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

Jiangbo Wu [a], Caroline G.M. de Theije [a], Sofia Lopes da Silva [a,b], Suzanne Abbring [a], Hilma van der Horst [a], Laus M. Broersen [a,b], Linette Willemsen [a], Martien Kas [c,d], Johan Garssen [a,b], Aletta D. Kraneveld [a,e,*]

[a] Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands
[b] Nutricia Research, Uppsalalaan 12, 3584 CT Utrecht, The Netherlands
[c] Department of Translational Neuroscience, Brain Center Rudolf Magnus, University Medical Center Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands
[d] Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, The Netherlands
[e] Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 104, 3584 CM Utrecht, The Netherlands

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ABSTRACT

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 current study demonstrated that the amino acids histidine, lysine, threonine inhibited mTOR signaling and IgE-mediated mast cell activation, while the amino acids leucine, isoleucine, valine had no effect on mTOR signaling in BMMCs. Based on these results, we designed an mTOR-targeting amino acid diet (Active 1 diet) and assessed the effects of dietary interventions with the amino acid 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 + Itpr3tf/J mice. Cow's milk allergic (CMA) or BTBR male mice were fed a Control, Active 1, or Active 2 diet for 7 consecutive weeks. CMA mice showed reduced social interaction and increased self-grooming behavior. Both diets reversed behavioral impairments and inhibited the mTOR activity in the prefrontal cortex and amygdala of CMA mice. In BTBR mice, only Active 1 diet reduced repetitive self-grooming behavior and attenuated the mTOR activity in the prefrontal and somatosensory cortices. 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.

1. 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 (Kohane, 2015; Matson and Goldin, 2014). The etiology of ASD is thought to be multifactorial and involves 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 (Carter and Scherer, 2013; Ehninger and Silva, 2009; De Rubeis et al., 2014; Iossifov et al., 2014). There is also increasing evidence indicating that immune dysfunction in ASD individuals may also play a role in the pathophysiology of ASD (Croen et al., 2015; de Theije et al., 2011; Onore et al., 2012; Zerbo et al., 2015). An increased intestinal permeability was found in patients with ASD. (de Magistris et al., 2010). Furthermore, it was demonstrated that food allergy in children with ASD is associated with higher scores of stereotyped behavior (Lyall et al., 2015). These studies indicate that intestinal immune dysfunction, including food allergy, may cause disturbances in the pathways underlying the so-called 'gut-immune-brain axis', which may fundamentally contribute to the development of ASD (Kraneveld et al., 2014; Onore et al., 2012).

The mammalian target of rapamycin (mTOR) signaling pathway is a critical regulator in various cellular processes including protein synthesis and synaptic plasticity (Ebrahimi-Fakhari and Sahin, 2015; Wang and Doering, 2013). Findings from various single-gene mutant mouse models suggest that enhanced mTOR signaling may play a central role in the pathophysiology of ASD

* Corresponding author at: Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands.
E-mail address: a.d.kraneveld@uu.nl (A.D. Kraneveld).

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and pharmacological attenuation of the mTOR signaling pathway rescued various ASD-relevant phenotypes ranging from behavioral impairments to morphological abnormalities in the brain (Ehninger et al., 2008; Gkogkas et al., 2013; Sharma et al., 2010; Zhou et al., 2009). In the BTBR inbred mouse model for ASD rapamycin improved several measures of sociability (Burket et al., 2014). In addition, inhibition of the mTORC1 pathway by rapamycin reversed the autistic-like behaviors in CMA mice that were associated with enhanced phosphorylation of mTOR downstream proteins p70 S6K and 4E-BP1 in the prefrontal cortex and amygdala (Wu et al., 2015). Despite the fact that rapamycin showed promising capacities to reverse behavioral deficits in various murine models of autism, due to the ubiquitous inhibition of mTOR pathway, the clinical use of rapamycin as an immunosuppressive drug leads to diverse side effects, ranging from acute side effects to long-term ones (Aguiar et al., 2015; Geissler, 2015).

An alternative approach targeting mTOR is using specific amino acids that are not only the basic building blocks for protein synthesis, but are also implicated in regulation of mTORC1 activation (Bar-Peled and Sabatini, 2014; Efeyan et al., 2012). In murine mammary epithelial cells, threonine, histidine, and lysine inhibited p70 S6K1 phosphorylation both individually and as a mix (Prizant and Barash, 2008). For screening purposes, the first part of the present study examined the effects of individual and combined amino acids on mTOR signaling, antigen-specific degranulation, and cytokine production in bone-marrow derived mast cells (BMMCs). Based on the results from the *in vitro* experiments, a specific amino acid diet for *in vivo* studies was designed (Active 1 diet), aimed to attenuate the mTOR signaling pathway in ASD-related brain regions and to improve behavioral impairments in a mouse model of CMA and in an inbred BTBR *T*$^{+}$*Itpr3*^{tf}/J (BTBR) mouse strain for ASD. The efficacy of this diet was compared to a control diet and a diet of low-glycemic index carbohydrates, dietary fibers, high trypthophan content and a lipid profile that predominantly differed in DHA and EPA content (Active 2 diet). The Active 2 diet was previously demonstrated to have neuroprotective and anti-inflammatory effect and to ameliorate behavioral impairments in a prenatal valproate-induced murine model for ASD (de Theije et al., 2014a,b; de Theije et al., 2015a,b).

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 with nutritional interventions may offer a new approach in the management of ASD.

2. Materials and methods

2.1. In vitro mast cell studies

2.1.1. Mouse bone-derived marrow cells (BMMCs) culture and treatment

BMMCs were generated from the femur bone marrow cells of male C3H/HeOuJ mice as previous described (Mortaz et al., 2008, 2006). In short, bone marrow cells were cultured for 3 weeks in RPMI 1640 with glutamine and phenol red (Lonza, Switzerland) supplemented with 10% FCS, 1% penicillin-streptomycin solution (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 IL-3; Prospec, USA) and 2 ng stem cell factor (mouse SCF; Prospec, USA), at 37 °C in a humidified atmosphere with 5% CO_2.

After 4 weeks BMMCs were sensitized with 15% anti-2,4-dinitrophenol (DNP) IgE from hybridoma clone 26.82 as previously described (Karimi et al., 2004; Mortaz et al., 2008). After overnight sensitization, BMMCs were washed and incubated at a concentration of 5 x 10^6 cells/ml for 30 min with:

1. 1, 200, 500, or 1000 nM rapamycin (Rapa, LC labs, Woburn, MA, USA) or
2. with 0.4 or 10 mM single amino acids, histidine (His), lysine (Lys), threonine (Thr), leucine (Leu), isoleucine (Ile) or valine (Val) (Sigma Aldrich, Zwijndrecht, the Netherlands), or amino acid combinations.

Afterwards, BMMCs were activated with 2,4-dinitrophenyl conjugated to human serum albumin (DNP-HAS), at indicated concentrations to assess acute degranulation or 4 hours' cytokine production. Supernatants were collected at several time points. 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 to the BMMCs to obtain cell lysates. Supernatants and cell lysates were stored at 20 °C until further analysis.

2.1.2. Mast cell degranulation

To measure acute degranulation, β-hexosaminidase assay was performed as previously described (Karimi et al., 2004; Mortaz et al., 2008). The percentage of degranulation was calculated as: $[(A - B)/(T - B)] \times 100$ where A is the amount of β-hexosaminidase released from stimulated cells, B is the release of β-hexosaminidase from unstimulated cells, and T is total cellular β-hexosaminidase content.

2.1.3. Mast cell cytokine production

Quantification of both IL-6 and tumor necrosis factor-α (TNF-α) was performed using the ELISA MAX™ Standard Sets (BioLegend, USA) according to the manufacturer's instructions.

2.2. In vivo mouse studies

2.2.1. 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 a 12 h light/dark cycle with free access to food and water *ad libitum* in groups of 4 or 5 mice per cage at the animal facility of the Utrecht University, Utrecht, The Netherlands.

