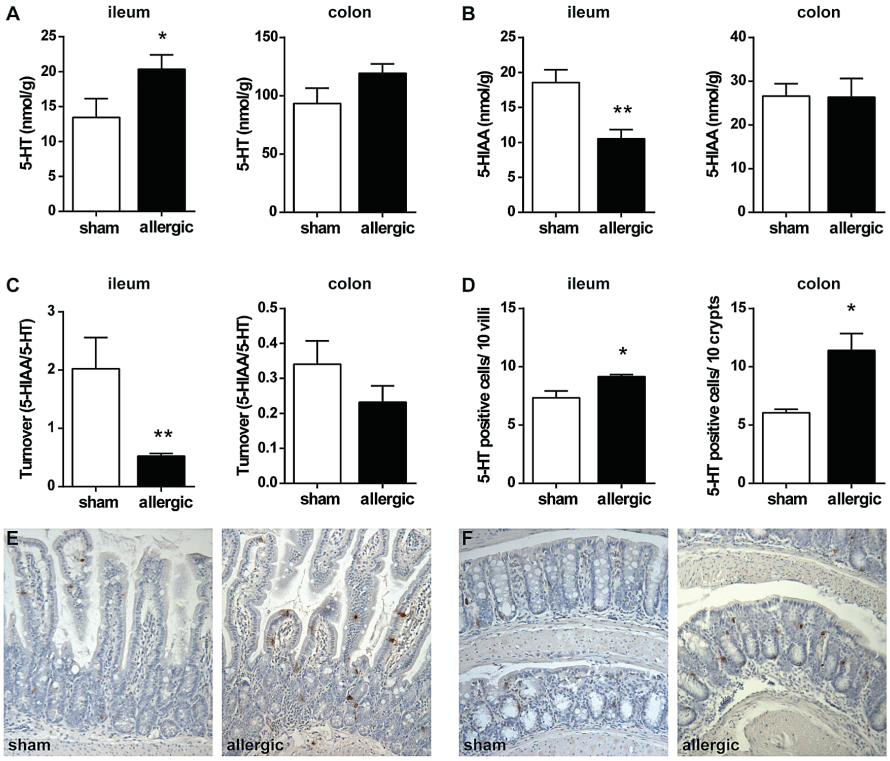

Fig. 6. Levels of serotonin (5-HT; 5-hydroxytryptamine) and 5-HIAA (5-hydroxyindoleacetic acid) and number of 5-HT positive cells in ileum and colon of food allergic mice. In ileum of whey-sensitized allergic mice, significantly increased concentrations of 5-HT (A) and decreased concentrations of 5-HIAA (B) were observed in the ileum, resulting in reduced 5-HT turnover rate (C), compared to sham-sensitized control mice. No significant differences were found in colon of allergic compared to control mice. The number of 5-HT positive cells in the epithelial layer was increased in both ileum (E) and colon (F) of allergic mice compared to control mice (D). Quantification was presented as number of 5-HT positive cells per 10 villi or crypts for ileum or colon, respectively. Student's t-test was conducted and data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, (A–C) n = 8 per group, (D–F) n = 4 per group.

4. Discussion

There is little fundamental evidence showing direct effects of food allergic immune responses on social and repetitive behaviour. In the present study, we showed that an IgE-mediated allergic immune response in the intestinal tract of mice, induced shortly after weaning, is associated with disturbed social interaction, without any overt signs of sickness. This effect was specific for food allergy, as social behaviour was not changed in DSS-induced colitis mice. Notably, the social interaction data may be confounded by the observation that DSS-treated mice had reduced locomotor activity, meaning that DSS treatment may actually stimulate social interaction. In addition to social behavioural deficits, food allergic mice showed increased self-grooming, indicative for increased repetitive behaviour, and reduced alternation in the T maze, which is not exclusively a test for spatial memory, but also involves the willingness to explore novel environmental stimuli [32] and [33] and repetitive behaviour [31] and [34].

A food allergic reaction in the intestine increased levels of 5-HT in the ileum of food allergic mice compared to controls. This increase in 5-HT levels may be explained by an increase in the number of enterochromaffin (EC) cells that was observed in food allergic mice, as the vast majority of intestinal 5-HT is stored in EC cells [35]. In colon of food allergic mice, the number of EC cells was doubled when compared to controls, while 5-HT was not significantly increased, suggesting that EC cells produce less 5-HT per cell. Mast cells are also important sources of 5-HT, in particular during an allergic reaction. Serum levels of mMCP-1 were significantly increased in food allergic mice and previous studies showed that this was accompanied by decreased mMCP-1 positive mast cells in the intestines [36], indicating that intestinal mast cells are degranulated after oral challenge. Although no increase in the number of lamina propria 5-HT positive cells in ileum and colon was observed in this model, increased numbers of intestinal mast cells have been described in food allergic mice [37]. Therefore, additional to the increased number of EC cells, mast cells may also contribute to the increased 5-HT levels observed in the intestines of allergic mice. Increased 5-HT levels were accompanied by decreased levels of 5-HIAA in ileum of food allergic mice. Intestinal 5-HT is taken up by the serotonin transporter (SERT) on epithelial cells, where most intestinal 5-HT is degraded into 5-HIAA [35]. Therefore, reduced levels of 5-HIAA may be explained by decreased SERT activity, or reduced availability of extracellular 5-HT. A decrease in SERT activity would predict lower 5-HT uptake and metabolism in the mucosa, decreasing intestinal 5-HIAA/5-HT ratio and diverting 5-HT to metabolism in the liver or lung or

uptake by platelets. A pro-inflammatory environment in the intestine is thought to decrease SERT activity, as activity was reduced in in vitro cultures of intestinal epithelial cells and immune cells upon exposure to lipopolysaccharides and cytokines such as tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-4 (IL-4) [38], [39] and [40]. EC cell hyperplasia and increased levels of intestinal 5-HT accompanied by depressed levels of 5-HIAA, and thus a reduced turnover rate, were also observed in intestinal biopsies of patients with coeliac disease [41] and IBS [42]. Interestingly, this disturbance in intestinal 5-HT metabolism in both diseases is associated with increased platelet 5-HT, a repeated finding in approximately 30% of patients with ASD [43].

Signalling molecules produced by the intestinal mucosal and immune cells, including cytokines, neuropeptides and neurotransmitters, can activate their respective receptors on extrinsic vagal and spinal afferents to signal to the brain stem and spinal cord, respectively [44]. 5-HT produced by EC cells and mast cells are an example of such a signalling molecule. Extrinsic vagal afferents are in sufficient proximity to the epithelial layer where EC cells are located [45] and to mast cells [46]. Vagal afferents respond to secrete 5-HT via 5-HT3 receptors by signalling to the nucleus tractus solitarius (NTS) and PVN [47]. Food allergy-induced c-Fos expression in rat brains was reduced after blockade of 5-HT3 receptors and vagotomy [48]. This suggests that 5-HT released during a food allergic response acts through 5-HT3 receptors on vagal afferents to affect neuronal activation in the brain. However, administration of a 5-HT3 receptor antagonist partially reversed food allergy-induced aversion to food [49], indicating that signalling to the brain is not solely mediated by release of 5-HT. Moreover, DSS-induced colitis mice did not show impaired social behaviour, while increased 5-HT release has been reported in these mice [50]. Therefore, we hypothesize that immune signalling to relevant brain regions involved in the regulation of social behaviour is mediated by a combination of various allergy-induced signalling molecules, including 5-HT. Indeed, for example mast cell release of histamines and prostaglandins were also shown to stimulate enteric neurons [51]. Furthermore, IgE can bind to the functional high-affinity FcεRI on neurons [52], resulting in an antigen-specific neuronal stimulation [53]. Unravelling which allergy-induced signalling molecules are responsible for impaired social behaviour would require further investigation of immune–nerve interactions in the intestines of food allergic mice.

In contrast to the profound effects of food allergy on the 5-HT system in the intestinal tract, changes in central 5-HT systems were less pronounced that changes observed in

the DA system. Of note is the practical limitation of measuring monoamines in tissue homogenates, omitting information about monoamine levels in the neuronal synaptic cleft. Microdialysis studies in this murine model could provide more insight into synaptic transmission of 5-HT in the brain. Nevertheless, pronounced changes in the mesocorticolimbic DA system were observed. Results suggest that DA and metabolite levels were decreased in the PFC and increased in amygdala of allergic mice compared to controls. While multiple neural systems undoubtedly underlie social–emotional behaviours, the mesocorticolimbic DA system seems a crucial pathway involved [54]. This mesocorticolimbic DA system originates in the ventral tegmental area (VTA) of the midbrain and projects to various forebrain regions including PFC, amygdala and nucleus accumbens. Socially rewarding stimuli activate the mesocorticolimbic DA system, resulting in feelings of desire, wanting and excitement [55] and [56]. Evidence for involvement of the mesocorticolimbic DA system in the pathophysiology of ASD is limited, but it is reported that children with ASD display reduced levels of DA in the medial PFC, as assessed with PET [57]. Furthermore, dampening of the DA system in the PFC was also observed in a fragile X mouse model for ASD [58]. Multiple studies revealed that social interaction in rodents is accompanied by increased DA levels in the PFC [59] and [60] and depletion of D in the PFC markedly reduces social interactions [61]. Moreover, abnormal vagal functioning inhibits the DA system, but not 5-HT, in various brain regions, including PFC [62], indicating that vagal input can affect the mesocorticolimbic DA system. Overall, this suggests that the observed dampening of the DA system in the PFC of food allergic mice may underlie their reduced social behaviour and that this attenuation of the DA system in the PFC could be mediated by intestinal signalling through the vagus nerve.

Marked neuronal activation was observed in the LO and VO regions of the PFC of food allergic mice, after exposure to a social target. The oPFC is involved in cognitive processing of decision making in response to emotional stimuli that can have a rewarding or punishing value. Therefore, it is also important in guiding social–emotional behaviour [63] and [64]. In line with our observations, patients with ASD showed increased oPFC activation in response to tasks involving facial recognition [65], motor function [66] and attention [67]. Furthermore, patients with orbitofrontal lesions have impaired abilities to recognize and interpret emotional expressions and to respond in a socially proper manner [68], [69], [70] and [71]. Since the oPFC guides social–emotional behaviour, enhanced neuronal activation in the oPFC of food allergic mice, as observed in the present study, may be implicated in their reduced social behaviour. However, based on

the c-Fos staining we cannot speculate about the subtype of these neurons that are activated in the oPFC of allergic mice, as many different subtypes exist in the oPFC, and whether these are excitatory or inhibitory neurons.
Basso et al. [72] reported increased neuronal activation of the PVN after sensitization and challenge with ovalbumin allergen. However, c-Fos expression was measured 90 min after oral challenge, whereas in our study, c-Fos expression was determined after a social interaction test. Exposure to a social target elicits a specific neuronal activation pattern, making it impossible to compare these studies. In out study, decreased neuronal activation was observed in the hypothalamic PVN of food allergic mice after exposure to a social target. PVN neurons release oxytocin, a hormone that has numerous effects in the body, but also acts as neuromodulation in the brain. Oxytocin is released upon positive social interactions and enables individuals to overcome their natural avoidance of social approach [73]. Deficits in oxytocin may partly underlie social impairments found in patients with ASD, since decreased levels of peripheral oxytocin in ASD patients have been observed [74] and oxytocin administration improved social cognition in ASD patients [34]. Furthermore, oxytocin injection into the VTA of rats caused increased DA and DOPAC levels in medial PFC, while levels of 5-HT and 5-HIAA remained unaffected [75] and [76]. Therefore, oxytocin production by the PVN can also regulate dopaminergic neurotransmission in the PFC [77], which could be of importance in our study, but requires further investigation.

In conclusion, this study provides evidence that a food allergic reaction reduces social behaviour, increased repetitive behaviour and impaired spontaneous alternation in mice, accompanied by neuronal and dopaminergic changes in the brain and serotonergic changes in the intestine. We hypothesize that an allergic response regulates complex, but critical, intestinal neuroimmune interactions involving 5-HT and consequently affecting brain circuits involved in social behaviour. Together with a genetic predisposition and multiple environmental factors, these effects of allergic immune activation may exacerbate behavioural abnormalities in patients with ASD. Therefore, the intestinal tract could be a potential target in the treatment of patients with ASD and comorbid food allergic symptoms.

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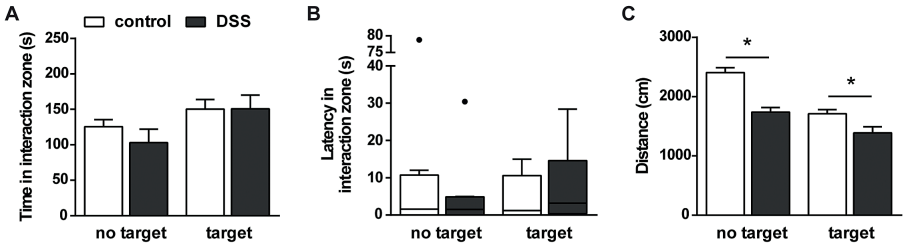
Supplementary information

DSS-induced colitis model

Colonic inflammation was induced in male C57Bl6 mice (8-12 weeks old, Charles River Laboratories) by administration 2% (w/v) dextran sodium sulfate (DSS, MW= 36.000-50.000, MP Biomedicals, Aurora, OH, USA) in tap water *ad libitum* for 5 consecutive days followed by 2 days of normal drinking water. The DSS solution was refreshed every other day. Control mice received drinking water without DSS. The presence of blood in stool, stool consistency and body weight were recorded daily for each animal. The morning after two days of drinking water, mice were exposed to a social interaction test (DSS-induced colitis model, n=8 per group).

Social interaction is not changed in DSS-induced colitis mice

To investigate whether deficits in social interaction are a general result of intestinal inflammation, social interaction was also measured in a murine model for DSS-induced colitis. At the day of the social interaction test, all DSS-treated mice, but no control mice, suffered from clinic-pathological signs of colitis such as blood in stool, diarrhea and loss of bodyweight, collectively resulting in a significant increase in disease activity score (median [IQR]: 0.0[0.0-0.0] and 3.0[3.0-3.8] for control and DSS respectively, P<0.001). In the social interaction test, DSS-induced colitis mice spent as much time in the interaction zone as control mice, regardless of the presence or absence of an unfamiliar social target (figure S1A). Also latency until first occurrence in the interaction zone (figure S1B) was not affected by DSS treatment. Locomotor activity of DSS mice, however, was significantly reduced both when a target mouse was absent (P<0.05) and present (P<0.05) compared to their healthy controls (figure S1C).



Supplementary figure S1. Social behaviour of DSS-induced colitis mice compared to control mice. DSS-induced colitis mice spent as much time in the interaction zone as control mice (**A**) and latency of first occurrence in the interaction zone was not significantly changed DSS mice compared to control mice (**B**). Locomotor activity, measured by total distance moved, was significantly reduced in DSS-induced colitis mice compared to controls (**C**). **A, C**: Student's t-test was conducted and data are presented as mean ± SEM. **B**: Mann-Whitney test was conducted and data are presented as box-and-whisker Tukey plots. * P<0.05, n=8 per group.

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CHAPTER FOUR

mTOR plays an important role in cow's milk allergy-associated behavioral and immunological deficits

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Abstract

Autism spectrum disorder (ASD) is multifactorial, with both genetic as well as environmental factors working in concert to develop the autistic phenotype. Immunological disturbances in autistic individuals have been reported and a role for food allergy has been suggested in ASD. Single gene mutations in mammalian target of rapamycin (mTOR) signaling pathway are associated with the development of ASD and enhanced mTOR signaling plays a central role in directing immune responses towards allergy as well. Therefore, the mTOR pathway may be a pivotal link between the immune disturbances and behavioral deficits observed in ASD. In this study it was investigated whether the mTOR pathway plays a role in food allergy-induced behavioral and immunological deficits.

Mice were orally sensitized and challenged with whey protein. Meanwhile, cow's milk allergic (CMA) mice received daily treatment of rapamycin. The validity of the CMA model was confirmed by showing increased allergic immune responses. CMA mice showed reduced social interaction and increased repetitive self-grooming behavior. Enhanced mTORC1 activity was found in the brain and ileum of CMA mice. Inhibition of mTORC1 activity by rapamycin improved the behavioral and immunological deficits of CMA mice. This effect was associated with increase of Treg associated transcription factors in the ileum of CMA mice.

These findings indicate that mTOR activation may be central to both the intestinal, immunological, and psychiatric ASD-like symptoms seen in CMA mice. It remains to be investigated whether mTOR can be seen as a therapeutic target in cow's milk allergic children suffering from ASD-like symptoms.

1. Introduction

The mammalian target of rapamycine (mTOR) is an evolutionarily conserved phosphatidylinositol-3-OH kinase (PI(3)K)-related kinase that plays a central role in the regulation of cell growth and metabolism (Wullschleger et al., 2006), protein synthesis, cell proliferation and survival (Hay and Sonenberg, 2004). mTOR acts as the core subunit of two functionally distinct multi-protein signaling complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Hay and Sonenberg, 2004; Sengupta et al., 2010; Wullschleger et al., 2006). The activation of mTORC1 by immunological and environmental cues is mediated through the PI3K/Akt signaling pathway leading to phosphorylation and inhibition of tuberous sclerosis complex 2 (TSC2) and the subsequent suppression of GTPase activity of RHEB (RAS homologue enriched in brain), which directly stimulates the catalytic activity of mTOR in mTORC1 (Fig. 1). Stimulation of mTOR in mTORC1 causes the phosphorylation of the two translational regulators: protein p70 S6 kinase (p70 S6K) and the eIF-4E binding protein 1(4E-BP1) (Fig. 1). In this way, mTORC1 regulates protein synthesis and cell growth (Fingar and Blenis, 2004a). Recent findings in animal models showed that mTOR activity gain-of-function mutations in tuberous sclerosis complex (TSC) (Ehninger et al., 2008), phosphatase and tensin homolog (PTEN) (Kwon et al., 2006; Zhou et al., 2009) and Fragile X mental retardation protein (FMRP) (Nimchinsky et al., 2001) were all strongly associated with neurodevelopmental disorders including autism spectrum disorders (ASD). Rapamycin treatment in these genetic murine models for ASD was shown to rescue the ASD-related behavioral deficits and several neurological impairments by targeting the altered mTOR signaling pathway (Ehninger et al., 2008; Zhou et al., 2009). The etiology of most neuropsychiatric disorders, including ASD, is considered to be caused by an interaction of genetic disturbances and environmental factors. Currently, disturbances in the immune system, such as those leading to food allergy, are proposed as an important environmental risk factor (Kennedy et al., 2012; Theoharides, 2013). Increasing evidence suggests that the immune disturbances in the gastrointestinal tract of patients with ASD can influence brain functioning via a number of pathways that connect the brain and the gut (Kennedy et al., 2012). Recently, it was also demonstrated that cow's milk allergic mice display ASD-like behavioural and neurochemical deficits (de Theije et al., 2014b). However, more research is needed to elucidate the underlying mechanism regarding the correlation of the gastrointestinal immune problems with ASD. Currently, more evidence is emerging that apart from the involvement in neurological disorders, mTOR signaling pathway also plays an important role in immunological function.

mTOR is known to have a role in macrophage polarization (Byles et al., 2013), regulating T cell balance and survival (Delgoffe et al., 2011), differentiation (Delgoffe et al., 2011), function and activation of mast cells (Kim et al., 2009). Therefore, the current study investigated the involvement of mTOR signaling pathway in behavioral changes as well as allergic immune responses and the development of Treg cells in CMA mice. The effect of rapamycin on enhanced mTOR signaling pathway in the brain as well as in the intestine was examined.

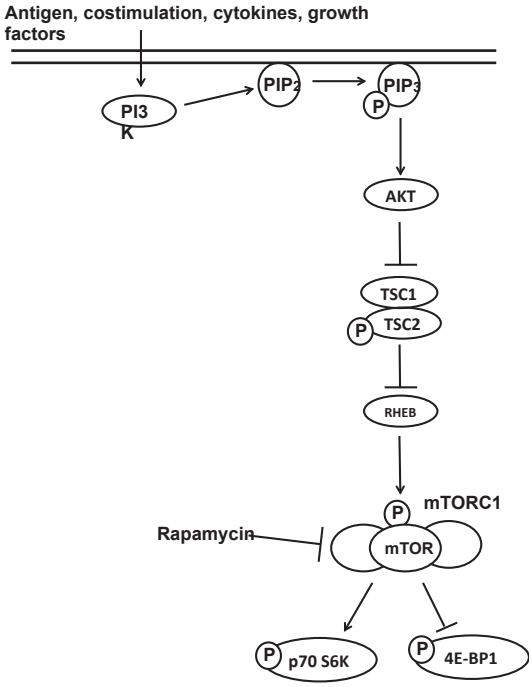


Fig. 1. Schematic illustration of mTORC1 pathway. mTORC1 activation starts with the binding of antigen, costimulatory molecules, cytokines, toll like receptor ligands, and growth factors to the receptor on the cell membrane, leading to the phosphorylation and activation of PI3K and AKT. AKT inhibits TSC2 activity by phosphorylation, which suppresses GTPase activity of RHEB. RHEB directly induces mTOR phosphorylation and thereby the formation of mTORC1, eventually leading to the phosphorylation of two downstream effectors: p70 S6K and 4E-BP1. In this figure, activating phosphorylation events are indicated by arrows, while inhibitory phosphorylation events are indicated by flat-ended lines.

2. Methods and Materials

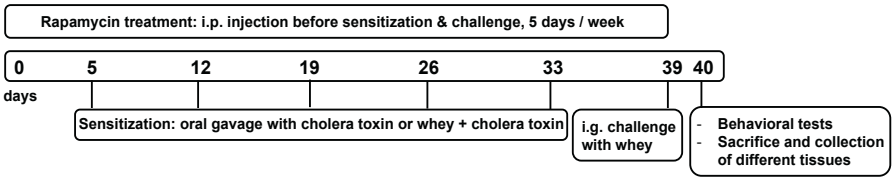
2.1 Animal model and treatment protocol*s*

Three-week-old pathogen free male C3H/HeOuJ mice were purchased from Charles River Laboratories (L'Arbresle Cedex, France) and housed at the animal facility of Utrecht

University. Mice were fed a cow's milk free diet (Special Diet Services, Witham, UK). The murine model of CMA was induced as described previously (Fig. 2A) (de Theije et al., 2014b; Schouten et al., 2010). In short, mice were sensitized intragastrically (i.g.) with 20 mg whey (DMV International, Veghel, The Netherlands) / 0.5 mL PBS (Cambrex Bio Science, Verviers, Belgium) containing 10 µg cholera toxin (CT, List Biological Laboratories, Campbell, CA, USA) as an adjuvant. Sham-sensitized mice received CT alone. Mice were sensitized once a week for 5 consecutive weeks. One week after the last sensitization, sham and whey- sensitized mice were challenged i.g. with 50 mg whey/ 0.5 mL PBS and behavioral tests were conducted the next day. Rapamycin (LC laboratories, Woburn, MA, USA) was dissolved in 100% ethanol, stored at a stock concentration of 20 mg/mL in aliquots at -20 °C. Prior to each administration, working solutions of rapamycin were prepared in 5% Tween-80 (Sigma-Aldrich, Zwijndrecht, The Netherlands), 5% PEG 400 (Sigma-Aldrich, Zwijndrecht, The Netherlands), and 4% ethanol (Sigma-Aldrich, Zwijndrecht, The Netherlands). Mice, CMA and control groups, were injected intraperitoneally with either rapamycin (0.5 mg/kg, 2 mg/kg or 4 mg/kg body weight) or vehicle once per day for 5 consecutive days per week. The results described in this report were obtained from 2 independent experiments (Fig. 2B).

All animal procedures were approved by and conducted in accordance with the guidelines of the Animal Ethics Committee of Utrecht University (approval number: 2011.I.04.045 and 2012.I.04.054).

A



B

Sensitization	Challenge	Treatment	Sample size per group	
			Experiment 1	Experiment 2
Cholera toxin + whey	Whey	0.5 mg/kg rapamycin	n = 7	n = 10
Cholera toxin + whey	Whey	2 mg/kg rapamycin	n = 7	n = 10
Cholera toxin + whey	Whey	4 mg/kg rapamycin	n = 7	–
Cholera toxin	Whey	2 mg/kg rapamycin	–	n = 10
Cholera toxin	Whey	4 mg/kg rapamycin	n = 7	–
Cholera toxin + whey	Whey	Vehicle	n = 7	n = 10
Cholera toxin	Whey	Vehicle	n = 7	n = 10

Fig. 2. Schematic representation of experimental design (A) and overview of treatment groups (B)

2.2 Behavioral tests

Social interaction test and grooming tests were performed as described previously (de Theije et al., 2014b). In short, mice were individually placed in a 45 x 45 cm open field, with a small perforated Plexiglass cage (10 cm diameter) located against one wall allowing visual, olfactory and minimal tactile interaction (Fig. 3A). Mice were habituated to the open field for 5 min. and an age- and gender-matched unfamiliar target mouse was introduced in one of the cages for an additional 5 min. By using video tracking software (EthoVision 3.1.16, Noldus, Wageningen, The Netherlands), an interaction zone around the cage was digitally determined. Time spent in the interaction zone, latency until first occurrence in the interaction zone, and total distance moved were measured. In addition, the mice were scored for spontaneous grooming behaviors as described earlier (Crawley, 2012; Kas et al., 2014). Each mouse was placed individually in an empty home cage (35 cm x 20 cm) without bedding and video recordings were used for behavioral scorings of frequency and cumulative time spent grooming all body regions. After a 5 min habituation period in the cage, each mouse was scored for 5 min by two independent researchers who were blinded for treatment schedule. Inter-rater reliability was 99%. Open field was cleaned with water followed by 70% ethanol after each test.

2.3 Measurements of serum mMCP-1 and whey-specific immunoglobulins

16 Hours after oral challenge, blood of mice was collected and centrifuged for 20 min at 14,000 rpm. Serum was collected and stored at -70 °C. Concentration of mouse mast cell protease-1(mMCP-1) in serum was measured by commercially available ELISA kits (Moredun Scientific Ltd., Penicuik, UK) according to the manufacturer's protocol. Concentrations of whey-specific immunoglobulins IgE, IgG, and IgG2a in serum were measured by ELISA according to the protocol described previously (de Theije et al., 2014b). Biotin-labeled rat anti-mouse IgE, IgG1 and IgG2a were purchased from BD Biosciences (Alphen aan den Rijn, The Netherlands). Microlon plates were purchased from Greiner (Alphen aan den Rijn, The Netherlands). Carbonate/bicarbonate buffer was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Streptavidin-horse radish peroxidase was purchased from Sanquin (Amsterdam, The Netherlands). O-phenylendiamine was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Microplate reader was obtained from Bio-Rad (Veenendaal, The Netherlands).

2.4 Western blotting

After sacrificing, brains and intestinal tissues were immediately isolated from mice and snap frozen in 2-methylbutane (Sigma-Aldrich, Zwijndrecht, The Netherlands) and dry ice, stored at -70 °C after the dissection. Coronal slices of 500 µm were sectioned using a cryostat (Model700, Laméris Instruments, Utrecht, The Netherlands). Then bilateral brain regions (prefrontal cortex, amygdala, dorsal hippocampus, somatosensory cortex) were isolated from the coronal slices using a scalpel. To prepare lysates, frozen tissues were sonicated in lysis buffer containing RIPA buffer (Fisher Scientific, Landsmeer, The Netherlands), complete mini protease inhibitor cocktail tablets (Roche, Almere, The Netherlands), benzonase nuclease (Calbiochem, Amsterdam, The Netherlands), AEBSF (Calbiochem, Amsterdam, The Netherlands), and phosphatase inhibitor cocktail (Calbiochem, Amsterdam, The Netherlands). Homogenate was centrifuged at 14,000 rpm for 20 min and supernatant was collected. Protein concentration was determined using BCA kit (Pierce, Rockford, USA). For western blotting, 30 µg of sample was loaded onto Criterion TGX precast gel (Bio-Rad, Veenendaal, The Netherlands), and blotted overnight onto PVDF membrane (Bio-Rad, Veenendaal, The Netherlands), which was blocked in 5% nonfat dry milk for 1 hour. Subsequently, membranes were washed with TBS/0.1% Tween-20 (Sigma-Aldrich, Zwijndrecht, The Netherlands) 3x10 min and incubated overnight with the primary antibodies (1:1000) at 4°C. The primary antibodies against phospho-mTOR (Ser2448, #5536), mTOR (#2972), phospho-AKT (Ser473, #4060), AKT (#9272), phospho-p70 S6K (Thr389, #9205), p70 S6K (#9202), phospho-4E-BP1 (Thr37/46, #9459), and 4E-BP1 (#9452) were from Cell Signaling, Leiden, The Netherlands. The primary antibody against GAPDH (#3777R-100) was from Biovision, Uithoorn, The Netherlands. Afterwards, the membranes were washed with TBS/0.1% Tween-20 3x10 minutes and incubated with the secondary antibody polyclonal HRP-conjugated goat anti-rabbit immunoglobulins (1:5000, DAKO, Eindhoven, The Netherlands). Finally the immunoreactive bands were detected by ECL prime kit (Health Care, Amsterdam, The Netherlands) and the results were normalized with GAPDH or the non-phosphorylated corresponsing protein using quantitative densitometry (Bio-Rad, Veenendaal, The Netherlands) and reported as relative band densities. Membranes were reprobed for a maximal of 3 times with different primary antibodies after stripping the membranes with Restore Western Blot Stripping buffer (Pierce, Rockford, USA)).

As shown in figure 6 and 7, phospho-p70 S6K (Thr389) detects endogenous p70 S6K as well as p85 S6K. P70 S6K is functionally revalvant for mTOR signaling pathway as this protein is required for cell growth and G1 cell cycle grogression (Pullen and Thomas,1997). We measured the phosphorylation of p70 S6K as an readout for mTORC1 activation.

2.5 mRNA expression analysis

After sacrificing, the distal part of the jejunum and peyer's patches were isolated and stored at -70 °C until further analysis. The total RNA was isolated using the RNAeasy kit (Qiagen, Germantown, MD, USA) and stored at -20°C. Afterwards, the total RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). After cDNA synthesis, real-time PCR was performed using iQ SYBR Green supermix kit (Bio-Rad, Hercules, CA USA) with the CFX 96 Real-time system (BioRad, Hercules, CA USA). Ribosomal protein S13 (Rps13) was used as reference gene. Relative target mRNA was calculated by applying the formula: relative mRNA expression = $2^{Ct[Rps13] - Ct[target\ mRNA]}$ (García-Vallejo et al., 2004). Primers for interleukin (IL) 10, transforming growth factor (TGF)-ß and Foxp3 were commercially purchased from SABiosciences-QiagenGmbH (Hilden, Germany).

2.6 Statistical analysis

Experimental results are expressed as mean ± S.E.M. In general, differences between groups were statistically determined with a one-way ANOVA followed by a Bonferroni's multiple comparisons test. Log transferred data were used to obtain normality for one-way ANOVA when analyzing the following data: whey-specific IgE level, whey-specific IgG1 level, whey-specific IgG2a level, and mouse mast cell protease-1. As latency in interaction zone (social interaction test) did not have normal distribution, data were analysed with Kruskal-Wallis test followed by a Dunn's multiple comparisons test. Results were considered statistically significant when $P < 0.05$. Analyses were performed using GraphPad Prism, version 6.02.

3. Results

3.1 mTOR signaling pathway is implicated in the reduced social interaction and increased repetitive behavior of cow's milk allergic mice.

Previous studies using mice mutant for genes in the mTOR signaling pathway, have shown that activation of mTOR can contribute to the development of autistic phenotypes observed in ASD and related neurodevelopmental disorders (Ehninger et al., 2008; Kwon et al., 2006; Nimchinsky et al., 2001; Zhou et al., 2009). In a previous study using a murine model of CMA, de Theije and coworkers showed that whey-allergic mice displayed reduced social interaction and increased repetitive behavior compared to sham-sensitized control mice (de Theije et al., 2014b). To investigate the involvement

of the mTOR signaling pathway in the behavioral changes seen in whey-allergic mice, CMA mice were treated with different doses of rapamycin and it was analysed whether this treatment did influence behavioral tests after oral challenge with whey. Both social interaction as well as self-grooming behavior were investigated. CMA mice spent less time with the interaction mouse compared to the control mice and rapamycin treatment normalized the reduced social interaction (Fig. 3B). CMA mice also showed an increased latency of first approach to the interaction mouse compared to control mice and rapamycin reversed this increased latency (Fig. 3C). In both the habituation phase (no target) and the interaction phase (target), induction of allergy did not affect locomotor activity which was unaltered upon rapamycin treatment of the allergic mice as well (Fig. 3D). In addition, the data of control groups were analyzed separately from CMA groups. In both the habituation phase (no target) and the interaction phase (target), rapamycin had no significant effects on latency in interaction zone and total distance moved in control groups.

Besides social interaction, the novelty-induced self-grooming behavior of the mice was scored, which is a representative of repetitive behavior (Crawley, 2012, 2007; McFarlane et al., 2008). Grooming was assessed in the second experiment in which 0.5 and 2 mg/kg rapamycin have been tested. Grooming was not assessed in mice treated with the higher dose of rapamycin (4 mg/kg) because no additional effects were expected based on previous proof of concept studies that were focused on immune parameters (see Results section 3.2) and social interaction (see Fig. 3). CMA mice displayed increased cumulative grooming time and grooming frequency compared to sham-sensitized control mice. Rapamycin reversed the grooming duration dose dependently (Fig. 4A) and reduced grooming frequency (Fig. 4B) of CMA mice.

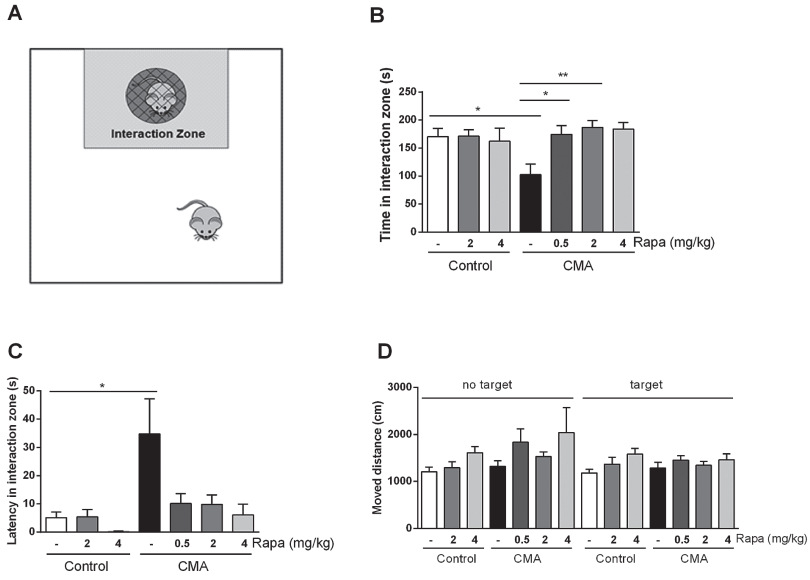


Fig. 3. (A) Schematic representation of the social interaction test. CMA mice showed reduced social interaction (B) and it took significantly more time for CMA mice to approach the interaction mouse for the first time as compared to control (C). The mobility of these mice was hardly affected in both habituation phase (no target) and interaction phase (target) (D). Rapamycin treatment reversed the social behavior of CMA mice. One-way ANOVA followed by a Bonferroni's multiple comparisons test was conducted and data are presented as mean time (s) ± SEM for (B) and mean distance moved (cm) ± SEM for (D) from two independent experiments. Kruskal-Wallis test followed by a Dunn's multiple comparisons test was conducted for (C) as latency in interaction zone was not normally distributed. $^{*}P < 0.05$. $^{**}P < 0.01$. n = 7–17 per group. (C) CMA vs CMA with 0.5 mg/kg: $P = 0.5879$; CMA vs CMA with 2 mg/kg Rapa: $P = 0.5051$; CMA vs CMA with 4 mg/kg: $P = 0.4177$.

