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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 homolog 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, 2004). 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

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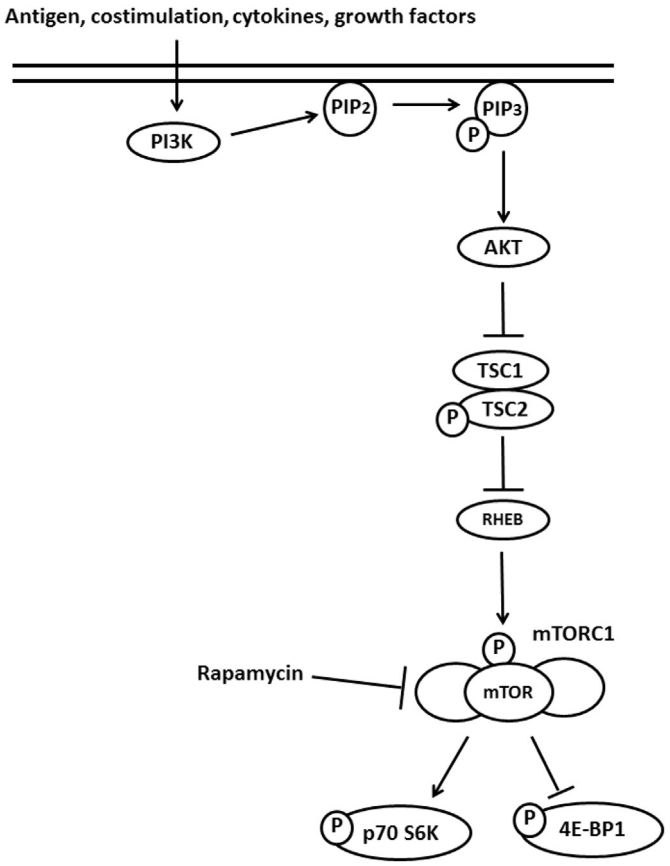


Fig. 1. Schematic illustration of mTORC1 pathway. mTORC1 activation starts with the binding of antigen, costimulatory molecules, cytokines, toll like receptor ligands, and growth factors to the receptor on the cell membrane, leading to the phosphorylation and activation of PI3K and AKT. AKT inhibits 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.

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 behavioral 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.

2. Methods and materials

2.1. Animal model and treatment protocols

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).

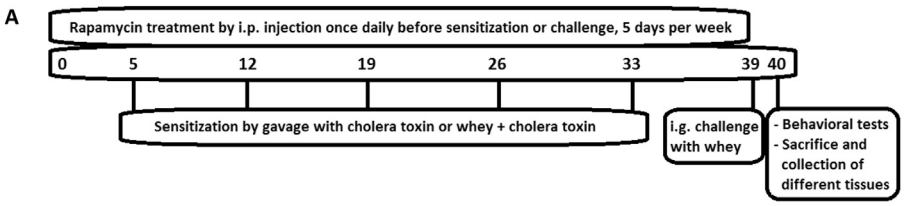
All animal procedures were approved by and conducted in accordance with the guidelines of the Animal Ethics Committee of Utrecht University (approval number: 2011.I.04.045 and 2012.I.04.054).

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 × 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 × 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).



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).

2.4. 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 h. Subsequently, membranes were washed with TBS/0.1% Tween-20 (Sigma–Aldrich, Zwijndrecht, The Netherlands) 3 × 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-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 Technology, Leiden, The Netherlands. The primary antibody against GAPDH (#3777R-100) was from Biovision, Uithoorn, The Netherlands. Afterward, the membranes were washed with TBS/0.1% Tween-20 3 × 10 min and incubated with the secondary antibody polyclonal HRP-conjugated goat anti-rabbit