2.2.2. Induction of cow's milk allergy in mice

The CMA mouse model (Fig. 1A) was conducted as described previously (Schouten et al., 2010; Wu et al., 2015). 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,

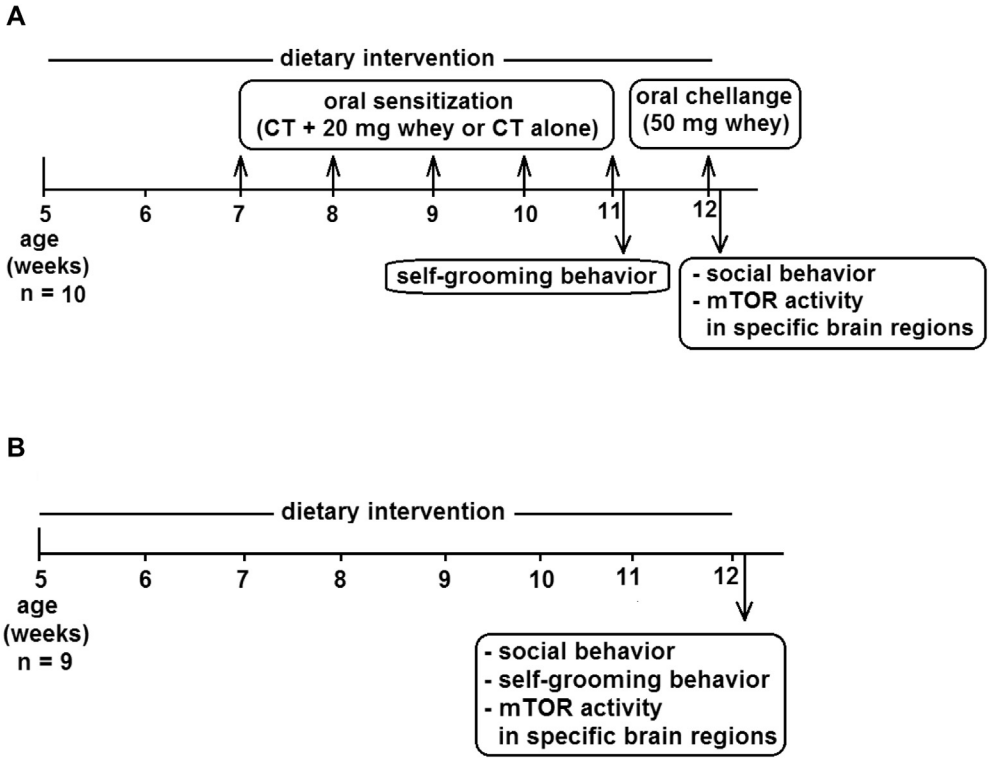

Fig. 1. Schematic overview of two animal experiments. (A) The mouse model of CMA, n = 10 mice per group. (B) The BTBR mouse model of autism, n = 9 mice per group.

confirming the allergic sensitization to whey. One day after challenge, social interaction of the mice was assessed. Afterwards, mice were sacrificed to collect brain tissues for further analysis.

2.2.3. Dietary intervention

The iso-caloric diets were produced by Research Diet Services (Wijk bij Duurstede, the Netherlands) and were based on standard food for laboratory rodents AIN-93G (Reeves et al., 1993). The compositions of the Control, Active 1, Active 2 diets are presented in Table S1 (Supplementary data). Compared to Control diet, Active 1 diet contained relative high levels of the amino acids His, Lys, and Thr and relatively low levels of the amino acids Leu, Ile, and Val. 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 Leu, Ile, and Val as compared to His, Lys, and Thr. The weight to weight ratios of [Leu + Ile + Val]/[His + Lys + Thr] for the respective diets were 1.53 (Control), 0.67 (Active 1), and 1.32 (Active 2).

To study the effects of dietary intervention in the CMA model, 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 (Fig. 1A).

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 7 consecutive weeks (Fig. 1B). Around 7 weeks of dietary treatment, all mice were subjected to social interaction and grooming tests as indicated in Fig. 1. Subsequently, mice were sacrificed and serum and tissues were collected for further analysis.

2.2.4. Social interaction test

Social interaction test was performed as described previously (de Theije et al., 2014a,b; Wu et al., 2015). In short, test mice were individually 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 under day light conditions (Fig. 4A). Test mice were habituated to the open field for 5 min (habituation phase; no target) and a strain- and age-matched unfamiliar target male mouse was introduced in the small cage for an additional 5 min (interaction phase; target). 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 by test mice were measured. Test mice were subjected to one social behavioral test. Strain- and age-matched unfamiliar target males were used in maximal 3 trials. Arenas were cleaned with water followed by 70% ethanol after each test.

2.2.5. Self-grooming

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 length × 20 cm wide × 13 cm high) without bedding under day light conditions for 15 min 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 arenas were cleaned with water followed by 70% ethanol after each test.

2.2.6. Brain tissues

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

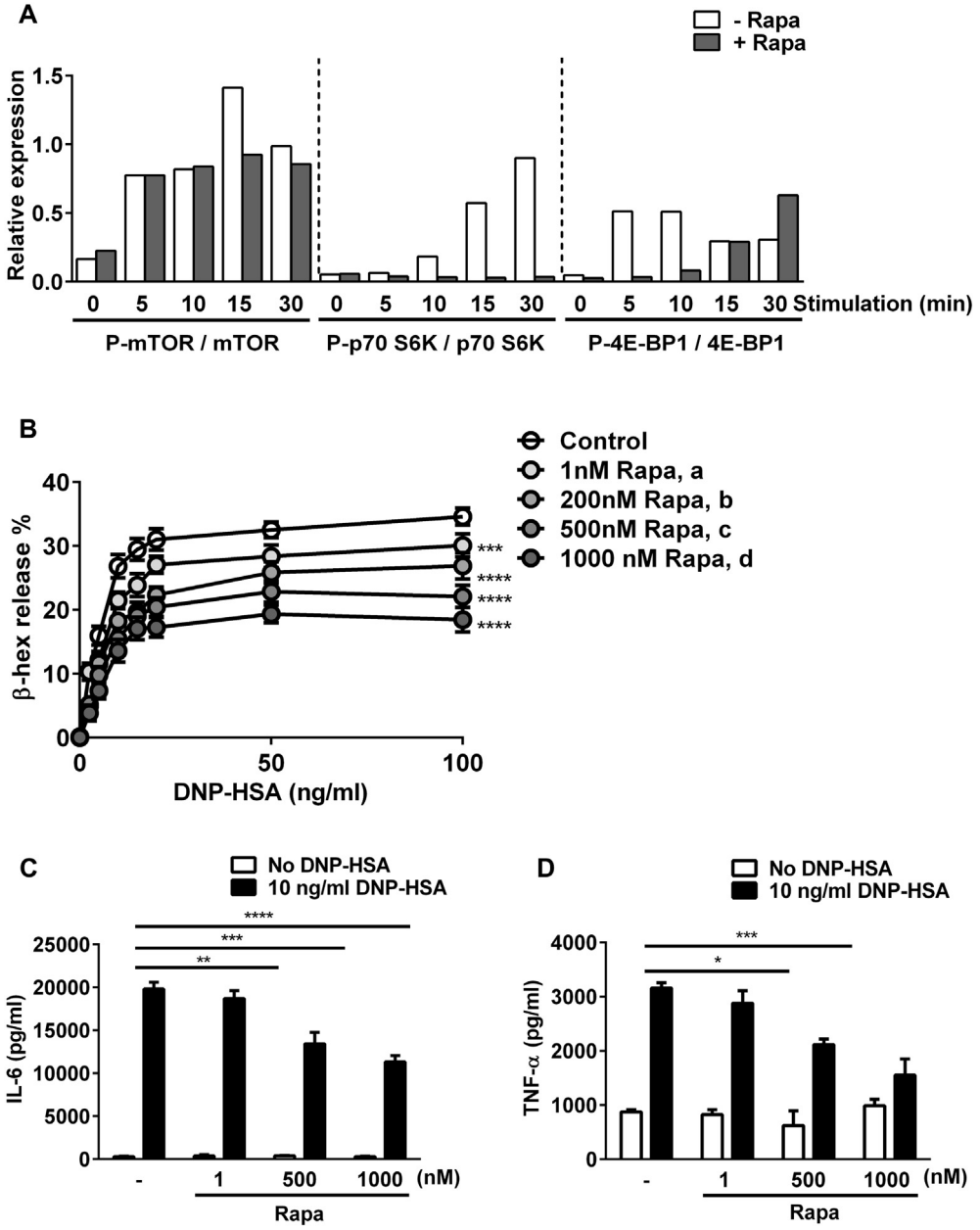

Fig. 2. Effect of rapamycin on mTOR signaling pathway, acute degranulation, and cytokine production of antigen-specific mast cell activation. (A) The densities of phosphorylation of mTOR, p70 S6K and 4E-BP1 were divided by the corresponding density of the non-phosphorylated corresponding protein. (B) Rapamycin inhibited acute degranulation of IgE-sensitized BMMCs induced by several concentrations of DNP-HSA in a dose-dependent manner. Data are presented as the mean ± SEM, n = 3, two-way ANOVA followed by a Bonferroni's multiple comparisons test with repeated measures was conducted. Curve **a** VS control: $P = 0.0002$, **b** VS control: $P < 0.0001$, **c** VS control: $P < 0.0001$, **d** VS control: $P < 0.0001$. Rapamycin inhibited IL-6 (C) and TNF-α (D) productions of IgE-activated BMMCs (10 ng/ml DNP-HSA) in a dose-dependent manner. Data are presented as the mean ± SEM, n = 3, one-way ANOVA followed by a Bonferroni's multiple comparisons test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

slices of approximate 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 cell 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. Then supernatant was collected and stored at −20 °C until further analysis.