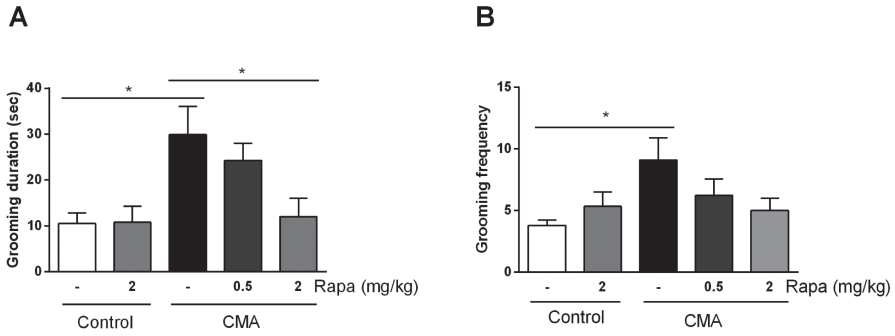


Fig. 4. CMA mice showed significantly enhanced grooming duration (A) and frequency (B) as compared to control, indicating the enhanced repetitive behavior of CMA mice. Rapamycin treatment normalized the grooming duration dose-dependently and reduced the grooming frequency of CMA mice. One-way ANOVA followed by a Bonferroni's multiple comparisons test was conducted and data are presented as mean duration (s) ± SEM for A and mean frequency ± SEM for B. $^{*}P < 0.05$, n = 10 per group. (B) CMA vs CMA with 0.5 mg/kg: $P = 0.2274$.

3.2 mTOR signaling pathway and CMA immune responses

To assess whether the mTOR signaling pathway was involved in whey-induced allergic immune responses, whey-specific serum immunoglobulins have been analysed. The whey-specific serum IgE, IgG1, and IgG2a levels were significantly increased in whey-sensitized and challenged mice in comparison to that of sham (non)-sensitized mice. Rapamycin suppressed the increased whey-specific immunoglobulin serum levels in CMA mice (Fig. 5A-C).

To further investigate the effect of rapamycin on whey-induced allergic immune response, mucosal mast cell degranulation was analysed by measuring mMCP-1 concentrations in serum. mMCP-1 is a protease derived from the mucosal mast cells and is released in the blood stream after mast cell degranulation (Pemberton et al., 2006; Wastling et al., 1998). The mMCP-1 concentration was augmented in the serum of CMA mice in comparison to that of sham-sensitized control mice, indicating an enhanced mast cell degranulation in the whey-allergic mice. The inhibition of mTOR with rapamycin suppressed mast cell degranulation as indicated by reduced mMCP-1 serum levels in CMA mice (Fig. 5D).

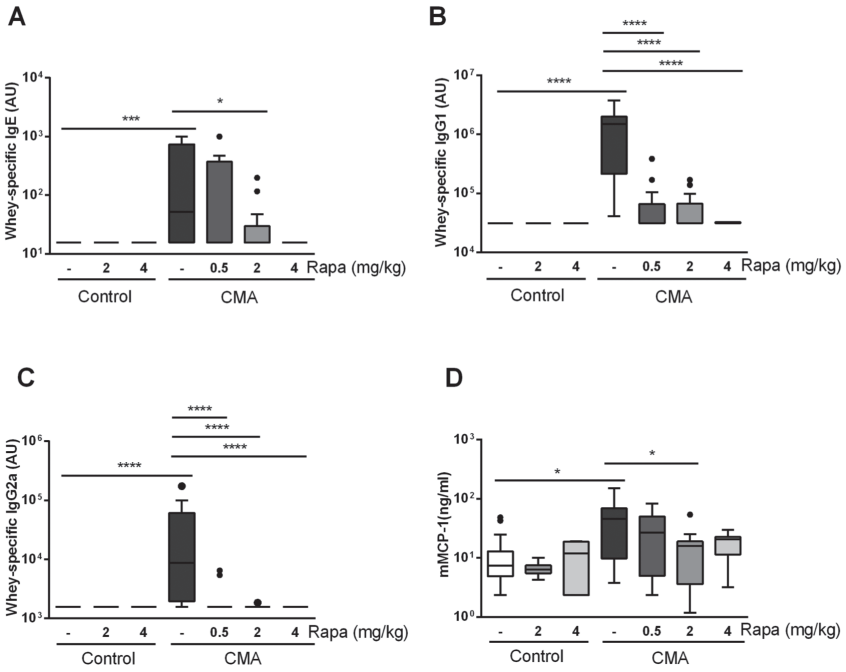


Fig. 5. Effect of rapamycin treatment on whey-induced allergic immune responses in CMA mice and sham-sensitized control mice. CMA mice showed significantly increased serum levels of arbitrary units (AU) of whey-specific immunoglobulin IgE (A), IgG1 (B), IgG2a (C) and serum concentration of mouse mast cell protease-1 (D).

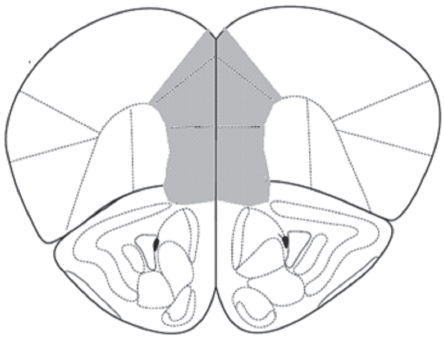
Rapamycin treatment inhibited the allergic immune responses of CMA mice. Data were log transferred to obtain normality. One-way ANOVA followed by a Bonferroni's multiple comparisons test was conducted and data are presented as Box-and-Whisker Turkey plots. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$, n = 7-17 per group. (A) CMA vs CMA with 4 mg/kg Rapa: $P = 0.1190$. (D) CMA vs CMA with 4 mg/kg Rapa: not significant.

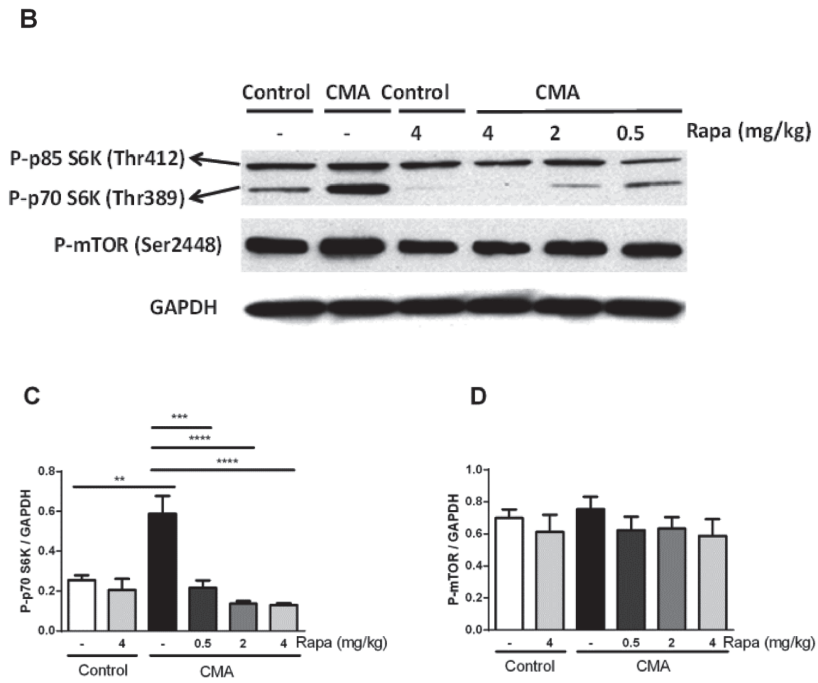
3.3 CMA is associated with enhanced mTOR signaling in the prefrontal cortex and amygdala.

Since enhanced mTOR signaling is implicated in a number of neurodevelopmental disorders including ASD, the question whether mTOR signaling was enhanced in specific brain regions of whey-allergic mice was raised. To address this question, western blotting technologies were used to measure the phosphorylation of mTOR-related proteins in the prefrontal cortex, amygdala, dorsal hippocampus, and somatosensory cortex of the mice. The phosphorylation of p70 S6K and 4E-BP1 was increased in prefrontal cortex and amygdala of CMA mice compared to sham-sensitized control mice (Fig. 6B-C & E-G; Fig. 7B-C & E-G). Rapamycin reduced the enhanced phosphorylation of p70 S6K and 4E-BP1 in prefrontal cortex and amygdala of CMA mice (Fig. 6B-C & E-G; Fig. 7B-C & E-G). The phosphorylation of mTOR (Fig. 6D & H; Fig. 7D & H) or AKT (Fig. 6I; Fig. 7I) was not significantly changed in the prefrontal cortex and amygdale of CMA mice as compared to that of sham-sensitized control mice.

The extent of phosphorylation of p70 S6K in the prefrontal cortex and amygdala is also shown to correlate with the extent of social interaction (Fig. 6J; Fig 7J). Overall, no effects of CMA or of rapamycin were observed on mTOR signaling pathway proteins in dorsal hippocampus and somatosensory cortex of the mice (data not shown).

A




B
Control
CMA
Control
CMA
- - 4 4 2 0.5 Rapa (mg/kg)
P-p85 S6K (Thr412)
P-p70 S6K (Thr389)
P-mTOR (Ser2448)
GAPDH
C
P-p70 S6K / GAPDH
0.0 0.2 0.4 0.6 0.8
- 4 - 0.5 2 4 Rapa (mg/kg)
Control
CMA
D
P-mTOR / GAPDH
0.0 0.2 0.4 0.6 0.8 1.0
- 4 - 0.5 2 4 Rapa (mg/kg)
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Control
CMA
Control
CMA
- - 2 0.5 2 Rapa (mg/kg)
P-p85 S6K (Thr412)
P-p70 S6K (Thr389)
S6K
P-4E-BP1 (Thr37/46)
4E-BP1
P-mTOR (Ser2448)
mTOR

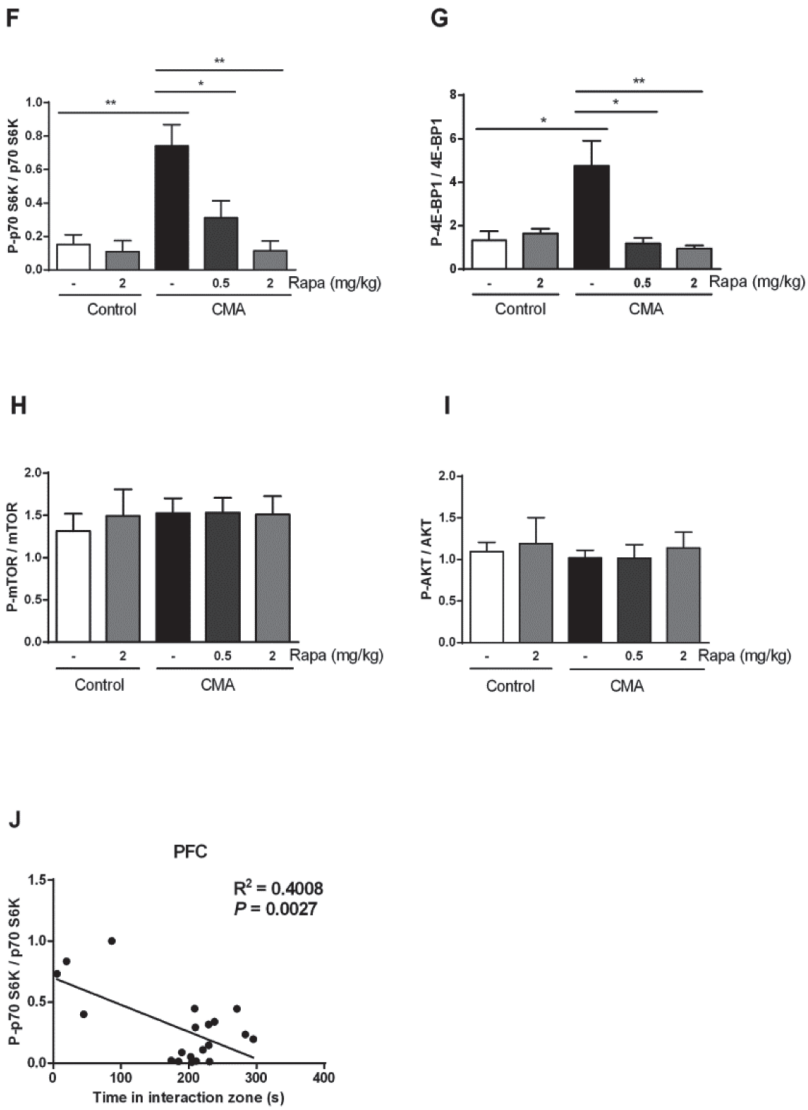
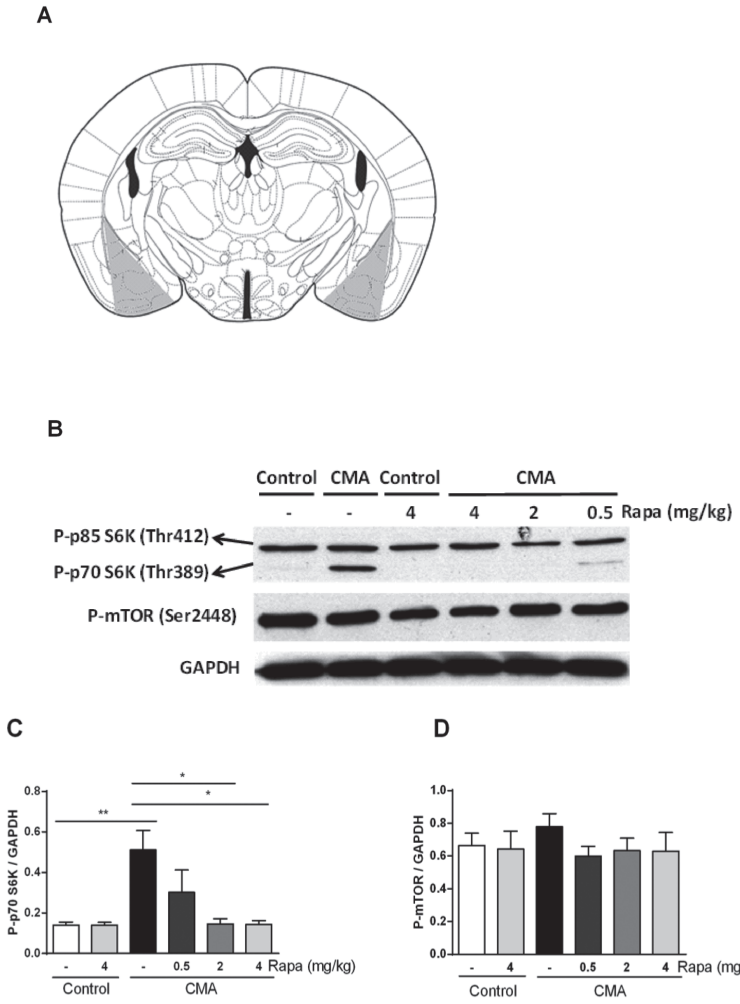


Fig. 6. Western blot analysis showed significantly increased phosphorylation of p70 S6K and 4E-BP1 in the prefrontal cortex of CMA mice and the phosphorylation of mTOR and AKT was hardly affected. The gray area in Figure A indicates prefrontal cortex. Figure B and E are typical examples of western blots. Rapamycin treatment inhibited the CMA-induced activation of p70 S6K and of 4E-BP1 in the prefrontal cortex. Densities of phosphorylation of p70-S6K, 4E-BP1, mTOR, and AKT were divided by the corresponding density of the GAPDH signal (C & D) or the non-phosphorylated corresponding protein (F-I). Figure J shows that the enhanced phosphorylation of p70 S6K in the prefrontal cortex is associated with reduced social interaction. One-way ANOVA followed by a Bonferroni's multiple comparisons test was conducted and data are presented as mean relative density ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, (C-D, F & H-I) n = 4 per group. (G) n = 3 per group.


A
B
Control
CMA
Control
CMA
- - 4 4 2 0.5 Rapa (mg/kg)
P-p85 S6K (Thr412)
P-p70 S6K (Thr389)
P-mTOR (Ser2448)
GAPDH
C
D
P-p70 S6K / GAPDH
P-mTOR / GAPDH
- 4 - 0.5 2 4 Rapa (mg/kg)
Control
CMA

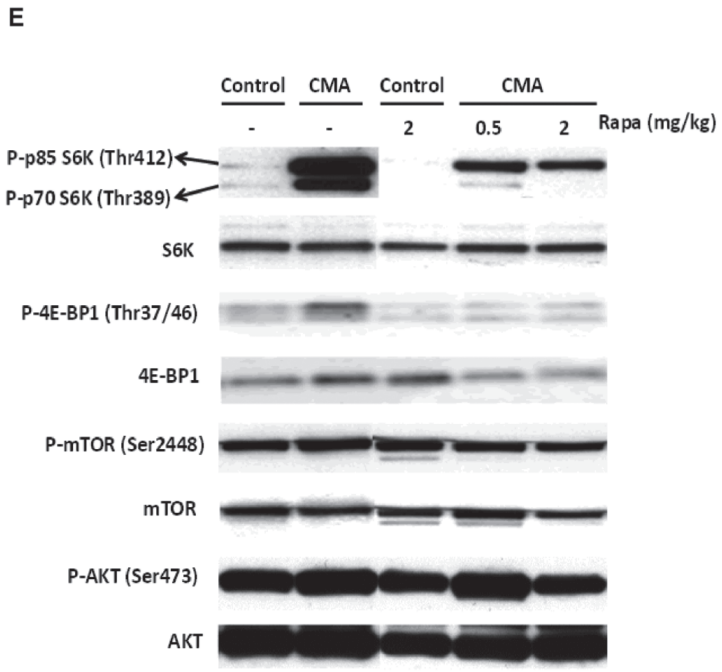


Fig. 7. Western blot analysis showed increased phosphorylation of p70-S6K and 4E-BP1 in the amygdala of CMA mice and rapamycin treatment inhibited the phosphorylation of p70-S6K in amygdala of CMA mice. The gray area in Figure A indicates amygdala. Figure B and C are typical examples of western blots. The phosphorylation of mTOR was not affected by either CMA or rapamycin treatment. Densities of phosphorylation of p70-S6K, mTOR, and AKT were divided by the corresponding density of the GAPDH signal (C & D) or the non-phosphorylated corresponding protein (F-I). Figure J shows that the enhanced phosphorylation of p70 S6K in the amygdala is associated with reduced social interaction. One-way ANOVA followed by a Bonferroni's multiple comparisons test was conducted and data are presented as mean relative density ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (C-D, F & H-I) n = 4 per group. (G) Control vs CMA: $P = 0.0560$. CMA vs CMA with Rapa 0.5 mg/kg: $P = 0.4614$. CMA vs CMA with Rapa 0.5 mg/kg: $P = 0.8803$. n = 3 per group.

3.4 Rapamycin inhibited the enhanced p70 S6K activity and increased the development of Treg cells in the ileum of CMA mice.

mTOR is known to play an essential role in directing immune responses. To investigate the activity of mTOR in the whey-induced allergic response, the phosphorylation of mTOR signaling proteins was examined in the ileum of CMA mice and compared to that of control mice. The phosphorylation of p70 S6K was significantly increased in ileum of CMA mice compared to that of sham-sensitized control mice (Fig. 8). Comparable to the observations in the brain, rapamycin inhibited the enhanced phosphorylation of p70 S6K in the distal ileum of CMA mice (Fig. 8A & B). The phosphorylation of mTOR was not significantly affected by CMA or rapamycin (Fig. 8A & C). To examine the

involvement of mTOR signaling pathway in the differentiation and in the development of the regulatory immune responses, mRNA expression of Treg associated transcription factor Foxp3 was analysed in ileum and Peyer's patches of mice. The mRNA expression level of Foxp3 in the ileum, but not in the Peyer's patches, of CMA mice was lower compared to that in control mice, although not significantly (Fig. 9). Treatment with rapamycin significantly increased the mRNA expression level of Foxp3 both in the ileum (Fig. 9A) and in the Peyer's patches of CMA mice (Fig. 9B). To further examine the effect of rapamycin on the production of Treg cell associated anti-inflammatory cytokines, the production of specific cytokines was analysed in the ileum. Levels of anti-inflammatory interleukin (IL)-10 (Fig. 9C) and transforming growth factor (TGF)-ß (Fig. 9D) were elevated by rapamycin treatment, although not significantly. Furthermore, a correlation between the extent of phosphorylation of p70 S6K and of Foxp3 mRNA expression in the ileum was demonstrated (Fig. 9E). Foxp3 mRNA expression in the ileum also positively correlates with social interaction (Fig. 9F).

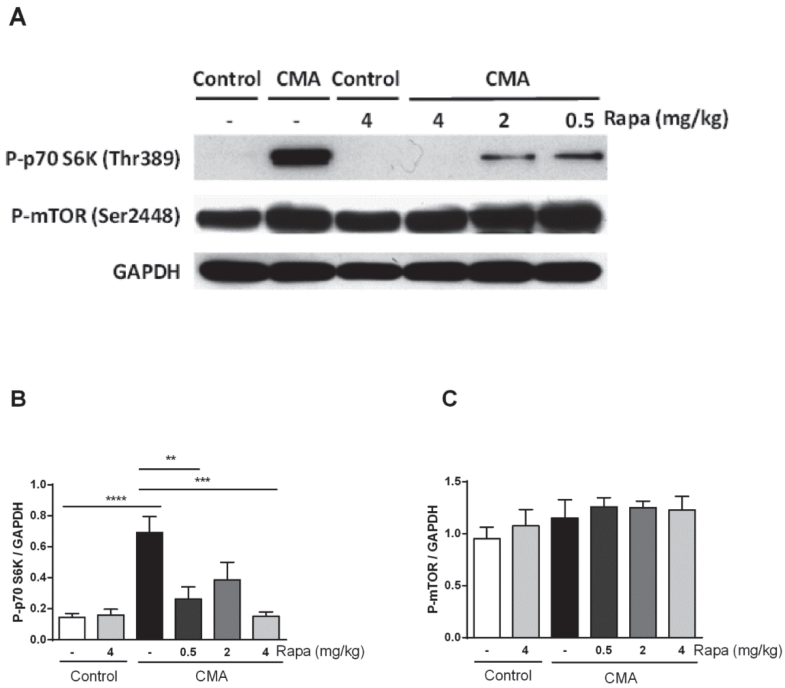


Fig. 8. CMA mice showed significantly increased phosphorylation of p70 S6 kinase in ileum and rapamycin treatment inhibited the phosphorylation of p70 S6 kinase (B). No difference was observed between the groups regarding the phosphorylation of mTOR (C). Figure A is a typical example of western blots. Density of phosphorylation of p70-S6K and mTOR was divided by the corresponding density of the GAPDH signal. One-way ANOVA followed by a Bonferroni's multiple comparisons test was conducted and data are presented as mean relative density ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, n = 5 per group.

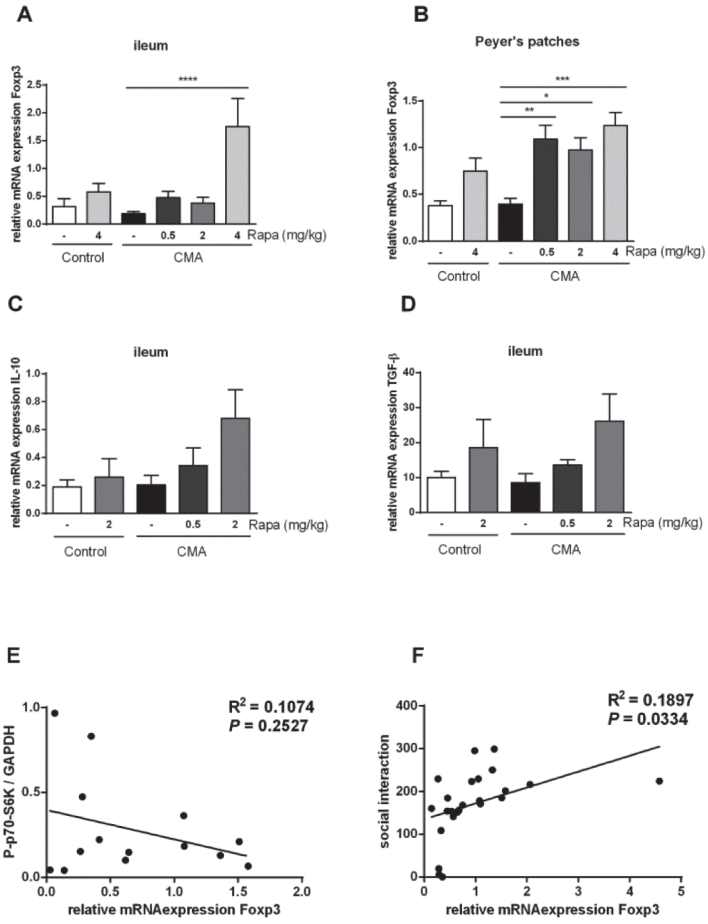


Fig. 9. In the ileum of mice undergoing CMA a reduced expression of Foxp3 mRNA was found (A). Suppression of mTOR signaling by rapamycin induced upregulation of Foxp3 mRNA expression in both ileum (A) and Peyer's patches (B) in CMA mice. Rapamycin treatment promoted anti-inflammatory IL-10 (C) and TGF-ß (D) production in the ileum of CMA mice. Reduced Foxp3 mRNA expression in the ileum is associated with enhanced phosphorylation of p70-S6 kinase in the ileum (E) as well as social interaction (F). (A) and (B) One-way ANOVA followed by a Bonferroni's multiple comparisons test was conducted and data are presented as mean relative abundance mRNA ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. (A) n = 7-12. (B) n = 7. (C) and (D) One-way ANOVA followed by a Bonferroni's multiple comparisons test was conducted to examine the effect of different doses of rapamycine in CMA. (C) CMA vs CMA with 2 mg/kg Rapa: $P = 0.1616$, n = 4-5. (D) CMA vs CMA with 2 mg/kg Rapa: $P = 0.0730$, n = 5.

4. Discussion

The current study investigated the involvement of the mTOR signaling pathway in the autistic-like behavior as well as in the immunological changes induced by cow's milk allergy in mice. It was demonstrated that induction of CMA induces reduced social

behavior and increased repetitive behavior. Rapamycin inhibited the enhanced mTOR signaling pathway both in the brain and in the intestine and improved the ASD-like behavioral symptoms. The inhibition of mTOR signaling pathway by rapamycin treatment also resulted in the suppression of allergic immune responses and resulted in an enhanced number of Treg cells in the ileum of CMA mice. By showing improvement of the ASD-like phenotype upon treatment with rapamycin, it was validated that mTOR plays a pivotal role in causing the behavioral phenotype and immunological changes seen in CMA mice. ASD are characterized by a series of behavioral deficits including reduced social behavior, stereotyped or repetitive behavior (Lord et al., 2000). Previous studies demonstrated that the induction of cow's milk allergy in mice, characterized by the induction of whey-specific immunoglobulin levels as well as by mast cell degranulation, can cause ASD-like behavioral symptoms including reduced social interaction and increased repetitive behavior (de Theije et al., 2014b). The present study demonstrated that rapamycin treatment improved the autistic-like behavior of CMA mice. The induction of allergy was accompanied by biochemical changes in the prefrontal cortex and amygdala as assessed by monoamine and its metabolite levels (de Theije et al., 2014b). The present study demonstrated that these biochemical changes also involve the enhanced mTOR signaling pathway, which has recently emerged as a central regulator of ASD-like behavioral symptoms.

Emerging evidence suggests that dysregulation of the brain-gut communication can result in gastrointestinal disorders, and in behavioral problems as well (Kennedy et al., 2012). The involvement of gastrointestinal disorders in ASD has been suggested (de Theije et al., 2014a, 2014b). Studies showed that both IgE-mediated and non-IgE-mediated allergic immune responses are associated with ASD symptoms (Theoharides et al., 2012). The exact patho-physiological relationship between the gastrointestinal and behavioral co-morbidities is yet unknown. In ASD, several risk genes have been identified that are part of or directly linked to the mTOR signaling pathway (Ehninger et al., 2008; Kwon et al., 2006; Nimchinsky et al., 2001; Zhou et al., 2009). Furthermore, enhanced mTOR activity plays a central role in directing immune responses towards allergy (Kim et al., 2009). Rapamycin inhibited the enhanced levels of whey-specific IgE, IgG1, and IgG2a in the serum of CMA mice. It needs to be further investigated whether this also leads to a relevant decrease in allergic symptoms. The mTORC1 pathway has been demonstrated to be involved in the function of mast cells and controls cell survival and/or growth (Kim et al., 2009). In a separate *in vitro* study we observed that antigen-IgE-mediated mast cell activation resulted in enhanced mTOR signaling and that rapamycin was able to reduce the acute degranulation as well as the cytokine production at 4 h (personal observation). Similar results were shown *in vivo* in this present study,

demonstrating that rapamycin inhibited the CMA-associated mast cell degranulation. Morphological abnormalities and dysfunctions in various brain regions, including prefrontal cortex and amygdala, have been found in autistic individuals in numerous clinical and preclinical studies (Baron-Cohen et al., 2000; Courchesne et al., 2011; Haws et al., 2014; Wegiel et al., 2014). The prefrontal cortex is known to play an important role in the process of cognitive control and the control of goals-directed thought and behavior (Miller, 2000; Watanabe and Sakagami, 2007). Damage to corticostriatal circuits in prefrontal cortex can result in abnormal repetitive behavior (Langen et al., 2011), which has been seen in CMA mice. Prefrontal cortex lesions in monkeys and humans can also lead to impairments in social and emotional behavior (Szczepanski and Knight, 2014). Amygdala plays an essential role in social behavior and guiding the emotions. It was shown that amygdala volume positively correlates the size and complexity of social network in adult humans (Bickart et al., 2011). Amygdala lesions impaired social anxiety and social recognition in mice (Wang et al., 2014). In the current study, upregulation of mTORC1 pathway was found in the prefrontal cortex and amygdala of CMA mice, which may be asscociated with enhanced repetitive behavior and disturbed social behavior observed in CMA mice. Pharmacologic administration of rapamycin inhibited the mTORC1 pathway in the prefrontal cortex and amygdala and reversed autistic-like behavior in CMA mice. In addition, Ehninger et al. reported that in $Tsc2^{+/-}$ mice hyperactive mTOR signaling was shown in hippocampus and that this led to deficits in hippocampal-dependent learning (Ehninger et al., 2008). Moreover, it was demonstrated that mTORC1 was highly activated in Pten mutant mice and rapamycin treatment effectively reduced mTORC1 signaling in both hippocampus and cortex (Zhou et al., 2009). The mTORC1 pathway plays central roles in synaptic protein synthesis (Hay and Sonenberg, 2004; Hoeffer and Klann, 2011). In the current study, the phosphorylation of mTOR effector proteins was examined in several brain regions including prefrontal cortex, amygdala, dorsal hippocampus, and somatosensory cortex. P70 S6K and 4E-BP1 are the most important downstream effector proteins of mTORC1 and regulates protein synthesis. Upon induction of whey allergy in mice, the phosphorylation of p70 S6K and 4E-BP1 seemed to be enhanced in both amygdala and prefrontal cortex, indicating that the mTORC1 signaling pathway is enhanced in both brain regions. A hyperactive mTOR pathway leading to aberrant protein synthesis can result in synaptic dysfunction (Wang and Doering, 2013). The enhanced phosphorylation of p70-S6K and 4E-BP1 could induce excessive synthesis of synaptic proteins including neuroligin (NLGN) synthesis (Südhof, 2008; Wang and Doering, 2013). It has been demonstrated that increased translation of NLGNs leads to increased ratio of synaptic excitation to inhibition (E/I), which may

eventually be involved in the development of autistic phenotypes in CMA mice (Südhof, 2008; Wang and Doering, 2013). The enhanced phosphorylation levels of p70 S6K at Thr389 or 4E-BP1 at Thr37/46 also indicate enhanced mTOR activity, because these epitopes on p70 S6K and 4E-BP1 are directly phosphorylated by mTOR. It is known that mTOR phosphorylation at Ser2448 does not always reflect mTOR activity and mTOR activity is routinely determined by measuring the phosphorylation levels of p70 S6K at Thr389 or 4E-BP1 at Thr37/46 (Caccamo et al., 2010; Das et al., 2008; Guertin and Sabatini, 2007; Hay and Sonenberg, 2004; Hay, 2005). The regulation of mTOR has been shown to occur via multiple phosphorylation sites, namely Ser1261, Thr2446, Ser2448, and Ser2481 (Acosta-Jaquez et al., 2009). In the current study we evaluated only the Ser2448 phosphorylation of mTOR as Ser2448 is involved in the formation of mTORC1 (Copp et al., 2009). However, Ser2448 was shown to be a feedback site on mTOR from its downstream target, p70 S6K, which means that p70 S6K is able to phosphorylate mTOR at Ser2448 and thereby restore Ser2448-specific phosphorylation (Chiang and Abraham, 2005). Therefore, no significant change of mTOR phosphorylation on Ser2448 was observed in CMA mice. mTOR phosphorylation on other sites such as Ser1261 might be affected more significantly after induction of CMA, because mTOR phosphorylation on Ser1261 is also required for mTORC1 function and mTORC1-mediated substrate phosphorylation, e.g. p70 S6K and 4E-BP1 (Acosta-Jaquez et al., 2009). Furthermore, rapamycin forms a complex with FK506-binding protein of 12 kDa (FKBP12) and this complex then binds to mTOR (Fingar and Blenis, 2004a; Wullschleger et al., 2006). The binding site of FKBP12-rapamycin complex in mTOR is different to the phosphorylation site of mTOR. Essentially, FKBP12-rapamycin complex binds to FRB domain of mTOR, while the phosphorylation site is located at ser 2448 close to C-terminal (Fingar and Blenis, 2004b; Wullschleger et al., 2006). FKBP12-rapamycin complex binds directly to the FRB domain of phosphorylated mTOR, blocks the binding of other structural proteins of mTOR complex1 and thereby the formation of mTOR complex1 (de Theije et al., 2011; Fingar and Blenis, 2004b; Wullschleger et al., 2006). Therefore, the phosphorylation of mTOR was barely affected by rapamycin treatment while the phosphorylation of mTORC1 downstream effector proteins, namely p70 S6K and 4E-BP1, was inhibited in the prefrontal cortex and amygdala of CMA mice, which was directly associated with the improvement of the behavioral deficits in CMA mice. Because of the direct inhibition of p70 S6K and 4E-BP1-dependent synaptic protein synthesis in the prefrontal cortex and amygdala of CMA mice, rapamycin treatment in the low dose showed more profound effects on behavioral changes as compared to CMA-associated mast cell degranulation, which involves a complex interplay of various intracellular signaling pathways and

mTOR signaling pathway is part of the complex intracellular signaling network (Gilfillan and Tkaczyk, 2006; Sibilano et al., 2014).