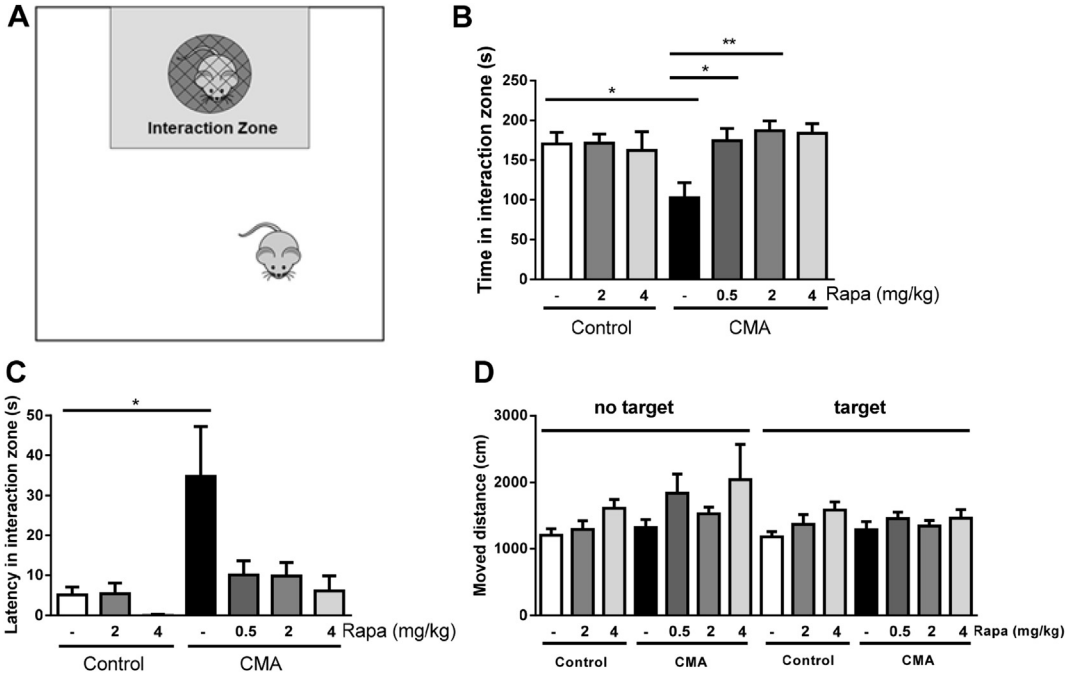


Fig. 3. (A) Schematic representation of the social interaction test. CMA mice showed reduced social interaction (B) and it took significantly more time for CMA mice to approach the interaction mouse for the first time as compared to control (C). The mobility of these mice was hardly affected in both 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: $P = 0.4177$.

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 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 Figs. 6 and 7, 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 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. Afterward, 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)-β 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 analysing 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 analyzed 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 analyzed 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.

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 analyzed. 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 analyzed 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

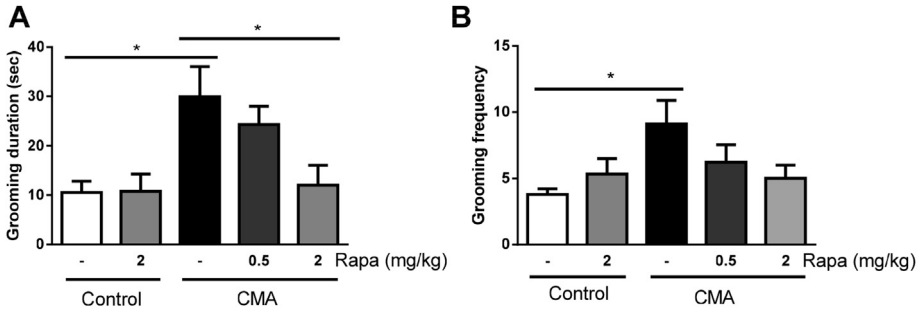


Fig. 4. CMA mice showed significantly enhanced grooming duration (A) and frequency (B) as compared to control, indicating the enhanced repetitive behavior of CMA mice. Rapamycin treatment normalized the grooming duration dose-dependently and reduced the grooming frequency of CMA mice. One-way ANOVA followed 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: $P = 0.2274$.

