

SYSTEMATIC REVIEW

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Animal studies reveal downregulation of the Beclin-1 autophagy pathway as shared mechanism in Autism Spectrum Disorder: a systematic review and meta-analysis

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BACKGROUND: Autism Spectrum Disorder (ASD) is a heterogeneous neurodevelopmental condition with complex etiology, involving genetic and environmental influences on brain development and behavior. Dysregulation of mammalian target of rapamycin (mTOR) signaling alters neuronal growth and synaptic plasticity, and has emerged as a potential underlying pathway in ASD.

GOAL AND METHODS: To investigate mTOR dysregulation as a common mechanism in ASD, we performed a systematic review, and a meta-analysis of 192 studies examining mTOR signaling in diverse genetic and environmental animal models.

RESULTS: Our random-effects model identified significant alterations in mTOR pathway-related proteins. For several proteins (p-AKT, PTEN, p-mTOR, p-EIF4e, LC3-II, p-S6K and p-S6), subgroup analyses revealed clear species-, sex-, age-, or brain region-specific effects. Interestingly, Beclin-1 was consistently downregulated across all subgroups.

CONCLUSION: Our findings support mTOR-pathway dysregulation in ASD. The observed consistent downregulation of Beclin-1 highlights autophagy as a common mechanism, and provides new leads for novel ASD biomarker and treatment development.

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INTRODUCTION

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by differences in communication and social interaction, as well as restricted and repetitive behaviors and interests [1, 2]. It is a heterogeneous disorder that varies in severity and type of symptoms across individuals and is prevalent across all ethnic and economic groups [3, 4].

Both genetic and environmental factors are believed to play a role in the development of ASD. The heritability of ASD is estimated to be around 80%, with multiple genes and gene-environment interactions contributing to its etiology [5]. Environmental factors, including prenatal exposure to chemicals such as valproic acid and maternal infection have also been associated with an increased risk of ASD [6]. It is thought that these genetic and environmental factors interfere with neurodevelopment, resulting in structural and functional abnormalities which lead to altered synaptic function, neuronal connectivity and communication between brain regions in individuals with ASD [7, 8]. ASD-associated genes reported by genome-wide association studies encode proteins related to synaptic formation, transmission, and plasticity [9, 10]. Still, the etiology of ASD is not well understood and is considered highly heterogeneous given the diversity in underlying factors and the individual variation in phenotypic expression [3]. Although studies suggest the involvement of synaptic plasticity [11], research has not yet led to the discovery of a common mechanism underlying ASD development.

In search of such a common mechanism, a potential role for the mammalian target of rapamycin (mTOR) pathway has emerged [12, 13]. The mTOR pathway is a highly conserved signaling pathway involved in a range of physiological processes, including protein synthesis, cell growth, metabolism, and autophagy and, as such, it is an important regulator of synaptic plasticity [14]. The mTOR pathway consists of two mTOR complexes, mTORC1 and mTORC2, and its regulation is controlled by several upstream signaling pathways, including GSK3β, TSC1/TSC2, PI3K/Akt and various growth factors and nutrients [15]. While mTORC1 mediates protein synthesis through S6K and 4E-BPs and autophagy through the Ulk-1/Beclin/LC-3 autophagy pathway, mTORC2 regulates processes related to cell survival, proliferation and actin cytoskeleton regulation. mTORC2 also regulates Akt upstream of mTORC1 (see Fig. 1) [14, 15].

Dysregulation of the mTOR pathway is associated with diseases such as cancer, Alzheimer's disease, diabetes, and epilepsy [14], and recently has been implicated in neuropsychiatric disorders as well. Several studies suggest that mTOR signaling plays a role in the etiology of ASD through neuronal spine density regulation mechanisms such as synaptic pruning [16, 17]. Variations in genes involved in the mTOR pathway have been identified in individuals with ASD [18, 19], and abnormalities in mTOR signaling have been demonstrated in studies where ASD-characteristics are modeled in laboratory animals [20, 21]. However, it remains unclear whether mTOR-pathway dysregulation is a fundamental shared mechanism in ASD, and if so, how mTOR signaling is involved.