2.2.7. Intestinal tissue

After sacrificing C3H/HeOuJ mice, the proximal and distal segments of small intestine were isolated and stored in RNAlater (Qiagen GmbH, Hilden, Germany) at −70 °C until further analysis (Kerperien et al., 2014).

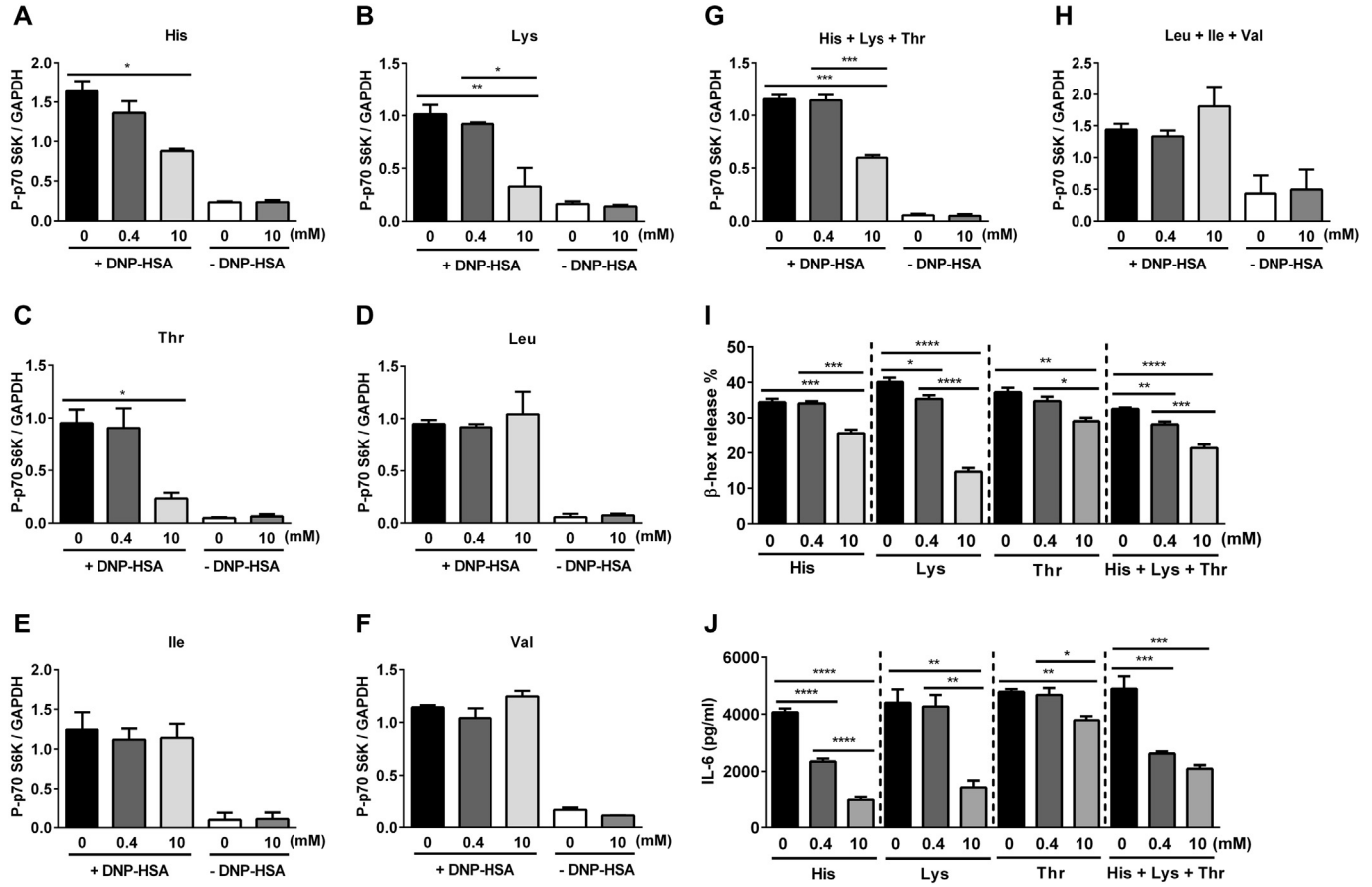

Fig. 3. Effect of single amino acids and amino acid combinations on mTOR signaling pathway, acute degranulation, and cytokine production of antigen-IgE-activated BMMCs. The specific amino acids His, Lys, Thr, and the amino acid combination of His, Lys, Thr inhibited the phosphorylation of p70-S6K in antigen-induced activation of BMMCs (A–C and G). Leu, Ile, Val, and the combination of Leu, Ile, Val had no effect on the phosphorylation of p70-S6K in antigen-IgE-activated BMMC (D-F and H). The densitometric data are presented as mean relative density ± SEM, n = 3 of two independent experiments. The single amino acids His, Lys, Thr, and the combination of His, Lys, Thr inhibited antigen-induced degranulation of BMMCs in a dose-dependent manner (I). The single amino acids Lys, His, Thr and the amino acid combination of His, Lys, Thr inhibited antigen-induced IL-6 production of BMMCs at 4 h (J). All mast cell activity data shown represent the mean ± SEM, n = 4. One-way ANOVA followed by a Bonferroni's multiple comparisons test was conducted. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

2.3. Western blotting

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 western blotting was performed as described before (Wu et al., 2015). 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. The immunoreactive bands were detected by ECL prime kit (Health Care, Amsterdam, The Netherlands) and the results were normalized to GAPDH or to 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).

2.4. mRNA expression analysis

The total RNA of the small intestine of C3H/HeOuJ mice was isolated using the RNAeasy kit (Qiagen, Germantown, MD, USA) and 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 = $100 \times 2^{Ct[Rps13] - Ct[target\ mRNA]}$ (García-Vallejo et al., 2004). Primers for *interleukin (IL) 10*, *transforming growth factor (TGF)-β*, and T cell-associated transcription factors *T-bet*, *Gata-3*, *RORγ* and *FoxP3*, were commercially purchased from SABiosciences-QiagenGmbH (Hilden, Germany).

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

16 Hours after oral challenge, serum of C3H/HeOuJ mice was collected. To assess mast cell activation concentration of mouse mast cell protease-1 (mMCP-1) in 10 times diluted serum was measured by commercially available ELISA kits (BD Biosciences, Alphen aan de Rijn, The Netherlands) according to the manufacturer's protocol. Concentrations of whey-specific immunoglobulins IgE, IgG1 and IgG2a in 10, 100 or 1000 times diluted serum were measured by ELISA according to the protocol described previously (de Theije et al., 2014a,b).