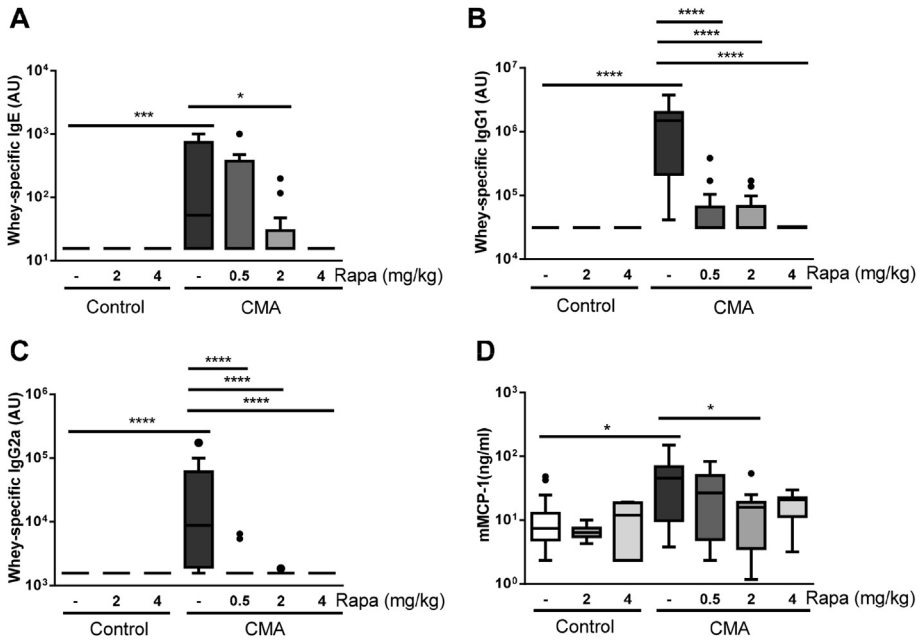


Fig. 5. Effect of rapamycin treatment on whey-induced allergic immune responses in CMA mice and sham-sensitized control mice. CMA mice showed significantly increased serum levels of arbitrary units (AU) of whey-specific immunoglobulin IgE (A), IgG1 (B), IgG2a (C) and serum concentration of mouse mast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

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).

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 (Figs. 6B,C & E–G and 7B,C & E–G). Rapamycin reduced the enhanced phosphorylation of p70 S6K and 4E-BP1 in prefrontal cortex and amygdala of CMA mice (Figs. 6B,C & E–G and 7B,C & E–G). The phosphorylation of mTOR (Figs. 6D & H and 7D & H) or AKT (Figs. 6I and 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 (Figs. 6J and 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).

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 and B). The phosphorylation of mTOR was not significantly affected by CMA or rapamycin (Fig. 8A and 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 analyzed 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 analyzed in the ileum. Levels of anti-inflammatory interleukin (IL)-10 (Fig. 9C) and transforming growth factor (TGF)-β (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).