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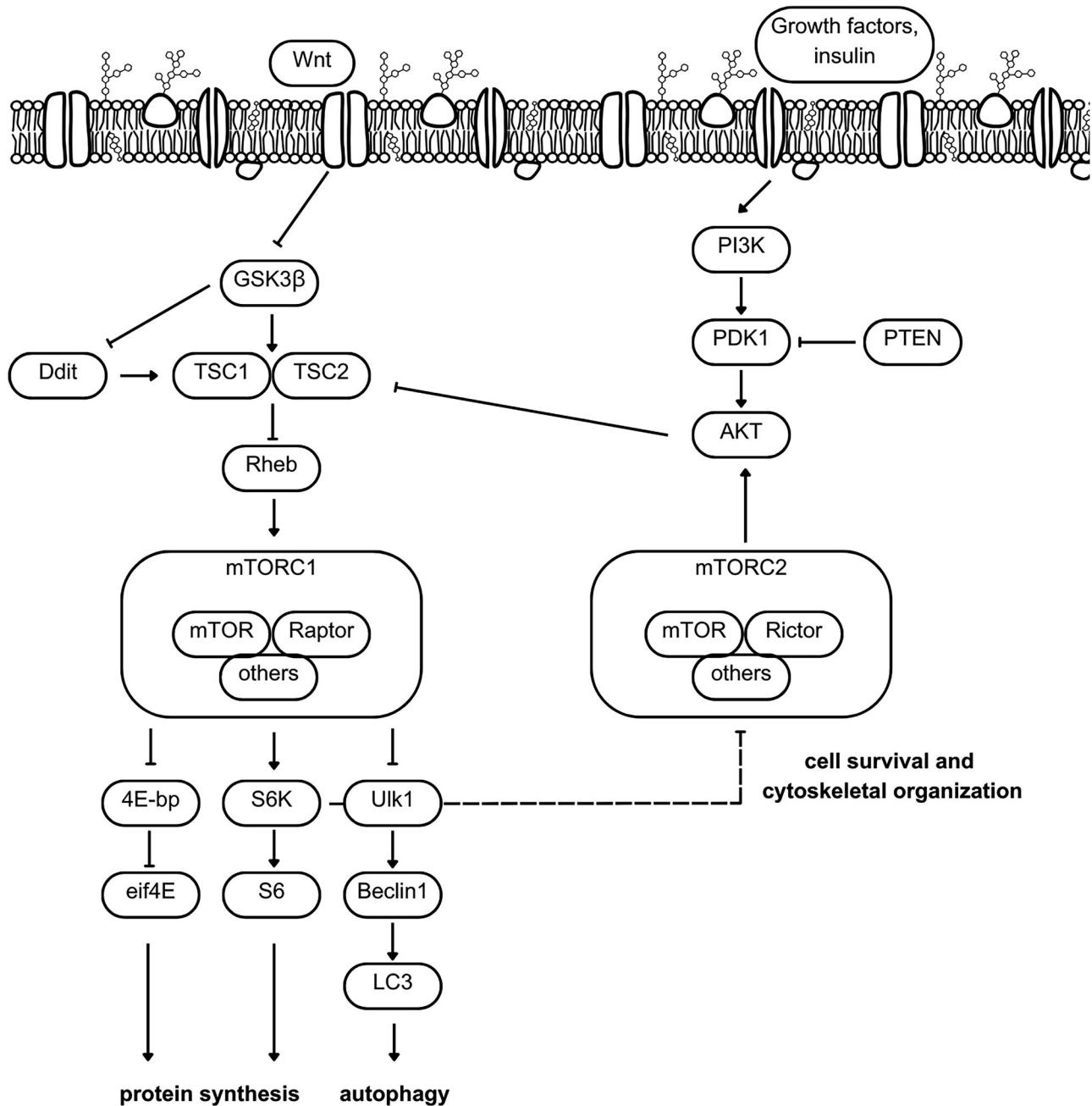


Fig. 1 Schematic diagram of the mTOR pathway with selected proteins. Phosphorylated versions are not shown.

Animal studies are essential for understanding how specific genetic and environmental changes impact brain function and behavior [22]. In line with the heterogeneity observed in ASD, animal studies investigating ASD etiology are highly diverse as well, including a wide range of environmentally and genetically induced models [23]. Integrating knowledge from these animal studies may provide new insights into shared biological mechanisms underlying ASD etiology.

Therefore, we aim to compare mTOR regulation across a diverse range of genetic and environmental animal models for ASD by conducting a systematic review and meta-analysis. The effects of species, age, sex, model type, and brain region are taken into account by using subgroup analyses. Finding common alterations in mTOR regulation can lead to a better understanding of ASD etiology. Alternatively, differential regulation of mTOR across

different animal models can also help understanding the heterogeneity of ASD.