A variety of environmental factors have been implicated in the development of ASD, of interest is intestinal immune distrubances (de Theije et al., 2014a; Kennedy et al., 2012; Kraneveld et al., 2014; Meldrum et al., 2012). The mTOR pathway may be the link between the immune disturbances and behavioral deficits observed in ASD. The current study described that phosphorylation of downstream effector protein p70 S6K was enhanced in the ileum of CMA mice and rapamycin inhibited the CMA-induced mTOR activation in the ileum. Delgoffe GM, et al reported that the low mTORC1 and mTORC2 activity is required for the development of regulatory T cells (Delgoffe et al., 2011). In the present study it was found that rapamycin treatment enhanced regulatory T cell associated transcription factor Foxp3, mRNA expression level in the ileum and Peyer's patches of CMA mice. Previous studies showed that IL-10 and TGF-ß were able to suppress T cell activity and support regulatory T cells in suppressing airway hyperreactivity and inflammation (Jutel et al., 2003; Presser et al., 2008). Elevated mRNA expression of anti-inflammatory IL-10 and TGF-ß was demonstrated in the ileum of CMA mice with rapamycin treatment, indicating that possibly IL-10 and TGF-ß are involved in the rapamycin induced suppression of cow's milk allergy in mice. Rapamycin treatment induces IL-10 and TGF-ß production in the ileum of CMA mice. These anti-inflammatory cytokines might be able to get into the circulation to reach the brain. Subsequently, the anti-inflammatory cytokines might cross the blood brain barrier via direct transport or cytokine transporters/receptors on the cell surface and thereby directly interact with brain tissue in the specific brain regions (Banks et al., 2002; Banks, 2005). Through this mechanism the anti-inflammatory cytokines might positively regulate the function of central nervous system, eventually leading to the improvement of disturbed brain functions and the alleviation of autistic-like behaviors in CMA mice. Furthermore, altered serum cytokine levels such as IL-4 and interferon gamma (IFNy) in response to maternal immune activation (MIA) have been found in MIA mouse model for autism and were shown to play a critical role in manifestation of behavioral deficits caused by MIA (Onore et al., 2012, 2014). Of interest is IL4, which is a typical Th2 cytokine released during allergic responses, such as CMA. Future studies to examine the role of IL4 and other allergy-associated cytokines in CMA-induced ASD-like behavior might be of interest, but beyond the scope of this study. Overall, our results provide additional and new knowledge in the mechanism of how immune regulatory T cell responses and T cell activity after rapamycin treatment are linked to gut-immune-brain axis, showing

potential for the increased production of anti-inflammatory cytokines IL-10 and TGF-ß in the gut of CMA mice to alleviate behavioral deficits.

4.1 Conclusions

In conclusion, the current studies provide strong and first evidence that the enhanced mTOR signaling pathway in the brain as well as in the intestines plays an pivotal role in the behavioral and immunological changes in CMA mice. mTOR might be the linking pin involved in gut-immune-brain axis in ASD and the intestinal tract could be a potential target in the treatment of patients with ASD and comorbid intestinal symptoms. It is a compelling hypothesis that an enhanced mTOR activity throughout the body may account for both the behavioral as well as the gastrointestinal dysfunctions in patients with ASD. Whether inhibition of mTOR is able to treat both allergic and behavioral deficits of ASD patients remains to be further investigated. Importantly, increased gastrointestinal deficits and in particular behavioral abnormalities are commonly reported in other neurodevelopmental diseases such as attention deficit hyperactivity disorder (ADHD) (Verlaet et al., 2014), multiple sclerosis (Lin et al., 2014), schizophrenia(Severance et al., 2014), Parkinson's disease (Pfeiffer, 2011), however the role of mTOR needs to be investigated. Our findings on the gut-immune-brain connection in a murine model of CMA indicate that targeting mTOR signaling pathway might be applicable to various neurological disorders. Future studies focusing on the mTOR signaling pathway should shed more light on the effective treatment of ASD and other neurodevelopmental disorders.

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Conflict of interest

Prof. Dr. Johan Garssen is a parttime employee at Nutricia Research, Utrecht, The Netherlands. Dr. Laus Broersen is an employee of Nutricia Research, Utrecht, The Netherlands. Dr. Sofia Lopes da Silva was an employee of Nutricia Research, Utrecht, The Netherlands, at the time of the study. This study is part of the Utrecht University 'Focus en Massa' program and financially supported by Nutricia Research and Utrecht University.

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CHAPTER FIVE

Effect of specific amino acids or amino acid combinations on activation of mTOR signaling pathway in mast cells: consequences for acute degranulation and cytokine production

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Abstract

Previous studies have shown that mTOR signaling pathway is involved in directing immune responses including allergic reactions. In addition, amino acids were shown to modulate the activation of mTORC1 pathway in various tissues and cell lines. In the present study, we investigated the role of the mTORC1 pathway in degranulation and cytokine production of murine bone marrow-derived mast cells (BMMCs). We also examined the effect of specific amino acids or amino acid combinations on activation of mTORC1 pathway during antigen-specific activation of mast cells and consequent degranulation and cytokine production. ß-Hexosaminidase release was measured as marker for degranulation and IL-6 and TNF-α as products of gene transcription. The effects of rapamycin or amino acids on mTOR signalling were analysed by western blot analysis. The mTORC1 signaling pathway was shown to be involved in IgE-mediated degranulation and cytokine production of BMMCs. A combination of leucine, isoleucine, and valine enhanced phosphorylation of the mTORC1 downstream protein p70 S6K in antigen-IgE-activated BMMCs while a combination of lysine, histidine, and threonine inhibited phosphorylation of p70 S6K. Furthermore, both amino acid combinations inhibited antigen-specific degranulation and cytokine production in BMMCs.

In conclusion, our results suggest that mTORC1 pathway is partly involved in IgE-mediated mast cell activation and the inhibitory effects of amino acid combinations on IgE-mediated mast cell activation may also be independent on the mTORC1 pathway. Dietary amino acid supplementations, either or not via the inhibition of mTOR signaling pathway, might be beneficial for the treatment of mast cell-mediated diseases.

1. Introduction

The mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine protein kinase and is a key component of two different multi-protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2)[1,2]. mTORC1 is sensitive to the antifungal metabolite rapamycin and can be activated through the phosphatidylinositol-3-OH kinase/AKT (PI3K/AKT) signaling pathway in response to a wide range of environmental cues, including growth factors, stress as well as nutrients[1,2]. mTORC1 promotes cell growth and metabolism by stimulating the phosphorylation of p70 S6 kinase (p70 S6K) and the eIF-4E binding protein 1 (4E-BP1) that are involved in protein synthesis[1,3].

Amongst various stimuli regulating mTORC1, amino acids are the most crucial for mTORC1 signaling because they not only effectively control mTORC1 activity, but are also required for mTORC1 activation by other stimuli[4,5]. The exact molecular mechanism for activation of mTORC1 in response to amino acid signals is still not fully elucidated. Recently, there has been a growing interest in the effect of specific amino acids on mTOR signaling and the possible mechanisms involved[6,7]. Previous studies in various tissues and cell types have shown that branched-chain amino acids (BCAAs), including leucine (Leu), isoleucine (Ile), and valine (Val), induced protein synthesis through activated mTORC1 signaling pathway[6–10]. In contrast to BCAAs that positively regulate mTOR signaling pathway, the individual supplementation of lysine (Lys), histidine (His), and threonine (Thr) and the combination of Lys, His, Thr were shown to exert an inhibitory effect on phosphorylation of mTORC1 downstream proteins p70 S6K and 4E-BP1 in mammary epithelial cells and this effect was associated with a reduced total protein synthesis and ß-casein synthesis[10], indicating that the combination of Lys, His and Thr could be tested as a potential inhibitor of activated mTORC1 signaling.

Mast cells play a critical role in allergy and autoimmunity[11,12]. Antigen-specific mast cell activation can be induced by cross-linking of IgE-bound FcεR1, leading to activation of multiple signal transduction pathways that trigger the release of pre-stored immune mediators such as histamine and protease from granules and that trigger cytokine synthesis and secretion[11,13]. Previous *in vitro* studies have pinpointed the critical role of mTORC1 pathway in regulation of mast cell activation and survival[14,15]. Furthermore, the involvement of the mTOR signaling pathway in IgE-mediated allergic immune responses has been investigated in the mouse model of food allergy induced by oral ovalbumin. Pretreatment with the mTOR inhibitor rapamycin reduced the

concentration of mouse mast cell protease-1 (MMCP-1) released from mucosal mast cells and the accumulation of mast cells in the intestine of allergic mice[14].
Despite these *in vitro* and *in vivo* findings suggesting an importance of the mTOR pathway in regulating mast cell function, it is presently unclear if this pathway could be influenced by specific amino acids to modulate IgE-mediated mast cell activation. The current study first investigated the effect of rapamycin on IgE-mediated mast cell activation and the activation of mTORC1 pathway in murine bone marrow-derived mast cells (BMMCs). Given the inhibitory effect of Lys, His, Thr and the stimulating effect of BCAAs on mTORC1 signaling pathway shown in mammary epithelial cells[10], we further investigated the effect of individual supplementation of Lys, His, Thr, Leu, Ile, Val and the amino acid combination of Lys, His, Thr and the amino acid combination of Leu, Ile, Val on activation of mTORC1 pathway in antigen-IgE activated BMMCs.

2. Materials & Methods

2.1. Isolation of mouse bone marrow cells

After sacrificing the mice, intact femurs and tibia were removed and bone marrow cells were isolated from the femurs and tibia of adult male C3H/HeOuJ mice according to the following protocol: the bone shaft was repeatedly flushed by sterile PBS using a syringe with a needle. Cell suspensions of bone marrow were collected using a 70 µM nylon cell strainer (BD Biosciences, USA). The cells were centrifuged at 1,300 rpm and 22°C for 4 min, resuspended in PBS and centrifuged again. Subsequently, the pellet was resuspended in a red blood cell lysis buffer containing NH_4Cl (MERCK, Darmstadt Germany), KHCO3 (Sigma, USA) and 5% ethylenediaminetetraacetic acid (EDTA, MERCK, Darmstadt Germany) in demineralized water and cells were centrifuged at 1,460 rpm and 22 °C for 4 min. The pellet was resuspended in PBS and the total amount of cells was determined using a haemocytometer (Assistant, Germany). Afterwards, the cells were centrifuged again at 1,460 rpm and 22 °C for 4 min. After centrifugation, the supernatant was removed and the cells were resuspended (dropwise) in ice-cold medium containing 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Zwijndrecht, The Netherlands) and 90% heat-inactivated fetal calf serum (FCS, Hyclone Laboratories, USA) and frozen at -70°C for 24 hours and then stored in liquid nitrogen until further use.

2.2. Cultures of mouse bone marrow cells

Bone marrow-derived mast cells were generated from the bone marrow cells of male C3H/HeOuJ mice according to the following protocol: after thawing the cells at the room temperature, bone marrow cells were cultured in a 175 cm^2 tissue culture flask with filter (Cellstar, Germany) with 100 ml of culturing medium, consisting of RPMI 1640 with glutamine and phenol red (Lonza, Switzerland) supplemented with 10% FCS, 1% penicillin-streptomycin solution (pen-strep, Sigma-Aldrich, Zwijndrecht, The Netherlands), 2 µM sodium pyruvate (Sigma-Aldrich, Zwijndrecht, The Netherlands), 6 µl 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 20 ml MEM non-essential amino acid solution (GIBCO, USA) combined with 1 ng interleukin 3 (mouse IL3; Prospec, USA) and 2 ng stem cell factor (mouse SCF; Prospec, USA), at 37°C in a humidified atmosphere with 5% CO_2. The amount of mouse IL3 and SCF were reduced to 0.5 ng of IL3 and 1 ng of SCF in culture weeks 1, 2, and 3. Nonadherent cells were centrifuged and resuspended in fresh medium once a week. Bone marrow cells were cultured for 3 weeks to develop BMMCs and cells were used in week 4 for the experiments.

2.3. Degranulation assay

BMMCs were transferred to a new 175 cm^2 tissue culture flask with filter (Cellstar, Germany) and overnight sensitized with 15% anti-2,4-dinitrophenol (DNP) IgE from hybridoma clone 26.82 at 37°C and 5% CO_2. After overnight sensitization, BMMCs were washed twice with Tyrode buffer (GIBCO, USA)/0.1% BSA and then transferred to a Costar sterile 96-well flat bottom plate (Corning Life Sciences, USA) in a concentration of 100,000 cells/well. Subsequently, BMMCs were incubated with 1 nM, 200 nM, 500 nM, and 1000 nM rapamycin (LC labs, Woburn, MA, USA) or with 0.4 mM or 10 mM single amino acids or amino acid combinations for 30 min. Afterwards, BMMCs were activated with 0, 2.5, 5, 10, 15, 20, 50 and 100 ng/ml of 2,4-dinitrophenyl conjugated to human serum albumin (HSA), DNP-HSA, at 37°C and 5% CO^2 for 10 min for ß-hexosaminidase release or 4 hours for cytokine production. 10% Triton X-100 (TX-100; Merck, Germany) was used to obtain 100% release of ß-hexosaminidase. To stop the reaction, cells were centrifuged at 3,000 rpm and 4°C for 5 min and the supernatant was then transferred to a new 96-well flat bottom plate. The supernatant was incubated with 4-methylumbelliferyl-N-acetyl-ß-D-glucosaminide (4-MUG, Sigma-Aldrich, Zwijndrecht, The Netherlands) solution in 0.1 M citrate buffer (Merck, Germany) (pH = 7.2), as a substrate for ß-hexosaminidase, at 37°C and 5% CO^2 for 1 hour. The reaction was stopped with 0.2 M glycine buffer (MP, USA) (pH = 10.7). ß-hexosaminidase release

was measured by fluorescence (excitation: 351 nm, emission: 462 nm). The percentage of degranulation was calculated as: [(A-B)/(T-B)] x 100 where A is the amount of ß-hexosaminidase released from stimulated cells, B is the release of ß-hexosaminidase from unstimulated cells, and T is total cellular content.

2.4. Cytokine production

To examine the stimulation time of maximal IL-6 release, BMMCs were stimulated with 20 ng/ml DNP-HSA at different time points (2, 4, 6, or 16 hours). To assess the effect of rapamycin or amino acids or amino acid combinations, BMMCs were stimulated with 10 ng/ml DNP-HSA for 4 hours. The supernatant was transferred to a 96-well flat bottom plate. Quantification of IL-6 and TNF-α was performed using the ELISA MAX™ Standard Sets (BioLegend, USA) according to the manufacturer's instructions.

2.5. Western blotting

BMMCs were transferred to a new 175 cm^2 tissue culture flask with filter (Cellstar, Germany) and overnight sensitized with 15% anti-2,4-dinitrophenol (DNP) IgE from hybridoma clone 26.82 at 37°C and 5% CO_2. Subsequently, the cells were washed twice with tyrode buffer (GIBCO, USA)/0.1% BSA and incubated for 4 hours in RPMI 1640 with glutamine and phenol red (Lonza, Switzerland). After incubation, the cells were centrifuged and seeded onto a Costar sterile 24-well flat bottom plate (Corning Life Sciences, USA) at a concentration of 5 x 10^6 cells/ml. BMMCs were incubated with the specific amino acids or amino acid combinations or 100 nM rapamycin for 30 min. In order to observe both the stimulating and inhibitory effect of the single amino acids or amino acid combinations on the phosphorylation of p70 S6K, BMMCs were stimulated with 5 ng/ml DNP-HSA, which is the concentration causing approximately 50% of the maximum level of ß-hexosaminidase release in BMMCs, at 37°C and 5% CO^2 for 15 min. Subsequently, lysis buffer containing SDS loading buffer, complete mini protease inhibitor cocktail tablets (Roche, Almere, The Netherlands), phosphatase inhibitor cocktail (Calbiochem, Amsterdam, The Netherlands), 5% dithiothreitol (DTT, Sigma, USA), benzonase nuclease (Calbiochem, Amsterdam, The Netherlands) was added and then the cell lysates were centrifuged at 4,000 rpm at 4°C for 10 min and stored at -20°C until further analysis.

Western blotting was performed as described previously[16]. Briefly, the cell lysates were preheated in boiling water for 5 min and 30 µl of each lysate was loaded onto Criterion TGX precast gel (Bio-Rad, Veenendaal, The Netherlands), electrophoresed and subsequently electroblotted onto PVDF membrane (Bio-Rad, Veenendaal, The

Netherlands), which was blocked in 5% nonfat dry milk for 1 hour. Subsequently, membranes were washed with TBS/0.1% Tween-20 (Sigma-Aldrich, Zwijndrecht, The Netherlands) 3 x 10 min and incubated overnight with the primary antibodies (1:1000) at 4°C. The primary antibodies against phospho-mTOR (Ser2448, #5536), mTOR (#2972), phospho-p70 S6K (Thr389, #9205), p70 S6K (#9202), phospho-4E-BP1 (Thr37/46, #9459), and 4E-BP1 (#9452) were obtained from Cell Signaling, Leiden, The Netherlands. The primary antibody against GAPDH (#3777R-100) was obtained from Biovision, Uithoorn, The Netherlands. Then the membranes were washed with TBS/0.1% Tween-20 3 x 10 minutes and incubated with the secondary antibody polyclonal HRP-conjugated goat anti-rabbit immunoglobulins (1:5000, DAKO, Eindhoven, The Netherlands). The signals were detected by using ECL prime kit (Health Care, Amsterdam, The Netherlands).

2.6. LDH assay

A cytotoxicity detection kit (Roche, Penzberg, Germany) was used to quantify cell death in culture with or without treatments of rapamycin or amino acid. We determined the cytoplasmic enzyme activities released into culture supernatant. The release of enzyme lactate dehydrogenase (LDH) from antigen-IgE-stimulated mast cells was measured at 490 nm on a microplate reader (Bio-Rad, Veenendaal, The Netherlands) and the percentage of cytotoxicity was calculated according to the following formula:

Cytotoxicity (%) = ((exp. value – low control) / (high control – low control)) x 100

Low control: the LDH activity released from the untreated antigen-IgE-stimulated mast cells.

High control: the maximum releasable LDH activity in antigen-IgE-stimulated mast cells.

2.7. Statistical analysis

Experimental results are expressed as mean ± S.E.M. In general, differences between groups were statistically determined with a one-way ANOVA followed by a Bonferroni's multiple comparisons test. For time-response curve of acute degranulation in IgE-mediated BMMCs and effect of different rapamycin concentrations on acute degranulation of IgE-mediated BMMCs, data were analyzed with two-way ANOVA followed by a Bonferroni's multiple comparisons test with repeated measures. Results were considered statistically significant when $P < 0.05$. Analyses were performed using GraphPad Prism, version 6.02.

3. Results

3.1. Effect of rapamycin on IgE-mediated mast cell degranulation and cytokine production

Since mast cell degranulation is an immediate response, we first assessed IgE-dependent mast cell degranulation by measuring ß-hexosaminidase release after triggering with DNP-HSA for the indicated times. After 10 min stimulation with DNP-HSA, BMMCs were shown to reach the maximal ß-hexosaminidase release at each concentration of DNP-HSA ($F_{7, 28}$ = 224.4, P < 0.0001, Fig. 1A). To examine whether mTOR pathway is involved in IgE-mediated degranulation of mast cells, the IgE-primed BMMCs were incubated with rapamycin in different concentrations for 30 min prior to stimulating with DNP-HSA. Mast cell degranulation was significantly inhibited by the pretreatment with rapamycin in a dose-dependent manner ($F_{7, 7}$ = 1162, P < 0.0001, Fig. 1B).

In contrast to mast cell degranulation, the release of the cytokine IL-6 in response to antigen/IgE stimulation requires gene transcription, which reaches maximum levels approximately 4 hours after stimulation with DNP-HSA (data not shown). To further investigate the effect of rapamycin on cytokine production of IgE-mediated mast cell activation, we pretreated BMMCs with increasing concentrations of rapamycin and then measured IL-6 and TNF-α release after 4 hours stimulation with DNP-HSA. Rapamycin dose-dependently inhibited IL-6 and TNF-α production ($F_{4, 10}$ = 886.8, P < 0.0001 for IL-6 production; $F_{4, 15}$ = 9.661, P < 0.001 for TNF-α production; Fig. 1C and D). Wortmannin, which is a specific inhibitor of mTOR upstream protein PI3K and was used as a positive control in the current study, inhibited both acute degranulation and IL-6 production in IgE-activated BMMCs (Fig. 1B-D). Furthermore, to examine the effect of rapamycin concentrations on viability of BMMCs, lactate dehydrogenase (LDH) activity was measured in cell supernatant. The tested rapamycin concentrations did not increase LDH activity (data not shown).

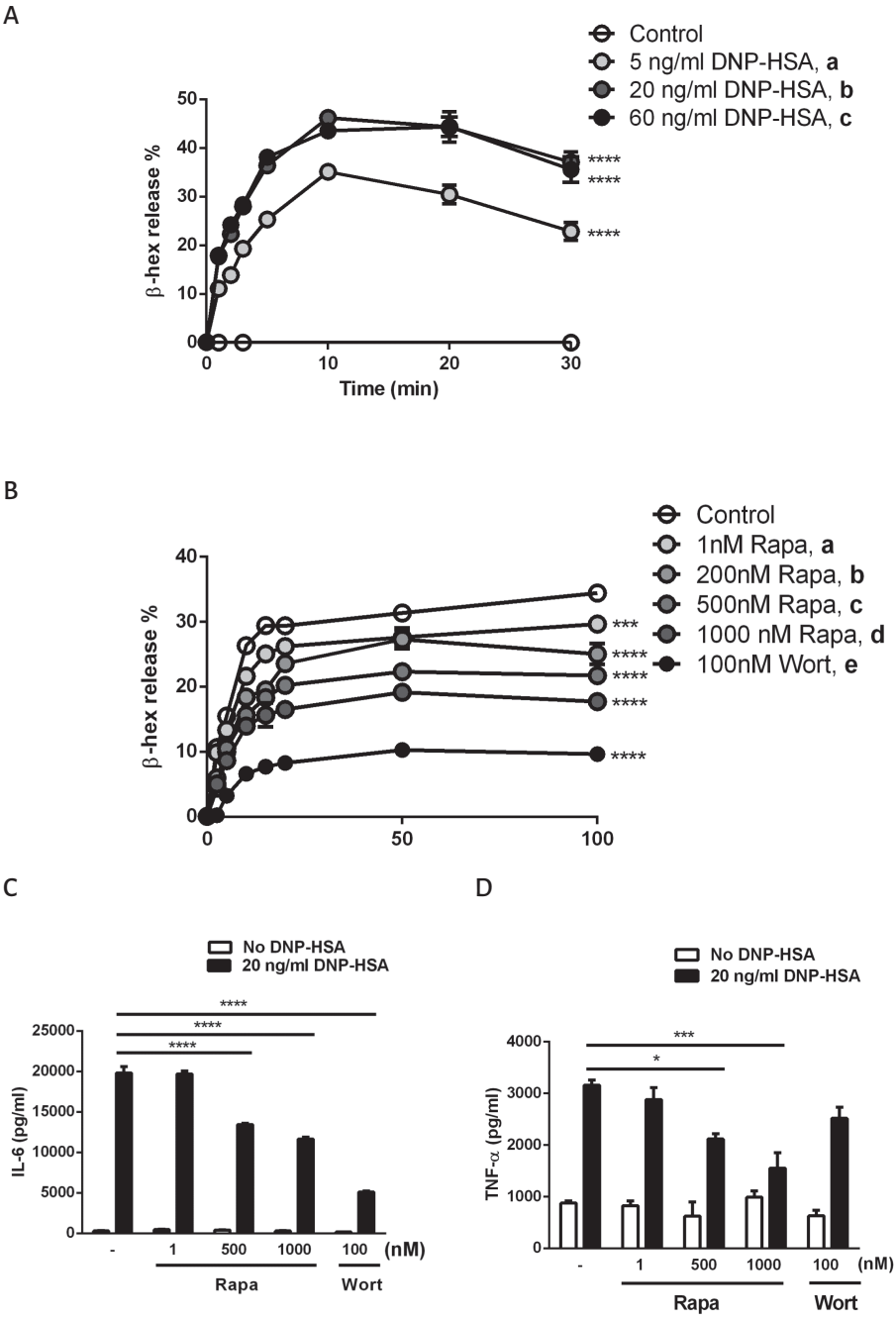


Fig. 1. Effect of rapamycin on acute degranulation and cytokine production of antigen-IgE activated BMMCs. (A) Time-response curves of acute degranulation in IgE-activated BMMCs induced by DNP-HSA at three different concentrations: 5 ng/ml, 20 ng/ml, and 60 ng/ml. Mast cell degranulation reaches the maximum level after 10 min

stimulation with the tested DNP-HSA concentrations. Data are presented as the mean ± SEM, n = 5. Curve **a** VS control: $P < 0.0001$, **b** VS control: $P < 0.0001$, **c** VS control: $P < 0.0001$, **a** VS **b**: $P < 0.0001$, **a** VS **c**: $P < 0.0001$, **b** VS **c**: not significant. (B) Rapamycin inhibited acute degranulation of IgE-activated BMMCs induced by DNP-HSA in a dose-dependent manner. Data are presented as the mean ± SEM, n = 3. Curve **a** VS control: $P = 0.0002$, **b** VS control: $P < 0.0001$, **c** VS control: $P < 0.0001$, **d** VS control: $P < 0.0001$, **e** VS control: $P < 0.0001$. (C) Dose-response curve of IL-6 production in IgE-activated BMMCs induced by 5 ng/ml DNP-HSA. IL-6 production reaches the maximum level after 4 hours stimulation with DNP-HSA. Data are presented as the mean ± SEM, n = 5. (D) Rapamycin inhibited IL-6 and TNF-**ε**productions in a dose-dependent manner of DNP-HSA-IgE-activated BMMCs. Data are presented as the mean ± SEM, n = 3. For time-response curve of acute degranulation in IgE-mediated BMMC activation and effect of different rapamycin concentrations on acute degranulation of IgE-activated BMMCs, data were analyzed with two-way ANOVA followed by a Bonferroni's multiple comparisons test with repeated measures. For effect of rapamycin on IL-6 and TNF-**ε**productions, data were analyzed with one-way ANOVA followed by a Bonferroni's multiple comparisons test. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

3.2. Effect of rapamycin on mTOR signaling pathway in IgE-mediated BMMCs

Previously, it was demonstrated that the mTORC1 pathway is activated in FcεR1-stimulated human mast cells as key protein components of this pathway including mTOR and two downstream effectors of mTORC1, p70 S6K and 4E-BP1, are phosphorylated[15]. Since rapamycin was able to inhibit the mast cell degranulation as well as the cytokine production after antigen challenge, we further examined effect of rapamycin on the phosphorylation of protein components of mTORC1 signaling pathway in IgE-mediated BMMCs. The phosphorylation of mTOR and its downstream protein 4E-BP1 is a rapid response. Within 5 minutes after triggering with DNP-HSA phosphorylation of mTOR and its downstream protein 4E-BP1 reached maximum levels (Fig. 2A & B). In contrast to mTOR and 4E-BP1, the phosphorylation of p70 S6K, another downstream protein of mTORC1, was slower and took 30 minutes to reach maximum levels (Fig. 2A & B). Rapamycin did not affect the phosphorylation of mTOR while it inhibited the phosphorylation of 4E-BP1 and p70 S6K. Interestingly, rapamycin showed an acute and short-term inhibition on phosphorylation of 4E-BP1 for 30 minutes (Fig. 2A & B).

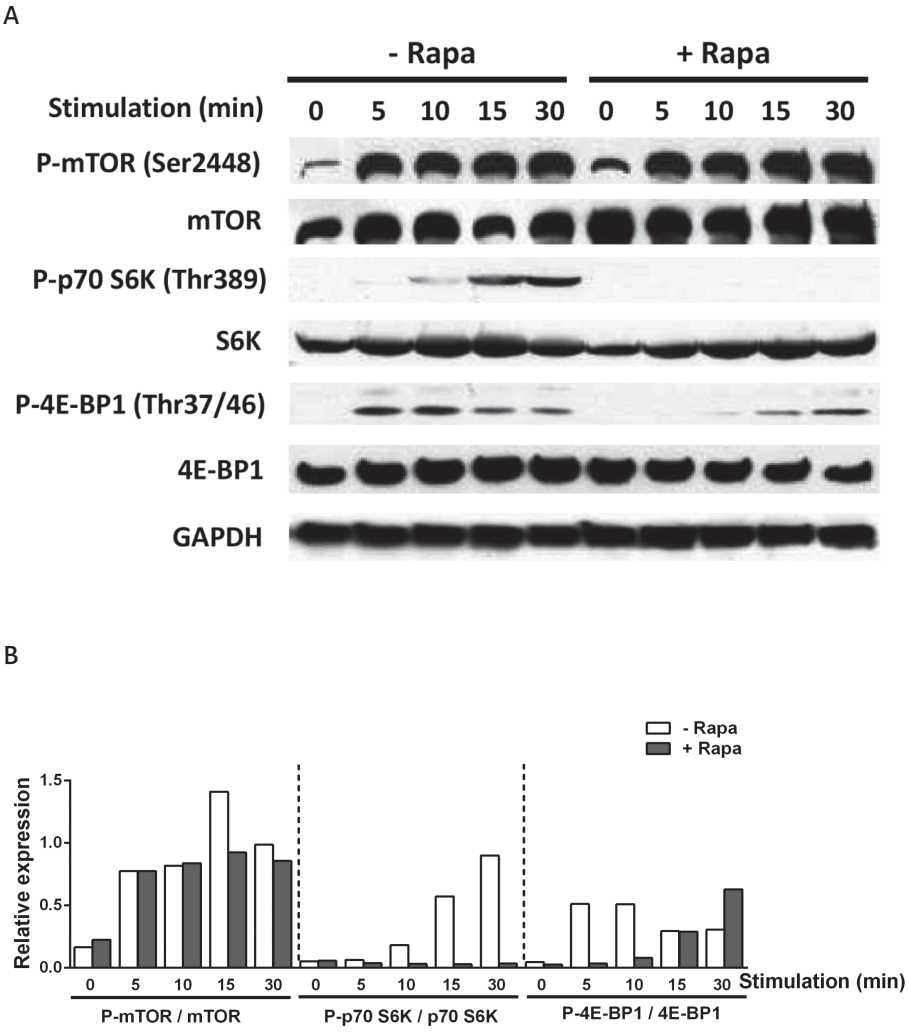
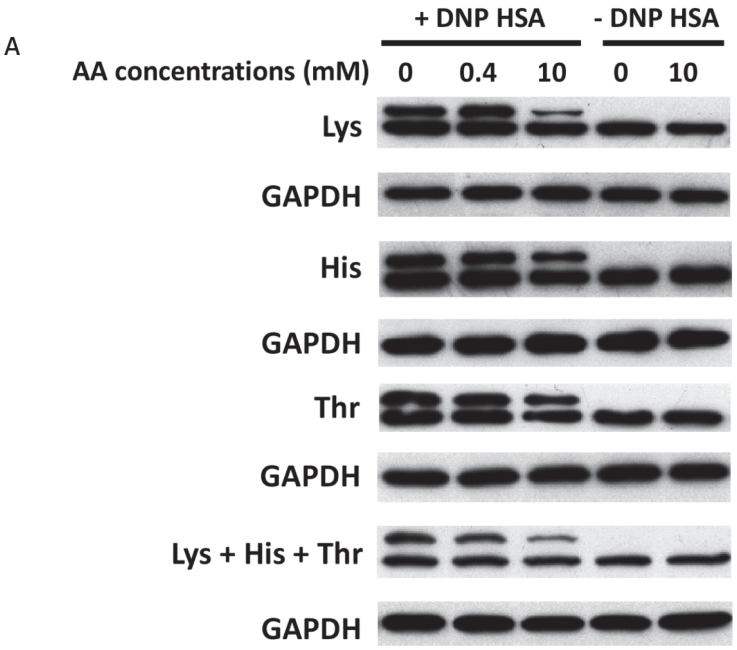


Fig. 2. Effect of rapamycin on phosphorylation of protein components involved in mTOR signaling pathway in DNP-HSA-stimulated and IgE-sensitized BMMCs. Mouse BMMCs were overnight sensitized with DNP-specific IgE, incubated with 100 nM rapamycin for 30 min and triggered with 5 ng/ml DNP-HSA. The phosphorylation of mTOR, p70 S6K, and 4EBP-1 was examined at the indicated time points after stimulation. Figure A is the typical example of western blots. (B) Densities of phosphorylation of mTOR, p70 S6K and 4E-BP1 were divided by the corresponding density of the non-phosphorylated corresponding protein.

3.3. Effect of specific amino acids or amino acid combinations on phosphorylation of p70-S6K in IgE-mediated BMMCs

Since inhibition of mTOR signaling by rapamycin results in the inhibition of IgE-mediated mast cell activation, we next investigated the effects of specific amino acids or amino acid combinations on the mTOR signaling in antigen-IgE stimulated BMMC. P70 S6K and 4EBP-1 are the best characterized downstream targets of mTORC1. Both proteins are key elements for protein synthesis and regulates cell growth and proliferation[17–20]. However, the phosphorylation of p70 S6K versus 4E-BP1 is differentially affected by rapamycin. Previous studies in various cell types showed that rapamycin inhibited p70 S6K phosphorylation throughout the duration of the treatment while prolonged rapamycin treatment induced 4E-BP1 phosphorylation despite initial inhibition[21]. In the current study, it was also shown that rapamycin treatment inhibited p70 S6K phosphorylation throughout 30 min incubation, but 4E-BP1 recovered in phosphorylation within 15 min in IgE-mediated mast cells (Fig. 2). Therefore, we used phosphorylation of p70 S6K as a readout for mTORC1 signaling. The single amino acids Lys, His, Thr and the combination of Lys, His, Thr inhibited p70 S6K phosphorylation ($F_{2, 3} = 63.76$, $P < 0.01$ for the combination of Lys, His, and Thr, Fig. 3A & C), while the single amino acids Leu, Ile, Val and the combination of Leu, Ile, Val enhanced p70 S6K phosphorylation in IgE-mediated BMMCs ($F_{2, 3} = 19.85$, $P < 0.05$ for the combination of Leu, Ile, and Val, Fig. 3B &D)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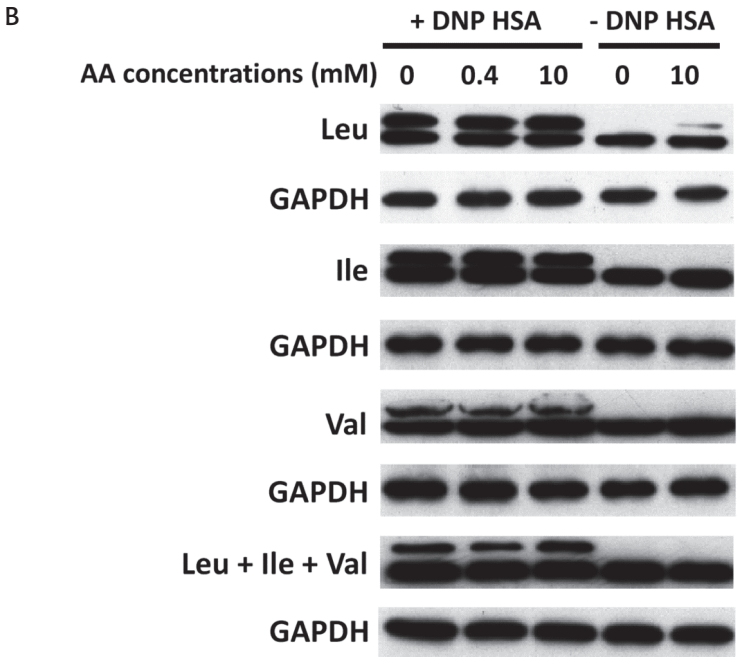
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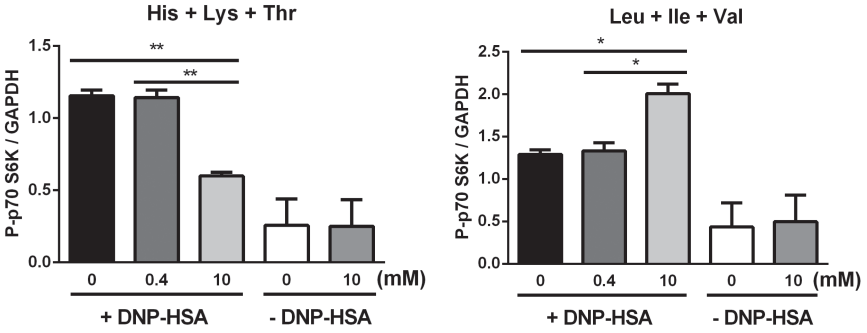

Fig. 3. Effect of specific amino acids on mTOR signaling pathway in antigen-activated mast cells. (A & C) The specific amino acids His, Lys, Thr, and the amino acid combination of His, Lys, Thr inhibited the phosphorylation of p70-S6K, a downstream protein of mTORC1, in antigen-induced activation of BMMCs, (B & D) while Leu, Ile, Val, and the combination of Leu, Ile, Val augmented the phosphorylation of p70-S6K in antigen-IgE-activated BMMC. IgE-sensitized BMMCs were incubated with the single amino acids or amino acid combinations at different concentrations, followed by stimulation with 5 ng/ml DNP-HSA for 15 min. Densities of phosphorylation of p70-S6K was divided by the corresponding density of the GAPDH signal. One-way ANOVA followed by a Bonferroni's multiple comparisons test were conducted and densitometric data are presented as means ± SEM of two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. The blots are representative of two independent experiments.