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

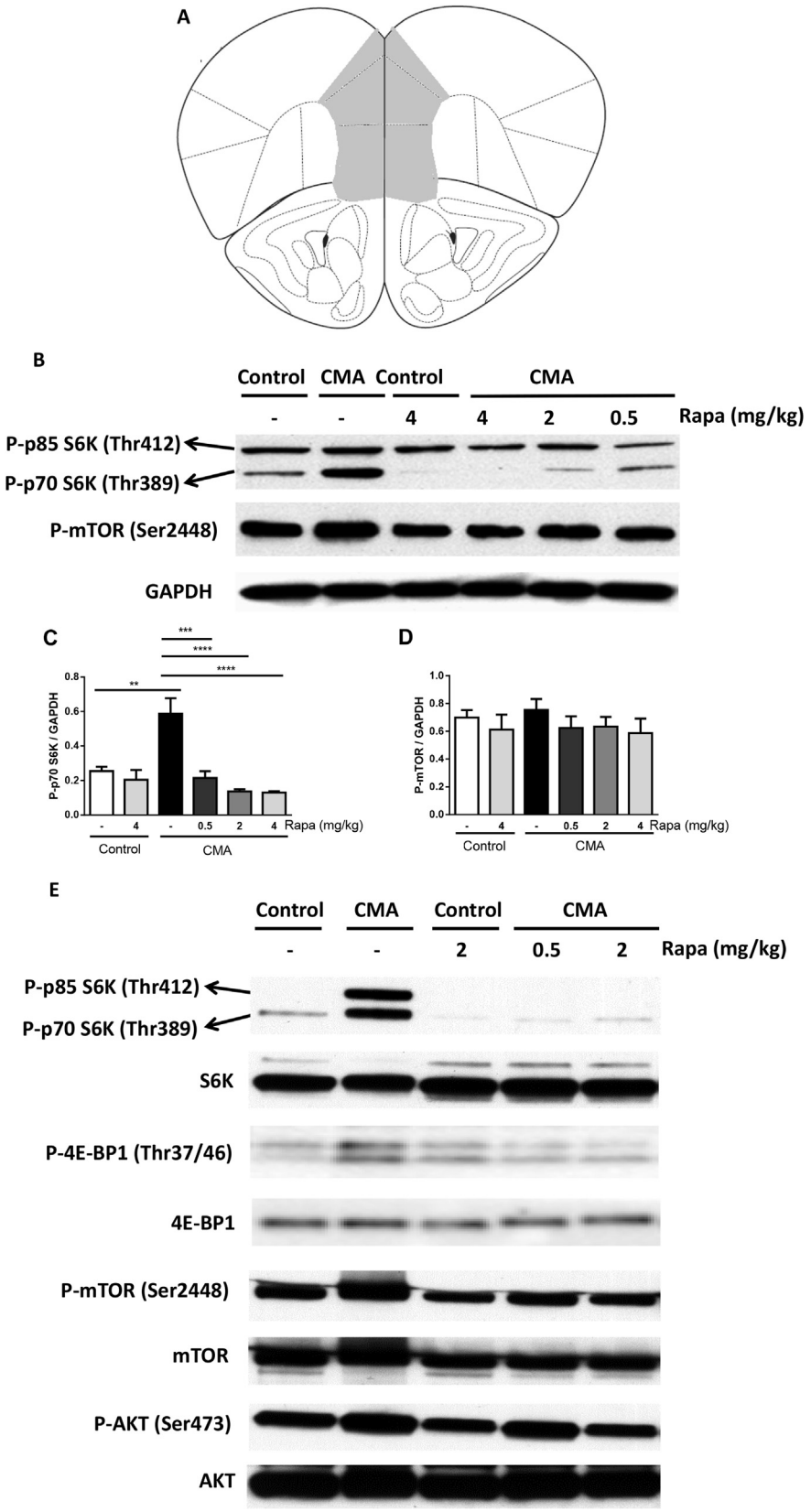


Fig. 6. Western blot analysis showed significantly increased phosphorylation of p70 S6K and 4E-BP1 in the prefrontal cortex of CMA mice and the phosphorylation of mTOR and AKT was hardly affected. The gray area in Figure A indicates prefrontal cortex. Figure B and E are typical examples of western blots. 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.