This review and meta-analyses provide: (1) a comprehensive overview of relevant experimental animal studies, (2) insights into differentially expressed proteins in the mTOR pathway, (3) an analysis of how various model and study characteristics impact mTOR pathway regulation, and (4) the identification of consistently dysregulated mTOR pathway proteins in ASD animal studies that could serve as potential future biomarkers and/or new targets for treatment development.

METHODOLOGY

This review seeks to answer the question how proteins of the mTOR pathway are altered in the brains of animal models of ASD in

comparison to wild-type animals. The review protocol was preregistered on PROSPERO (www.crd.york.ac.uk/prospero/; CRD42023392578). The reporting in this systematic review adheres to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist [24] (Supplementary File 3).

Search strategy

PubMed and Web of Science were the two bibliographic databases that were searched for relevant studies. The search consisted of three components: one for animal models [25], one for Autism Spectrum Disorder and the third for the mTOR pathway (for full strategy see Supplementary File 1). On March 27th, 2023, the comprehensive search was carried out, and updated September 18th, 2024. Both indexed terms (i.e., MeSH) and title abstract words were employed, when available. Utilizing Rayyan [26] and Endnote software (version 21), bibliographic records were imported and duplicates were removed before the remaining references proceeded to blinded eligibility screening. Additionally, all included studies' reference lists were reviewed for published research that the comprehensive bibliographic search had missed.

Eligibility screening

Studies were eligible for inclusion if they compared animal models with an ASD-like phenotype to wild-type animals or animals where the ASD-like phenotype is not induced. AA, CMD and CL independently screened all identified records in duplicate in two stages using Rayyan software [26] in blinded mode. Disagreements were resolved by discussion after each stage.

During the first stage, titles and abstracts of the articles were screened according to a predefined set of criteria by AA and CMD. Studies were excluded if they were: (i) not an original primary study (e.g., review, editorial or conference abstract), (ii) not examining an animal model of ASD / ASD-like phenotype (see explanation below), (iii) not analyzing molecular measurements of proteins or a gene expression level related to the mTOR pathway in the brain. In case the abstract screening gave no clear answer, it was included in the following full text screening. In the second stage, the full texts of the remaining articles were screened by AA, CMD and CL in a blinded manner. Articles were excluded for one or more of the reasons from stage one, plus the following additional reasons: (iv) The study did not examine a genetic or induced animal model for ASD / ASD-like phenotype (see explanation below), (v) There was no comparison with wild type or control animals, (vi) There were no molecular measurements for at least one protein or gene related to the mTOR pathway in the brain (see Supplementary File 1 for a list), (vii) There was no full text available.

Regarding criterion ii) and iv), the definition of a genetic ASD model was determined by using the SFARI database [27] to identify genes that have been associated with ASD. Genes from the categories S, 1 and 2 were included, while genes from category 3 or genes that were not in the database at all were not considered a model for ASD and therefore excluded. For these included genetic models, the included studies did not need to show a behavioral phenotype. For environmentally-induced models, we included established models for ASD such as valproic acid (VPA) treatment, propionic acid (PPA) treatment or maternal immune activation. These were considered established models with enough evidence and did therefore not need to show behavioral phenotypes in the included study. However, we also included more novel models that showed behavioral outcomes suggesting autistic-like traits.

Extraction of study characteristics and outcome data

The extraction of study characteristics (if available) was carried out by CL (for the initial set of studies and by AA for the studies included after the search update). The following study characteristics were collected:

- (i) Study Information: This included the study's ID, title, authors, publication year, journal and the digital object identifier (DOI).
- (ii) Animal Model Details: This category encompassed information about the species, genetic background, sex, age, housing conditions, model induction method, type of control and analyzed brain areas.
- (iii) Outcome Measures: This involved compiling a list of relevant proteins from the mTOR pathway used in the study and a list of the specific molecular methods used.
- (iv) Measurement Characteristics: This involved documenting the specific molecular method employed and identifying the type of molecule under examination, including proteins, DNA, or mRNA. This encompassed details regarding the molecule being measured, the timing of the measurement (age at which it was conducted), and the precise brain area in which the measurement took place.

For each included study, outcome data extraction for all outcome measures was conducted by a single reviewer (either CL or AA) for both the ASD model group and the control group, encompassing all molecular measurements undertaken throughout the included studies. The mean, standard deviation (SD), or standard error of the mean (SEM), as well as the total number of animals (n) in both the experimental and control group were extracted.