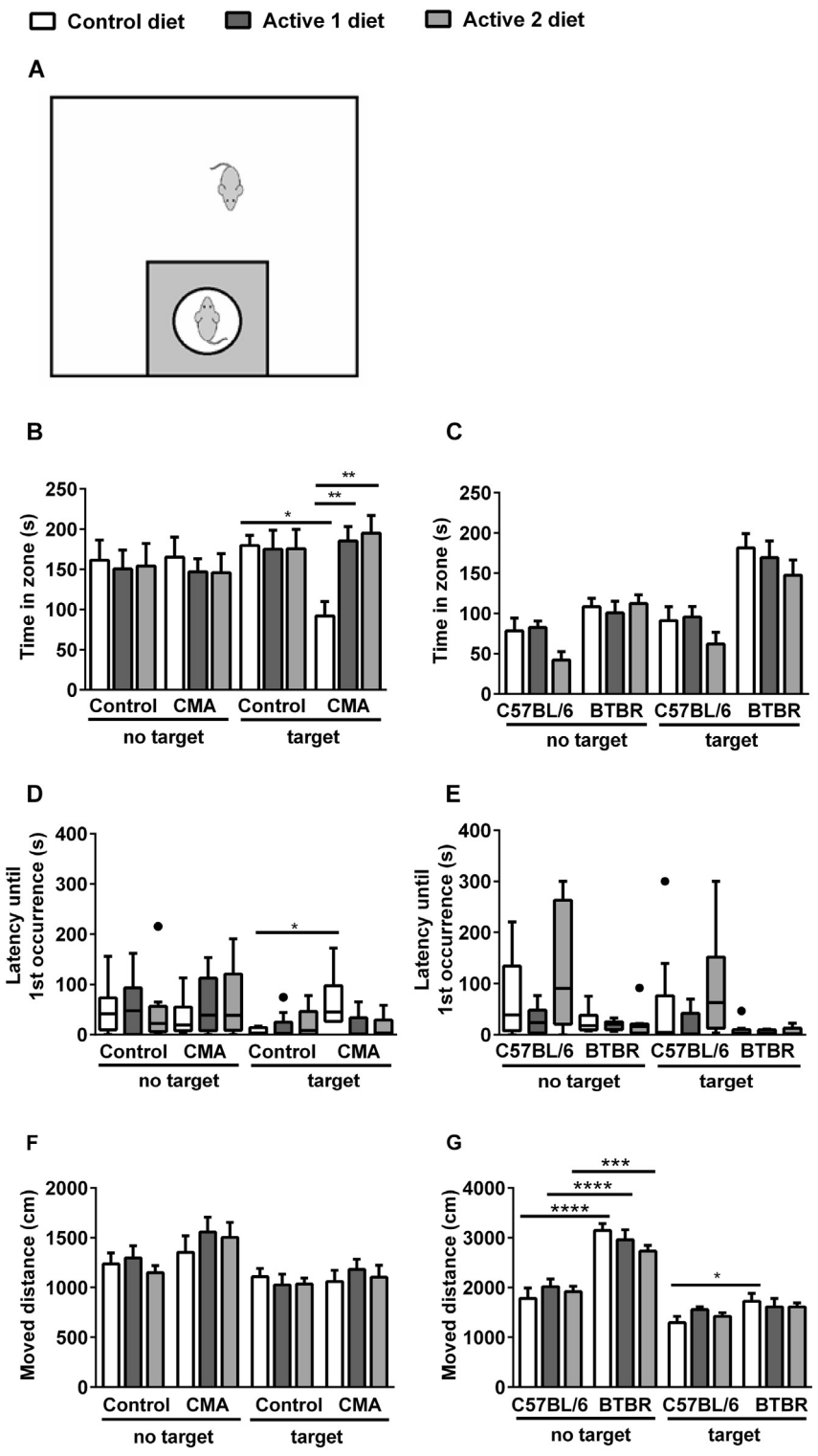

Fig. 4. Effect of dietary intervention with either Active 1 or Active 2 diet on social behavior in CMA mice and BTBR mice. (A) Schematic representation of the social interaction test. (B) CMA mice exhibited lack of sociability, defined as reduced time spent in the interaction zone compared to time of 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 either the habituation phase (no target) or 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. During the habituation phase or the interaction 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 as Box-and-Whisker Turkey plots for (D and E) and mean distance moved (cm) for (F and G). Dots in (D and E) are outliers but they were not excluded from analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. 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 and F) n = 10 per group; (C, E and G) n = 9 per group.

2.6. Statistical analysis

Experimental results are expressed as mean ± S.E.M. For effect of different rapamycin concentrations on acute degranulation of IgE-mediated BMMCs, data were analyzed with two-way ANOVA. For effect of single amino acids and amino acid combinations on phosphorylation of p70-S6K, acute degranulation, and IL-6 production of antigen-IgE-activated BMMCs, differences between groups were statistically determined with a one-way ANOVA. For effect of dietary interventions on phosphorylation of mTOR-associated proteins in specific brain regions of CMA and BTBR mice, 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 (habituation phase: no target and interaction phase: target), was added as a repeated measure (three-way ANOVA). Due to the fact that latency time data were not normally distributed, Kruskal-Wallis test followed by Dunn's multiple comparisons test was conducted for latency of first approach to the interaction zone in the social interaction test. For effect of dietary intervention on expression of T cell-associated factors, data were analyzed with two-way ANOVA. For effect of dietary interventions on serum immunoglobulin and mMCP-1 levels, data were analyzed with two-way ANOVA. Log transformed data were used to obtain normality for serum immunoglobulin and mMCP-1 levels. For all ANOVAs, significant main effects and interactions were followed by Bonferroni's multiple comparisons test. Differences were considered statistically significant when $P < 0.05$. Analyses were performed using either IBM SPSS Statistics 20 for three-way ANOVA or GraphPad Prism, version 6.02 for other statistical analysis.

3. Results

3.1. Effect of single amino acids or amino acid combinations on mTOR signaling and activity of IgE-sensitized mast cells after antigen stimulation, in vitro studies

To examine the importance of mTOR signaling in antigen-specific mast cell activation, we first investigated the effect of rapapmycin on the phosphorylation of key elements of mTORC1 pathway including mTOR, p70 S6K, and 4E-BP1 in IgE-sensitized BMMCs after antigen stimulation. It was shown that mTOR, p70 S6K, and 4E-BP1 are phosphorylated in response to antigen stimulation within 30 min (Fig. 2A). Rapamycin did not affect the phosphorylation of mTOR while it inhibited the phosphorylation of p70 S6K and 4E-BP1 (Fig. 2A and Fig. S1 in Supplementary Material). We further investigated the effect of rapamycin on antigen-specific degranulation and cytokine production in BMMCs. It was shown that inhibition of mTORC1 activity by rapamycin resulted in an inhibition of antigen-specific mast cell degranulation (Fig. 2B) and IL-6 and TNF-α production (Fig. 2C and D) in a dose-dependent manner. The tested rapamycin concentrations had no effect on cell viability of antigen-IgE activated BMMCs (Fig. S2A).

To investigate whether the regulation of mast cell activation by specific amino acids or amino acid combinations involves the mTORC1 pathway, we examined the effect of single amino acids or amino acid combinations on the mTORC1 pathway in antigen-IgE stimulated BMMCs. At a concentration of 10 mM, the single amino acids His, Lys, Thr and the combination of His, Lys and Thr inhibited antigen-induced enhancement of p70 S6K phosphorylation ($F_{4,\ 5} = 49.89$, $P = 0.0003$ for His, Fig. 3A; $F_{4,\ 5} = 44.53$, $P = 0.0004$ for Lys, Fig. 3B; $F_{4,\ 5} = 18.25$, $P = 0.0035$ for Thr, Fig. 3C; $F_{4,\ 5} = 284.6$, $P < 0.0001$ for the combination of His, Lys, Thr, Fig. 3G). The single amino acids Leu, Ile, Val and the combination of Leu, Ile, Val did not affect the antigen specific upregulation of p70 S6K phosphorylation (Fig. 3D, E, F, H, and Fig. S2 in the Supplementary Material).