3.4. Effect of specific amino acids or amino acid combinations on IgE-mediated mast cell degranulation and cytokine production

To investigate the effect of amino acids on IgE-mediated mast cell degranulation, IgE-primed BMMCs were incubated for 30 min with single amino acids or amino acid combinations in different concentrations and then triggered by DNP-HSA for 10 minutes followed by the measurement of ß-hexosaminidase release. We examined the effect of single amino acids Lys, His, Thr, Leu, Ile, Val, and the combination of Lys, His, Thr or Leu, Ile, Val on IgE-mediated mast cell degranulation. The single amino acids Lys, His, Thr, Leu, Ile, and the combination of Lys, His, Thr or Leu, Ile, Val were shown to inhibit IgE-mediated mast cell degranulation in a dose-dependent manner, while Val showed no effect on IgE-mediated mast cell degranulation ($F_{2,9} = 68.32$, $P < 0.0001$ for Leu; $F_{2,9} = 6.399$; $P < 0.05$ for Ile; $F_{2,9} = 2.818$, $P = 0.1121$ for Val; $F_{2,9} = 59.93$, $P < 0.0001$ for the combination of Leu, Ile, and Val; $F_{2,9} = 147.7$, $P < 0.0001$ for Lys; $F_{2,9} = 30.92$, $P < 0.0001$ for His; $F_{2,9} = 12.48$, $P < 0.01$ for Thr; $F_{2,9} = 50.83$, $P < 0.0001$ for the combination of Lys, His, and Thr.; Fig. 4A & B). To further investigate the effect of single amino acids or amino acid combinations on cytokine production of activated BMMCs, we incubated IgE-primed BMMCs with single amino acids Lys, His, Thr, Leu, Ile, Val and the combination of Lys, His, Thr or Leu, Ile, Val for 30 min followed by stimulation with DNP-HSA for 4 hours and IL6 production was measured. The single amino acids Lys, His, Thr and

the amino acid combination of Lys, His, Thr were shown to inhibit IL-6 production in a dose-dependent manner ($F_{2,9} = 18.73$, $P < 0.001$ for Lys; $F_{2,9} = 167.9$, $P < 0.0001$ for His; $F_{2,9} = 10.05$, $P < 0.01$ for Thr; $F_{2,8} = 26.24$, $P < 0.001$ for the combination of Lys, His, and Thr; Fig. 5A). IL-6 production was also inhibited by single amino acids Leu, Ile, Val and the amino acid combination of Leu, Ile, Val ($F_{2,9} = 71.63$, $P < 0.0001$ for Leu; $F_{2,9} = 19.95$, $P < 0.001$ for Ile; $F_{2,9} = 85.43$, $P < 0.0001$ for Val; $F_{2,9} = 32.84$, $P < 0.0001$ for the combination of Leu, Ile, and Val; Fig. 5B). Supplementation of different concentrations of single amino acids or amino acid combinations had no effect on cell viability as measured by LDH release (data not shown).

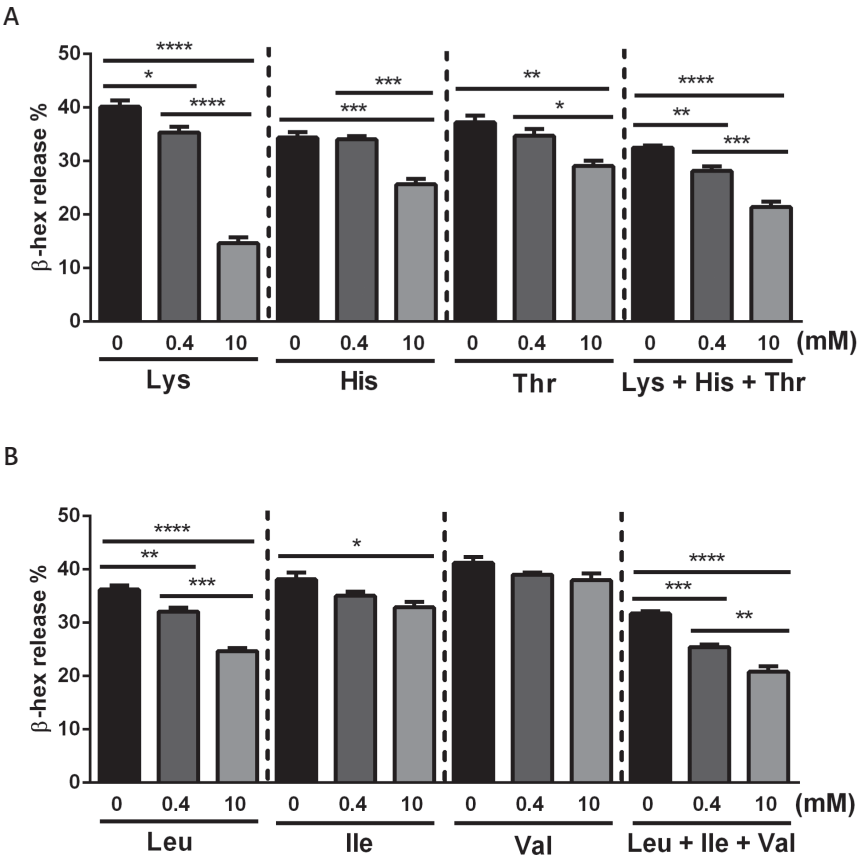

Fig. 4. Effect of single amino acids and amino acid combinations on the acute antigen-specific mast cell degranulation. (A) The specific amino acids Lys, His, Thr, and the combination of His, Lys, Thr inhibited antigen-induced degranulation of BMMCs in a dose-dependent manner. (B) The specific amino acids Leu, Ile, and the combination of Leu, Ile, Val inhibited antigen-induced degranulation of BMMCs, while Val had no effect on acute degranulation of BMMCs. IgE-sensitized BMMCs were incubated with the specific amino acids or amino acid

combinations at different concentrations for 30 min, followed by stimulation with 10 ng/ml DNP-HSA for 10 min. Acute degranulation was assessed by measuring ß-hexosaminidase (ß-hex) release in the supernatant after stimulation with 10 ng/ml DNP-HSA. One-way ANOVA followed by a Bonferroni's multiple comparisons test were conducted and data are presented as the mean ± SEM, n = 4. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

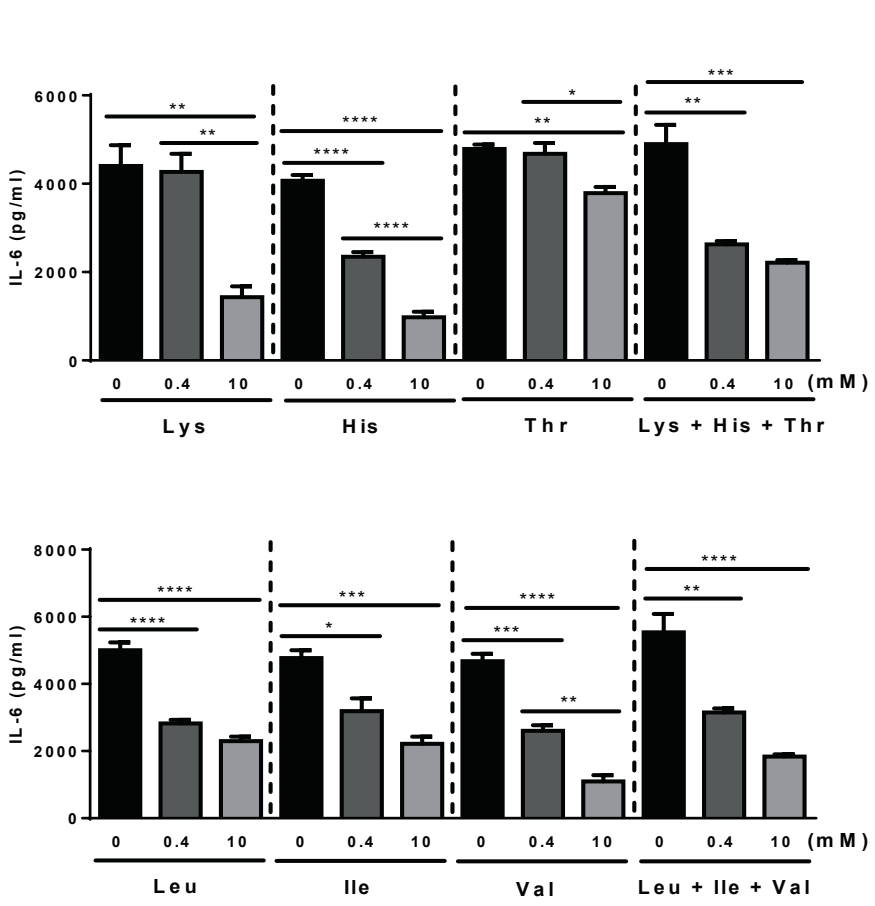

Fig. 5. Effect of single amino acids and amino acid combinations on antigen-induced IL-6 production of BMMCs. (A) The specific amino acids Lys, His, Thr and the amino acid combination of His, Lys, Thr inhibited antigen-induced IL-6 production of BMMCs. (B) The specific amino acids Leu, Ile, and Val and the amino acid combination of His, Lys, Thr enhanced antigen-induced IL-6 production of BMMCs. IgE-sensitized BMMCs were incubated with the specific amino acids or the amino acid combination for 30 min at different concentrations and then incubated in the absence or presence of 10 ng/ml DNP-HSA for 4 hours. IL-6 levels in the supernatant were measured by ELISA. One-way ANOVA followed by a Bonferroni's multiple comparisons test were conducted and data are presented as the mean ± SEM, n = 4. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

4. Discussion

In the present study, we showed that rapamycin suppressed mast cell activation and mTORC1 activation in antigen-IgE-activated BMMCs, which is consistent with the findings from previous studies[14,15]. We demonstrated that the amino acid combination of Lys, His, and Thr inhibited the phosphorylation of mTORC1 downstream target p70 S6K while the amino acid combination of Leu, Ile, and Val enhanced the phosphorylation of p70 S6K in antigen-IgE-activated BMMCs. Furthermore, the single amino acids Lys, His, Thr, Leu, and Ile and the amino acid combinations of Lys, His, Thr or Leu, Ile, and Val were shown to inhibit specific antigen-induced acute degranulation and cytokine production in BMMCs. Our results suggest that mTORC1 pathway is partly involved in IgE-mediated mast cell activation and the inhibitory effects of amino acid combinations on IgE-mediated mast cell activation may also be independent on the mTORC1 pathway.

mTORC1 pathway regulates numerous cellular processes including protein synthesis, cell growth, and proliferation[22,23]. Amino acids are key environmental stimuli that modulate mTORC1 activity. Apart from their role as indispensable building blocks for proteins, amino acids appear to regulate protein synthesis via a number of signaling transduction pathways including the mTOR signaling pathway[19,24]. The BCAAs, Leu, Ile, and Val are the most abundant essential amino acids. Leu, Ile, and Val have been shown to stimulate translation initiation and protein synthesis via activated mTORC1 pathway in various tissues and cell types including adipose tissue[8,25], liver[26], pancreas[27], and mammary epithelial cells[10]. Apart from the stimulating effects of Leu, Ile, and Val on mTORC1 pathway, the individual supplementation of Lys, His, Thr or as a mixture exerts inhibitory effects on mTORC1 pathway in mammary epithelial cells[10]. Hyperactivity of the mTORC1 pathway contributes to the development of ASD phenotypes, presumably involving the increased synaptic protein translation[28]. Besides a genetic background, based on the fact that about 50% of ASD patients suffer from gastrointestinal problems, it is hypothesized that food allergy might be an environmental triggers in ASD[29,30]. We and others have demonstrated that enhanced mTORC1 signaling is associated with autism-related behavioral impairments in various genetically modified mouse models for autism as well as in food allergy-induced ASD-like behaviors[16,31–33]. To develop a diet containing mTOR-targeting amino acids for the treatment of ASD, the current *in vitro* study investigated the effects of Leu, Ile, Val as well as Lys, His, Thr on mTORC1 pathway in antigen-IgE-activated BMMCs. The results reveal that mTORC1 pathway is activated in antigen-IgE-stimulated BMMCs. The amino acid combination of Lys, His, Thr inhibited the mTORC1 pathway, while the amino acid combination of Leu, Ile, Val enhanced the mTORC1 pathway in antigen-IgE-activated

mast cells. Dietary intervention with a diet containing increased relative amounts of Lys, His, Thr could serve as a potential approach to inhibit enhanced mTOR signaling.
Mast cells are the main effector cells implicated in the pathogenesis of IgE-mediated allergic diseases and in inflammatory responses[11,34,35]. Mast cell activation is regulated by a complex series of intracellular signaling cascades that are initiated by cross-linking of IgE-bound FcεR1, leading to an increase in calcium mobilization, and eventually mast cell degranulation and cytokine production[13,36]. The current study demonstrated that the amino acid combination of Lys, His, Thr and the amino acid combination of Leu, Ile, Val suppressed degranulation and cytokine production in antigen-IgE-activated BMMCs. It is of interest to note that both amino acid combinations had no effect on the phosphorylation of p70 S6K at the concentration of 0.4 mM, while they significantly inhibited degranulation and cytokine production in antigen-IgE-activated BMMCs at the same concentration, suggesting that the regulation of IgE-mediated mast cell activation by the amino acid combinations is not completely mediated by an inhibition of the mTORC1 activation. FcεRI-induced activation of mast cells is regulated by a complex interplay of positive signaling pathways such as Lyn-Syk pathway, and negative signaling pathways such as the molecular pathways involving Src homology 2-containing inositol phosphatase (SHIP) and Src-homology-2-domain-containing protein tyrosine phosphatase (SHP)[13,36]. Previous studies showed that treatment with high doses of arginine and glutamine reduced IgE dependent activation of human mast cells and the mechanism of action involved a decreased activation of MAPK/ERK, p38, JNK, and AKT[37]. Further research is needed to elucidate the mechanism of inhibition of mast cell activation by the amino acid combinations Lys, His, Thr and Leu, Ile, Val.
In conclusion, our current study suggests that dietary amino acid supplementations might be beneficial for the treatment of mast cell-mediated diseases as well as food allergy-associated ASD phenotypes.

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Conflict of interest

Prof. Dr. Johan Garssen is a part-time employee at Nutricia Research, Utrecht, The Netherlands. Dr. Laus M Broersen is an employee of Nutricia Research, Utrecht, The Netherlands. Dr. Sofia Lopes da Silva was an employee of Nutricia Research, Utrecht, The Netherlands, at the time of the study.

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CHAPTER SIX

Dietary interventions that reduce mTOR activity rescue autistic-like behavioral deficits in mice

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Abstract

Introduction: Enhanced mammalian target of rapamycin (mTOR) signaling in the brain has been implicated in the pathogenesis of autism spectrum disorder (ASD). Inhibition of the mTOR pathway improves behavior and neuropathology in mouse models of ASD containing mTOR-associated single gene mutations. The aim of the current study was to assess the effects of dietary interventions with an mTOR-targeting amino acid diet (Active 1 diet) or a multi-nutrient supplementation diet (Active 2 diet) on autistic-like behavior and mTOR signaling in food allergic mice and in inbred BTBR *T*$^{+}$ *Itpr3*^{tf}*/J* mice.
Method: Male BTBR or C3H mice were fed a Control, Active 1, or Active 2 diet for 8 consecutive weeks, followed by behavioral tests. After two weeks of diet, C3H mice were orally sensitized and challenged with whey protein to induce cow's milk allergy (CMA). Brain samples were collected for western blotting analysis of mTOR signaling pathway.
Results: CMA mice showed reduced social interaction and increased self-grooming behavior. Both diets reversed behavioral impairments in CMA mice and inhibited the mTOR activity in the prefrontal cortex and amygdala of CMA mice. In BTBR mice, Active 1 diet reduced repetitive self-grooming behavior and attenuated the mTOR activity in the prefrontal and somatosensory cortices.
Conclusion: The current results suggest that activated mTOR signaling pathway in the brain may be a convergent pathway in the pathogenesis of ASD bridging genetic background and environmental triggers (food allergy) and that mTOR over-activation could serve as a potential therapeutic target for the treatment of ASD.

Introduction

Autism spectrum disorder (ASD) is characterized by severe and pervasive impairments in communication and social interaction, and by the presence of stereotyped or repetitive behaviors and interests*(1)*. The etiology of ASD is thought to be multifactorial and involves an interaction of a complex genetic background but also environmental triggers. Genome-wide association studies, copy number variation screening, and SNP analyses have identified several ASD candidate genes, and a large number of genetic mutations have been proposed to cause genetic predisposition to ASD*(2, 3)*. There is also increasing evidence indicating that immune dysfunction in ASD individuals may play a role in the pathophysiology of ASD*(4)*. An increased intestinal permeability was found in 36.7% of patients with ASD, compared to 4.8% of adult normal subjects and none of the child controls*(5)*. Furthermore, it is demonstrated that autistic patients on cow's milk elimination diet for 8 weeks showed marked improvement in behavioral disturbances*(6)*. These studies indicate that intestinal immune dysfunction, including food allergy, as an environmental trigger may cause disturbances in the pathways underlying so-called 'gut-immune-brain axis', which may fundamentally contribute to the development of ASD*(4, 7)*.

The mTOR signaling pathway is a critical regulator in various cellular processes including protein synthesis and synaptic plasticity*(8, 9)*. mTOR activation depends on several extracellular stimuli such as nutrients (amino acids), energy status, and growth factors. mTOR activation involves activation of phosphoinositide 3-kinase (PI3K)/AKT pathway and eventually leads to the phosphorylation of p70 S6 kinase (p70 S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1)*(10, 11)*. Both p70 S6K and 4E-BP1 are centrally involved in protein translation*(12, 13)*. Findings from various single-gene mutant mouse models suggest that enhanced mTOR signaling may play a central role in the pathophysiology of ASD and pharmacological attenuation of the mTOR signaling pathway by rapamycin rescued diverse ASD-relevant phenotypes ranging from behavioral impairments to morphological abnormalities in the brain*(14–16)*. Our previous studies in a murine model of cow's milk allergy (CMA) have demonstrated also that phosphorylation of mTOR complex 1 (mTORC1) downstream proteins p70 S6K and 4E-BP1 is enhanced in the prefrontal cortex and amygdala, which are brain regions relevant for ASD-related behaviors. Indeed, behavioral abnormalities including reduced social interaction and increased repetitive behavior were found in CMA mice. Pharmacological inhibition of the mTORC1 pathway by rapamycin reversed the autistic-like behaviors in CMA mice*(17)*. In addition, a recent study using BTBR inbred mouse

model for ASD showed that rapamycin improved several measures of sociability in BTBR mice as well*(18)*.

Amino acids are not only the basic building blocks for protein synthesis, but also implicated in regulation of mTORC1 activation. In murine mammary epithelial cells cultured under lactogenic conditions, threonine, histidine, and lysine inhibited S6K1 phosphorylation both individually and as a mix *(19)*. For screening purposes the effects of individual and combined amino acids on mTOR signaling, antigen-specific degranulation and cytokine production in bone-marrow derived mast cells (BMMCs) were tested*(20)*. Mast cells have a critical role in inflammatory processes including intestinal allergic reactions*(21, 22)*. It has been shown that key elements of mTORC1 pathway including mTOR, p70 S6K, and 4E-BP1 are phosphorylated in response to antigen stimulation, suggesting that the mTORC1 pathway is activated in antigen-induced BMMCs*(23)*. Indeed, this was confirmed and it was further demonstrated that inhibition of mTORC1 activity by rapamycin resulted in an inhibition of antigen-specific mast cell degranulation and cytokine production*(20)*. Furthermore, the individual amino acids threonine, histidine, and lysine or a mix of these amino acids inhibited the mTORC1 pathway and reduced mast cell activation, while the individual amino acids leucine, isoleucine, and valine or a mix of these amino acids enhanced the mTORC1 pathway in antigen-IgE-activated mast cells*(20)*. Therefore, for the present *in vivo* studies, a specific diet was designed that contained increased relative amounts of threonine, histidine, and lysine and reduced relative amounts of leucine, isoleucine, and valine, to attenuate the mTOR signaling pathway.

The current study investigated the ability of this mTOR-targeting amino acid diet (Active 1 diet) and a multi-nutrient supplementation diet (Active 2 diet) to improve behavioral impairments and inhibit the mTOR signaling pathway in ASD-related brain regions in a mouse model of CMA and in an inbred BTBR *T*$^{+}$ *Itpr3*^{tf}*/J* (BTBR) mouse strain for ASD. BTBR is a commercially available inbred mouse strain that exhibits several core symptoms of autism including low levels of reciprocal social interaction*(24–26)* and high levels of repetitive self-grooming behavior*(27)*. The Active 2 diet contained specific anti-inflammatory and neuroprotective ingredients and was previously shown to ameliorate behavioral impairments in a prenatal valproate-induced murine model for ASD*(28)*. The current results suggest that both the mouse model of CMA as well as the BTBR mouse model for ASD can serve as an attractive translational platform to evaluate the therapeutic efficacy of mTOR-targeting interventions including specific medical diets. Targeting the dysregulated mTOR signaling pathway may shed more light on potential therapies for food allergy-associated ASD.

Methods & Materials

Animals

All animal procedures were approved by and conducted in strict compliance with the guidelines of the Animal Ethics Committee of Utrecht University (approval number: 2012.I.04.054 and 2014.I.02.009). Four-week-old specific pathogen free male C3H/HeOuJ mice were purchased from Charles River Laboratories (L'Arbresle Cedex, France). Four-week-old male BTBR *T*$^{+}$ *Itpr3*^{tf}/J and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All animals were housed under standard conditions at the animal facility of the Utrecht University, Utrecht, The Netherlands.

Induction of cow's milk allergy in mice

The CMA mouse model (Figure 1) was conducted as described previously*(17, 29)*. Briefly, male C3H/HeOuJ mice were intragastrically (i.g.) sensitized with 20 mg whey (DMV International, Veghel, The Netherlands), with 10 µg cholera toxin (CT, List Biological Laboratories, Campbell, CA, USA) as an adjuvant in 0.5 ml PBS. Control mice received CT alone. Mice were sensitized once a week for 5 consecutive weeks. One day after the last sensitization, self-grooming behavior of the mice was assessed. One week after the last sensitization, mice were challenged i.g. with 50 mg whey/0.5 ml PBS. Increased whey-specific IgE, IgG1, and IgG2a levels were measured in the serum of CMA mice, confirming the allergic reaction to whey*(30)*. One day after challenge, social interaction of the mice was assessed. Afterwards, mice were sacrificed to collect brain tissues for further analysis.

Dietary intervention

The compositions of the Control, Active 1, Active 2 diets are presented in table 1. The Active 1 diet contained relative high levels of the amino acids lysine, histidine, and threonine and relatively low levels of the amino acids isoleucine, leucine, and valine. The Active 2 diet consisted of low-glycemic index carbohydrates, dietary fibers, high tryptophan content and a lipid profile that predominantly differed in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) content. The diets differed in the relative amounts of the amino acids leucine (Leu), isoleucine (Ile), and valine (Val) as compared to histidine (His), lysine (Lys), and threonine (Thr). The weight to weight ratio of [Leu + Ile + Val] / [His + Lys + Thr] for the respective diets were 1.53 (Control), 0.67 (Active 1), and 1.32 (Active 2). These diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands) and were based on standard animal food for laboratory rodents

AIN93-G(*31*). C3H/HeOuJ mice were fed the Control, Active 1 or Active 2 diet, starting 2 weeks prior to the first sensitization and continued during the entire experiment.
For dietary intervention study in the BTBR mouse model for ASD, BTBR mice or C57BL/6 control mice were fed the Control, Active 1, or Active 2 diet for 8 consecutive weeks. After 8 weeks of dietary treatment, both BTBR and C57BL/6 mice were subjected to social interaction and grooming tests. Subsequently, mice were sacrificed and brain tissues were collected for western blot analysis.

Table 1. Compositions of Control, Active 1, and Active 2 diet.

	Control diet components (g/kg)	**Active 1 diet** components (g/kg)	**Active 2 diet** components (g/kg)
Carbohydrates			
Cornstarch	322.48	331.89	260.84
Dextrinized cornstarch	132.00	132.00	
Sucrose	100.00	100.00	
Dextrose	100.00	100.00	100.00
Maltodextrin (DE6)			101.35
Galactose			34.94
Fructose			2.32
Isomaltulose			104.34
Fibers			
Fiber source (cellulose)	50.00	50.00	**21.29**
Rice fiber			**82.28**
GOS			**16.00**
FOS			**0.87**
Protein			
Alanine	7.7	7.8	6.6
Arginine	13.3	13.5	7.3
Aspartic acid	20.1	20.4	19.9
Cystine	2.0	2.1	3.3
Glutamine	13.5	13.7	10.4
Glutamic acid	21.5	21.7	19.3
Glycine	7.5	7.6	5.0
Histidine	**4.3**	**6.3**	**3.7**
Iso-leucine	**8.4**	**5.4**	**9.4**
Leucine	**14.5**	**9.4**	**14.5**
Lysine HCl	**9.6**	**14.2**	**12.0**
Methionine	5.0	5.0	4.7
Phenylalanine	9.3	9.3	6.8
Proline	8.4	8.5	8.3
Serine	8.8	8.9	8.5
Threonine	**6.7**	**9.8**	**9.0**

Table 1 continued

	Control diet components (g/kg)	**Active 1 diet** components (g/kg)	**Active 2 diet** components (g/kg)
Tryptophan	2.1	2.2	5.2
Tyrosine	6.7	6.7	5.6
Valine	**8.7**	**5.6**	**8.6**
Total	178.1	168.8	168.0
Fat			
Soy oil	26.6	26.6	
Coconut oil	12.6	12.6	
Corn oil	30.8	30.8	**8**
MCT oil			**10.7**
Fish oil (DHA 25)			**6.5**
Fish oil (EPA 28/12)			**27.5**
Phospholipids			**7.6**
Others			
Mineral mix	35.0	35.0	35.0
Vitamin mix	10.0	10.0	10.0
extra Vitamin A			**4000 IU**
extra Vitamin B6 (pyridixine)			**6 mg**
extra foliumzuur			**2 mg**
extra Vitamin B12			**25 µg**
extra Vitamin D			**1000 IU**
Choline bitartrate	2.50	2.50	2.50
Tert-butylhydroquinone (TBHQ)	0.014	0.014	0.014

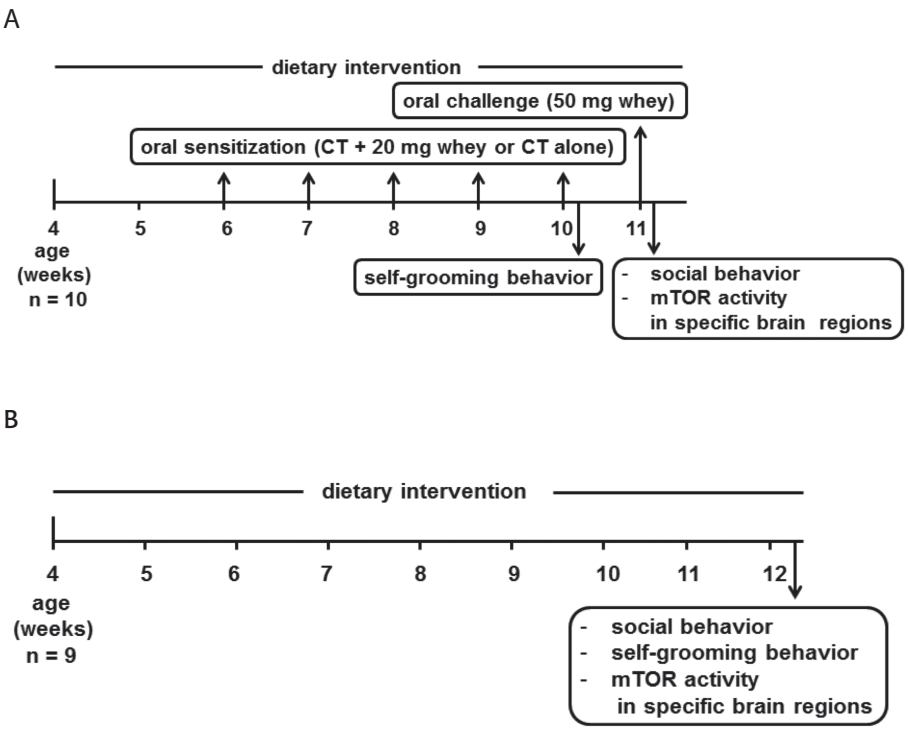


Fig. 1. Schematic overview of two animal experiments. (A) The mouse model of CMA, n = 10 mice per group. (B) The BTBR mouse model of autism, n = 9 mice per group.

Behavioral tests

Social interaction test

Social interaction test was performed as described previously*(17)*. In short, mice were individually placed in a 45 x 45 cm open field, with a small perforated Plexiglas cage (10 cm diameter) located against one wall allowing visual, olfactory and minimal tactile interaction (Fig. 2A). Mice were habituated to the open field for 5 min and an age- and gender-matched unfamiliar target mouse was introduced in one of the cages for an additional 5 min. By using video tracking software (EthoVision 3.1.16, Noldus, Wageningen, The Netherlands), an interaction zone around the cage was digitally determined (Figure 2A, the grey square area indicates an interaction zone). Time spent in the interaction zone, latency until first occurrence in the interaction zone and total distance moved were measured.

Self-grooming

The mice were scored for spontaneous grooming behaviors as described earlier *(32, 33)*. Each mouse was placed individually in an empty home cage (35 cm length x 20 cm wide x 13 cm high) without bedding and video recordings were used for behavioral scorings of frequency and cumulative time spent grooming all body regions. After a 5 min habituation period in the cage, each mouse was scored blindly for 10 min by two independent researchers using The Observer XT software (Noldus Information Technology, Wageningen, The Netherlands). Inter-rater reliability for the self-grooming tests in the mouse model of CMA was 96% and in the BTBR mouse model was 98.7%. The cage was cleaned with water followed by 70% ethanol after each test.

Western blotting

After decapitation, brain tissues were immediately isolated from mice, snap frozen in 2-methylbutane (Sigma-Aldrich, Zwijndrecht, The Netherlands) and dry ice, and stored at -70 °C. Coronal slices of 500 µm were sectioned using a cryostat (Model700, Laméris Instruments, Utrecht, The Netherlands). Bilateral brain regions (prefrontal cortex, amygdala, somatosensory cortex) were isolated from the coronal slices using a scalpel. The coronal slices for the isolation of prefrontal cortex, amygdala, and somatosensory cortex were sectioned from bregma 2.34 mm to bregma 1.34 mm, from bregma -0.58 mm to bregma -2.06 mm, and from bregma 0.50 mm to bregma -1.06 mm, respectively. To prepare lysates, frozen tissues were sonicated in lysis buffer containing RIPA buffer (Fisher Scientific, Landsmeer, The Netherlands), complete mini protease inhibitor cocktail tablets (Roche, Almere, The Netherlands), benzonase nuclease, AEBSF, and phosphatase inhibitor cocktail (Calbiochem, Amsterdam, The Netherlands). Homogenate was centrifuged at 14,000 rpm for 20 min and supernatant was collected. Protein concentration was determined using BCA kit (Pierce, Rockford, USA). For western blotting, 30 µg of sample was used to detect the phosphorylation of mTOR and AKT, or 55 µg of sample was used to detect the phosphorylation of p70 S6K. The lysates were loaded onto Criterion TGX precast gel (Bio-Rad, Veenendaal, The Netherlands), and blotted overnight onto PVDF membrane (Bio-Rad, Veenendaal, The Netherlands), which was blocked in 5% nonfat dry milk for 1 hour. Subsequently, membranes were washed with TBS/0.1% Tween-20 (Sigma-Aldrich, Zwijndrecht, The Netherlands) 3x10 min and incubated overnight with the primary antibodies at 4°C. The primary antibodies against phospho-mTOR (Ser2448, #5536), mTOR (#2972), phospho-AKT (Ser473, #4060), AKT (#9272), phospho-p70 S6K (Thr389, #9205), p70 S6K (#9202), phospho-4E-BP1 (Thr37/46, #9459), and 4E-BP1 (#9452) were obtained from

Cell Signaling Technology, Leiden, The Netherlands. Afterwards, the membranes were washed with TBS/0.1% Tween-20 3 x 10 minutes and incubated with HRP-conjugated goat anti-rabbit secondary antibody (DAKO, Eindhoven, The Netherlands). Finally the immunoreactive bands were detected by ECL prime kit (Health Care, Amsterdam, The Netherlands) and the results were normalized with the non-phosphorylated corresponding protein using quantitative densitometry (Bio-Rad, Veenendaal, The Netherlands) and reported as relative band densities. Membranes were reprobed for a maximal of 3 times with different primary antibodies after stripping the membranes with Restore Western Blot Stripping buffer (Pierce, Rockford, USA).

As shown in Fig. S1 and S2, the primary antibody against phospho-p70 S6K (Thr389) detects endogenous p70 S6K as well as p85 S6K. P70 S6K is functionally relevant for mTOR signaling pathway as this protein plays a critical role in regulating protein translation through the phosphorylation of ribosomal protein S6*(34)*. In addition, it was previously demonstrated that the phosphorylation of 4E-BP1 and p70 S6K is differentially regulated as long-term suppression of mTORC1 activity by rapamycin recovered the phosphorylation of 4E-BP1 in various cell types*(20, 35)*. Therefore, the phosphorylation status of p70 S6K is considered a better indicator for mTORC1 activity compared to that of 4E-BP1 and has been used in the previous animal studies as the most reliable readout for mTORC1 activation*(14, 15)*. In the current study, the phosphorylation of mTOR, AKT, and p70 S6K as readouts for the activation of mTOR signaling pathway have been measured.