When exact values were readily available within the publications, they were directly extracted. In cases where such values were not explicitly provided, the WebPlotDigitizer [28] (version 4), a semi-automated web-based tool, was employed to reverse-engineer numerical data from graphical figures. When standard deviation (SD) was not explicitly indicated, it was calculated from the available standard error of the mean (SEM) and sample size (n) ($SD = SEM * \sqrt{n}$). If necessary data were missing for a comparison, the corresponding author of the paper was contacted via email and reminded after three weeks. If necessary data (mean, SD, n) were still missing three weeks after the reminder, the study was excluded from the meta-analysis due to missing data, except when only the SD/SEM of one of the groups was missing. In that case, the SD/SEM was assumed to be the same as the SD/SEM of the experimental group.

Following data extraction, a standardization process was employed, categorizing data by age groups (prenatal, juvenile (<6 weeks), adolescent (6 - 9 weeks for mice and 6 - 21 weeks for rats), and adult (>9 weeks for mice and >21 weeks for rats)) [29–31] and specific brain regions (as detailed in Supplementary File 2).

Risk of bias assessment

The assessment of potential risk of bias was conducted to evaluate the internal validity of the studies included in the meta-analysis. Given the substantial number of papers encompassed within this systematic review and the anticipation of a significant proportion having 'unclear risk of bias' due to inadequate reporting, a random sample of 50 papers (26%) underwent bias analysis using SYRCLE risk of bias tool for animal studies [32]. Responses to these items were categorized as "Yes" for low risk of bias, "No" for high risk of bias, or "Unclear" in situations where bias assessment was hindered by insufficient information. Item one and three from the tool, concerning randomization and blinding of treatment or group allocation respectively, were only assessed for studies without genetic models for ASD in the current meta-analysis, since randomization and blinding for group allocation in genetically modified animals is not applicable.

Additionally, three questions regarding reporting quality were assessed. The studies were checked for reporting on power analyses, randomization at any level, and blinding at any level. Yes indicated reported, and no indicated not reported. The risk of bias assessment was conducted independently in duplicate by two

individuals, AA and CL, with any disagreements resolved through discussion.

Meta-analysis

We conducted a meta-analysis for each outcome with data from at least two studies. The standardized mean difference (SMD) using Hedges' g correction, and 95% confidence interval (95% CI) for each comparison was calculated. In case the same control group was used in multiple comparisons, we corrected for multiple uses of the control group by dividing the number of animals in the control group by the number of times the control group was used and rounding up to the nearest whole number (but never below 2). When multiple similar outcomes were reported from the same cohort, we included the outcome with the highest effect size. If sample sizes were reported as a range, the smallest number was used in the meta-analysis. We employed a random-effects model, which accounts for the precision of individual studies and the variation between studies by weighting each study accordingly. Meta-analyses were performed using Comprehensive Meta-Analysis [33] (version 4.0). Forest plots were used to illustrate the mean overall effect sizes, and the heterogeneity levels (I^2). Possible subgroup analyses were predefined in the protocol and conducted only if at least 10 independent comparisons per subgroup were available. Differences between subgroups were assessed using the Q test for heterogeneity between subgroups.

Publication bias assessment

To detect the potential presence of publication bias, funnel plots were created in R and evaluated on symmetry when more than 15 studies per outcome measure were present, using Duval and Tweedie's trim and fill analysis and Egger's regression analysis for small-study effects [34, 35]. As standardized mean differences (SMDs) may cause funnel plot distortion, the SMD was plotted against a sample size-based precision estimate ($1/\sqrt{n}$) [36].

RESULTS

Comprehensive search

After conducting the comprehensive search in PubMed and Web of Science according to our predefined keyword search string (see Supplementary File 1), the elimination of duplicate records left a total of 1,137 distinct and unique records, which then underwent a rigorous screening process to determine their eligibility. Through a meticulous assessment of titles and abstracts, 437 records were identified as meeting the criteria for further evaluation (see definitions in the methods section). After eligibility screening of the full articles, 213 articles met the inclusion criteria for the systematic review and 192 scholarly articles were suitable for inclusion in the meta-analyses and the subsequent assessment of potential bias (Fig. 2).