The effect of single His, Lys, Thr and the combination on antigen-specific mast cell degranulation and cytokine production was further investigated. The single amino acids His, Lys, Thr and the combination inhibited antigen-mediated mast cell degranulation in a dose-dependent manner, as assessed by means of β-hexosaminidase release ($F_{2,\ 9} = 30.92$, $P < 0.0001$ for His; $F_{2,\ 9} = 147.7$, $P < 0.0001$ for Lys; $F_{2,\ 9} = 12.48$, $P < 0.01$ for Thr; $F_{2,\ 9} = 50.83$, $P < 0.0001$ for the combination; Fig. 3I). To investigate the effect of amino acids on cytokine production of antigen-specific activated BMMCs, IL-6 production was measured 4 h after stimulation of IgE-sensitized BBMCs with DNP-HSA. The single His, Lys, Thr and the combination inhibited IL-6 production in a dose-dependent manner ($F_{2,\ 9} = 167.9$, $P < 0.0001$ for His; $F_{2,\ 9} = 18.73$, $P < 0.001$ for Lys; $F_{2,\ 9} = 10.05$, $P < 0.01$ for Thr; $F_{2,\ 8} = 26.24$, $P < 0.001$ for the combination; Fig. 3J). Supplementation of different concentrations of single amino acids or amino acid combinations had no effect on cell viability as measured by LDH release (Fig. S1B and C). Based on these results, a specific amino acid diet was designed for *in vivo* studies that contained increased relative amounts of His, Lys, and Thr and reduced relative amounts of Leu, Ile, and Val, to attenuate the mTOR signaling pathway (Table S1 in the Supplementary Material, Active 1 diet).

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

CMA mice were fed the Active 1 or Active 2 diet and exposed to a social interaction test one day after oral challenge with the allergen whey. A two-way ANOVA showed a significant interaction between Model and Diet ($F_{2,\ 53} = 4.184$, $P = 0.0205$). CMA mice on the Control diet spent significantly less time with the interaction mouse compared to non-allergic control mice ($P = 0.0125$, Fig. 4B). 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 fed Control diet (Kruskal-Wallis test: $P = 0.0106$, Dunn's multiple comparison test: $P = 0.0196$, Fig. 4D). 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 three-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 ($F_{2,\ 54} = 0.364$, $P = 0.696$). Thus, locomotor activity of the control and CMA mice fed different diets did not significantly differ between groups during either the habituation phase (no target) or the interaction phase (target) (Fig. 4F).

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 7 consecutive weeks of dietary treatment. For social interaction, a three-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 (habituation phase, no target: $F_{1,\ 46} = 16.48$, $P = 0.0002$) and even more so in the presence (interaction phase, target: $F_{1,\ 46} = 36.00$, $P < 0.0001$) of an interaction mouse (Fig. 4C). No significant differences were observed in latency of first approach to the interaction zone with or without target comparing C56BL/6J and BTBR mice (Kruskal-Wallis test: $P = 0.0547$, Fig. 4E). For distance travelled, a

three-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. 4G) and albeit to a lesser extent in the presence (target: $F_{1, 46} = 5.548$, $P = 0.0228$) of an interaction mouse (Fig. 4G). Introduction of an interaction mouse (target) induced a significant reduction of distance moved by BTBR mice ($F_{3, 92} = 68.43$, $P < 0.0001$, Fig. 4G) and this was associated with spending more time in the interaction zone (Fig. 4C).

3.3. Active 1 diet normalized repetitive behavior of both CMA mice and BTBR mice, while Active 2 diet normalized repetitive behavior of CMA mice only.

Next, the effect of Active 1 diet and Active 2 diet on novelty-induced repetitive self-grooming behavior of CMA and BTBR mice was assessed. The two-way ANOVA showed a significant main effect of Model ($F_{1, 54} = 15.30$, $P = 0.0003$) and Diet ($F_{2, 54} = 5.402$, $P = 0.0073$) for the duration of self-grooming. When fed the Control diet, CMA mice showed increased duration of self-grooming compared to control mice ($P = 0.0015$, Fig. 5A). 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. 5A). A significant interaction between Model and Diet was observed for the frequency of self-grooming ($F_{2,54} = 4.869$, $P = 0.0114$), with CMA mice on the Control diet showing an increase in frequency of self-grooming compared to control mice on the Control diet ($P = 0.0027$, Fig. 5C). Self-grooming frequency was normalized when CMA mice were fed the Active 1 diet or Active 2 diet (Active 1 diet: $P = 0.0037$; Active 2 diet: $P = 0.0363$, Fig. 5C).

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. 5B; frequency: $F_{2,45} = 6.009$, $P = 0.0049$, Fig. 5D). Consistent with previous findings (McFarlane et al., 2008; Pearson et al., 2011; Silverman et al., 2010a), BTBR mice fed the Control diet engaged in much longer bouts of self-grooming ($P = 0.0169$, Fig. 5B) and showed a higher self-grooming frequency ($P = 0.0146$, Fig. 5D) 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 significantly alter duration of self-grooming and reduced frequency of self-grooming in BTBR mice (Fig. 5B and D).

3.4. Both Active 1 and Active 2 diets attenuated mTORC1 signaling in the brain of CMA and BTBR mice

To examine the effect of Active 1 diet and Active 2 diet on mTOR signaling pathway in the brain of CMA and BTBR mice, the phos-

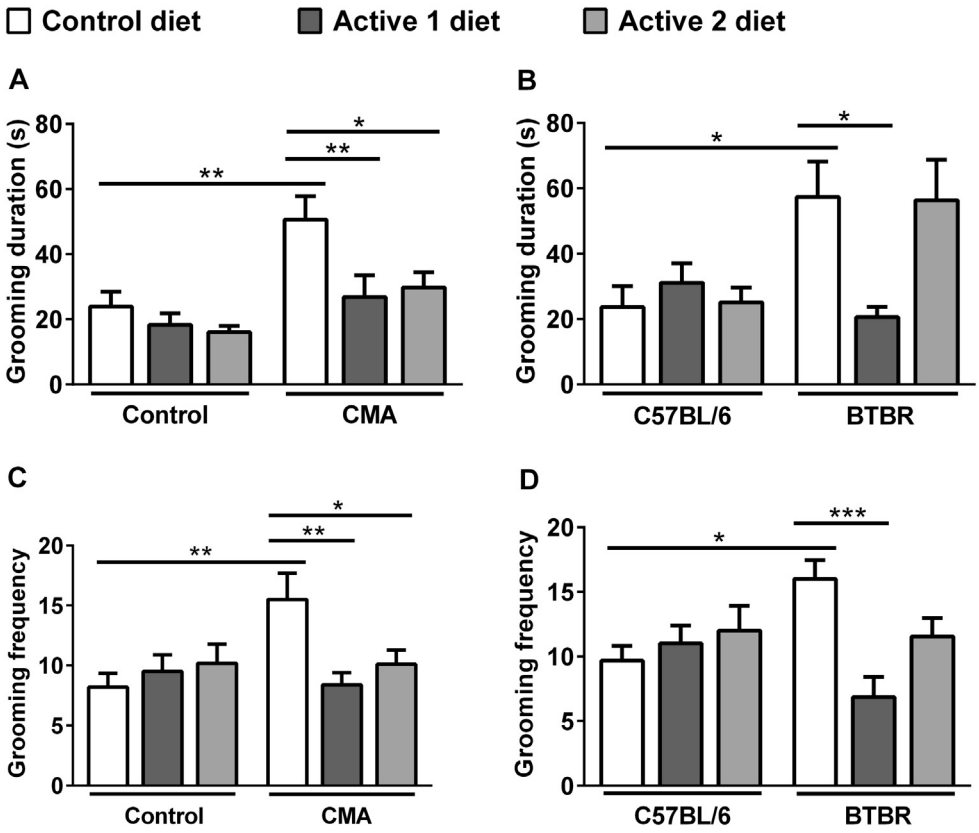

Fig. 5. Effect of dietary intervention with either Active 1 or Active 2 diet on repetitive self-grooming behavior in CMA and BTBR mice. (A) CMA mice displayed significantly increased amount of time spent self-grooming compared to control mice and this effect was reduced by dietary intervention with either 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 and C) n = 10 per group; (B and D) n = 9 per group.