Statistical analysis

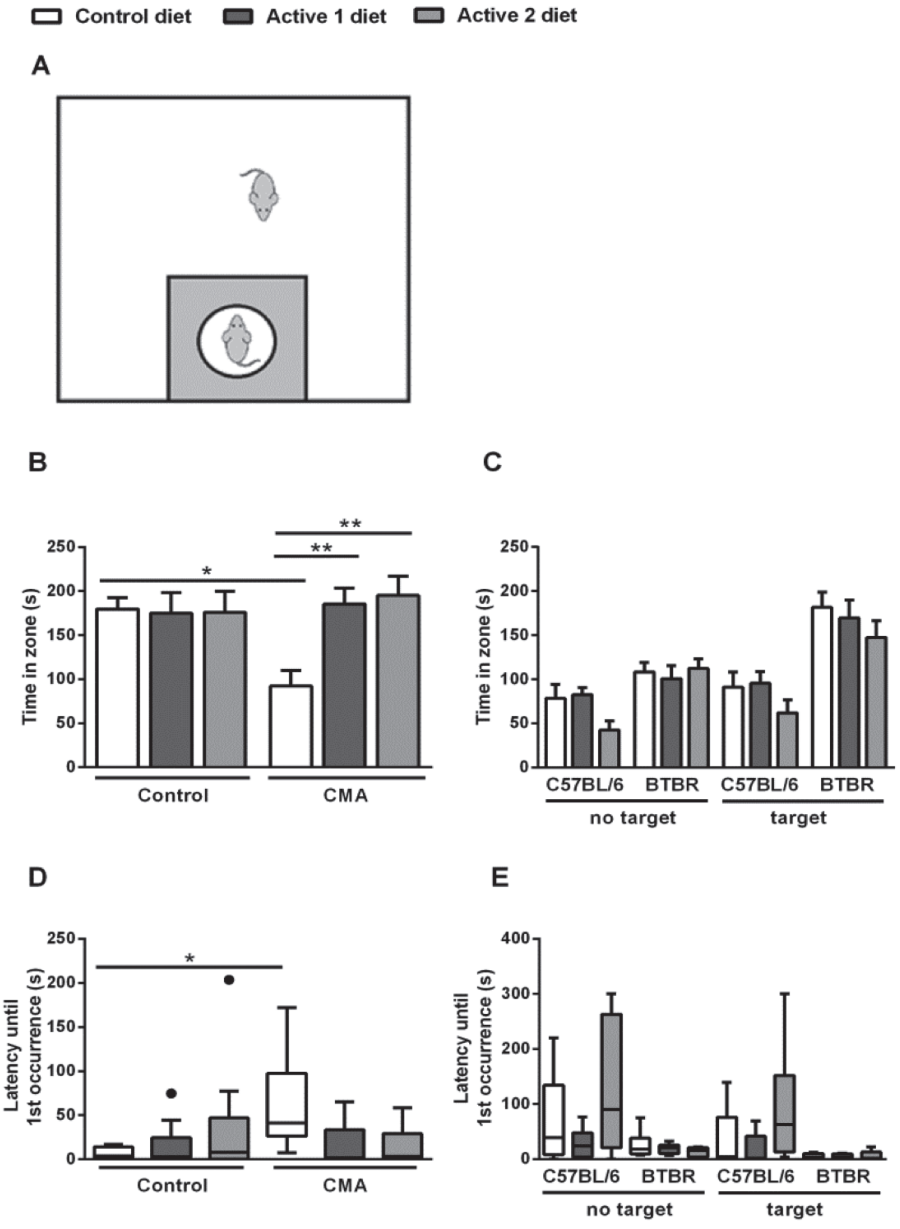
In general, results were statistically analyzed using a two-way ANOVA with between-subject factors Model, having two levels (Control-CMA, or C57BL/6-BTBR), and Diet, having 3 levels (Control-Active 1-Active 2). Where appropriate, the within-subject factor Phase, having 2 levels (no target-target), was added as a repeated measure. For all ANOVAs, significant main effects and interactions were followed by Bonferroni's multiple comparisons test. For latency of first approach to the interaction zone in the social interaction test, Kruskal-Wallis test followed by Dunn's multiple comparisons test was conducted. Differences were considered statistically significant when $P < 0.05$. Analyses were performed using IBM SPSS Statistics 20.

Results

Both Active 1 and Active 2 diets improved social behavior of CMA mice

Previous studies have demonstrated that rapamycin, a specific mTOR inhibitor, improved social behavior of CMA mice*(17)*, and improved several measures of sociability in the BTBR mouse model for ASD*(18)*. In the current study, CMA mice were exposed to a social interaction test one day after challenge (Fig 2A). A two-way ANOVA showed a significant interaction between Model and Diet ($F_{2,53}$ = 4.184, P = 0.0205, Fig 2B). CMA mice on the Control diet spent significantly less time with the interaction mouse compared to control mice (P = 0.0125). Social behavior was normalized when CMA mice were fed the Active 1 (P = 0.0055) or Active 2 diet (P = 0.0020). Latency of first approach to the interaction mouse was significantly increased in CMA mice compared to control mice (Kruskal-Wallis test: P = 0.0243, Dunn's multiple comparison test: P = 0.0424, Fig. 2D). The increased latency in CMA mice was not observed when CMA mice were fed the Active 1 or Active 2 diet. For distance travelled, a 3-way ANOVA showed a significant interaction between Phase and Model ($F_{1, 54}$ = 4.219, P = 0.045), but no significant interaction between Phase and Diet ($F_{2, 54}$ = 0.530, P = 0.592) or between Phase, Model, and Diet ($F_{2, 54}$ = 0.364, P = 0.696). The locomotor activity of the control and CMA mice fed different diets in both habituation phase (no target) and interaction phase (target) did not significantly differ between groups (Fig. 2F).

To investigate the effect of the Active 1 diet and Active 2 diet on social behavior in the BTBR mouse model for ASD, social interaction of BTBR mice was determined after 8 consecutive weeks of dietary treatment. For social interaction, a 3-way ANOVA showed a significant interaction between phase and model ($F_{1, 46}$ = 15.708, P < 0.001), but no significant interaction between Phase and Diet ($F_{2, 46}$ = 0.771, P = 0.469) or between Phase, Model, and Diet ($F_{2, 46}$ = 1.661, P = 0.201). BTBR mice spent more time in the interaction zone compared to C57BL/6 both in the absence (no target: $F_{1, 46}$ = 16.48, P = 0.0002) and presence (target: $F_{1, 46}$ = 36.00, P < 0.0001) of an interaction mouse (Fig. 2C). No significant differences were observed in latency of first approach to the interaction mouse comparing C56BL/6J and BTBR mice, although the BTBR mice tended to display shorter latencies (Kruskal-Wallis test: P = 0.0547, Fig. 2E). For distance travelled, a 3-way ANOVA showed a significant interaction between Phase and Model ($F_{1, 46}$ = 53.3010, P < 0.001), but no significant interaction between Phase and Diet ($F_{2, 46}$ = 0.6360, P = 0.534) or between Phase, Model, and Diet ($F_{2, 46}$ = 0.8210, P = 0.446). BTBR mice displayed significantly increased locomotor activity compared to C57BL/6 mice in the absence (no target: $F_{1,46}$ = 64.61, P < 0.0001, Fig. 2G) and in the presence (target: $F_{1,46}$ = 5.548, P = 0.0228) of an interaction mouse (Fig. 2G).


Control diet
Active 1 diet
Active 2 diet
A
B
Time in zone (s)
250
200
150
100
50
0
*
**
**
Control
CMA
C
Time in zone (s)
250
200
150
100
50
0
C57BL/6
BTBR
C57BL/6
BTBR
no target
target
D
Latency until 1st occurrence (s)
250
200
150
100
50
0
*
Control
CMA
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Latency until 1st occurrence (s)
400
300
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BTBR
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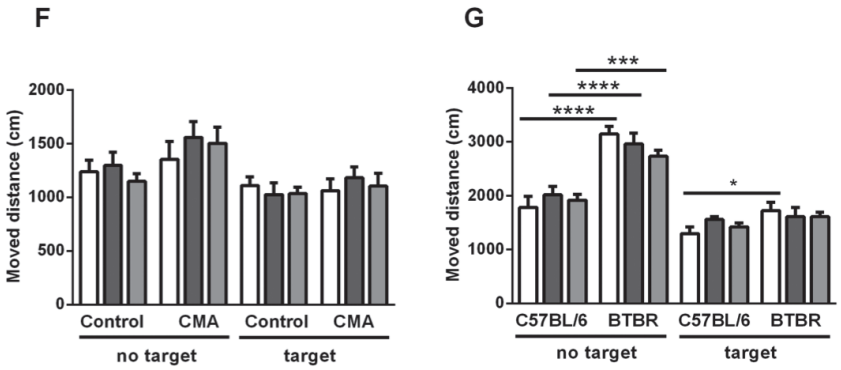

Fig. 2. Effect of dietary intervention with either Active 1 or Active 2 diet on social behavior in CMA mice and BTBR mice. (A) Schematic representation of the social interaction test. (B) CMA mice exhibited lack of sociability, defined as reduced time spent in the interaction zone compared to time of control mice spent in the interaction zone. Dietary intervention with either Active 1 or Active 2 diet rescued the social deficit in CMA mice. (C) BTBR mice spent significantly more time in the interaction zone compared to C57BL6 mice during the interaction session, irrespective of the dietary interventions. (D) CMA mice exhibited lack of sociability as the latency of first approach to the interaction zone was significantly increased in CMA mice compared to that in control mice. Dietary intervention with either Active 1 or Active 2 diet ameliorated the increased latency in CMA mice. (E) It took less time, but not significantly, for BTBR mice to approach the interaction mouse compared to C57BL/6 mice. Dietary intervention with either Active 1 or Active 2 diet did not affect the latency in both C57BL/6 mice and BTBR mice. (F) The locomotor activity, measured by moved distance, during both the habituation phase (no target) as well as the interaction phase (target) was barely affected in both CMA mice and control mice, irrespective of the dietary interventions. (G) BTBR mice displayed significantly increased locomotor activity during the habituation phase. Dietary intervention with either Active 1 or Active 2 diet did not affect the locomotor activity in both C57BL/6 mice and BTBR mice. Two-way ANOVA followed by a Bonferroni's multiple comparisons test was conducted and data are presented as mean time (s) ± SEM for (B and C) and mean distance moved (cm) for (F and G). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. For latency, Kruskal-Wallis test followed by a Dunn's multiple comparisons test was conducted and data are presented as mean time (s) ± SEM, $^{*}P < 0.05$. (B, D & F) n = 10 per group; (C, E & G) n = 9 per group.

Active 1 diet ameliorated repetitive behavior of both CMA mice and BTBR mice and Active 2 diet ameliorated repetitive behavior of CMA mice

As it was previously shown that administration of rapamycin reduced repetitive self-grooming behavior in CMA mice*(17)*, the current study examined the effect of Active 1 diet and Active 2 diet on novelty-induced repetitive self-grooming behavior of CMA mice.

The two-way ANOVA showed a significant main effect of Model ($F_{1, 54}$ = 15.30, P = 0.0003) and a significant main effect of Diet ($F_{2, 54}$ = 5.402, P = 0.0073) for the duration of self-grooming (Fig 3A). CMA mice showed increased duration of self-grooming compared to control mice and both the Active 1 diet and Active 2 diet normalized the increased duration of self-grooming (Active 1 diet: P = 0.0053; Active 2 diet: P = 0.0167, Fig. 3A). A

significant interaction between Model and Diet was observed for the frequency of self-grooming ($F_{2,54}$ = 4.869, *P* = 0.0114, Fig. 3C), with CMA mice on the Control diet showing an increase in frequency of self-grooming compared to control mice on the Control diet (*P* = 0.0027), which was not observed in CMA mice that were fed the Active 1 diet or Active 2 diet (Active 1 diet: *P* = 0.0037; Active 2 diet: *P* = 0.0363).

Significant interactions between Model and Diet on duration and frequency of repetitive self-grooming were also observed in BTBR mice (duration: $F_{2,45}$ = 4.240, *P* = 0.0207, Fig. 3B; frequency: $F_{2,45}$ = 6.009, *P* = 0.0049, Fig. 3D). Consistent with previous findings*(27, 36, 37)*, BTBR mice fed the Control diet engaged in much longer bouts of self-grooming (*P* = 0.0169, Fig. 3B) and showed a higher self-grooming frequency (*P* = 0.0146, Fig. 3D) compared to control C57BL/6 mice on the Control diet. Dietary intervention with Active 1 diet significantly reduced repetitive self-grooming scores in BTBR mice (self-grooming duration: *P* = 0.0140; self-grooming frequency: *P* = 0.0007) while dietary intervention with Active 2 diet did not alter duration of self-grooming and reduced frequency of self-grooming in BTBR mice.

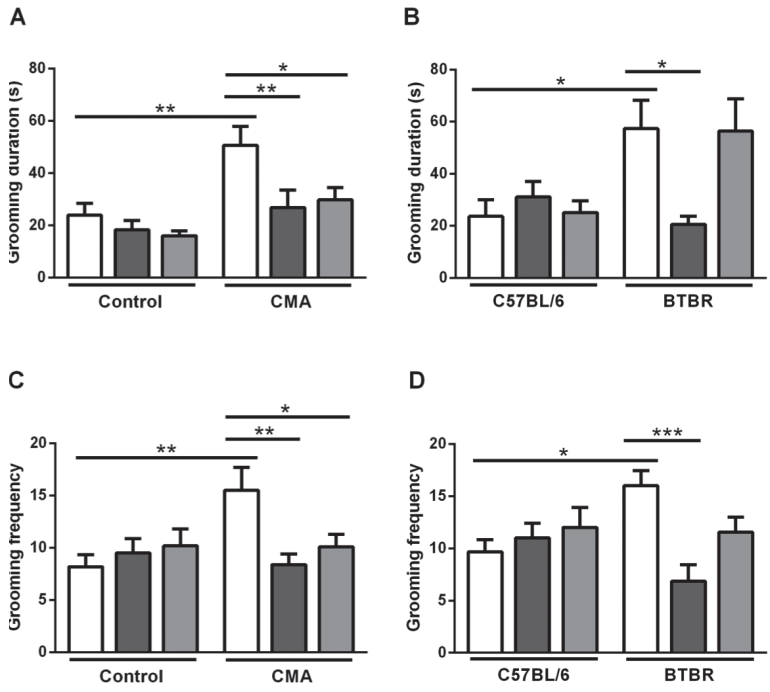

Fig. 3. Effect of dietary intervention with either Active 1 or Active 2 diet on repetitive self-grooming behavior in CMA and BTBR mice. Cumulative time spent self-grooming behavior and self-grooming frequency were scored over a 10-min period in an empty, clean home cage after a 5-min habituation period. (A) CMA mice displayed

significantly increased amount of time spent self-grooming compared to control mice and this effect was reduced by dietary intervention with either Active 1 or Active 2 diet. (B) BTBR mice displayed significantly increased amount of time spent self-grooming compared to C57BL/6 mice. Dietary intervention with Active 1 diet, but not Active 2 diet, reduced the amount of time spent self-grooming in BTBR mice. (C) CMA mice fed either Active 1 or Active 2 diet displayed significant reductions in high levels of self-grooming frequency. (D) High levels of self-grooming frequency were significantly reduced in BTBR mice fed the Active 1 diet, but not when fed the Active 2 diet. Two-way ANOVA followed by a Bonferroni's multiple comparisons test was conducted and data are presented as mean duration (s) ± SEM for (A and B) and mean frequency for (C and D), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A & C) n = 10 per group; (B & D) n = 9 per group.

Both Active 1 and Active 2 diets attenuated mTORC1 signaling in the prefrontal cortex and in the amygdala of CMA mice

Previous studies have demonstrated that rapamycin treatment effectively blocks the enhanced mTOR signaling in the prefrontal cortex and amygdala of CMA mice*(17)*. To examine the effect of Active 1 diet and Active 2 diet on enhanced mTOR signaling pathway in the brain of CMA mice, the phosphorylation of mTOR-related proteins was measured in the prefrontal cortex (PFC, Fig. 4A), amygdala (Fig. 4B), and somatosensory cortex (SSC, Fig. 4C) via western blotting analysis. The phosphorylation of mTOR (Fig. 4D-F) and AKT (Fig. 4G-I) was not affected by CMA or diets in the examined regions of the brain. In contrast, a significant two-way ANOVA interaction was observed between Model and Diet on the phosphorylation of p70 S6K in the PFC ($F_{2,18} = 8.775$, $P = 0.0022$, Fig. 4J) and amygdala ($F_{2,18} = 4.724$, $P = 0.0234$, Fig. 4K). The phosphorylation of p70 S6K, a downstream protein of mTORC1, was significantly enhanced in the PFC ($P = 0.0001$) and amygdala ($P = 0.0047$) of CMA mice on Control diet compared to control mice on Control diet, which is consistent with previous findings*(17)*. Treatment of CMA mice with either Active 1 or Active 2 diet effectively reduced the levels of phosphorylated p70 S6K in the PFC (Active 1 diet: $P = 0.0006$, Active 2 diet: $P = 0.0002$) and amygdala (Active 1 diet: $P = 0.0067$, Active 2 diet: $P = 0.0072$). The phosphorylation of p70 S6K (Fig. 4L) was not affected in the SSC of CMA mice. Photos of the Western blot experiments are presented in the Supplementary Results (Fig. S1).

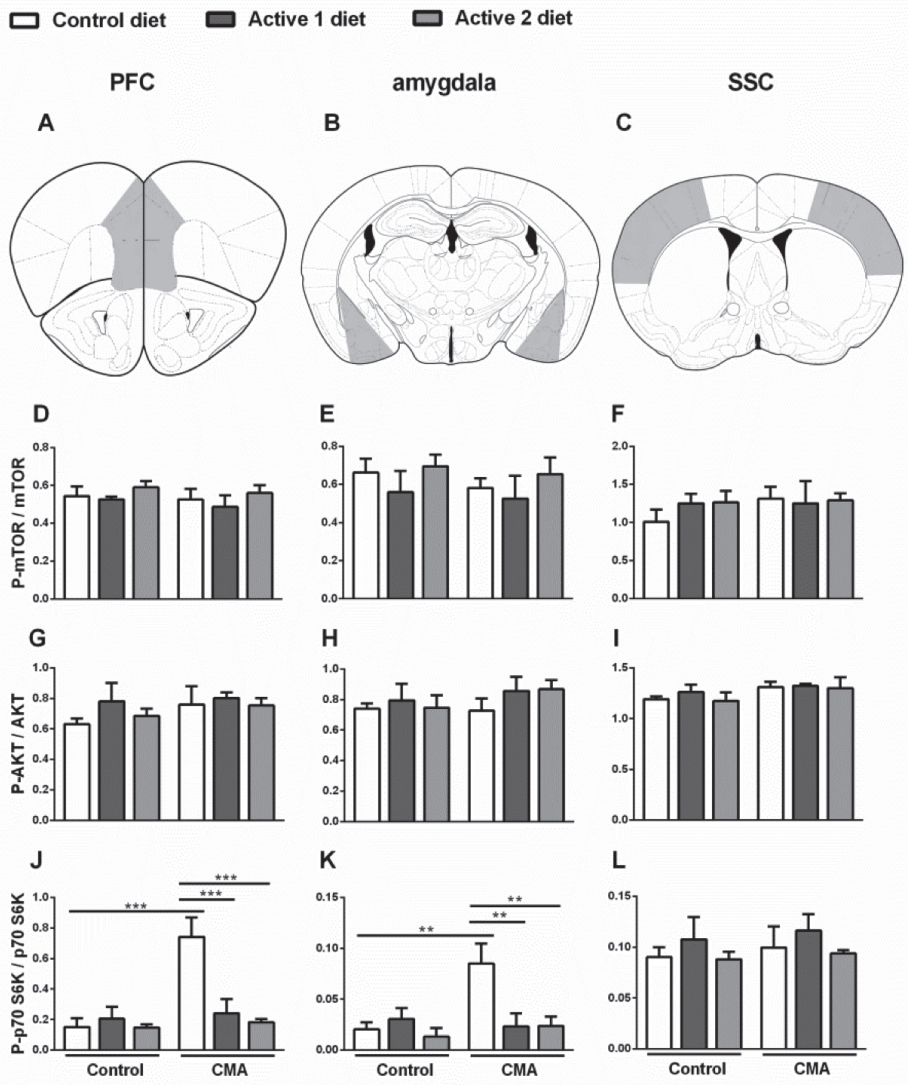

Fig. 4. Effect of dietary intervention with either Active 1 or Active 2 diet on mTOR signaling in the prefrontal cortex (PFC), amygdala, and somatosensory cortex (SSC) in CMA mice measured by western blot analysis. Figure A, B, and C are schematic illustrations of a typical brain slice for the isolation of each brain region. The gray areas in figure A, B, and C indicate PFC, amygdala, and SSC, respectively. (D to I) The phosphorylation of mTOR and AKT in the PFC, amygdala, and SSC was not affected by either the induction of CMA or dietary interventions. (J to K) A significant increase of the phosphorylation of p70 S6K was measured in the PFC and amygdala in CMA mice. CMA mice fed either Active 1 or Active 2 diet exhibited significant reductions in the phosphorylation of p70 S6K in the PFC and amygdala. (L) The phosphorylation of p70 S6K in the SSC was barely affected by either the induction of CMA or dietary interventions. Densities of phosphorylation of mTOR, AKT, and p70 S6K were divided by the non-phosphorylated corresponding protein. Two-way ANOVA followed by a Bonferroni's multiple comparisons test was conducted and data are presented as mean relative density ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$. (A to B, D to E, G to H) n = 4 per group; (C, F, and I) n = 2 to 4 per group.

Active 1 diet attenuated mTOR signaling pathway in the prefrontal and somatosensory cortices of BTBR mice

Activation of the mTOR signaling pathway was examined in male BTBR mice as well. As observed in CMA mice, no significant differences in the phosphorylation of mTOR (Fig. 5D-F) or AKT (Fig. 5G-I) in PFC, amygdala, and SSC were found when comparing BTBR and C57BL/6 mice, irrespective of dietary intervention. For the phosphorylation of p70 S6K, a two-way ANOVA showed no significant interaction between model and diets in the PFC ($F_{2,17}$ = 1.946, P = 0.1734, Fig. 5J). In the amygdala and the SSC, a two-way ANOVA showed a significant interaction between Model and Diets for the phosphorylation of p70 S6K (amygdala: $F_{2,17}$ = 4.081, P = 0.0357, Fig. 5K; SSC: $F_{2,18}$ = 4.421, P = 0.0274, Fig 5L). The Active 1 diet significantly reduced phosphorylation of p70 S6K in the PFC ($F_{2,17}$ = 47.77, P < 0.0001, Fig 5J), which was observed both in C57BL/6 mice (P = 0.0004) and in BTBR mice (P < 0.0001). The Active 2 diet had no effect on the phosphorylation of p70 S6K in the PFC of C57BL/6 mice and BTBR mice. In the amygdala, BTBR mice fed the Active 2 diet showed a significant reduction in phosphorylation of P70 S6K, compared to C57BL/6 mice fed the Active 2 diet (P = 0.0339). In the SSC, the phosphorylation of p70 S6K is significantly reduced in BTBR mice compared to that in C57BL/6 mice (P = 0.0008). Low level of phosphorylation of p70 S6K was significantly further reduced by the dietary intervention with Active 1 diet (P = 0.0050). A similar effect of Active 1 diet was also observed in the SSC of C57BL/6 mice (P < 0.0001). Active 2 diet did not affect the phosphorylation of p70 S6K in the SSC in both C57BL/6 mice and BTBR mice. Photos of Western blot experiments are provided in the Supplementary Results (Fig. S2).

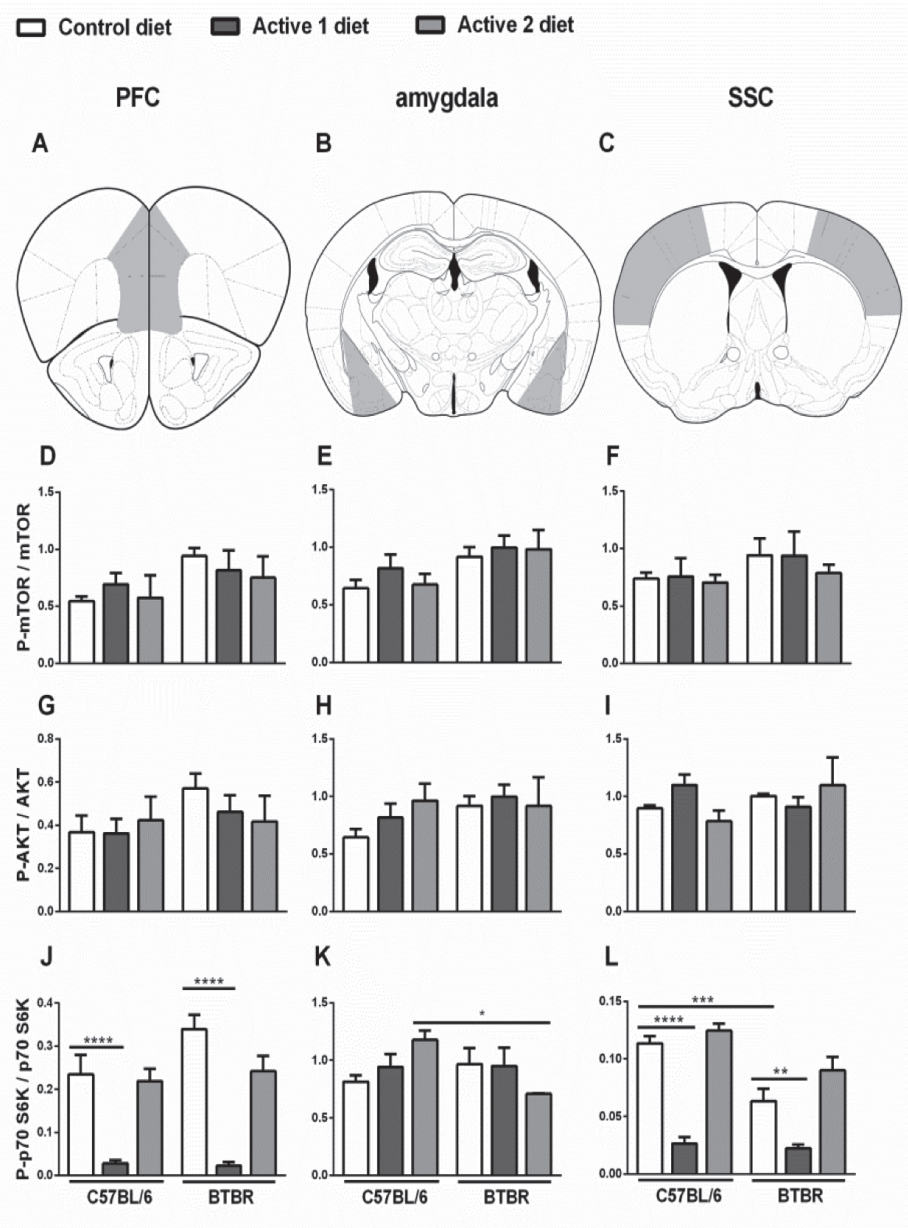

Fig. 5. Effect of dietary intervention with either Active 1 or Active 2 diet on mTOR signaling in the prefrontal cortex (PFC), amygdala, and somatosensory cortex (SSC) in BTBR mice measured by western blot analysis. Figure A, B, and C are schematic illustrations of a typical brain slice for the isolation of each brain region. The gray areas in figure A, B, and C indicate PFC, amygdala, and SSC, respectively. (D to I) The phosphorylation of mTOR and AKT in the PFC, amygdala, and SSC was not significantly altered in BTBR mice compared to that in C57BL/6 mice. (J and L) BTBR mice displayed a significant decrease in the phosphorylation of p70 S6K in the SSC compared to C57BL/6 mice. Dietary intervention with Active 1 diet, but not Active 2 diet, significantly inhibited the phosphorylation of

p70 S6K in the PFC and SSC in both C57BL/6 mice and BTBR mice. (K) The phosphorylation of p70 S6K is significantly reduced in amygdala of BTBR mice fed Active 2 diet compared to that in amygdala of C57BL/6 mice fed Active 2 diet. Densities of phosphorylation of mTOR, AKT, and p70 S6K were divided by the non-phosphorylated corresponding protein. Two-way ANOVA followed by a Bonferroni's multiple comparisons test was conducted and data are presented as mean relative density ± SEM, n = 4 per group.

Discussion

The current study investigated whether activation of mTOR signaling in the brain might be a novel therapeutic target for the treatment of ASD-like behavioral deficits by using dietary interventions. For this goal, two different mouse models displaying ASD-like behavior were used. The ability of dietary interventions to improve behavioral impairments by attenuating mTOR activity in the brains of CMA mice as well as of BTBR mice was investigated by using two diets: a diet containing a specific ratio of amino acids to target mTOR, indicated as Active 1 diet, and a multi-nutrient supplementation diet containing specific neuroprotective and anti-inflammatory ingredients, indicated as Active 2 diet. The present study demonstrated that dietary intervention with either Active 1 diet or Active 2 diet normalized food allergy-induced autistic-like behavioral deficits in a mouse model of CMA, which may be attributed to the attenuation of the enhanced mTOR signaling in the PFC and amygdala of CMA mice. In the BTBR mouse model of autism, it was demonstrated that BTBR mice manifested more intensive self-grooming behavior compared to C57BL/6 mice, which is consistent with previous findings*(25, 27, 36)*. In addition, we demonstrated no clear increased activation of mTOR signaling in the brain of BTBR mice compared to that of C57BL/6 mice. Dietary intervention with the Active 1 diet normalized the repetitive self-grooming behavior while dietary intervention with Active 2 diet had no effect on behavioral deficits in BTBR mice. The mTOR signaling in the PFC and SSC of mice was attenuated by Active 1 diet, but not affected by Active 2 diet.

The current study demonstrated that the phosphorylation of mTORC1 downstream protein p70 S6K was enhanced in the PFC and amygdala in CMA mice, which is consistent with previous findings*(17)*. The enhanced phosphorylation levels of p70 S6K at Thr389 reflect enhanced mTOR activity, because this epitope on p70 S6K is directly phosphorylated by mTOR*(34)*. It is known that mTOR phosphorylation itself is a poor indicator for mTOR activity and mTOR activity is routinely determined by measuring the phosphorylation status of p70 S6K at Thr389*(11, 38–41)*. AKT is not only the upstream protein of mTORC1, but also the downstream protein of mTORC2*(11)*. No significant difference was observed in all groups regarding the AKT phosphorylation,

suggesting that mTORC2 activity may be not involved in the development of ASD-related phenotypes in CMA mice. Given the fact that p70 S6K plays a crucial role in protein translation*(34, 42, 43)*, enhanced p70 S6K activity in the PFC and amygdala of CMA mice leads to increased translation of synaptic cell-adhesion molecules including neuroligins (NLGNs), which cause increased excitation/inhibition (E/I) ratio, potentially leading to the development of ASD phenotypes*(8, 44)*. The PFC plays a central role in acquiring and representing both cognitive and motivational context information to generate the goal-directed behavior, which allows us to cope with complex and novel situations*(45, 46)*. Abnormal repetitive behavior can be attributed to damage to the corticostriatal circuits and disturbances in direct and indirect neurobiological pathways in the prefrontal cortex*(47)*. The amygdala has been implicated in mediating social behavior and dealing with emotions*(48)*. Destruction of amygdala impaired social recognition in mice*(49)*. In the current study, the augmentation of the mTOR signaling in the prefrontal cortex and amygdala may correlate with abnormal repetitive behavior and reduced social behavior found in CMA mice. Dietary attenuation of the mTOR signaling in the brain with either Active 1 or Active 2 diet improved the autistic-like behavioral alterations in CMA mice. Interestingly, systemic humoral immune response and mucosal mast cell degranulation were not affected by the dietary intervention with either Active 1 or Active 2 diet, but in the small intestine both diets restored the disturbed balance of T helper and regulatory T cells transcription factors*(30)*. These results suggest that the dietary interventions may exert its beneficial effects directly on the brain or via the intestinal immune system.

Unlike the Active 1 diet that is designed to attenuate the mTOR signaling pathway by modulating the relative amount of specific amino acids, the Active 2 diet is composed of various specific ingredients that play important roles in the development, maintenance, and function of the nervous system and/or influence (neuro)inflammatory responses. For instance, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) belong to omega-3 long-chain polyunsaturated fatty acids (n-3 LCPUFAs), which play a critical role in the development and the function of the brain and nervous system*(50–53)*. The dietary supplementation with n-3 LCPUFAs was shown in our previous studies to restore decreased prefrontal dopamine and metabolite levels and normalize social behavior in allergic mice*(54)*. Vitamins, especially B vitamins, are essential for neurodevelopment and neuronal function, and severe deficiencies have been linked to increased risk of neurodevelopmental disorders including ASD*(55, 56)*. Moreover, vitamin B6 has anti-inflammatory properties in the intestinal tract*(57)*. Previous studies have shown that both LCPUFAs and dietary fibers supplemented in the Active 2 diet are implicated

in modulating the mucosal immune activation to prevent allergic symptoms. For instance, n-3 LCPUFAs were shown to reduce the humoral response and acute allergic symptoms and enhance local intestinal and systemic Treg cell responses in the mouse model of CMA*(58)*. Dietary supplementation with the mixture of short-chain galacto-oligosaccharides (GOS) and long-chain fructo-oligosaccharides (FOS) effectively alleviated allergic symptoms in CMA mice*(59)* and reduced immunoglobulin free light-chain plasma concentrations, which might be involved in the pathophysiology of allergic disease, in infants at high risk for allergies*(60)*. The rice fibers were also shown to reduce inflammation by modulating colonic physiology and regulating T cell differentiation in a mouse model for colitis*(61)*. In the current study, the Active 2 diet reduced food allergy-enhanced mTOR signaling in CMA mice while it did not affect the mTOR signaling in BTBR mice containing genetic mutations, suggesting that the Active 2 diet is more likely to attenuate the local immune disturbances in intestinal tract of CMA mice and thereby indirectly down-regulates the mTOR signaling in the brain, eventually leading to the improvement of brain functions and behavioral alterations in CMA mice. In contrast to the Active 2 diet, the present data indicate that Active 1 diet may have directly exerted its effect on the mTOR signaling pathway in the brain of CMA and BTBR mice, ameliorating behavioral impairments in CMA and BTBR mice.

Unlike knockout mice, whose autistic phenotypes may be induced by single gene depletion, the BTBR mouse carries multiple naturally occurring mutations and their behavioral and neurological impairments may result from a network of genes*(27, 62)*. The genetic mechanisms underlying the behavioral impairments or autistic phenotypes of BTBR mice may, therefore, more closely resemble the multi-genic forms of ASD. The current study explored the effects of both the Active 1 and the Active 2 diets on social behavior of BTBR mice. Previously it was demonstrated that BTBR mice exhibit lower levels of sociability compared to C57BL/6 mice in a three-chamber sociability test*(25, 27)*. In the current study, we were not able to assess social behavior of BTBR mice using our experimental set-up. It is known that high exploration in a novel environment is one of the characteristic behavioral phenotypes in BTBR mouse strain*(25)*. BTBR mouse strain exhibited highest levels of exploratory activity in a novel environment, including highest levels of locomotor activity, among 10 inbred mouse strains*(25)*. Results from another study showed that total distance traveled in an open field test was initially higher in BTBR mice than C57BL/6 mice and both mouse strains exhibited similar levels of total distance traveled after habituation*(27)*. This was also observed in the social interaction test of this study, where BTBR mice showed higher locomotor activity compared to C57BL/6 during the habituation phase, but not after a second exposure to

the open field when a social target was present. It was also shown that BTBR mouse strain spent significantly more time with a novel target than with a familiar one and displayed highest level of preference for social novelty among 10 inbred mouse strains*(25)*. In the current study, the BTBR mice also spent more time in proximity to the novel interaction mouse compared to C57BL/6 mice, presumably due to high level of preference for social novelty in BTBR mice. Therefore, we could not explore the effects of the Active 1 diet and Active 2 diet using our experimental set-up on social behavior of BTBR mice.

The current study showed that dietary intervention with Active 1 diet reduced both self-grooming duration as well as self-grooming frequency while dietary intervention with Active 2 diet did not affect the repetitive self-grooming behaviors in BTBR mice. The attenuation of the repetitive self-grooming behavior by dietary intervention with Active 1 diet may be attributed to the inhibitory effect of mTOR signaling in the PFC in BTBR mice as the dysregulation of molecular pathways in the PFC is known to be implicated in abnormal repetitive behavior in mice*(47)*. It is of interest to note that the self-grooming behavior of C57BL/6 mice was not affected by dietary intervention with Active 1 diet although the dietary intervention significantly reduced the activation of the mTORC1 pathway in the PFC of the C57BL/6 mice. It is more likely that the mTORC1 activity in the PFC of C57BL/6 mice did not reach the threshold and thereby higher levels of self-grooming behavior was not observed in C57BL/6 mice. Because the mTORC1 activity may be below the threshold, the inhibition of mTORC1 activity in the PFC of C57BL/6 mice by dietary intervention with Active 1 diet did not result in the reduction of self-grooming behavior in C57BL/6 mice.

The SSC is a brain region that processes tactile information from the body. It integrates various sensory information, including touch, pain, temperature, and spatial attention, allowing people to receive and interpret a wide variety of sensations*(63–65)*. It was reported that 44-88% of individuals with ASD have abnormal responsibility to tactile stimuli*(66)* and children with ASD have reduced somatosensory response*(67)*. In the current study, dietary intervention with Active 1 diet significantly reduced the phosphorylation of mTORC1 downstream protein p70 S6K in the SSC in both C57 BL/6 mice and BTBR mice, suggesting that protein translation related to disturbed brain functioning in the BTBR mice diminished in the SSC and this may contribute to the behavioral improvements in BTBR mice.