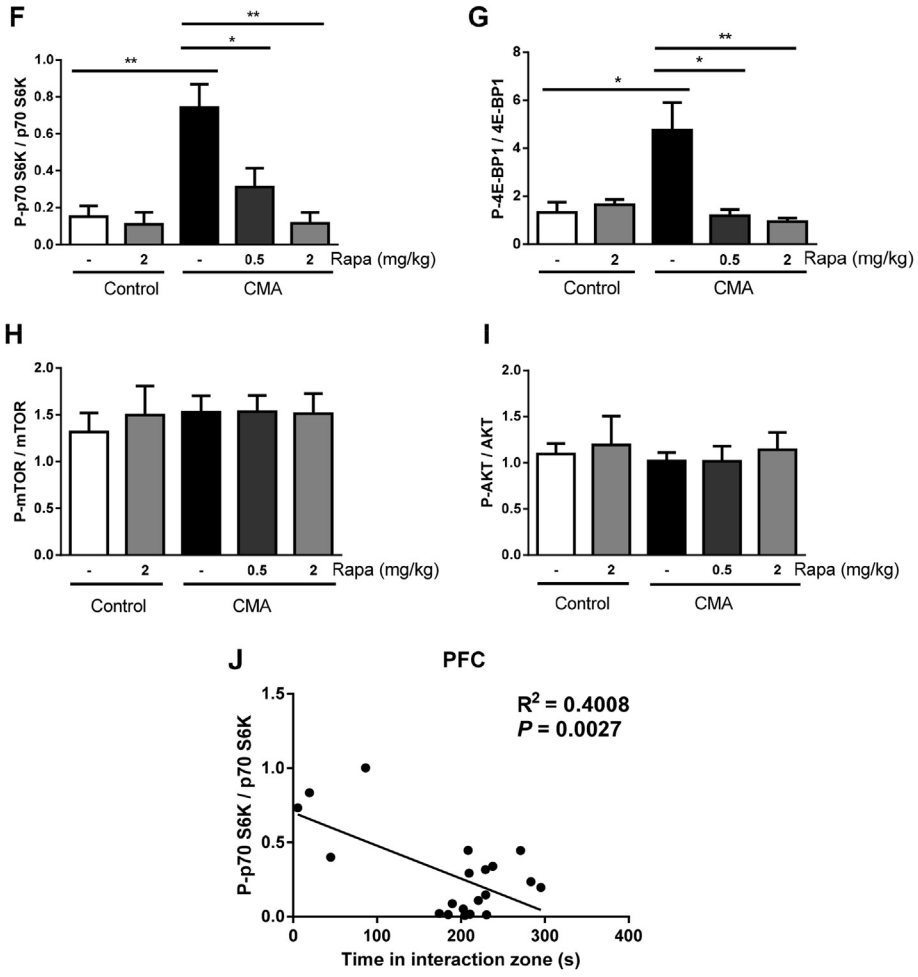


Fig. 6. (*continued*).