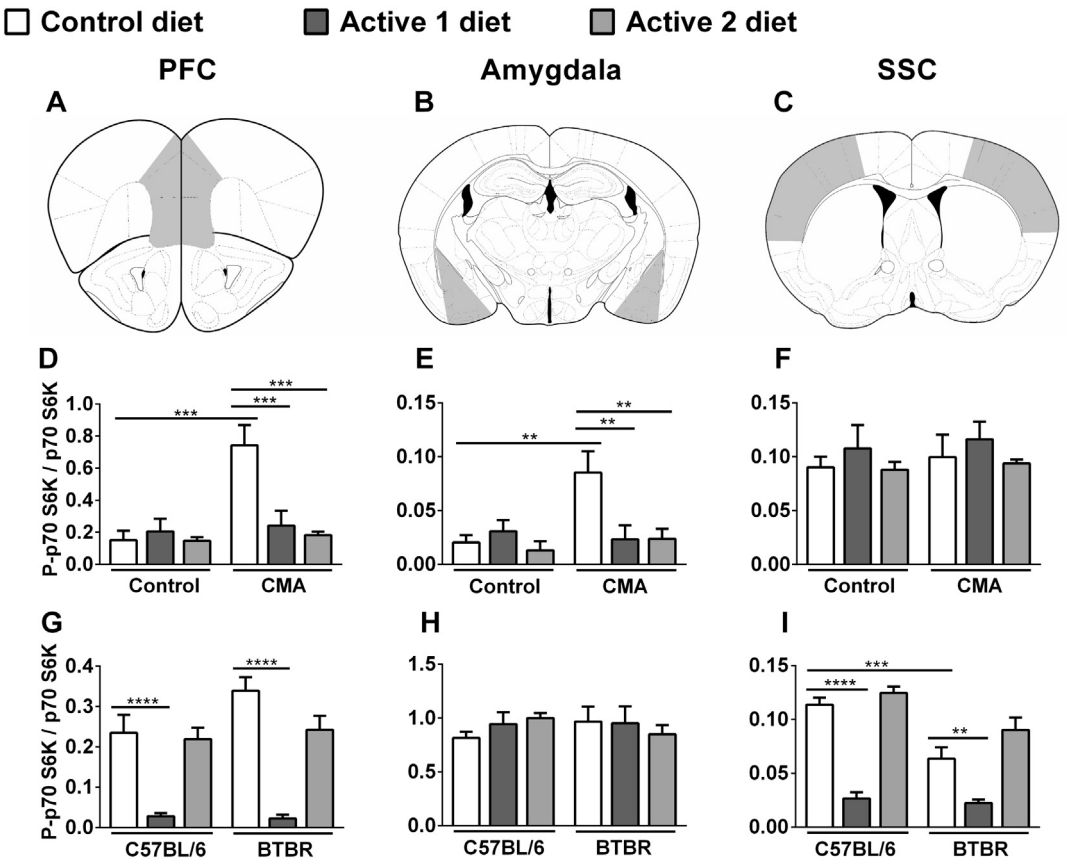

Fig. 6. Effect of dietary intervention with either Active 1 or Active 2 diet on mTOR signaling in the prefrontal cortex (PFC, A), amygdala (B), and somatosensory cortex (SSC, C) in CMA and BTBR mice measured by western blot analysis. (D and E) A significant increase of the phosphorylation of p70 S6K was measured in the PFC and amygdala in CMA mice on Control diet. CMA mice fed either Active 1 or Active 2 diet exhibited significant reductions in the phosphorylation of p70 S6K in the PFC and amygdala. (F) The phosphorylation of p70 S6K in the SSC was not affected by either the induction of CMA or dietary interventions. (G and I) 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. (H) The phosphorylation of p70 S6K in the amygdala was not affected by dietary interventions. Density of phosphorylation of p70 S6K were divided by the non-phosphorylated p70 S6K. Two-way ANOVA followed by a Bonferroni's multiple comparisons test was conducted and data are presented as mean relative density ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (D–H) n = 4 per group; (I) n = 3–4 per group.

phorylation of mTOR-related proteins was measured in the prefrontal cortex (PFC, Fig. 6A), amygdala (Fig. 6B), and somatosensory cortex (SSC, Fig. 6C) using western blotting analysis. The phosphorylation of mTOR and AKT was not affected by CMA or diets in the examined regions of the brain (Fig. S4A–F). 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. 6D) and amygdala ($F_{2,18} = 4.724$, $P = 0.0234$, Fig. 6E). The phosphorylation of p70 S6K, a downstream protein of mTORC1, was significantly enhanced in the PFC ($P = 0.0001$, Fig. 6D) and amygdala ($P = 0.0047$, Fig. 6E) of CMA mice on Control diet compared to control mice on Control diet, which is consistent with previous findings (Wu et al., 2015). 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 in the SSC (Fig. 6F) was not affected in CMA mice. Images of the Western blot experiments are presented in the Supplementary Material (Fig. S5).

Activation of the mTOR signaling pathway was examined in BTBR mice as well. As observed in CMA mice, no significant differences in the phosphorylation of mTOR or AKT in PFC, amygdala, and SSC were found when comparing BTBR and C57BL/6 mice, irrespective of dietary intervention (Fig. S4G–L). For the phosphorylation of p70 S6K, a two-way ANOVA showed no significant interaction between Model and Diet in the PFC ($F_{2,17} = 1.946$, $P = 0.1734$, Fig. 6G). The Active 1 diet significantly reduced phosphorylation of p70 S6K in the PFC ($F_{2,17} = 47.77$, $P < 0.0001$, Fig. 6G), 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. The phosphorylation of p70 S6K in the amygdala was not affected by dietary interventions both in C57BL/6 mice and in BTBR mice (Fig. 6H). In the SSC, a two-way ANOVA showed a significant interaction between Model and Diet for the phosphorylation of p70 S6K ($F_{2,18} = 4.421$, $P = 0.0274$, Fig. 6I). The phosphorylation of p70 S6K in the SSC is significantly reduced in Control diet fed BTBR mice compared to that in Control diet fed C57BL/6 mice ($P = 0.0008$, Fig. 6I). The phosphorylation of p70 S6K was significantly further reduced by the dietary intervention with Active 1 diet ($P = 0.0050$, Fig. 6I). A similar effect of Active 1 diet was also observed in the SSC of C57BL/6 mice ($P < 0.0001$, Fig. 6I). Active 2 diet did not affect the phosphorylation of p70 S6K in the SSC in both C57BL/6 mice and BTBR mice. Images of Western blot experiments are provided in the Supplementary Material (Fig. S6).

3.5. Both Active 1 and Active 2 diets suppress whey-induced Th17 cell associated transcriptional markers and enhance Treg cell-associated transcription marker in the proximal part of 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 no effect for expression of Th1-associated transcription factor Tbet or Th2-associated transcription factor GATA3 in the small intestine of CMA mice. For the ratio of Th2 (GATA3) to Th1 (Tbet), a two-way ANOVA showed a significant interaction between Model and Diet in the proximal small intestine ($F_{2,\ 15} = 3.806$, $P = 0.0460$, Fig. 7A). The ratio of Th2 to Th1 associated T cell transcription factor mRNA expression tended to be increased in the proximal small intestine