In conclusion, dysregulation of mTOR signaling contribute to the pathogenesis of genetically and food allergy-induced autistic phenotypes. The current results from both the murine model of CMA and the BTBR mouse model of ASD indicate that dietary attenuation of the mTOR signaling pathway or ASD-associated immune

dysfunction has promising consequences on autistic-like behavioral impairments in mice. These findings reveal more details of the molecular mechanisms underlying the interaction of environmental triggers with a complex genetic predisposition and suggest a possible therapeutic strategy for patients with ASD suffering from intestinal problems that might be related to food allergy.

Acknowledgements

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Conflict of interest

Prof. Dr. Johan Garssen is a part-time employee at Nutricia Research, Utrecht, The Netherlands. Dr. Sofia Lopes da Silva was an employee of Nutricia Research, Utrecht, The Netherlands, at the time of the study. Dr. Laus Broersen is an employee of Nutricia Research, Utrecht, The Netherlands.

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Supplementary Materials for:

Dietary interventions that reduce mTOR activity rescue autism-like behavioral deficits in mice

Methods and Materials

Animals
The animals used are described in the main article (see Methods & Materials).

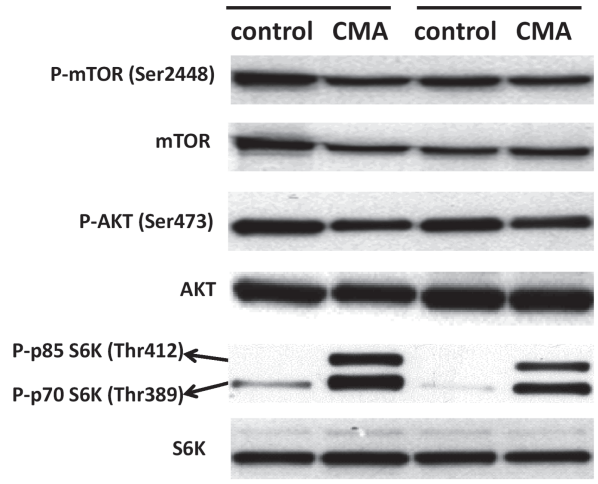
Induction of cow's milk allergy in mice
The mouse model of cow's milk allergy was conducted as described in the main article (see Methods & Materials and Figure 1A in the main article).

Dietary intervention
The compositions of the Control, Active 1, and Active 2 diets are represented in table 1 in the main article. The dietary interventions in the mouse model of CMA and the BTBR mouse model of autism were conducted as described in the main article (see Methods & Materials and Figure 1A & B in the main article).

Western blotting
Western blotting was performed as described in the main article (see Methods & Materials).

Statistical analysis
Statistical analysis was performed as described in the main article (see Methods & Materials in the main article).

A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CMA
control
CMA
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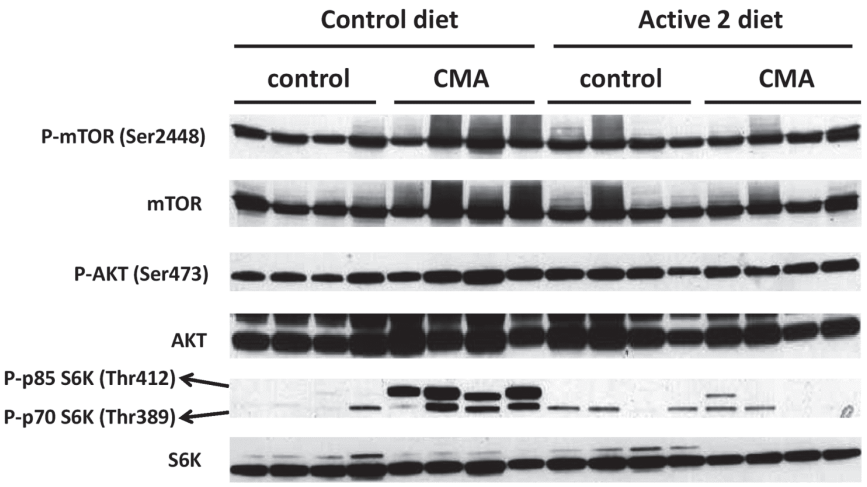
mTOR
P-AKT (Ser473)
AKT
P-p85 S6K (Thr412)
P-p70 S6K (Thr389)
S6K

B


Control diet
Active 2 diet
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CMA
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CMA
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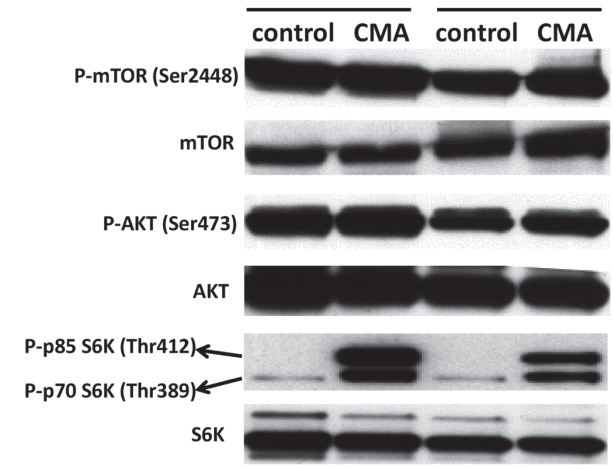
P-AKT (Ser473)
AKT
P-p85 S6K (Thr412)
P-p70 S6K (Thr389)
S6K

C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CMA
control
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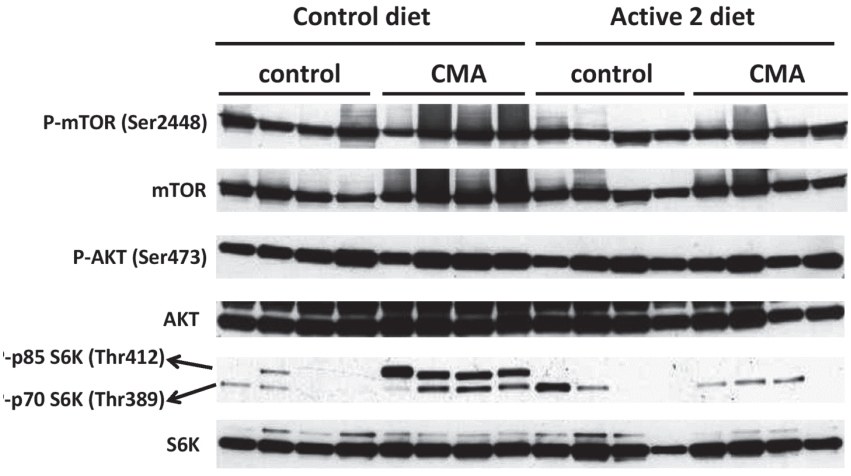
P-p70 S6K (Thr389)
S6K

D


Control diet
Active 2 diet
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CMA
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CMA
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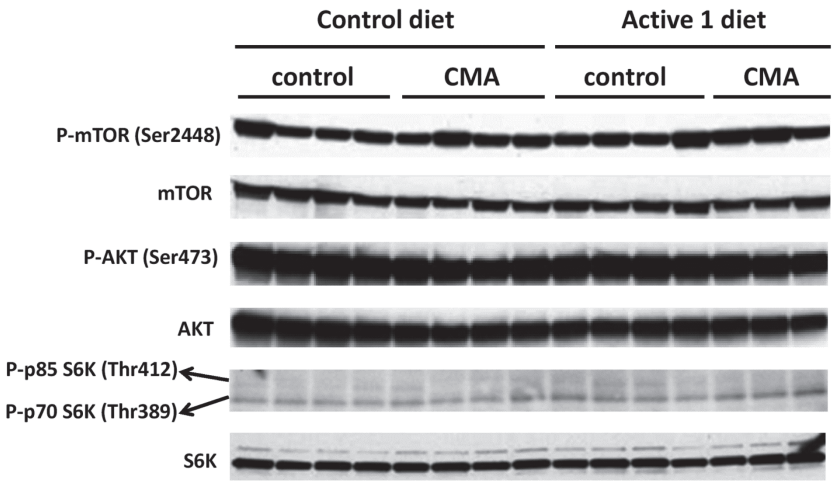
-p70 S6K (Thr389)
S6K

E



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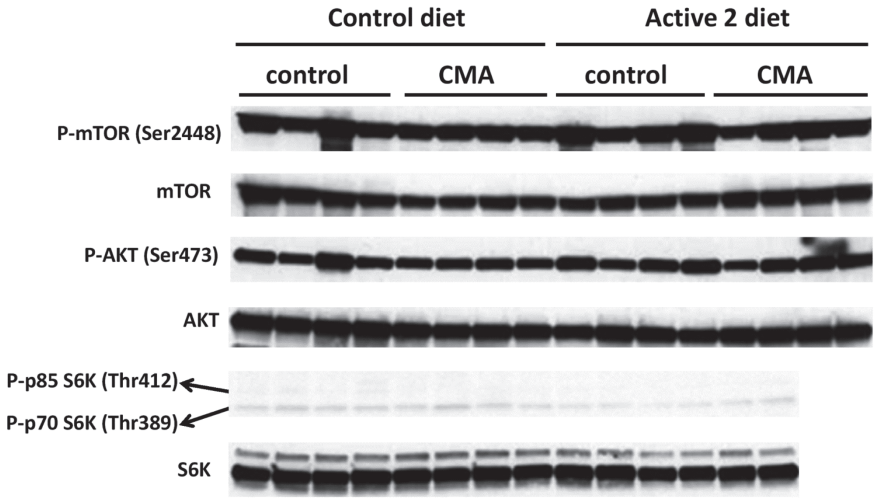
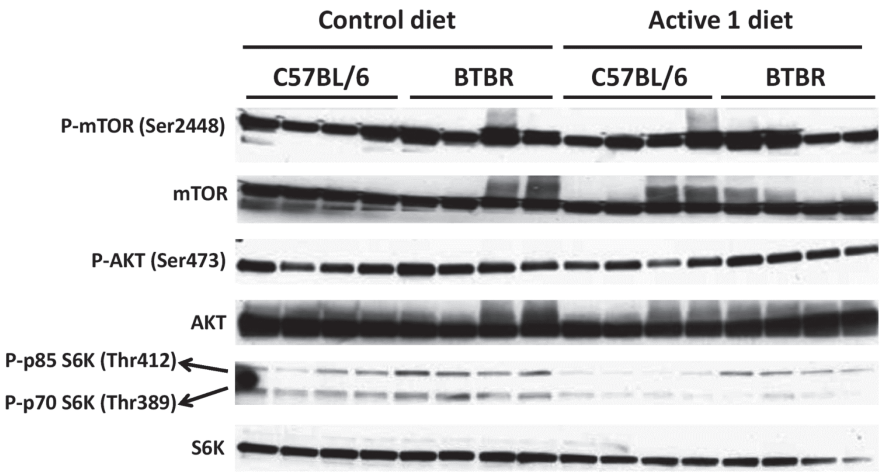


Fig. S1. Photos of western blot experiments for effect of dietary intervention with either Active 1 or Active 2 diet on the phosphorylation of mTOR-related proteins in the PFC, amygdala, and SSC in CMA mice. (A to B) Photos of western blot experiments for effect of dietary intervention with either Active 1 or Active 2 diet on the phosphorylation of mTOR, AKT, and p70 S6K in the PFC in CMA mice. N = 4. Figure A is a typical example of western blots. (C to D) Photos of western blot experiments for effect of dietary intervention with either Active 1 or Active 2 diet on the phosphorylation of mTOR, AKT, and p70 S6K in the amygdala in CMA mice. N = 4. Figure C is a typical example of western blots. (E to F) Photos of western blot experiments for effect of dietary intervention with either Active 1 or Active 2 diet on the phosphorylation of mTOR, AKT, and p70 S6K in the SSC in CMA mice. (E) N = 3 - 4; (F) n = 2 – 4.

A


Control diet
Active 1 diet
C57BL/6
BTBR
C57BL/6
BTBR
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P-AKT (Ser473)
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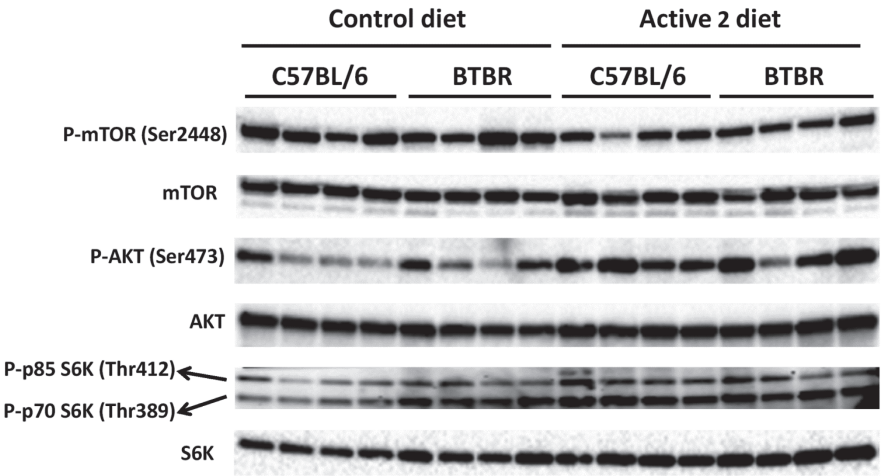
P-p85 S6K (Thr412)
P-p70 S6K (Thr389)
S6K

B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Active 2 diet
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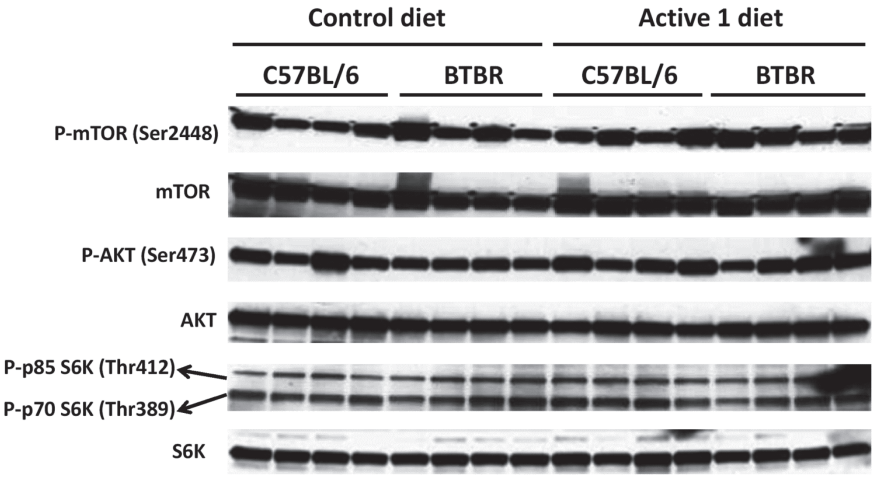
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S6K

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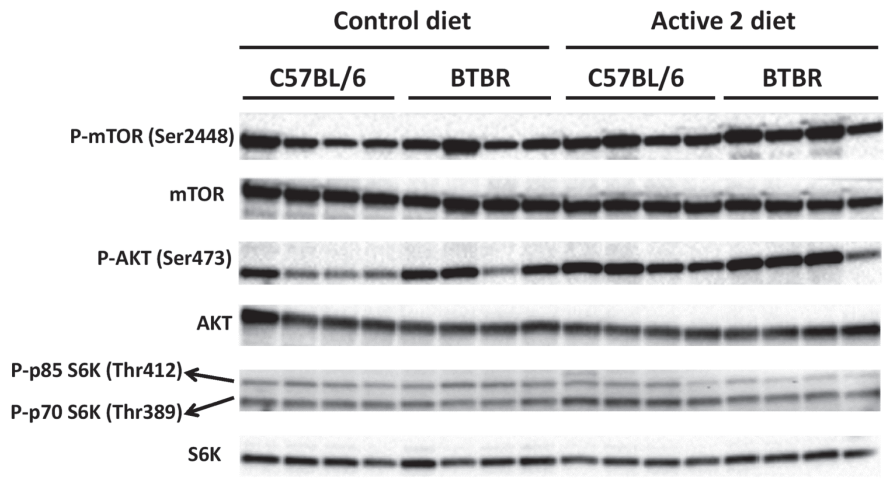
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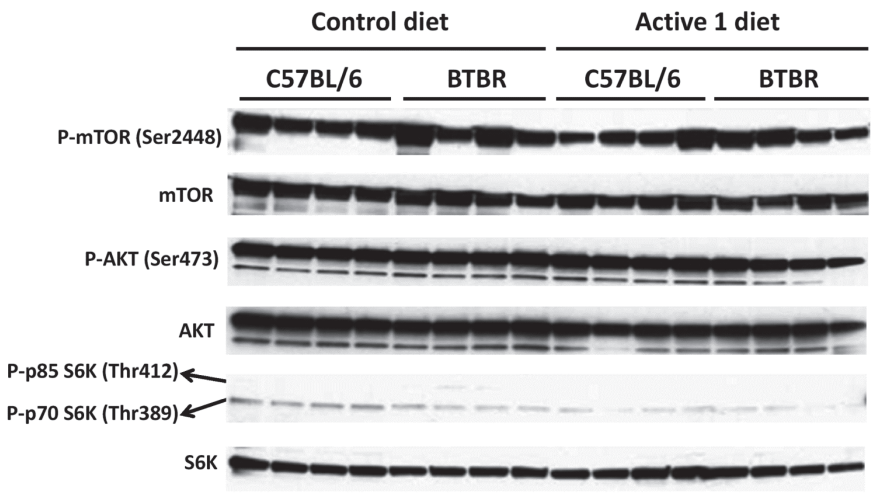

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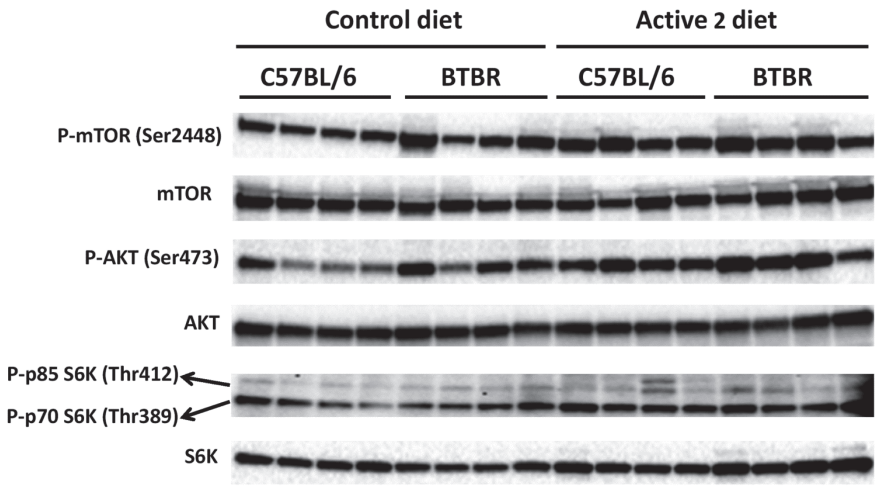

Fig. S2. Photos of western blot experiments for effect of dietary intervention with either Active 1 or Active 2 diet on the phosphorylation of mTOR-related proteins in the PFC, amygdala, and SSC in BTBR mice. (A to B) Photos of western blot experiments for effect of dietary intervention with either Active 1 or Active 2 diet on the phosphorylation of mTOR, AKT, and p70 S6K in the PFC in BTBR mice. N = 4. (C to D) Photos of western blot experiments for effect of dietary intervention with either Active 1 or Active 2 diet on the phosphorylation of mTOR, AKT, and p70 S6K in the amygdala in BTBR mice. N = 4. (E to F) Photos of western blot experiments for effect of dietary intervention with either Active 1 or Active 2 diet on the phosphorylation of mTOR, AKT, and p70 S6K in the SSC in BTBR mice. N = 4.

CHAPTER SEVEN

Dietary interventions modulate allergy-associated mucosal immune activation by changing T cell-associated transcription markers in the small intestine of food allergic mice

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Abstract

The etiology of autism spectrum disorders (ASD) encompasses a complex interplay of genetic factors and environmental contributions. Immune dysregulation including food allergy may contribute to the pathophysiology of ASD. We have demonstrated previously that food allergy induces behavioral changes in mice. Dietary intervention with an amino acid diet (Active 1 diet) targeting mTOR or with a multi-nutrient supplementation diet (Active 2 diet) containing neuroprotective and anti-inflammatory ingredients, restored both behavioral impairments in food allergic mice. The aim of the present study is to further investigate the effects of both diets on humoral immune response, mucosal mast cell degranulation, and on mucosal immune activation by determining effects on T cell related transcription factors in the small intestine of food allergic mice. Mice were fed Control diet or Active 1 or Active 2 diet starting two weeks prior to the induction of food allergy by sensitization with whey protein. Both the Active 1 and Active 2 diets did not affect the humoral immune responses and antigen-specific mucosal mast cell degranulation. In whey allergic mice Th2 (GATA-3) and Th17 (ROR-γ cell related transcription factor expression was increased in the proximal small intestine compared to control mice. Both diets normalized ROR-γ expression levels while GATA-3 followed a same pattern. By contrast FoxP3, a regulatory T-cell (Treg) related transcription factor was enhanced by the dietary interventions in the proximal small intestine of allergic mice. In conclusion, although the diets did not affect allergic sensitization, the intestinal T cell-associated transcription factor expression in whey allergic mice was modulated by the diets. This suppression of allergy-associated intestinal immune activation may relate to the improved social interaction and inhibition of repetitive behavior of these mice by acting via the gut-brain axis. Our findings provide an indication that dietary intervention with either one of the Active diets may prevent local immunological changes in the small intestine of food allergic mice.

Introduction

Autism spectrum disorders (ASD) are characterized by several behavioral impairments including impaired social behavior and the presence of repetitive behavior. The complex interplay between genetic basis and environmental factors including food allergy is considered to play a fundamental role in the development of ASD. Currently, the gut-immune-brain axis is receiving more and more attention within the field of research focusing on ASD. Emerging evidence suggests that gastrointestinal (GI) disturbances are more prevalent in children suffering from ASD[1]. The GI disorders are manifested by various symptoms including increased intestinal permeability[2,3] and food allergic reactions[4]. An altered intestinal permeability was found in 43% of autistic patients, while this occurred in none of the control individuals[2]. Children with ASD have been more frequently reported to suffer from food allergies compared to healthy children[5] and a significantly higher levels of immunoglobulins IgA, IgG, and IgM specific for cow's milk allergens have been found also in children suffering from ASD[6]. Previously, we have demonstrated that food allergic reactions induced neurochemical changes in the brain and intestinal tract of cow's milk allergic (CMA) mice, including altered neuronal activation in the prefrontal cortex (PFC) and paraventricular nucleus of hypothalamus (PVN), altered dopaminergic activity in the PFC and amygdala, and increased levels of serotonin in the intestinal tract[7]. In addition, CMA mice demonstrated autistic-like behaviors[7]. Additionally, it was demonstrated that the induction of food allergy enhanced the mTOR signaling pathway in the brain and intestinal tract of CMA mice, which may centrally contribute to the development of autistic-like behaviors found in food allergic mice[8]. Pharmacological inhibition of the mTOR signaling pathway by rapamycin improved autistic-like behaviors in CMA mice[8]. These findings suggest that intestinal allergic responses may affect brain functions via direct or indirect pathways and targeting these immune disturbances in the intestinal tract may serve as a novel therapeutic approach to alleviate autistic symptoms.

Food allergy is an increasing human health problem and mast cells play a critical role in food allergic reactions[9]. In general, IgE-mediated food allergy is characterized by T helper-2 (Th2) polarization and class-switching towards antigen-specific IgE producing plasma cells during and after sensitization. Upon a second encounter to the identical food allergens mast cell degranulation is induced in the intestinal tract, resulting in allergic symptoms in the intestine[10,11]. The mTOR signaling pathway has been shown to play a key role in regulation of mast cell function[12] as well as directing the development of T cells[13]. The mTOR signaling pathway serves as a nutritional sensor and is regulated

by a.o. amino acids[14,15]. The amino acid combination histidine (His), lysine (Lys), and threonine (Thr) inhibited the phosphorylation of p70 S6K, a mTOR downstream protein in antigen-IgE-activated mast cells[16]. Moreover, it was previously shown that dietary attenuation of the mTOR signaling pathway by a mTOR-targeting amino acid diet (Active 1 diet) containing the increased relative amount of His, Lys, Thr and the reduced relative amount of Leu, Ile, Val restored the social impairment and reduced the repetitive self-grooming behavior of CMA mice[17]. A multi-nutrient supplementation diet (Active 2 diet) containing specific neuroprotective and anti-inflammatory ingredients was shown to have similar effects on behavioral alterations in CMA mice[17]. To further explore the ability of Active 1 diet and Active 2 diet to attenuate the immunological disturbances in CMA mice, the current study investigated the effects of both diets on humoral immune responses and mucosal mast cell degranulation and mucosal immune activation in the small intestine of CMA mice.

Materials & methods

Animals

All animal procedures were approved by and conducted in strict compliance with the guidelines of the Animal Ethics Committee of Utrecht University (approval number: 2012.I.04.054). Four-week-old specific pathogen free male C3H/HeOuJ mice were purchased from Charles River Laboratories (L'Arbresle Cedex, France). All animals were housed under standard conditions at the animal facility of the Utrecht University, Utrecht, The Netherlands.

Experimental design

The compositions of the Control, Active 1, and Active 2 diet are represented in table 1. The Active 1 diet contained relative high levels of the amino acids lysine, histidine, and threonine and relatively low levels of the amino acids isoleucine, leucine, and valine. The Active 2 diet consisted of low-glycemic index carbohydrates, dietary fibers, high tryptophan content and a lipid profile that predominantly differed in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) content. The weight to weight ratio of [Leu + Ile + Val] / [His + Lys + Thr] for the respective diets were 1.53 (Control), 0.67 (Active 1), and 1.32 (Active 2).These diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands) and were based on standard animal food for laboratory rodents AIN93-G[18].

Two weeks prior to the first sensitization, the dietary intervention was started and continued during the entire experiment. The mouse model of cow's milk allergy was conducted as described previously (Figure 1)[7,19]. Briefly, male C3H/HeOuJ mice were intragastrically (i.g.) sensitized with 20 mg whey (DMV International, Veghel, The Netherlands) with 10 µg cholera toxin (CT, List Biological Laboratories, Campbell, CA, USA) as an adjuvant in 0.5 ml PBS. Control mice received CT alone. Mice were sensitized once a week for 5 consecutive weeks. One week after the last sensitization, mice were challenged i.g. with 50 mg whey/0.5 ml PBS. Afterwards, mice were sacrificed to collect serum and other tissues for further analysis of several immune parameters.

Table 1. Compositions of Control, Active 1, and Active 2 diet.

	Control diet components (g/kg)	**Active 1 diet** components (g/kg)	**Active 2 diet** components (g/kg)
Carbohydrates			
Cornstarch	322.48	331.89	260.84
Dextrinized cornstarch	132.00	132.00	
Sucrose	100.00	100.00	
Dextrose	100.00	100.00	100.00
Maltodextrin (DE6)			101.35
Galactose			34.94
Fructose			2.32
Isomaltulose			104.34
Fibers			
Fiber source (cellulose)	50.00	50.00	**21.29**
Rice fiber			**82.28**
GOS			**16.00**
FOS			**0.87**
Protein			
Alanine	7.7	7.8	6.6
Arginine	13.3	13.5	7.3
Aspartic acid	20.1	20.4	19.9
Cystine	2.0	2.1	3.3
Glutamine	13.5	13.7	10.4
Glutamic acid	21.5	21.7	19.3
Glycine	7.5	7.6	5.0
Histidine	**4.3**	**6.3**	**3.7**
Iso-leucine	**8.4**	**5.4**	**9.4**
Leucine	**14.5**	**9.4**	**14.5**
Lysine HCl	**9.6**	**14.2**	**12.0**
Methionine	5.0	5.0	4.7
Phenylalanine	9.3	9.3	6.8

Table 1. Continued

	Control diet components (g/kg)	**Active 1 diet** components (g/kg)	**Active 2 diet** components (g/kg)
Proline	8.4	8.5	8.3
Serine	8.8	8.9	8.5
Threonine	**6.7**	**9.8**	**9.0**
Tryptophan	2.1	2.2	5.2
Tyrosine	6.7	6.7	5.6
Valine	**8.7**	**5.6**	**8.6**
Total	178.1	168.8	168.0
Fat			
Soy oil	26.6	26.6	
Coconut oil	12.6	12.6	
Corn oil	30.8	30.8	**8**
MCT oil			**10.7**
Fish oil (DHA 25)			**6.5**
Fish oil (EPA 28/12)			**27.5**
Phospholipids			**7.6**
Others			
Mineral mix	35.0	35.0	35.0
Vitamin mix	10.0	10.0	10.0
extra Vitamin A			**4000 IU**
extra Vitamin B6 (pyridixine)			**6 mg**
extra foliumzuur			**2 mg**
extra Vitamin B12			**25 µg**
extra Vitamin D			**1000 IU**
Choline bitartrate	2.50	2.50	2.50
Tert-butylhydroquinone (TBHQ)	0.014	0.014	0.014

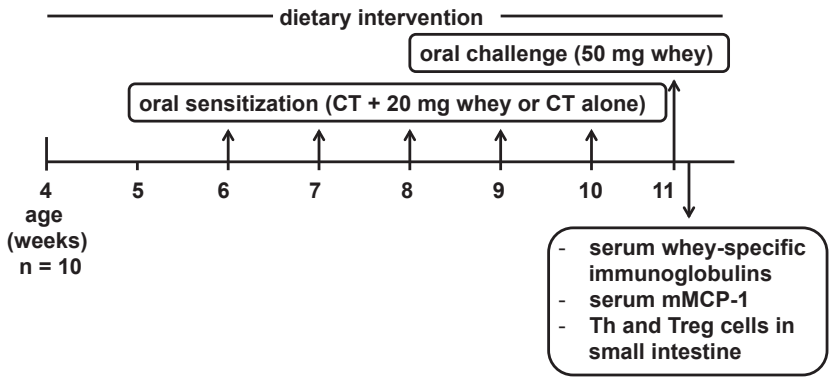


Fig. 1. Schematic representation of experimental design.

Measurements of serum mMCP-1 and whey-specific immunoglobulins

16 Hours after oral challenge, blood of mice was collected and centrifuged for 20 min at 14,000 rpm. Serum was collected and stored at -70 °C. Concentration of mouse mast cell protease-1(mMCP-1) in serum was measured by commercially available ELISA kits (Moredun Scientific Ltd., Penicuik, UK) according to the manufacturer's protocol. Concentrations of whey-specific immunoglobulins IgE, IgG1 and IgG2a in serum were measured by ELISA according to the protocol described previously[7].

mRNA expression analysis

After sacrificing, the proximal and distal segments of small intestine were isolated and stored in RNAlater (Qiagen GmbH, Hilden, Germany) at -70 °C until further analysis[20]. The total RNA was isolated using the RNAeasy kit (Qiagen, Germantown, MD, USA) and stored at -20°C. Afterwards, the total RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). After cDNA synthesis, real-time PCR was performed using iQ SYBR Green supermix kit (Bio-Rad, Hercules, CA USA) with the CFX 96 Real-time system (BioRad, Hercules, CA USA). Ribosomal protein S13 (Rps13) was used as reference gene. Relative target mRNA was calculated by applying the formula: relative mRNA expression = $100 \times 2^{Ct[Rps13] - Ct[targetmRNA]21}$. Primers for interleukin (IL) 10, transforming growth factor (TGF)-ß, and T cell-associated transcription factors were commercially purchased from SABiosciences-QiagenGmbH (Hilden, Germany).

Statistical analysis

Experimental results are expressed as mean ± S.E.M. In general, differences between groups were statistically determined with a two-way ANOVA followed by a Holm-Sidak's multiple comparisons test. For serum immunoglobulin and mMCP-1 levels, log transferred data were used to obtain normality. Results were considered statistically significant when $P < 0.05$. Analyses were performed using GraphPad Prism, version 6.02.

Results

Both diets had no effect on humoral antigen specific-immune responses and mucosal mast cell degranulation

To investigate the effect of the Active 1 and Active 2 diet on humoral immune response, whey-specific immunoglobulin levels were measured in the serum. Increased serum levels of whey-specific IgE, IgG1, and IgG2a were observed in CMA mice compared to control mice (Figure 2A-C). Both Active 1 and Active 2 diets had no effect on whey-specific IgE, IgG1, and IgG2a levels in serum of CMA mice. The concentration of mMCP-1 was measured in the serum as marker for mucosal mast cell degranulation in the intestinal tract. CMA mice showed significantly augmented mMCP-1 concentration in serum of CMA compared to control mice and both dietary interventions did not affect this (Figure 2D).