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

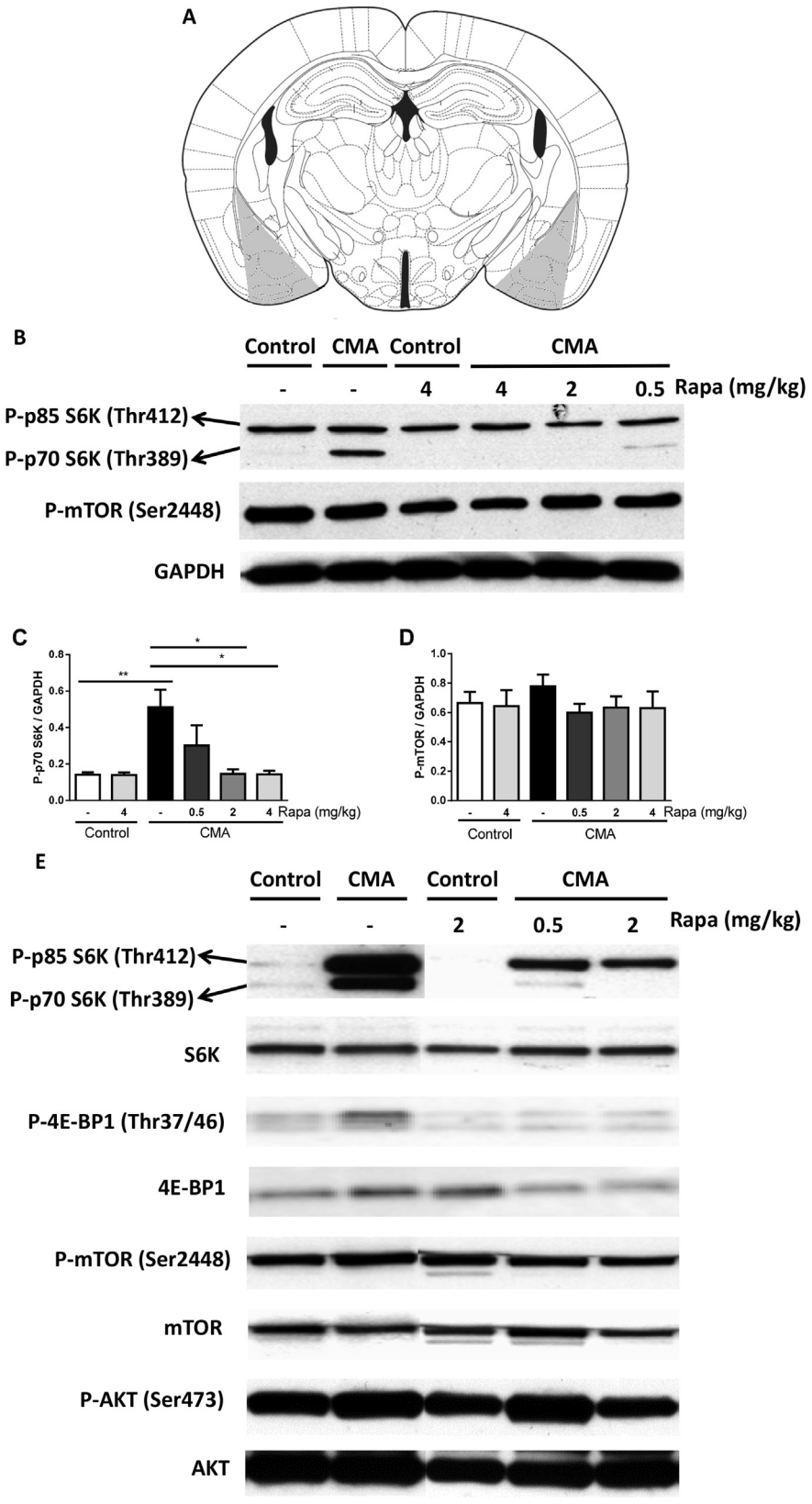


Fig. 7. Western blot analysis showed increased phosphorylation of p70-S6K and 4E-BP1 in the amygdala of CMA mice and rapamycin treatment inhibited the phosphorylation of p70-S6K in amygdala of CMA mice. The gray area in Figure A indicates amygdala. Figure B and C are typical examples of western blots. 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.