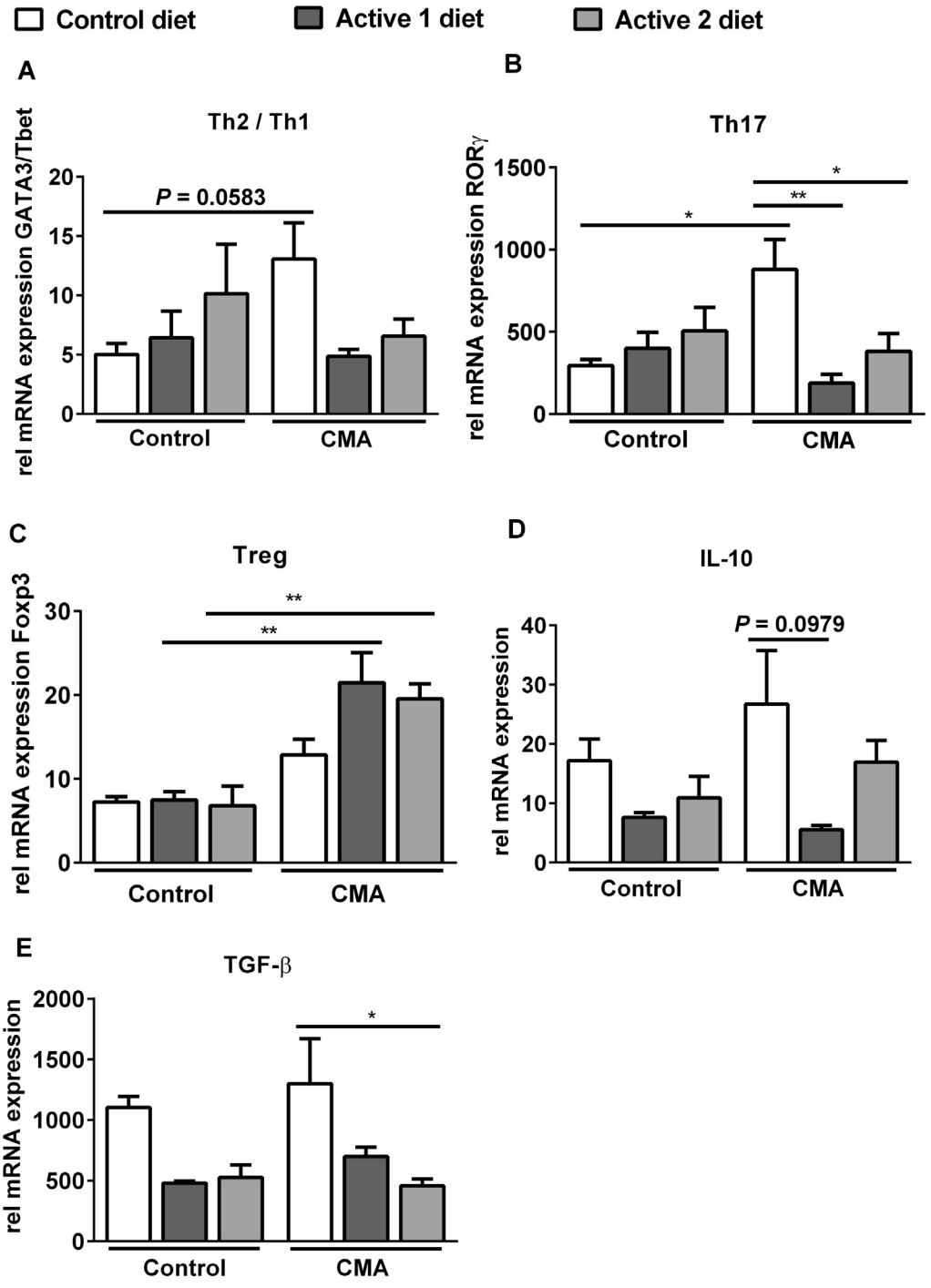

Fig. 7. The effects of Active 1 diet and Active 2 diet on T cell differentiations in the proximal part of small intestine of CMA mice. (A) The ratio of Th2 to Th1 tended to be increased in the proximal part of small intestine of CMA mice and both diets suppressed this ratio. (B) CMA mice showed increased mRNA expression levels of Th17-associated transcription factor RORγ and both diets inhibited Th17 development in the proximal part of the small intestine. (C) Both the Active 1 and Active 2 diets significantly enhanced Treg-associated transcription factor Foxp3 in the proximal part of small intestine of CMA mice. (D) Expression of mRNA levels for IL-10 in the proximal part of the small intestine were suppressed by Active 1 diet in CMA mice. (E) Expression of mRNA levels for TGF-β in the proximal part of the small intestine were suppressed by Active 2 diet in CMA mice. Data were analyzed by a two-way ANOVA followed by a Bonferroni's multiple comparisons test. $^{*}P < 0.05$, $^{**}P < 0.01$. N = 4–5 per group.

of CMA mice ($P = 0.0583$, Fig. 7A). This was suppressed by dietary intervention with either Active 1 ($P = 0.0786$) or Active 2 diet ($P = 0.1552$). A two-way ANOVA showed a significant interaction between Model and Diet for expression of Th17-associated transcription factor RORγ in the proximal small intestine of CMA mice ($F_{2,\ 21} = 6.049$, $P = 0.0084$, Fig. 7B). The relative mRNA expression levels of RORγ were enhanced in the proximal small intestine of CMA mice compared to control mice fed Control diet ($P = 0.0103$, Fig. 7B). Dietary intervention with either Active 1 or Active 2 diet reduced the relative mRNA expression levels of RORγ in the proximal small intestine (Active 1 diet: $P = 0.0025$; Active 2 diet, $P = 0.0322$). For expression of Treg cell-associated transcription factor Foxp3, a two-way ANOVA showed a significant effect of Model in the proximal small intestine of CMA mice ($F_{1,\ 19} = 42.39$, $P < 0.0001$, Fig. 7C). Dietary intervention with Active 1 diet enhanced Treg cell-associated transcription factor Foxp3 in the proximal small intestine of CMA mice compared to control mice ($P = 0.0011$, Fig. 7C). This effect was also found by dietary intervention with Active 2 diet ($P = 0.0039$, Fig. 7C). For expression of IL-10 and TGF-β, a two-way ANOVA showed a significant effect of Diet in the proximal small intestine (IL-10: $F_{2,\ 21} = 4.782$, $P = 0.0194$, Fig. 7D; TGF-β: $F_{2,\ 23} = 10.03$, $P = 0.0006$, Fig. 7E). Dietary intervention with Active 1 diet tended to reduce IL-10 expression ($P = 0.0979$, Fig. 7D) and dietary intervention with Active 2 diet reduced TGF-β expression in the proximal small intestine of CMA mice ($P = 0.0264$, Fig. 7E).

3.6. 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 (Fig. 8A–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 (Fig. 8D).

4. Discussion

This study aimed to assess whether activation of mTOR signaling in the brain could serve as a novel therapeutic target for the treatment of ASD-like behavioral deficits by using dietary interventions. In the first part of this study, antigen-induced mast cell degranulation was used as a screening method to investigate the mTORC1-modulatory capacity of different amino acids. Mast cell activation is a critical process in the allergic response and is regulated by a complex series of intracellular signaling cascades that also involve phosphorylation of key elements of the mTORC1

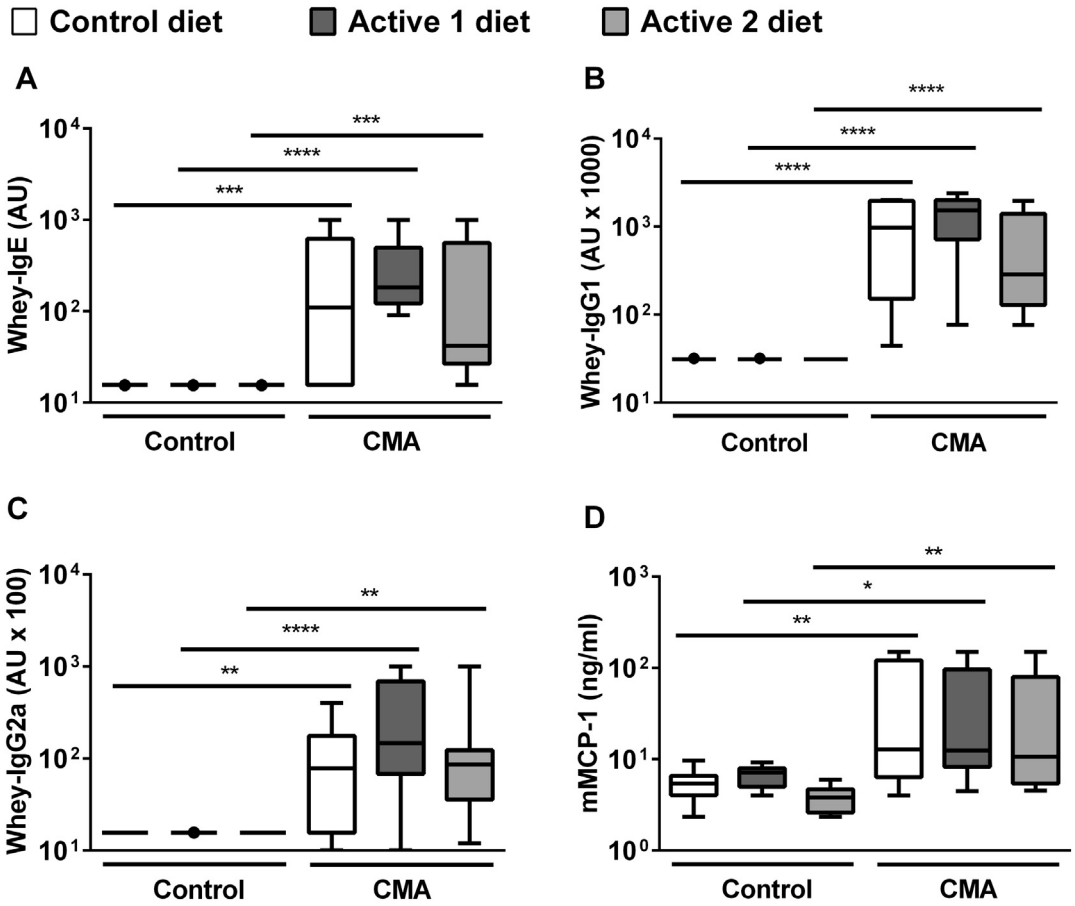

Fig. 8. Effect of Active 1 diet and Active 2 diet on humoral response and mucosal mast cell degranulation in CMA mice. Serum levels of whey-specific IgE (A), IgG1 (B), IgG2a (C) as well as mMCP-1 (D) were increased in allergic mice compared to control. Dietary intervention with either Active 1 or Active 2 diet had no effect on humoral response and mucosal mast cell degranulation in CMA mice. Data were log transformed 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.