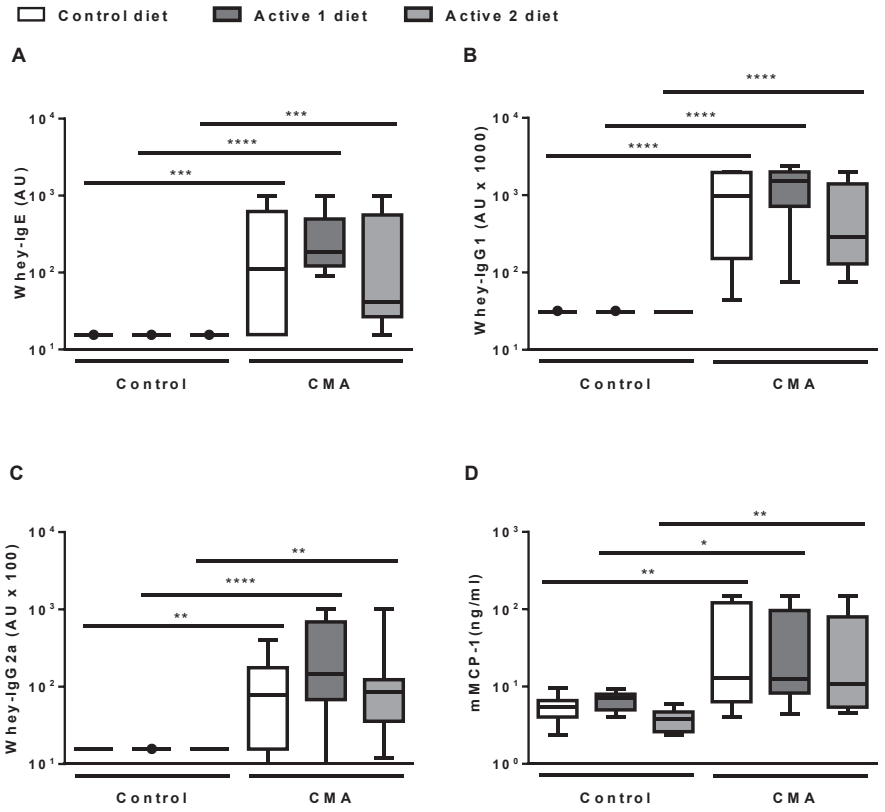
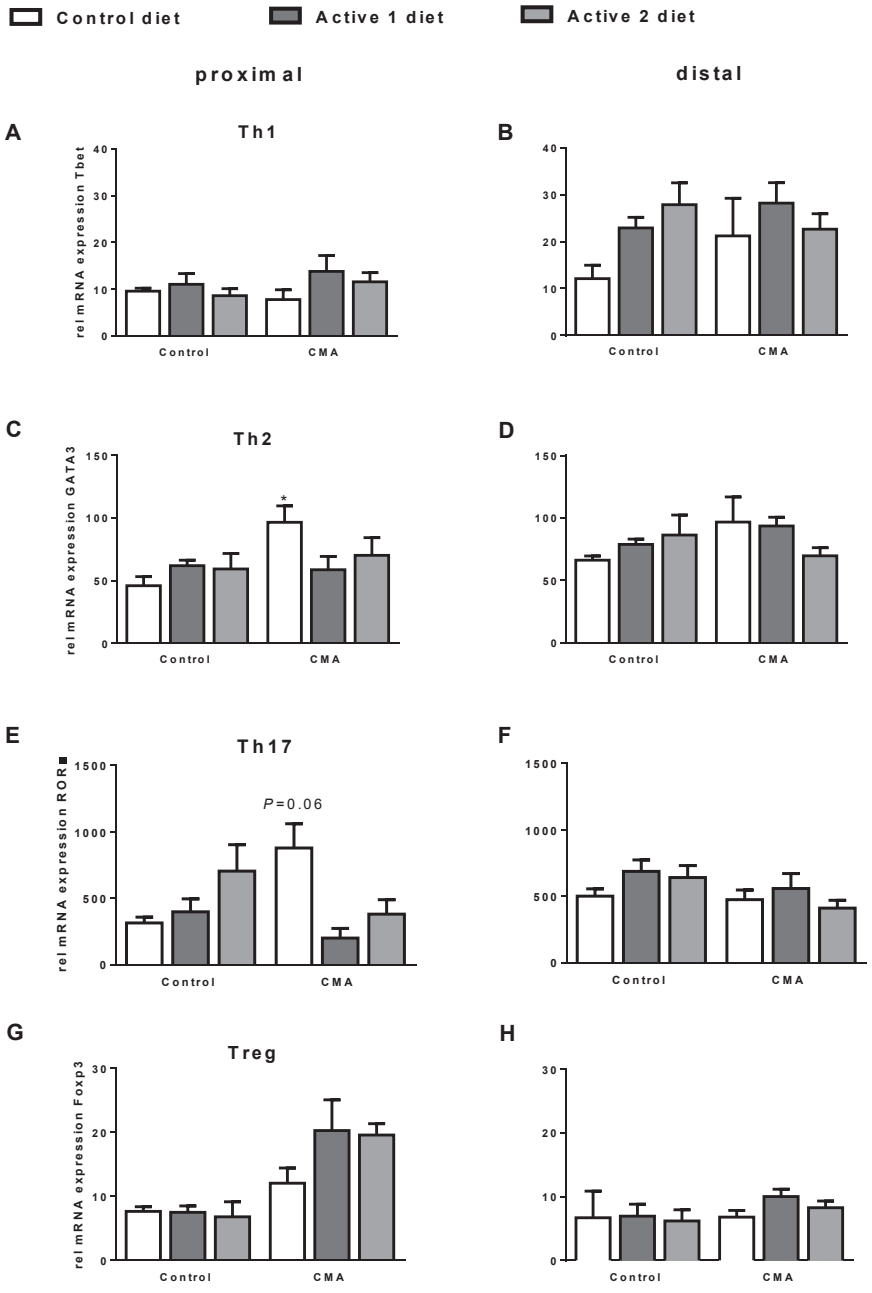


Fig. 2. The effects of Active 1 diet and Active 2 diet on humoral response and mucosal mast cell degranulation in CMA mice. Serum levels of whey-specific IgE (A), IgG1 (B), IgG2a (C) as well as mMCP-1 (D) were increased in allergic mice compared to control. Dietary intervention with either Active 1 or Active 2 diet had no effect on

humoral response and mucosal mast cell degranulation in CMA mice. Data were log transferred to obtain normality. Two-way ANOVA was conducted followed by a Bonferroni's multiple comparisons test and data are presented as Box-and-Whisker Turkey plots. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, n = 10 per group.

Active 1 diet and Active 2 diet suppress whey-induced Th2 and Th 17-associated transcriptional markers and enhance Treg-associated transcription marker Foxp3 in the small intestine of CMA mice

To investigate the effect of both dietary interventions on allergy associated mucosal immune activation , mRNA analysis of transcription factors specific for T cell populations in the small intestine of CMA mice was performed. A two-way ANOVA showed a significant interaction between model and diets for expression of Th17-associated transcription factor in the proximal small intestine of CMA mice ($F_{2, 19} = 4.674$, $P = 0.0223$, Fig. 3E). For expression of Th2 and Treg-associated transcription factors, a two-way ANOVA showed a significant effect of model in the proximal small intestine (Th2: $F_{1, 16} = 5.093$, $P = 0.0384$; Treg: $F_{1, 16} = 28.47$, $P < 0.0001$). For the ratio of Th2 to Th1 and expression of IL-10 and TGF-ß, a two-way ANOVA showed a significant effect of diets in the distal small intestine (Th2/Th1: $F_{2, 23} = 21.03$, $P < 0.0001$; IL-10: $F_{2, 21} = 4.782$, $P = 0.0194$; TGF-ß: $F_{2, 23} = 10.03$, $P = 0.0006$). The relative mRNA expression levels of Th2 cell-associated transcription factor GATA3 (Figure 3C) and Th17 associated RORɣ (Figure 3B) were enhanced in the proximal small intestine of CMA mice compared to control mice fed control diet. No differences were found in the distal small intestine (Figure 3D & 3F). The CMA-induced increase in GATA3 and RORɣ mRNA expression in the proximal small intestine was no longer observed after dietary intervention with either Active 1 or Active 2 diet (Figure 3C & 3E). The ratio of Th2 (GATA-3) to Th1 (Tbet) associated T cell transcription factor mRNA expression tended to be increased in the distal small intestine of CMA mice and both dietary interventions seem to suppress this (Figure 3J). In addition, the Active 1 diet as well as the Active 2 diet enhanced Treg cell-associated transcription factor Foxp3 in the proximal small intestine of CMA mice (Figure 3G). Furthermore, we examined the effects of dietary intervention with either Active 1 or Active 2 diet on mRNA expression of regulatory type cytokines IL10 (Figure 3K) and TGF-ß (Figure 3L) in the distal small intestine. No significant differences were observed in the relative expression levels for IL-10 and TGF-ß when comparing CMA and control mice fed the control diet. The mRNA levels of IL10 and/or TGF-ß in the intestine of both control mice and CMA mice were suppressed by dietary intervention with either Active 1 or Active 2 diet, respectively (Figure 3K & 3L).


Control diet
Active 1 diet
Active 2 diet
proximal
distal
A
Th1
rel mRNA expression Tbet
B
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Th2
rel mRNA expression GATA3
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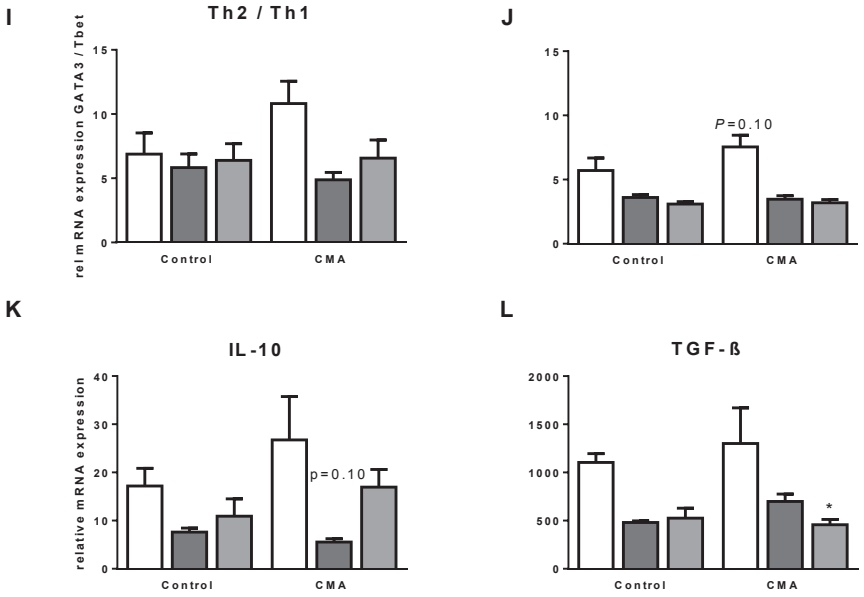

Fig. 3. The effects of Active 1 diet and Active 2 diet on T cell differentiations in small intestine of CMA mice. A, C, E & G represent data obtained in the proximal part of the small intestine, while B, D, F , H, K & L represent data from the distal part. CMA mice showed increase mRNA expression levels of Th2-associated transcription factor GATA3 (A) and Th17-associated transcription factor RORγ (C) and both diets inhibited Th2 and Th17 development in the proximal part of the small intestine. No significant differneces were found in the distal part. Both the Active 1 and Active 2 diets significantly enhanced Treg-associated transcription factor Foxp3 in the proximal part of the small intestine of CMA mice (E). This response was not found in the distal part (F). The ratio of Th2 to Th1 tended to be increased in the distal, but not proximal, part of the small intestine and both diets suppressed this ratio (I & J). Furthermore, expression of mRNA levels for IL-10 and TGF-ß in the distal part of the small intestine were suppressed by either Active 1 or Active 2 diet in control mice and CMA mice (K & L). Data were analyzed by a two-way ANOVA followed by a Bonferroni's multiple comparisons test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0{,}001$, n = 10 per group. (A, C & D) Data from proximal small intestine were combined with data from distal small intestine. n = 5 per group. (B) Data from distal small intestine. n = 5 per group. (E-F) Data from distal small intestine. n = 4-5 per group. (E) Control vs control with Active 1 diet: $P = 0.5676$; control vs control with Active 2 diet: $P > 0.9999$; CMA vs CMA with Active 2 diet: $P = 0.4703$. (F) Control vs control with Active 2 diet: $P = 0.0703$; CMA vs CMA with Active 1 diet: $P = 0.0789$.

Discussion

The current study investigated the effect of the Active 1 and Active 2 diet on the humoral immune response, mucosal mast cell degranulation and mucosal immune activation in the small intestine of CMA mice. We demonstrated that both diets had no effect on humoral immune response and intestinal mucosal mast cell degranulation. However, both diets inhibited enhanced expression of Th2 and Th17 cell-associated

transcriptional markers in the small intestine of CMA mice. The current results suggest that allergy associated mucosal immune activation occurring in the proximal small intestine of CMA mice can be alleviated by dietary intervention with either the Active 1 or Active 2 diet.

IgE mediated food allergy is characterized by a series of immunological reactions during the sensitization and challenge phase including the development of Th2 cells from naïve T cells, isotype switching in B cells followed by the production of antigen-specific IgE, cross-linking of cell-bound IgE leading to degranulation of mast cells after allergen challenge, and release of mediators that cause clinical symptoms[22]. In this study we found the most pronounced CMA-induced effects on T cell-associated transcriptional markers in the proximal part of the small intestine indicative for a Th2 and Th17 response. Although the Active 1 and Active 2 diet did not affected the humoral immune response and intestinal mucosal mast cell degranulation in CMA mice (current study), both diets did improve the autistic-like behavior and inhibited the enhanced mTOR signaling pathway in the brain of CMA mice[17]. Thus, the Active 1 diet may directly inhibit the enhanced mTOR signaling pathway in the brain of CMA mice. In addition, the behavioral effects of Active diet 1 may also be indirectly induced via targeting mTOR in the intestinal mucosal immune system and thereby reducing the mucosal inflammatory response in CMA mice. Previously, it was demonstrated that the induction of CMA also augmented mTOR signaling pathway in the small intestine[8]. The mTOR signaling pathway is known to play a central role in the development and differentiation of Th cells and Treg cells[13]. It is also possible that the effects of the Active 1 diet on transcriptional markers indicative for Th and Treg cells in the small intestine of CMA mice is regulated via the mTOR signaling pathway and thereby indirectly affects mTOR-related processes in the brain of CMA mice.

Unlike the Active 1 diet that is designed to attenuate the mTOR signaling pathway, the Active 2 diet contains various ingredients that predominantly provide anti-inflammatory and neuroprotective effects, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)[23], galaco-oligosaccharides (GOS) and fructo-oligosaccharides (FOS)[20,24], vitamins[25,26], and rice fibers[27]. The Active 2 diet attenuated the local allergic immune activation in the proximal small intestine, which may have indirectly contributed to the observed inhibition of the mTOR signaling pathway in the brain of CMA mice. Therefore, the effects of the Active 2 diet on transcriptional markers indicative for Th2, Th17 and Treg cells in the proximal small intestine of CMA mice may be attributed to the anti-inflammatory effects of the ingredients.

Th2 cells are important effector T cells involved in the sensitization of allergic immune responses. The Th17 cell subset is generated from naïve T cells in response to TGF-ß and IL-6[28]. Th17 cells are not only implicated in the development of autoimmune diseases such as multiple sclerosis, but also contribute to the pathogenesis of Th2-mediated allergic disorders[28,29]. A significant increase in Th17 cell response has been found in allergic asthmatic patients after allergen challenge compared to control subjects[30]. As previously described by, in the current study, we found an upregulation of Th2 and Th17-associated transcriptional markers in the small intestine of CMA mice[20]. However, there seems to be an difference in location: we observed the most pronounced effects of CMA in the proximal part of the small intestine and Kerperien and co-workers in the middle part of the small intestine. Since we only divided the small intestine in proximal and distal parts, it could be reasonable to speculate that our proximal tissue samples are more or less the same as the middle small intestinal samples studied by Kerperien and colleagues. Dietary intervention with either Active 1 or Active 2 diet suppressed this CMA-induced response in the small intestine. Regulatory T cells are a population of T cells that plays a critical role in maintenance of tolerance to innocuous antigens, preventing the development of autoimmune diseases and allergies[31,32]. In previous studies it was shown that whey-specific $CD25^+$ regulatory T cells suppressed allergic immune responses in mice and rapamycin, a specific mTOR inhibitor, induced regulatory T cell responses in CMA mice[8,19]. This indicates that suppression of whey-induced allergic immune responses by regulatory T cells involves modulation of mTOR signaling pathway. Both the Active 1 and Active 2 diet were also shown to induce an increase in regulatory T cell-associated transcriptional marker Foxp3 specifically in the small intestine of CMA mice, which may suggest the diets to enhance the Treg cell response. However, Active 1 and Active 2 diets inhibited the expression on mRNA encoding for IL-10 and TGF-ß, respectively, in the distal small intestine of CMA mice, which are anti-inflammatory cytokines that are produced by a wide variety of immune cell types including Th2, Th17 as well as Treg cells[33–35]. The reduced IL-10 and TGF-ß mRNA levels found in the distal small intestine of CMA mice fed the Active 1 diet and Active 2 diet may be attributed to the suppression of transcriptional markers indicative for Th2 and Th17 type mucosal immune activation by dietary intervention with either Active 1 or Active 2 diet. However, the expression of these regulatory cytokines needs to be examined in the proximal small intestine as well, to drawn proper conclusions. Naïve T cells generate from bone marrow and continually circulate through the spleen and secondary lymphoid organs in search of pathogens. During sensitization, naïve T cells in the mesenteric lymph nodes and Peyers patches differentiate into effector T cells such as

Th2 and Th17 cells upon recognition of antigen-MHC complex on the surface of antigen-presenting cell[36]. Some Th2 cells travel to the B-cell follicle to induce isotype switching in B cells, leading to the development of antigen-specific plasma cell and consequent production of IgE antibodies that are released in the blood vessels[37]. The effector T-cells generated in the mesenteric lymph nodes traffic via the bloodstream and home back to the lamina propria. Here the locally infiltrated antigen-specific T tells play a role in the effector phase of the allergic response in intestine. The current study demonstrated that no effect of dietary interventions on whey-specific IgE, IgG1, and IgG2a was observed in the serum while possibly Th2 and Th17 cell responses in the small intestine were inhibited by dietary intervention with either Active 1 or Active 2 diet in CMA mice. This suggests that the effect of Active 1 diet and Active 2 diet on immune responses is site-specific and that dietary intervention significantly affect T cell responses in the small intestine but cannot overcome the systemic whey-induced allergic immune responses. In conclusion, the current study demonstrates that mucosal immune activation in the proximal small intestine of whey- allergic mice can be regulated by dietary intervention with the mTOR-targeting Active 1 diet or Active 2 diet containing neuroprotective and anti-inflammatory ingredients.

Acknowledgements

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Conflict of interest

Prof. Dr. Johan Garssen is a part-time employee at Nutricia Research, Utrecht, The Netherlands. Dr. Sofia Lopes da Silva was an employee of Nutricia Research, Utrecht, The Netherlands, at the time of the study. Dr. Laus M Broersen is an employee of Nutricia Research, Utrecht, The Netherlands.

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CHAPTER EIGHT

General discussion

Autism spectrum disorder (ASD) is a series of heterogeneous neurodevelopmental disorders that display communication and social impairments, repetitive and stereotyped behaviors. ASD affects about 1 in 68 children and is approximately 5 times more common among boys than among girls[1]. Genome-wide association studies, copy number variation screening and SNP analyses have revealed a number of ASD-associated genetic mutations including single-gene mutations, sex chromosome aneuploidy, and recurrent copy number variants[2]. Nowadays, ASD are considered to be induced by a complex interplay of genetic and environmental factors. Dysregulated immune system with a propensity for inflammation has been frequently found in patients with ASD, suggesting that immunological disturbances may act as an environmental trigger that contributes to the pathophysiology of ASD[3].

The single-gene mutations in the mammalian target of rapamycin (mTOR) signaling pathway, including TSC1 or TSC2 (Tuberous Sclerosis)[4], FMR1 (Fragile X Syndrome)[5], Nf1 (Neurofibromatosis type 1)[6], eIF4E[7], and PTEN[8], are highly associated with ASD. A dysregulated mTOR signaling pathway has been found in various mouse models for ASD[4,5,8–11]. Moreover, a growing body of evidence suggests that the mTOR signaling pathway plays a critical role in the regulation of T cell differentiation and mast cell function as well[12–14]. Therefore, it is suggested that the mTOR signaling pathway might serve as a potential therapeutic target for the treatment of ASD and associated intestinal problems. Throughout this dissertation research, I have investigated the role of the gut-immune-brain axis in ASD-like behavior by concentrating on a common denominator, the mTOR signaling pathway, which plays a central role in both the intestinal tract, its associated immune system, and the brain.

I investigated the role of the mTOR signaling pathway in the relationship between immune disturbance (in particular related to food allergy) and ASD-like behavior both in a mouse model for food allergy as well as in mouse models for ASD due to genetic mutations. Moreover, I have studied the therapeutic effect of several nutritional interventions on behavioral impairments and immune disturbances in these mouse models. In this final chapter, I have summarized the major findings of my dissertation research and describe the potential of therapeutics targeting the mTOR signaling pathway using dietary interventions for the treatment of ASD.

Intestinal disturbances and autism spectrum disorders

Gastrointestinal problems have been reported in patients with ASD frequently. The reported prevalence ranges from 9% to 91.4%. The immense range is probably due to different interpretations of gastrointestinal problems and the communication impairment of autistic patients. Gastrointestinal symptoms observed in autistic patients are variable and diverse, including chronic constipation, diarrhea, abdominal pain, and food allergy (reviewed in **chapter 2**). The cause of these gastrointestinal symptoms include increased intestinal permeability[15], intestinal inflammation[16], changes in microbial composition[17], and allergic reactions[18] and are associated with the behavioral deficits[19,20]. These findings suggest a tight interaction between the immune disturbances in the gastrointestinal tract and behavioral changes due to brain dysfunction.

In addition to gastrointestinal disturbances associated with ASD, **chapter 2** summarizes several molecular pathways underlying the complex crosstalk in gut-brain interactions, including pathways of intestinal inflammation, serotonin system, and the mTOR signaling pathway. Food allergic reactions have been observed among autistic patients frequently. A previous study from Lucarelli, et al. demonstrated that a cow's milk free diet might lead to an improvement in behavioral symptoms associated with ASD. Additionally significantly higher serum levels of IgA, IgG, and IgM specific for cow's milk proteins were found in children suffering from ASD compared to healthy controls[21]. Furthermore, dietary intervention with a gluten-free, milk protein-free, or gluten- and milk protein-free diet can ameliorate some behavioral impairments and developmental outcomes in children suffering from ASD[22,23]. Based on these observations in ASD-patients we performed behavioral studies in cow's milk allergic (CMA) mice using whey as the major milk-derived allergen.

In **chapter 3**, we demonstrate that induction of whey-specific IgE-mediated allergic immune responses in the intestinal tract of mice caused autistic-like behavior such as reduced social interaction, increased repetitive behavior and disturbed spatial memory/reduced exploration behavior. CMA in mice, however, was shown not to affect anxiety-like behavior in mice (unpublished data).

To examine whether these autistic-like behavioral changes could be a general result of intestinal sickness, we conducted the similar behavioral tests in a mouse model for colitis. It was shown that social interaction in DSS-induced colitis mice was not affected (supplementary data of **chapter 3**), although DSS-induced colitis mice did display some behavioral signs of sickness such as less locomotor activity in the open field. In addition,

it is reported that LPS-induced sickness behavior in mice is accompanied with reduced self-grooming (less repetitive behavior)[24]. Taken together, all these findings indicate that autistic-like behavior found in CMA mice may specifically result from the allergic immune responses in the intestine and is not a general result of sickness behavior.

Neurochemical changes in CMA mice

To investigate neuronal activation in the brain of CMA mice, c-Fos expression was measured in the prefrontal cortex. Neuronal activation was demonstrated in the lateral and ventral orbital prefrontal cortex (oPFC) of CMA mice after being exposed to the interaction mouse in the social interaction test. The oPFC is important for guiding social-emotional behavior since this brain region is involved in cognitive processing of decision-making in response to emotional stimuli[25,26]. In addition, patients with ASD as well showed enhanced oPFC activation in response to tasks involving facial recognition[27] and attention[28].

Research continued with examination of the effects of the food allergic immune response on dopaminergic and serotonergic systems in the brain. In the serotonergic system changes were less pronounced than those observed in the dopaminergic system. Levels of dopamine and its metabolites 3,4-dihydroxyphenylacetic acid, 3-methoxytyramine, homovanillic acid were significantly decreased in the prefrontal cortex and increased in the amygdala of CMA mice. These neurochemical changes in the dopaminergic system of prefrontal cortex and amygdala of CMA mice may correlate with autistic-like behavioral impairments found in CMA mice. The prefrontal cortex plays a critical role in the process of cognitive control and the control of goals-directed thought and behavior[29,30]. Damage to the prefrontal cortex can result in abnormal repetitive behavior in mice[31] and in a fragile X mouse model for ASD dampening of the dopaminergic system in the prefrontal cortex was observed[32]. In addition, it has been shown that ASD children display lower levels of dopamine in the medial prefrontal cortex[33].

Besides the prefrontal cortex, the amygdala is an important forebrain region involved in the mesocorticolimbic dopamine system and plays an essential role in social behavior and guiding the emotions[34]. Amygdala volume positively correlates the size and complexity of social network in adult humans[35]. In addition, dopamine has been demonstrated to inhibit the activation of the amygdala during emotional processing[36] and in ASD reduced amygdala functioning was suggested[37]. The increased levels of dopamine observed in the amygdala of CMA mice might be involved in reducing the amygdala functioning resulting in ASD-like behavioural deficits.

A possible explanation for the reduced dopaminergic system in the prefrontal cortex in CMA could be abnormal functioning of the afferent vagus nerve fibers originated from the small intestine. Stimulation of the abdominal vagal nerve in rats led to inhibition of dopamine system in various brain regions including the prefrontal cortex[38]. In the small intestine of CMA mice increased levels of serotonin and numbers of serotonin-positive cells were found. Moreover, serotonin released from intestinal enteroendocrine cells has been shown to stimulate vagal afferent neurons[39].

In conclusion, the dampening of the dopaminergic system in the prefrontal cortex of food allergic mice may be involved in the deficits of social behavior. Furthermore, small intestinal serotonin acting upon vagal nerve endings might be responsible for the gut-brain signaling resulting in inhibition of the dopaminergic system in the brain of mice undergoing CMA.

Involvement of mTOR signaling pathway in behavioral and immunological changes in food allergic mice

The mTOR signaling pathway plays a pivotal role in sensing environmental cues and regulating various cellular processes[40,41]. As described in the introduction and **Chapter 2**, single-gene mutations that affect mTOR signaling pathway have been implicated in several neurodevelopmental disorders including ASD[4,8,9,42]. Dysregulated mTOR signaling pathway has been found in various murine models for ASD as well[10,43]. Additionally, it was shown that the mTOR signaling pathway is involved in mast cell activation and directing T cell differentiation[12,14]. Because of its dual role in both neuronal function and immune regulation, the mTOR signaling pathway has been considered as a convergent pathway underlying the complex interplay between the nervous system and the immune system.

Since whey-specific food allergy induced autistic-like behavioral deficits in mice (**chapter 3**), we raised the question whether mTOR signaling pathway could be implicated in both the behavioral deficits and immunological changes of CMA mice. To investigate this hypothesis mice were sensitized and challenged with the cow's milk protein whey. Meanwhile, the CMA mice received rapamycin treatment in different concentrations (**chapter 4**). Rapamcyin is a macrocyclic antibiotic produced by the bacterium *Streptomyces hygroscopicus*. It interacts with the intracellular receptor FK506 binding protein (FKBP12). FKBP12-rapapmycin complex binds to mTOR and dissociates mTOR complex1 (mTORC1). Rapamycin treatments were shown to inhibit the social behavioral

deficits and normalize the repetitive self-grooming behavior of CMA mice (**chapter 4**). The humoral immune responses and mucosal mast cell activation in the intestine were inhibited by rapamycin treatment as well (**chapter 4**). We further investigated the phosphorylation of mTOR signaling proteins in several specific brain regions including prefrontal cortex, amygdala, somatosensory cortex, and dorsal hippocampus. Both prefrontal cortex and amygdala perform a crucial role in social behavior, cognitive functions, and emotional expressions[29,44–46], which are mostly disturbed in patients with ASD. Somatosensory cortex is a brain region involved in processing sensory information such as touch, pain, temperature as well as spatial attention[47–49]. It was reported that 44-88% patients suffering from ASD express a disturbed function in somatosensory cortex[50] and reduced somatosensory response was frequently found in children suffering from ASD[51]. Dorsal hippocampus plays an important role in learning and spatial memory[52–54]. The phosphorylation of mTORC1 downstream proteins p70 S6K and 4E-BP1 was significantly increased or showed a trend in increase in the prefrontal cortex and amygdala in allergic mice. The enhanced phosphorylation of p70 S6K and 4E-BP1 in these brain regions suggests increased synthesis of synaptic proteins such as neuroligin (NLGN), leading to an increased synaptic excitation to inhibition (E/I) ratio in the prefrontal cortex and amygdala. The increased E/I ratio may eventually lead to the development of ASD phenotypes. Rapamycin inhibited the phosphorylation of p70 S6K and 4E-BP1 in both prefrontal cortex and amygdala of whey-induced allergic mice. However, both the CMA induction and/or rapamycin treatment had no effects on AKT and mTOR phosphorylation in the prefrontal cortex and amygdala. AKT is not only an upstream protein of mTORC1, but also the downstream protein of mTORC2. The lack of the effect of rapamycin treatment on AKT phosphorylation suggests that the activity of mTORC2 may not be involved in CMA. Furthermore, the mTOR signaling pathway in somatosensory cortex and dorsal hippocampus was not affected by CMA, indicating that the mTOR signaling pathway in these brain regions is not involved in the behavioral changes. However, other processes in these brain regions not assessed by us, could still be important for the behavior deficits observed in CMA mice.

An increased phosphorylation of p70 S6K was also found in small intestine of allergic mice, which was inhibited as well by rapamycin (**chapter 4**). The enhanced mTOR signaling correlated with low expression of the regulatory T (Treg) cell transcriptional marker Foxp3 in the ileum of CMA mice. In addition, rapamcyin treatment increased the expression of Treg cell-associated transcription factor as well as the expression of Treg cell-associated cytokines, interleukin 10 and transforming growth factor-ß, in the small intestine of CMA mice (**chapter 4**). Taken together, these results indicate that mTOR

may be central in the dysregulation of immune responses in the intestine and of brain function in CMA mice.

Targeting the mTOR signaling pathway with specific amino acids: *in vitro* studies using mast cells

Mast cells play a central role in mucosal allergy. Mast cells can be activated by cross-linking of allergen-bound IgE during the allergen challenge phase[55]. Mast cell activation is regulated by a complex network of intracellular signaling pathways that is initiated following aggregation of the IgE-receptor, FcεRI[56,57]. It has been shown that mTOR signaling pathway is activated by FcεRI-mediated mast cell stimulation and is also involved in cytokine production of FcεRI-mediated mast cell activation[14].

Amino acids are key environmental stimuli that modulate mTORC1 activity. Apart from their role as indispensable building blocks for proteins, amino acids appear to regulate protein synthesis via a number of signaling transduction pathways including the mTOR signaling pathway[58,59]. To develop a diet containing mTOR-targeting amino acids for the treatment of ASD in our *in vivo* studies, an *in vitro* study investigating the effects of several amino acids on mTORC1 pathway in antigen-IgE-activated bone marrow-derived mast cells (BMMCs) has been performed (**chapter 5**).

To investigate whether the mTOR signaling pathway is implicated in degranulation of IgE-mediated mast cells, IgE-primed BMMCs were incubated with rapamycin and consequently stimulated with DNP-HSA. Rapamycin inhibited acute degranulation of IgE-mediated mast cells in a dose-dependent manner. Furthermore, rapamycin inhibited both IL-6 and TNF-α production in a dose-dependent manner in antigen-IgE-stimulated mast cells. These findings confirm previous findings that the mTOR signaling pathway plays a critical role in activation of antigen-IgE-activated mast cells[60,61]. However, some studies suggested that rapamycin had no effect on acute degranulation of IgE-mediated mast cells[14,60]. This may be due to the lower concentrations of rapamycin used or to the way BMMCs have been stimulated (with OE-3/ovalbumin (OVA) instead of DNP-HSA) in these studies. In addition, intracellular calcium is involved in FcεRI-mediated mast cell acute release of pre-stored inflammatory mediators[56,57]. Therefore, to study the involvement of the mTOR signaling pathway in acute degranulation of mast cells, it would be reasonable to examine the effect of rapamycin on intracellular calcium release in IgE-mediated mast cells.

The involvement of the mTOR signaling pathway in activation of IgE-mediated mast cells was further assessed by examining the effect of rapamycin on phosphorylation of mTOR related proteins (**chapter 5**). mTOR, 4E-BP1 and p70 S6K were phosphorylated within 5 to 15 min in IgE-mediated mast cells after stimulation with DNP-HSA, indicating that activation of the mTOR signaling pathway is an immediate process in IgE-mediated mast cells. Rapamycin treatment inhibited the phosphorylation of mTORC1 downstream proteins p70 S6K and 4E-BP1.

Although amino acids are critical environmental stimuli, how exactly they are sensed and how they regulate mTORC1 activity is still not fully understood. It was hypothesized that a multi-protein complex involving RAG GTPases, Ragulator, and the v-ATPase on the lysosomal surface interacts with mTORC1, regulating various cellular processes[62,63]. A previous study demonstrated that leucine (Leu), isoleucine (Ile), and valine (Val) enhanced mTORC1 activity, while histidine (His), lysine (Lys), and threonine (Thr) suppressed mTORC1 activity in mammary epithelial cells[64].

Because of the critical role of mast cells in allergy and inflammation, we assessed the effects of individual amino acids and amino acid combinations on activation of antigen-IgE-stimulated mast cells (**chapter 5**). The individual amino acids Leu, Ile, Val and the combination of Leu, Ile, Val enhanced mTOR signaling pathway while the individual amino acids His, Lys, Thr and the combination of His, Lys, Thr inhibited mTOR signaling pathway in antigen-IgE-activated mast cells. Surprisingly, we demonstrated that both the combination of Leu, Ile, Val and the combination of Lys, His, Thr inhibited the acute degranulation and IL-6 production in antigen-IgE-stimulated mast cells. These results suggest that activation of mast cells involves a complex network of both positive and negative intracellular signaling pathways[56,57]. mTOR signaling pathway may be partly involved in acute degranulation and cytokine production of IgE-mediated mast cells.

Effects of dietary intervention with either an mTOR-targeting amino acid diet or a multi-nutrient supplementation diet on behavioral deficits of CMA mice and BTBR mice

Our *in vitro* studies with BMMCs demonstrated that the individual amino acids His, Lys, Thr and the combination of His, Lys, Thr had negative effects on mTOR signaling pathway in antigen-IgE-activated mast cells. While Leu, Ile, Val and the combination of Leu, Ile, Val positively regulated the mTOR signaling pathway in BMMCs (**chapter 5**). Based on the results from the *in vitro* studies, an mTOR-targeting amino acid diet containing

increased relative amounts of His, Lys, Thr and reduced relative amounts of Leu, Ile, Val was developed. Previously, we demonstrated that CMA mice exhibited reduced social behavior and increased repetitive self-grooming behavior. The mTOR signaling pathway was shown to be centrally involved in the behavioral deficits described for CMA mice (**chapter 3 and 4**). Therefore, we investigated the effects of the amino acid diet on behavioral alterations and on the activation of the mTOR signaling pathway inspecific brain regions of CMA mice (**chapter 6**). The mTOR-targeting amino acid diet was shown to restore the social interaction and normalize the repetitive behavior of CMA mice. Dietary intervention with the amino acid diet inhibited the enhanced mTOR signaling pathway in the prefrontal cortex and amygdala of CMA mice.

Furthermore, the effects of a multi-nutrient supplementation diet containing specific anti-inflammatory and neuroprotective ingredients on behavioral alterations of CMA mice were investigated (**chapter 6**). Similar to the mTOR-targeting amino acid diet, the multi-nutrient supplementation diet improved the social interaction and normalized the repetitive behavior of CMA mice. The activated mTOR signaling pathway in the prefrontal cortex and amygdala of CMA mice was also inhibited by the multi-nutrient supplementation diet. The multi-nutrient supplementation diet consisted of various ingredients that exert neuroprotective and anti-inflammatory effects, such as eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), galaco-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), vitamins, and rice fibers. For instance, EPA and DHA were shown to play a crucial role in the development of brain and nervous system[65–67]. Dietary supplementation with EPA and DHA was shown to prevent impaired social behavior and normalize brain dopamine levels in CMA mice[68]. Dietary supplementation with the mixture of GOS and FOS attenuated food allergic reactions in mice[69]. Vitamins, in particular B vitamins, play an essential role in the development and function of nervous system[70,71]. Moreover, it was shown that the rice fibers reduced colonic inflammation and regulated T cell differentiation in a mouse model of colitis[72]. Apart from the effects of both mTOR-targeting amino acid diet and multi-nutrient supplementation diet on behavioral changes of CMA mice, the effects of both diets were investigated also in an inbred mouse model for ASD, the BTBR *T+ Itpr3tf/J* mouse (**chapter 6**). BTBR mice display several core behavioral phenotypes of ASD including reduced social behavior and increased repetitive self-grooming behavior[73]. The BTBR mouse is considered a mouse model for ASD and frequently used in previous studies for ASD because it carries multiple spontaneous mutations. The behavioral and neurological impairments of BTBR mice may be induced by a complex genetic interplay[73,74]. BTBR mice spent more time in the interaction zone and displayed higher levels of locomotor

activity in the open field in the social interaction test compared to C57BL/6 mice. The high levels of locomotor activity of BTBR mice reduced when exposed to the interaction mouse, because they probably became habituated to the novel environment. High levels of locomotor activity of BTBR mice was frequently observed also in the open field tests or in some behavioral tests for anxiety as shown in previous studies[75–77]. Due to the inability to assess reduced social behavior in BTBR mice using our experimental set-up, we were unable to demonstrate clear effects of dietary intervention with either mTOR-targeting amino acid diet or multi-nutrient supplementation. However, a recent study has demonstrated that BTBR mice treated with rapamycin displayed a remarkable improvement in several measures of social behavior indicating a role for mTOR signaling[78]. A more validated, specific behavioral test such as three-chamber sociability test could be used for measuring some specific aspects regarding the sociability and social novelty of BTBR mice[75,78,79]. Furthermore, we demonstrated that the specific amino acid diet, but not the multi-nutrient supplementation diet, normalized both the increased self-grooming duration and self-grooming frequency of BTBR mice (**chapter 6**).