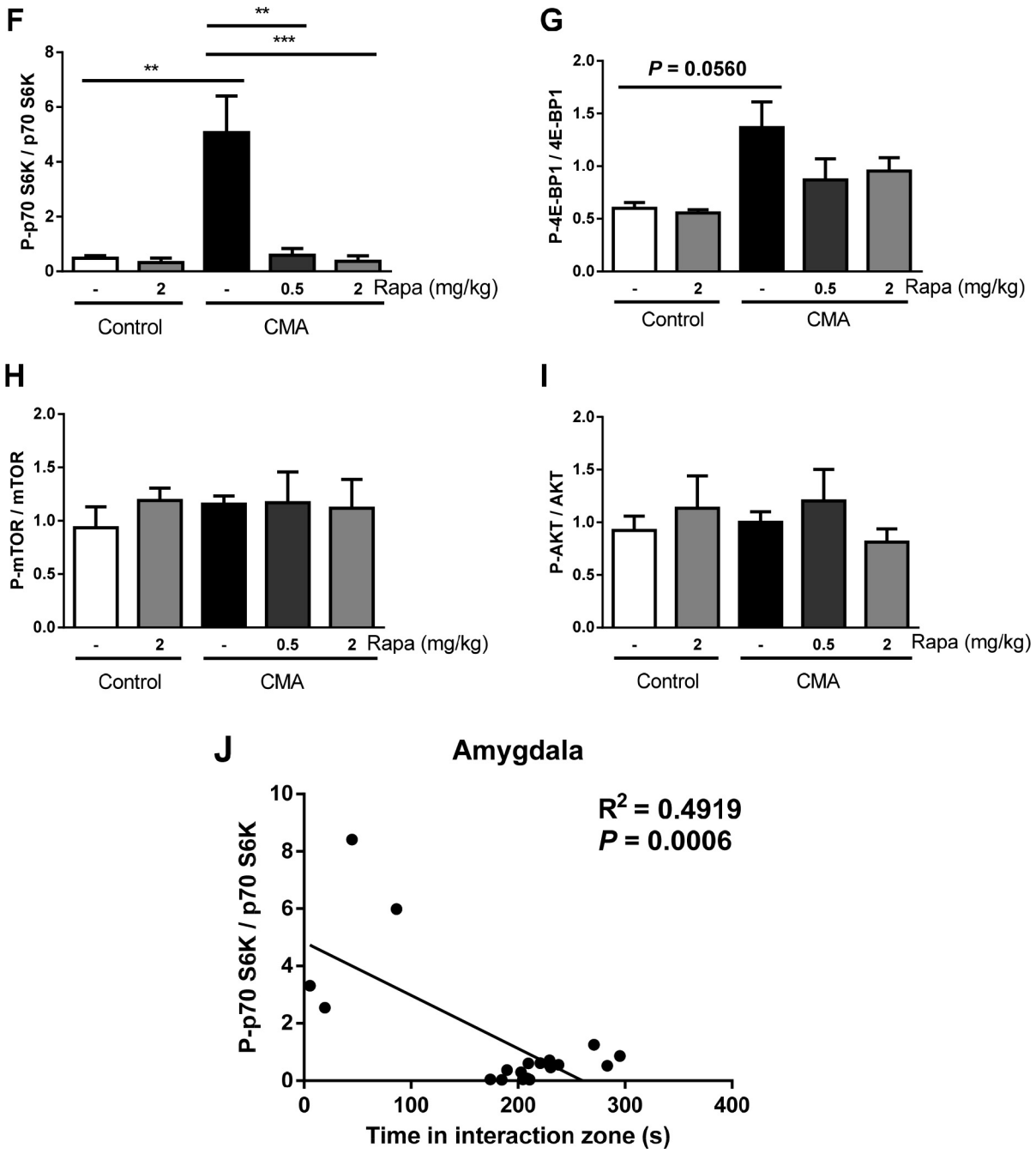


Fig. 7. (*continued*).

(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 associated 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).

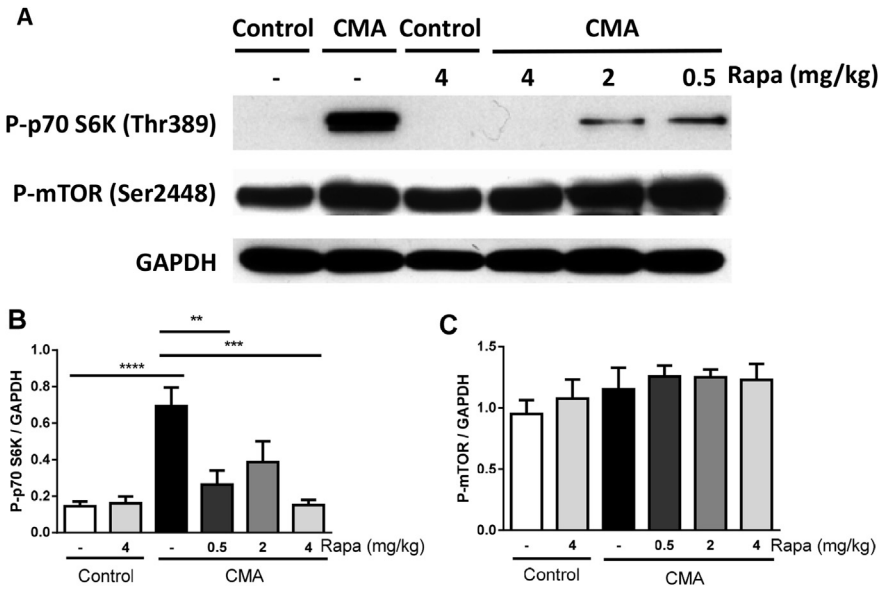


Fig. 8. CMA mice showed significantly increased phosphorylation of p70 S6 kinase in ileum and rapamycin treatment inhibited the phosphorylation of p70 S6 kinase (B). No difference was observed between the groups regarding the phosphorylation of mTOR (C). Figure A is a typical example of western blots. 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.