pathway including mTOR, p70 S6K, and 4E-BP1 (Kim et al., 2008; Yamaki and Yoshino, 2012). Our studies as well as from others in BMMCs (Hwang et al., 2013; Kim et al., 2008; Thomson et al., 2009) have shown that key elements of mTORC1 pathway including mTOR, p70 S6K, and 4E-BP1 are phosphorylated in response to antigen stimulation, and inhibition of mTORC1 activity by rapamycin resulted in an inhibition of antigen-specific mast cell degranulation and cytokine production, indicating that mTORC1 pathway plays a critical role in regulation of mast cell functions. Single amino acids His, Lys, Thr and amino acid combination of His, Lys, Thr inhibited the phosphorylation of mTORC1 downstream target p70 S6K, while the single amino acids Leu, Ile, Val and amino acid combination of Leu, Ile, Val had no effect on the phosphorylation of p70 S6K in antigen-IgE-activated mast cells *in vitro*. Furthermore, the single amino acids His, Lys, Thr and the amino acid combination of His, Lys, Thr inhibited specific antigen-induced acute degranulation and cytokine production of mast cells. Based on these *in vitro* results, an mTOR-targeting amino acid diet containing increased relative amounts of His, Lys, Thr and reduced relative amounts of Leu, Ile, Val was designed for *in vivo* purposes. For this goal, two different mouse models displaying ASD-like behavior were used. Previous study has demonstrated that early treatment with the ketogenic diet prevented the development of epilepsy by inhibiting the mTOR pathway in the hippocampus in the rat epilepsy model induced by kainic acid injection (McDaniel et al., 2011). We demonstrated ASD-like behavior in CMA mice, possibly representing a model for environmental triggered ASD (de Theije et al., 2014a,b; Wu et al., 2015). BTBR mice, used as a second model, carry multiple naturally occurring mutations (Ellegood and Crawley, 2015; Ellegood et al., 2014) and their behavioral and neurological impairments represent autistic phenotypes (McFarlane et al., 2008; Meyza et al., 2013; Silverman et al., 2010b). 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 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, only 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. Although mTOR signaling was not significantly altered in BTBR compared to C57BL/6 mice, mTOR signaling was reduced when fed the Active 1 diet, but not the Active 2 diet, in the PFC and SSC of both C57BL/6 and BTBR mice.

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 (Wu et al., 2015). The enhanced phosphorylation levels of p70 S6K at Thr389 reflect enhanced mTOR activity, because this epitope on p70 S6K is directly phosphorylated by mTOR (Magnuson et al., 2012). 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 (Caccamo et al., 2010; Das et al., 2008; Guertin and Sabatini, 2007; Hay and Sonenberg, 2004; Hay, 2005). AKT is not only the upstream protein of mTORC1, but also the downstream protein of mTORC2 (Toker and Marmiroli, 2014). 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 (Magnuson et al., 2012; Tavares et al., 2015), enhanced p70 S6K activity in the PFC and amygdala of CMA mice might lead 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 (Baudouin et al., 2012; Gkogkas et al., 2013; Penzes et al., 2011; Südhof, 2008; Wang and Doering, 2013). 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 social situations (Buschman and Miller, 2014; Chrysikou et al., 2014). Abnormal repetitive behavior can be attributed to damage to the corticostriatal circuits and disturbances in direct and indirect neurobiological pathways in the PFC (Langen et al., 2011). The amygdala has also been implicated in mediating social behavior and regulating emotions (Bickart et al., 2014; Hermans et al., 2014) and destruction of amygdala impaired social recognition in mice (Wang et al., 2014). Although it was previously demonstrated that BTBR mice exhibit lower levels of sociability compared to C57BL/6 mice in a three-chamber sociability test (McFarlane et al., 2008; Moy et al., 2007). We were not able to detect differences in social behavior of BTBR mice using our experimental set-up, which was fundamentally different: one chamber. The observed high locomotor activity of BTBR mice may have interfered with their social interaction, precluding the examination of social behavior in BTBR mice in the current experimental set-up.

In addition to the effects of the diets on social behavior in CMA mice, the Active 1 diet suppressed repetitive behavior in both CMA and BTBR mice and the Active 2 diet in CMA mice only. The attenuation of the self-grooming behavior by dietary intervention may be attributed to the inhibitory effect on mTOR signaling in the PFC, as the dysregulation of molecular pathways in the PFC is known to be implicated in abnormal repetitive behavior in mice (Langen et al., 2011).

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 (Pei and Bensmaia, 2014; Pei et al., 2011). It was reported that 44–88% of individuals with ASD have abnormal responsibility to tactile stimuli (Baranek and Parham, 2005) and some children with ASD have a reduced somatosensory response (Marco et al., 2012). In the current study, dietary intervention with Active 1 diet significantly reduced the phosphorylation of mTORC1 downstream protein p70 S6K in the SSC in BTBR mice, suggesting that altered mTOR signaling in the SSC may also contribute to the reduced repetitive grooming behavior in BTBR mice.

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 (Gharami et al., 2015; Martin, 2014; van Elst et al., 2014). 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 (de Theije et al., 2015a,b). A previous study showed that children with autism had significantly higher scores of essential fatty acid deficiency compared to controls. Supplementation with EPA-rich fish oils has been shown to result in improvements in general

health, sleep patterns, concentrations, sociability, and hyperactivity among autistic children (Bell et al., 2004). Vitamins are essential for neurodevelopment and neuronal function and severe deficiencies, in particular vitamin B and D deficiencies, have been linked to increased risk of neurodevelopmental disorders including ASD (Eyles et al., 2009; Mitchell et al., 2014). Moreover, vitamin B6 has anti-inflammatory properties in the intestinal tract (Selhub et al., 2013). 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 (van den Elsen et al., 2013). Dietary supplementation with the mixture of short-chain galacto-oligosaccharides (GOS) and long-chain fructo-oligosaccharides (FOS) effectively alleviated allergic symptoms in CMA mice (De Kivit et al., 2013; Kerperien et al., 2014; Schouten et al., 2010) 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 (Schouten et al., 2011; Van Hoffen et al., 2009). The rice fibers in Active 2 diet were also shown to reduce inflammation by modulating colonic physiology, restoring of serotonin levels and regulating T cell differentiation in a mouse model for colitis (Komiyama et al., 2011). Interestingly, in the small intestine of CMA mice in this study, both Active 1 and Active 2 diets restored the CMA-induced disturbed balance for T helper and regulatory T cells as indicated by transcriptional markers indicative for these cell types (Fig. 6). Additionally, both Active 1 and Active 2 diets exerted no effect on humoral response and mucosal mast cell degranulation in CMA mice (Fig. S6). Thus, the effects of the dietary interventions on CMA-induced ASD-like behavior as well as enhanced mTOR signaling in the brain can be the results of neuroprotective or anti-inflammatory properties, either via direct effects on mTOR signaling in the brain, or indirect via the gut-immune-brain axis.

Taken together, we demonstrated that inhibiting downstream mTOR cascades with dietary amino acid manipulation attenuated biochemical and behavioral influences of the allergenic and genetic models, while anti-inflammatory/neuroprotective diet was effective only in the allergen model. Our data indicate that Active 1 diet may have directly exerted its effect on the mTOR signaling pathway in the gut-immune-brain axis of CMA and brain of BTBR mice, ameliorating behavioral impairments in these mice. Active 2 diet only reduced food allergy-enhanced mTOR signaling in brains and associated ASD-like behavior while it did not affect the behavior or mTOR signaling in the brain of 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 conclusion, 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 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.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2016.09.016.

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