Given the effects of the mTOR-targeting amino acid diet on the behavioral impairments of BTBR mice, we raised the question whether the mTOR signaling pathway is enhanced in the specific brain regions of BTBR mice and the enhanced mTOR signaling pathway could be suppressed by dietary intervention with the specific amino acid diet (**chapter 6**). The mTOR signaling pathway was enhanced in the prefrontal cortex, which was inhibited by the specific mTOR-targeting amino acid diet. In contrast, the mTOR signaling pathway was found to be down-regulated in the somatosensory cortex of BTBR mice. A significantly reduced mTOR signaling was also found amongst other brain regions in the cortex of a Rett syndrome mouse model, which may be responsible for the dysregulated translational control in synaptic organization and the development of Rett syndrome[80]. The specific mTOR-targeting amino acid diet further down-regulated the reduced mTOR signaling pathway in the somatosensory cortex of BTBR mice.

Taken together, these findings provide a strong indication that the specific amino acid diet exerted its therapeutic effects on behavior by down-regulating the mTOR signaling pathway.

Effects of dietary intervention with either the mTOR-targeting amino acid diet or multi-nutrient supplementation diet on immune responses in CMA mice

Apart from its critical role in regulating cell growth and metabolism, mTOR signaling pathway is centrally involved in directing immune responses including mast cell activation[14] and differentiation of T cells[81]. Our *in vitro* studies revealed that activation of mTOR signaling pathway is required for mast cell function since both acute degranulation as well as cytokine production was inhibited by rapamycin treatment in a dose-dependent manner (**chapter 5**). The involvement of mTOR signaling pathway in mast cell activation has also been demonstrated in various previous studies[14,60]. In the current study we investigated the effects of both mTOR-targeting amino acid diet and multi-nutrient supplementation diet on humoral immune responses, mucosal mast cell activation and intestinal mucosal T cell responses in CMA mice (**chapter 7**). Dietary intervention with either mTOR-targeting amino acid diet or multi-nutrient supplementation diet was shown to have no effects on whey-specific IgE, IgG1, IgG2a, and mMCP-1 levels in serum of CMA mice. These results suggest that both the amino acid diet and multi-nutrient supplementation diet may bypass the immune system and exert their effects directly on brain functioning, improving the behavioral impairments found in CMA mice.

Previous studies have demonstrated that mTOR signaling pathway plays a critical role in the decision making process for differentiation of T cell populations[81,82]. mTORC1 activity is required for the differentiation of Th1 and Th17 cells. mTORC2 signaling is important for the differentiation of Th2 cells. The development of regulatory T cell is induced by the inhibition of both mTORC1 and mTORC2 activity. Given the critical role of mTOR signaling pathway in the differentiation of T cell populations, we assessed the effects of both diets on differentiation and development of T cell populations by examining the expression levels of T cell-associated transcription factors and Treg cell-associated cytokines in the small intestine of CMA mice (**chapter 7**). Based on examination of the expression of T cell-related transcription factors in the proximal small intestine, our results suggest that Th2 (GATA-3) and Th17 (ROR-_) cell development was induced in the small intestine of CMA mice and both diets inhibited this response. The ratio of Th2 to Th1 cell-associated transcription factors tended to be increased in the distal part of the small intestine of CMA mice, indicating a shift towards Th2 cell directed immune response as previous described by Kerperien et al 2014[73]. However, this CMA-induced Th2 cell shift was inhibited by dietary intervention with either mTOR-targeting amino acid

diet or multi-nutrient supplementation diet. Furthermore, dietary intervention with mTOR-targeting amino acid diet or multi-nutrient supplementation diet enhanced the Treg cell-related transcription factor (Foxp3) in the proximal small intestine of CMA mice, suggesting that Treg cells may play a suppressing role in allergic immune responses. A previous study also demonstrated that the whey-specific $CD25^+$ Treg cells are involved in the suppression of the allergic effector response induced by dietary intervention with prebiotics[83]. In addition, we have shown that the expression of mRNA encoding for IL-10 and TGF-ß in the distal part of the small intestine was inhibited by dietary intervention with either mTOR-targeting amino acid diet or multi-nutrient supplementation diet. Unfortunately, we did not examine the proximal part of the small intestine where the upregulation Treg cell transcriptional marker by both diets was observed. The cytokines IL-10 and TGF-ß, which are anti-inflammatory cytokines, are classically regarded to be produced by Treg cell, but a wide variety of immune cell types including Th2 and Th17 can also be a source of these cytokines[84–86]. The dietary intervention with the mTOR-targeting amino acid diet or multi-nutrient supplementation diet may have different effects on the cytokine production of different immune cells. Future studies, examining mRNA and protein levels of IL10 and TGF-ß in the proximal small intestine are necessary to further elucidated the role of intestinal Treg cells in CMA as well as the immunomodulating effects of both diets.

Overall, food allergic reactions in the intestine induced over-activation of the mTOR signaling pathway locally in the intestines as well as in the prefrontal cortex and amygdala, which eventually leads to the dysfunction of brain and autistic-like behavioral changes in mice (Fig. 1). Dietary interventions targeting over-activated mTOR signaling pathway in the brain or intestine or both improve brain function and autistic-like behavioral impairments as well as ASD-associated intestinal problems.

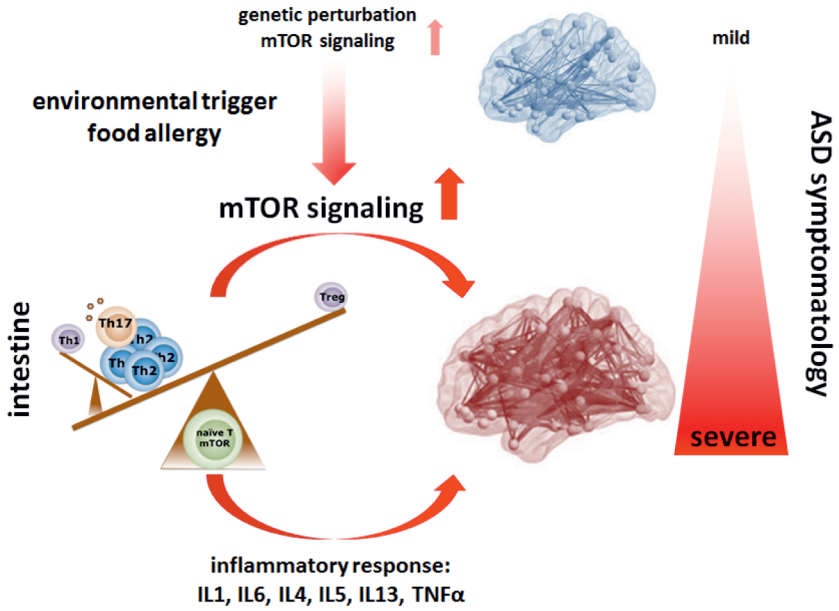

Fig. 1. Intestinal immune stress (by food allergy) results in a severe autistic phenotype in genetically predisposed subjects with a central role of mTOR. APC: antigen-presenting cells; ASD: autistic spectrum disorder; mTOR: mammalian target of rapamycin; Th1: Thelper 1 cells, Th2: Thelper 2 cells; Th17: Thelper 17 cells; Treg: regulatory T cells; IL: interleukin; TNF: tumor necrosis factor.

Concluding remarks and future perspectives

This thesis provides insight into the molecular mechanism underlying the relationship between intestinal immune dysregulation and the development of ASD. These results provide strong evidence supporting the hypothesis that mTOR signaling pathway could serve as a potential therapeutic target for the treatment of ASD associated with intestinal problems possibly related to food allergy using dietary interventions. We have demonstrated that food allergy induces reduced social interaction and increased repetitive self-grooming behavior in mice. The mTOR signaling pathway is enhanced in the brain and intestine of CMA mice. Given the positive and negative effects of amino acids on regulation of the mTOR signaling pathway, a specific amino acid diet that suppresses mTOR signaling was developed. This mTOR-targeting amino acid diet was effective in the prevention of behavioral deficits of CMA mice and inhibited the

mTOR signaling pathway in the brain. Furthermore, the mTOR-targeting amino acid diet improved the repetitive self-grooming behavior of BTBR autistic mice that contain spontaneous genetic mutations. These findings suggest that the mTOR signaling pathway may be a convergent pathway for the treatment of ASD in both food allergy-associated and genetically associated ASD-related situations.

Similar to the mTOR-targeting amino acid diet, a multi-nutrient supplementation diet containing specific anti-inflammatory and neuroprotective ingredients improved the behavioral deficits and inhibited mTOR signaling in the brain of CMA mice. However, this multi-nutritional diet had no effects on behavioral impairments of BTBR mice. These results suggest that the multi-nutrient supplementation diet may inhibit immune disturbances in the intestine, resulting in modulation of the disturbed gut-brain axis and thereby improve food allergy-induced autistic-like phenotypes.

Based on our preclinical results, we have collected promising evidence suggesting a role of the gut-immune-brain axis in ASD-like behavior regulated by a common denominator, the mTOR signaling pathway, which plays a central role in both the intestinal immune system, and brain.

The enhanced mTOR signaling pathway might also be observed in the (intestinal) immune system of patients suffering from ASD. Therefore, future investigations focusing on the mTOR signaling pathway in human tissues such as peripheral blood mononuclear cells or intestinal biopsies of autistic patients will uncover the critical role of enhanced the mTOR signaling pathway acting as therapeutic target for ASD as well as a useful biomarker for ASD.

Finally, our results with nutritional intervention targeting the enhanced mTOR signaling pathway in two distinct murine models for ASD-like behavior, valid prove of principle clinical trials in ASD patients. Whereas the multi-nutrients supplementation diet might also be useful for those ASD patients with a clear involvement of food allergy-associated intestinal problems.

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APPENDICES

Nederlandse samenvatting

Autisme wordt gekarakteriseerd door een serie gedragsafwijkingen, zoals verlaagd sociaal gedrag, verstoorde communicatie en de aanwezigheid van stereotiepe gedragingen. De etiologie van autisme is gerelateerd aan een complexe interactie tussen genetische aanleg en omgevingsfactoren. Voedselallergie, met name koemelkallergie wordt verondersteld een belangrijke omgevingsfactoren te zijn. Autistische patiënten hebben vaak ook maagdarmklachten gerelateerd aan voedselallergie, zoals buikpijn en problemen met de ontlasting.

mTOR (mammalian target of rapamycin) is een geconserveerd serine/threonine kinase dat de celgroei, het metabolisme en de eiwitsynthese in de cel reguleert. De mTOR remmer rapamycin is een antibioticum dat specifiek aan gefosforyleerd mTOR bindt en de vorming van mTORC1 complex blokkeert. Door deze blokkade wordt de activiteit van de 'downstream' mTOR-signaaltransductie in de cel geremd. Overactiviteit van de mTOR signaaltransductie in de cel door genetische afwijkingen leidt vaak tot neurologische aandoeningen die geassocieerd zijn met autisme. Defecten aan Tsc (Tubereuze Sclerosis Complex) en Pten (fosfatase en tensine homoloog) genen zijn bijvoorbeeld geassocieerd met de verhoogde mTOR signaaltransductie in het brein. Patiënten met defecten aan Tsc of Pten genen lijden vaak ook aan autisme of autisme gerelateerde aandoeningen. Onderzoek heeft aangetoond dat behandeling met de mTOR remmer rapamycin verstoord sociaal gedrag bij muizen met Tsc of Pten mutaties kan verbeteren.

De mTOR signaaltransductie is niet alleen betrokken bij de ontwikkeling van autisme-gerelateerde neurologische aandoeningen, maar ook bij de regulering van het immuunsysteem in het bijzonder T cellen. Eerdere onderzoeken hebben aangetoond dat mTORC1 activiteit belangrijk is voor de differentiatie van Th1 en Th17 cellen, en dat mTORC2 activiteit nodig is voor de differentiatie van Th2 cellen. Bovendien is uit eerder onderzoek gebleken dat mTOR signaaltransductie een cruciale rol speelt bij de mestcelfunctie. Th2 cellen en mestcellen spelen een belangrijke rol bij allergie. In dit proefschrift presenteren wij onderzoek naar de invloed van de mTOR signaaltransductie op de immunologische, neurologische en gedragsveranderingen in het koemelk-allergie muizenmodel en een genetisch muizenmodel voor autisme gerelateerde aandoeningen.

In **hoofdstuk 2** worden een aantal moleculaire mechanismen betrokken bij neuro-immune interacties en mogelijk betrokken bij autisme beschreven. Het gaat hierbij om moleculaire mechanismen in de darm, het serotoninesysteem, en de mTOR signaaltransductie in het brein en de darm. De verstoorde mTOR signaaltransductie wordt gezien als een therapeutische target voor de behandeling van (voedselallergie-geassocieerd) autisme. In **hoofdstuk 3** worden de immunologische, neurologische en gedragsveranderingen in koemelk-

allergische muizen beschreven. Onze resultaten hebben aangetoond dat koemelk-allergie resulteert in verlaagde sociale interactie en verhoogd repetitief poetsgedrag van de muizen. De gedragsveranderingen gaan gepaard met veranderingen in het dopaminesysteem in het brein. De concentraties van de neurotransmitter dopamine en zijn afbraakproducten zijn verlaagd in de prefrontale cortex en verhoogd in de amygdala van allergische muizen. Een verhoogde neuronale activiteit is ook waargenomen in de orbitale prefrontale cortex van allergische muizen. De prefrontale cortex en amygdala spelen een belangrijke rol bij diverse autistische gedragingen. De prefrontale cortex is betrokken bij het sociale en repetitieve gedrag. De amygdala speelt een cruciale rol bij emotionele uitdrukking en sociaal gedrag. Naast de neurochemische veranderingen in het brein is een verhoogde concentratie van serotonine in de dunne darm van allergische muizen waargenomen. Onze bevindingen suggereren dat allergische reacties in de darmen autisme-gerelateerde gedragsveranderingen induceren, en dat deze gedragsveranderingen geassocieerd zijn met de neurochemische veranderingen in het brein en de darmen.

In **hoofdstuk 4** hebben we de rol van mTOR in de immunologische, neurologische en gedragsveranderingen in koemelk-allergische muizen onderzocht. Allereerst hebben we aangetoond dat tijdens koemelk allergie de fosforylering van de mTOR signaaltransductie eiwitten p70 S6K en 4E-BP1 verhoogd is in de prefrontale cortex en amygdala en de dunne darm. Verhoogde fosforylering van p70 S6K en 4E-BP1 is geassocieerd met overactiviteit van mTOR. Het gevolg hiervan kan een verhoogde eiwitsynthese zijn, die uiteindelijk leidt tot een verstoorde activiteit en de ontwikkeling van autistische fenotypen. Behandeling met de mTOR remmer rapamycin remt de verhoogde mTOR signaaltransductie in het brein en de darm en verbetert de verstoorde gedragingen van de koemelk-allergische muizen. Rapamycin behandeling remt ook humorale immuun responsen en mucosale mestcel activiteit in de dunne darm van de allergische muizen. Naast het remmende effect van rapamycin op allergische immuun responsen is gebleken dat rapamycin behandeling de expressie van een Treg cel-geassocieerde transcriptie factor in de dunne darm van allergische muizen induceerde, wat suggereert dat rapamycin behandeling de allergische immuun responsen onderdrukte in de dunne darm van allergische muizen middels een effect op Treg cellen.

In **hoofdstuk 5** worden de effecten van individuele aminozuren en aminozuurcombinaties beschreven op de activiteit van de mTOR signaaltransductie in antigen-IgE geactiveerde mestcellen. Aminozuren zijn een belangrijke regulator voor de mTOR signaaltransductie. Uit onze studie is gebleken dar de mTOR signaaltransductie betrokken is bij antigen-IgE-gestimuleerde mestcel activiteit. De individuele aminozuren histidine, lysine, threonine en

de combinatie van deze aminozuren remmen de verhoogde mTOR signaaltransductie die is aangetoond op het niveau van de fosforylering van p70 S6K in antigen/IgE-geactiveerde mestcellen. De individuele aminozuren leucine, isoleucine, valine en de combinatie van deze aminozuren verhogen de fosforylering van p70 S6K in antigen/IgE-geactiveerde mestcellen. Alle geteste aminozuren en aminozuurcombinaties remmen de acute degranulatie en cytokine productie van antigen-IgE-geactiveerde mestcellen. Op basis van deze resultaten hebben we een aminozuur dieet ontwikkeld dat de mTOR signaaltransductie kan onderdrukken. Dit specifieke aminozuur dieet bevat verhoogde (relatieve) hoeveelheden van histidine, lysine en threonine en verlaagde (relatieve) hoeveelheden van leucine, isoleucine en valine.

Hoofdstuk 6 beschrijft de dierstudies waarbij de effecten van het aminozuur dieet en een multi-nutrient suppletie dieet zijn onderzocht op gedragsveranderingen en de mTOR signaaltransductie in het brein in het koemelk-allergie muizenmodel en het BTBR muizenmodel voor autisme. Het multi-nutrient suppletie dieet bevat verschillende componenten die neuro-protectieve en anti-inflammatoire effecten kunnen induceren. Uit onze studie is gebleken dat dieetinterventie met het aminozuur dieet of het multi-nutrient suppletie dieet de verlaagde sociale interactie en het verhoogde repetitieve poetsgedrag tijdens een koemelk-allergische reactie verbeterde. Dieetinterventie met het aminozuur dieet of het multi-nutrient suppletie dieet onderdrukte ook de verhoogde fosforylering van p70 S6K in de prefrontale cortex en amygdala van koemelk-allergische muizen. Naast het koemelk-allergische muizenmodel hebben we ook de effecten van de dieetinterventie met het aminozuur dieet of het multi-nutrient suppletie dieet op gedragsveranderingen en activatie van mTOR signering in het BTBR muizenmodel voor autisme onderzocht. BTBR muizen zijn muizen die spontane genetische mutaties bevatten. Ze vertonen een aantal typische autistische kenmerken in het gedrag, zoals verlaagd sociaal gedrag en verhoogd repetitief poetsgedrag. De dieetinterventie met het aminozuur dieet verbeterde het repetitieve poetsgedrag van BTBR muizen en onderdrukte de fosforylering van p70 S6K in de prefrontale cortex en somatosensorische cortex van BTBR muizen. De interventie met het multi-nutrient suppletie dieet had geen effect op het repetitieve poetsgedrag en op de activatie van de mTOR signaaltransductie in het brein. Deze bevindingen suggereren dat het aminozuur dieet de activatie van de mTOR signaaltransductie in het brein van BTBR muizen direct onderdrukte waardoor het verstoorde gedrag van BTBR muizen verbeterd kon worden. Het multi-nutrient suppletie dieet zou met name de allergische immuunreactie in het maagdarmkanaal kunnen onderdrukken waardoor de verstoorde functie van de hersenen en verstoorde gedragingen verbeterd kunnen worden. De effecten

van het multi-nutrient suppletie dieet op de activiteit van de mTOR signaaltransductie in het brein en op het verstoorde gedrag hebben we daarom alleen gezien in koemelk-allergische muizen.

Naast de effecten van de dieetinterventie op gedragsveranderingen en activiteit van de mTOR signaaltransductie in het brein, hebben we de effecten van het aminozuur dieet of het multi-nutrient suppletie dieet op humorale immuun responsen, en op mucosale mestcel activatie in de darm onderzocht (**hoofdstuk 7**). Uit onze studie is gebleken dat interventie met het aminozuur dieet of het multi-nutrient suppletie dieet geen effect had op de productie van koemelkeiwit-specifieke IgE, IgG1, IgG2a en op de concentratie van mMCP-1 in allergische muizen. Deze resultaten suggereren dat het aminozuur dieet of het multi-nutrient suppletie dieet het effect direct uitoefenen op het brein, waardoor de verstoorde gedragen van allergische muizen verbeterd kunnen worden. Aangezien de mTOR signaaltransductie een belangrijke rol speelt bij differentiatie van verschillen T cel populaties, hebben we ook de effecten van de interventie met het aminozuur dieet of het multi-nutrient suppletie dieet op de mucosale T cel balans in de dunne darm van allergische muizen onderzocht (**hoofdstuk 7**). Onze studie heeft aangetoond dat interventie met het aminozuur dieet of het multi-nutrient suppletie dieet de expressie van Th2cel en Th17cel-geassocieerde transcriptiefactoren onderdrukte en de expressie van Treg-geassocieerde transcriptiefactor verhoogde. Er is ook aangetoond dat interventie met het aminozuur dieet of het multi-nutrient suppletie dieet de mRNA expressie van cytokinen IL10 en TGF-ß in de dunne darm van allergische muizen onderdrukte. Dit komt waarschijnlijk doordat IL-10 en TGF-ß geproduceerd zouden kunnen worden door verschillende immune celtypen zoals Th2, Th17 en Treg cellen. Beide diëten zijn in staat om de verstoorde T cel balans tijdens koemelk allergie in de darm te moduleren.

Samenvattend geeft dit proefschrift inzicht in de moleculaire mechanismen die ten grondslag liggen aan de immunologische, neurologische en gedragsveranderingen in muizenmodellen voor koemelkallergie en autisme. Dit proefschrift heeft laten zien dat voedsel allergie een risicofactor zou kunnen zijn voor de ontwikkeling van autistische fenotypen. Dit proefschrift heeft ook aangetoond dat de activiteit van mTOR signaaltransductie verhoogd is in het brein en darmen van koemelk-allergische muizen, en dat dit betrokken is bij de gedragsveranderingen van allergische muizen. De mTOR signaaltransductie is ook centraal betrokken bij de immunologische veranderingen in de darm van allergische muizen. Een specifiek aminozuurdieet dat de mTOR signaaltransductie kan onderdrukken verbeterde de verstoorde gedragen en onderdrukte verhoogde activiteit van de mTOR signaaltransductie in allergische muizen en autistische muizen met genetische mutaties. Onze preklinische

data hebben aangetoond dat de mTOR signaaltransductie een centrale rol speelt in de darm-immuunsysteem-brein triangle. In autisme zou dit een waardevol aangrijpingspunt voor behandeling kunnen worden. Onderzoek in de mens zou de volgende stap moeten zijn om de centrale rol van mTOR in autisme aan te tonen.

Kortom, dit onderzoek vormt een essentiële stap in de zoektocht naar nieuwe aangrijpingspunten en methoden voor de behandeling van autisme.

Chinese summary

我过去四年研究的中心课题是食品过敏与自闭症的成因的相关性，我的研究主要专注于 gut-immune-brain axis 分子水平的作用原理以及这个分子作用原理对自闭症起因的影响。自闭症患者有一系列的行为方面的缺陷，比方说社会行为的降低，行为的重复性，语言交流的缺陷。而且很多自闭症患者有免疫方面的缺陷或者失调比如食品过敏，小肠渗透性的增加导致的腹泻等等。自闭症的起因被认为有外因以及内因的相互作用，内因主要指先天性的基因缺陷，外因则有各种各样，食品过敏比方说牛奶过敏已经越来越显示出其在自闭症成因中的重要性，被认为是最重要的外因之一。

mTOR 是一个非常重要的，高度保守的丝氨酸(serine)或者(threonine)羟基丁氨酸激酶，与各种细胞活动相关，比方说细胞生长，蛋白质合成等等。雷帕霉素(rapamycin)是特定的 mTOR 的抑制剂，能够有效抑制 mTORC1 的活动。已经有大量的研究表明，mTOR 信号传导路径的过度激活与自闭症或者一些自闭症的生理特征的形成息息相关，例如，结节性硬化症就是由于 mTOR 信号传导路径的蛋白质 TSC1 或者 TSC2 的基因突变引起的。大约 20-60%患有结节性硬化症的病人也有自闭症。有研究表明,TSC 功能敲除老鼠在大脑中显示了被过度激活的 mTOR 信号传导路径，并且也有自闭症行为的显现比如社会行为的降低。而雷帕霉素能够在 TSC 功能敲除老鼠的大脑中有效抑制 mTOR 信号传导路径并改善其自闭症行为特征。磷酸酯酶与张力蛋白同源物（Phosphatase and tensin homolog，简称为 PTEN）是由 *PTEN* 基因编码的蛋白质。*PTEN* 基因是一种肿瘤抑制基因， 该基因编码的蛋白质是一种磷脂酰肌醇-3,4,5-三磷酸 3-磷酸酯酶，能脱去磷酸肌醇底物上的磷酸。研究表明 PTEN 功能敲除老鼠也显示出自闭症特征包括自闭症的行为特征，大脑组织形态的改变。雷帕霉素能抑制 PTEN 功能敲除老鼠大脑中的 mTOR 信号传导路径，并改善其自闭症行为和改善大脑组织的形态。除此之外，有研究表明 mTOR 信号传导路径的其他的一些基因变异比如真核起始因子 4E（eIF4E）以及易脆 X 染色体症候群基因产物（FMRP）也能引发自闭症生理特征的形成。

mTOR 信号传导路径不仅仅与各种自闭症生理特征的形成相关，也能调节各种免疫系统的反应。有研究表明 mTORC1 的活动对辅助 T 细胞 Th1 和 Th17 的分化与形成不可缺失， mTORC2 的活动对辅助 T 细胞 Th2 的分化与形成不可缺失，而当 mTORC1 和 mTORC2 的活动被同时抑制时，又能促进调节 T 细胞 Treg 的分化与形成。另外有研究表明，mTOR 信号传导路径也与肥大细胞（mast cells）的激活和过敏反应的形成密切相关。基于 mTOR 信号传导路径这种对神经系统，免疫系统双重作用，而免疫系统的失调又能促进自闭症生理特征的形成与发展，我的研究主要着重于在牛奶过敏或者自闭症的老鼠模型中，mTOR 信号传导路径与神经系统，免疫系统以及行为方面的改变的相关性。通过这个研究我们希望能够找到治疗与食品过敏相关自闭症的新标靶，从而为治疗自闭症找到新方法，开辟新途径。

论文**第二章**是一篇关于在神经和免疫相互作用中的各种分子机制的综述,包括 mTOR 信号传导路径，并提出基于以前的研究成果，mTOR 信号传导路径可能是治疗与食品过敏相关的自闭症的新标靶。**第三章**描述了牛奶过敏老鼠的各种免疫，神经以及行为方面的改变。首先我们的研究表明有食品过敏反应的老鼠表现出一些类似于自闭症行为的行为改变，比如社群行为的降低，重复性的自我清洁行为的增加。伴随这些行为方面的改变，在过敏老鼠的大脑中和肠道中一些神经化学物质也发生了改变，比如神经传递介质多巴胺（dopamine）及分解物的浓度在前额叶皮层（prefrontal cortex）和杏仁核（amygdala）的改变。前额叶皮层和杏仁核都被认为与自闭症行为息息相关，

其中前额叶皮层与社群行为和重复性动作相关，杏仁核则与社群行为和情绪的表达相关。除了神经化学物质在大脑中的变化之外，在食品过敏老鼠的小肠中，我们也发现 5 羟色胺（serotonine）的浓度也有增加。这些在食品过敏老鼠的大脑特定区域和肠道中的发现表明食品过敏老鼠行为上的改变和神经化学物质在大脑以及肠道中的改变是息息相关的。

在**第四章**我们进一步阐述了引起食品过敏老鼠神经系统，免疫系统以及行为上改变的分子机制，尤其是 mTOR 信号传导路径的相关性。我们发现这些食品过敏的老鼠通过雷帕霉素的治疗其类似于自闭症行为的行为改变，比如社群行为的降低，重复性自我清洁动作的增加，得到了有效的改善。同时我们发现，mTORC1 的直接下游蛋白质 4E-BP1 和 p70 S6K 的磷酸化在食品过敏老鼠的前额叶皮层和杏仁核显著增加了，表明 mTOR 信号传导路径在食品过敏老鼠的上述脑部区域被过度激活了。由于 4E-BP1 和 p70 S6K 的活动和蛋白质的合成直接相关。4E-BP1 和 p70 S6K 在食品过敏老鼠的前额叶皮层和杏仁核的过度磷酸化将直接导致在这些脑部区域的过量蛋白质合成，包括突触蛋白质的过量合成。这将导致 excitation/inhibition 平衡的被打破，从而最终形成自闭症的生理特征。雷帕霉素的治疗能够在上述脑部区域有效抑制 4E-BP1 和 p70 S6K 的磷酸化。而且 p70 S6K 的磷酸化在食品过敏老鼠的小肠也被发现有显著的增加，通过雷帕霉素的治疗 p70 S6K 的磷酸化在小肠也能得到有效抑制。另外，雷帕霉素对食品过敏的老鼠的治疗也能有效抑制体液性的免疫反应和粘膜肥大细胞的激活。而且，我们的实验结果也显示雷帕霉素的治疗能够在食品过敏老鼠的小肠激发和促进调节 T 细胞的形成。

已经有大量的研究表明 mTOR 信号传导路径也通过氨基酸进行调节，比如有研究表明，在乳腺上皮细胞中离氨酸（lysine），组氨酸（histidine），苏氨酸(threonine)能够有效抑制 mTOR 信号传导路径，而白氨酸（leucine），异亮氨酸(isoleucine)，缬胺酸(valine)则能激发 mTOR 信号传导路径。由于 mTOR 信号传导路径的过渡激活能导致自闭症特征的形成，我们希望通过调节氨基酸在食品中的含量，设计出一种能抑制 mTOR 信号传导路径的食品，从而通过饮食干预达到治疗自闭症的目的。由于肥大细胞是过敏反应的一个非常重要的参与者，我们决定在肥大细胞中进行 mTOR 信号传导路径以及氨基酸对 mTOR 信号传导路径的调节的研究。**第五章**详细阐述了我们在肥大细胞中的这些研究。我们的实验结果表明，mTOR 信号传导路径在肥大细胞的脱颗粒作用和细胞因子的生成起了重要作用，因为经过雷帕霉素的培养后，这些肥大细胞在 IgE/antigen 激活后的脱颗粒作用和细胞因子的生成都得到了抑制。同时我们发现，离氨酸，组氨酸，苏氨酸的混合能有效抑制肥大细胞的激活以及激活后的肥大细胞的 mTOR 信号传导路径。白氨酸，异亮氨酸，缬胺酸的混合则进一步提升了 IgE/antigen 激活后的肥大细胞中的 mTOR 信号传导路径。有趣的是，白氨酸，异亮氨酸，缬胺酸的混合尽管能提升了 IgE/antigen 激活后的肥大细胞中的 mTOR 信号传导路径，但却能抑制肥大细胞在 IgE/antigen 激活后的脱颗粒作用和细胞因子的生成，这可能表明氨基酸对肥大细胞中在 IgE/antigen 激活后的脱颗粒作用和细胞因子的生成的调节是一个极其复杂的过程，是正面分子机制和负面分子机制相互作用的结果。基于这些实验结果，我们设计出了一种可以抑制 mTOR 信号传导路径氨基酸食品，在这种氨基酸食品中我们提升了离氨酸，组氨酸，苏氨酸的含量，降低了白氨酸，异亮氨酸，缬胺酸的含量。

第六章详细描述了用氨基酸食品和一种抗炎食品的饮食干预对牛奶过敏老鼠的类似自闭症行为和 BTBR 老鼠的自闭症行为以及大脑中 mTOR 信号传导路径的影响。抗炎食品中含有各种能改善肠道

炎症，保护和提高神经系统功能的营养成分，比如二十二碳六烯酸（DHA），二十碳五烯酸（EPA），低聚半乳糖(GOS)，低聚果糖 (FOS)，纤维素以及各种维他命。BTBR 老鼠是一种自发性基因突变的患有自闭症的老鼠。这种老鼠表现出了一系列的自闭症行为，比如社群活动的降低， 重复性自我清洁行为的增加，对新环境和新目标的高度敏感型等等。实验结果表明，在牛奶过敏老鼠中，氨基酸食品和抗炎食品的饮食干预都能改善过敏老鼠的类似自闭症行为，比如改善了牛奶过敏老鼠的社群行为，降低了它们的重复性自我清洁行为，并且抑制了 mTOR 信号传导路径在牛奶过敏老鼠的前额叶皮层和杏仁核的过度激活。在 BTBR 老鼠中，氨基酸食品的饮食干预降低了它们的重复性自我清洁行为，但抗炎食品对 BTBR 老鼠的重复性自我清洁行为却毫无作用。而且氨基酸食品的饮食干预有效抑制了 BTBR 老鼠大脑的前额叶皮层和躯体感觉皮质的 mTOR，抗炎食品对 BTBR 老鼠脑部区域的 mTOR 信号传导路径也没有任何作用。这些研究结果表明氨基酸食品是通过抑制老鼠脑部或者肠道的 mTOR 信号传导路径，从而达到改善牛奶过敏老鼠或者 BTBR 老鼠自闭症行为的目的，而抗炎食品则主要通过抑制肠道炎症，从而改善食品过敏老鼠的脑部功能和它们的自闭症行为，因此抗炎食品不能改善 BTBR 老鼠的自闭症行为。

在**第七章**我们进一步阐述了氨基酸食品和抗炎食品的饮食干预对牛奶过敏老鼠的过敏反应以及小肠内的免疫反应的影响。我们的实验结果显示，氨基酸食品和抗炎食品的饮食干预对牛奶过敏老鼠的体液性的免疫反应和粘膜肥大细胞的激活没有影响，这也表明氨基酸食品和抗炎食品对过敏老鼠行为的改善可能直接通过改善过敏老鼠的脑部功能而不是间接地通过抑制过敏反应。我们的实验结果也显示，氨基酸食品和抗炎食品的饮食干预在牛奶过敏老鼠的小肠内有效抑制了辅助 T 细胞 Th2 和 Th17 的分化和形成，并促进了调节性 T 细胞的分化和形成。同时，氨基酸食品和抗炎食品的饮食干预也抑制了细胞因子 IL-10 和 TGF-β 的生成。

总而言之，我的研究为与食品过敏相关的自闭症的形成的分子机制进行了透彻而详细的阐述，并为牛奶过敏老鼠免疫系统，神经系统以及行为方面的改变提供了分子机制方面的解释。我的研究结果显示食品过敏确实与自闭症的生理特征的形成相关。mTOR 信号传导路径的过度激活在导致与食品过敏相关的自闭症的形成起到了极其重要的作用，而通过饮食干预抑制 mTOR 信号传导路径的过度激活能有效改善食品过敏老鼠或者 BTBR 自闭症老鼠的行为缺陷。我的这些研究成果为将来治疗与食品过敏相关的自闭症找到了新标靶，开辟了一条新路径。

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Curriculum vitae

Jiang-bo Wu was born on October 26, 1982 in Xinning, Hunan Province, China. In 2002 he graduated from the Xinning No.1 Middle School in China, after which he started to study Chemistry in Central South University in China. In 2004 he came to The Netherlands and started to learn Dutch language at the University of Amsterdam. After one year he pass the NT2 State exam and received a diploma for the Dutch language. In 2005 he started to study Biomedical Sciences at the University of Amsterdam. He received his Bachelor's degree in 2008 and continue with the research Master Medical Biology at the University of Amsterdam. During the Master he performed an internship project to characterize the $S1P_5$ sphingolipid receptor and its related signaling pathways under the supervision of Dr. Astrid Alewijnse and Dr. Dennis Verzijl in the department of Pharmacology at the Academic Medical Center in Amsterdam. After receiving his Master's degree in 2010, he started his PhD project under the supervision of Dr. Aletta Kraneveld and Prof. Johan Garssen in the department of Pharmacology at Utrecht University. During his PhD, Jiang-bo investigated the involvement of mTOR signaling pathways in gut-immune-brain axis in the murine model of cow's milk allergy and the effects of dietary intervention on the mTOR signaling pathway in diverse murine models. He received a travel grant from NSFW for 8th World Congress on Developmental Origins of Health and Disease in Singapore and a travel grant from NSFW for 2nd European Conference of Microbiology and Immunology in Berlin. In August 2015, Jiang-bo started as a postdoctoral fellow in the laboratory of Prof. Joel Dore in French National Institute of Agriculture In Paris, France to continue his work in animal models with inflammatory bowel disease.

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