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, 2004; 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 ser2448 close to C-terminal (Fingar and Blenis, 2004; 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 complex 1 and thereby the formation of mTOR complex 1 (De Theije et al., 2011; Fingar and Blenis, 2004; 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 disturbances (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

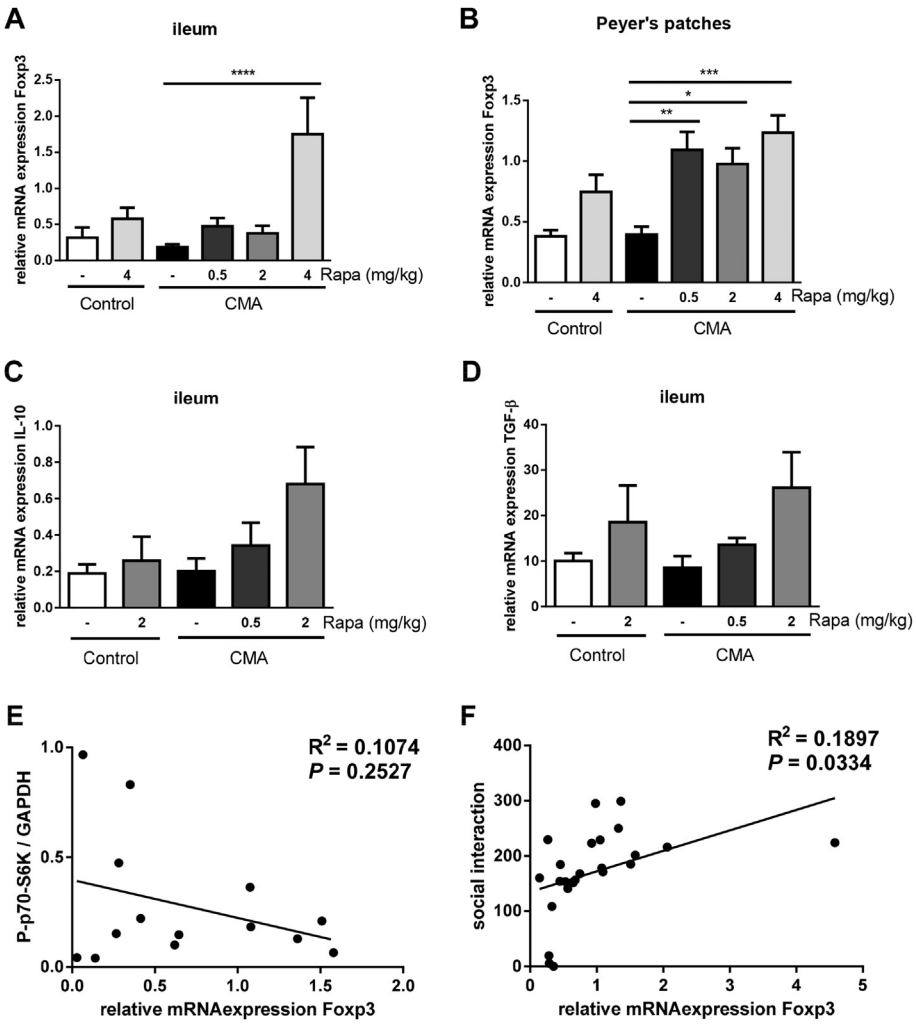


Fig. 9. In the ileum of mice undergoing CMA a reduced expression of Foxp3 mRNA was found (A). Suppression of mTOR signaling by rapamycin induced upregulation of Foxp3 mRNA expression in both ileum (A) and Peyer's patches (B) in CMA mice. Rapamycin treatment promoted anti-inflammatory IL-10 (C) and TGF-β (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 mRNA expression ± 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.

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-β 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-β was demonstrated in the ileum of CMA mice with rapamycin treatment, indicating that possibly IL-10 and TGF-β are involved in the rapamycin induced suppression of cow's milk allergy in mice. Rapamycin treatment induces IL-10 and TGF-β 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 (IFNγ) 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-β in the gut of CMA mice to alleviate behavioral deficits.

5. 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 a 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.

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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