Study characteristics

The characteristics of all 213 studies included in the review can be found in Supplementary File 2. From the 213 studies that were included in the review, 192 studies could also be included in the meta-analysis and resulted in 793 comparisons overall for all proteins investigated. Most comparisons were extracted for the proteins (p-)AKT (200), (p-)S6 (118) and (p-)mTOR (116), followed by LC3B (61), (p-)GSK3β (57) and Beclin-1 (47) (see Fig. 3c). From the 793 included comparisons, 636 used mice, 151 used rats and both zebrafish and drosophila were used 3 times as models (see Fig. 3b). Of the mice, 439 comparisons were based on a C57BL/6 strain, followed by the strains FVB (59), Balb/c (49), BTBR (16), CD-1 (11), C3H (10), KM (8), ICR (5) and NMRI (3). In 32 comparisons, the background strain was not reported. For the rat comparisons, Wistar rats were used 91 times, followed by Sprague-Dawley rats (55) and Eker rats (4).

In terms of models, 537 comparisons used genetic models, while ASD-like phenotypes were induced by valproic acid in 127

comparisons, followed by maternal immune activation (27) and disturbed maternal sleep (16), propionic acid (10) and inbred phenotype models (9). Other environmental models made up the rest (Fig. 3g). Data was reported either specifically for males (409) and females (67), or for the two sexes combined (83). The sex of the animals was not specified in 232 comparisons (see Fig. 3d). The majority of studies used adolescent animals (247), followed by adult (195), juvenile (180) and prenatal (22) animals. In 146 comparisons, the age of the animals was not reported (see Fig. 3f). In most studies animals were group-housed (44). In only 3 studies animals were individually housed during experiments and 121 studies did not report on housing conditions. Western blots (631) were the most prevalent molecular methods used for measuring the proteins of interest, followed by PCR (82), immunostaining (53), ELISA (23), and RNAseq (1) (see Fig. 3e). The analyses in the included comparisons comprised several different brain areas, with the biggest focus on the hippocampus (348) and the cortex (182) followed by total brain analyses (98) (see Fig. 3h).

Study quality

A risk of bias evaluation was conducted utilizing the SYRCLE's Risk of Bias tool [32] and the assessment encompassed a randomly selected subset of the included articles (Supplementary File 2). The overall findings regarding the risk of bias in these articles (Fig. 4a) indicated that randomized sequence generation as well as allocation concealment was only reported in one out of the 50 studies. Randomization of animal housing was not mentioned in any study. Blinded execution, random outcome assessment and blinded outcome assessment was explicitly performed at a slightly higher rate, but still lower than 20% of the sample. Our reporting questions regarding the reporting on blinding, randomization and power analysis (Fig. 4b) showed that only 4 out of 50 studies report the use of power analysis to determine appropriate sample sizes and only 17 studies report on some sort of randomization within the study. The reporting of blinding was slightly higher with over 50% of the studies mentioning some kind of blinding.

Meta-analysis

A separate meta-analysis was conducted for every protein with at least two comparisons from two independent studies. The number of included comparisons per protein alongside the descriptive statistics can be seen in Fig. 5. For the proteins of interest mTOR kinase, p-PTEN, p-Raptor, Rheb and TSC1, the minimum threshold of two independent studies per protein could not be reached; therefore there was no meta-analysis conducted for these proteins.

Out of the 31 proteins analyzed with our random-effects model, 13 proteins showed significant differences between the ASD model and the control group. Decreased expression was seen for PTEN, Beclin-1, LC3-II and Rictor, whereas increased expression was seen for p-Rictor, p-Ulk1, p-EIF4e, EIF4e, Ulk1, p-mTOR, p-S6K, p-AKT and p-S6 (phosphorylated at 235/236) (Fig. 5). Consequently, upstream proteins and both downstream parts of the pathway (mTORC1 and mTORC2) show relevant alterations.

Subgroup analyses were performed per protein if at least 10 comparisons per subgroup were available. For the proteins p-GSK3β (phosphorylated at Ser9), mTOR and p-S6 (phosphorylated at 240/244/245) some significant subgroup effects were found although the overall effect was not significant. An overview of all results can be found in Supplementary File 2. For eight of the 31 proteins of interest with a significant overall effect, results of the subgroup analyses are shown in Fig. 6.

Beclin-1 was significantly downregulated (Hedge's $G = -1.443$ [$-1.938; -0.942$], $n = 52$, $I^2 = 80.34$) and showed a consistently significant effect in all subgroups; the effects are significantly stronger ($p = 0.012$) in environmentally-induced ASD models (Hedge's $G = -2.251$ [$-3.028; -1.473$], $n = 21$, $I^2 = 86.54$) compared to genetic models (Hedge's $G = -0.937$ [$-1.541; -0.332$],

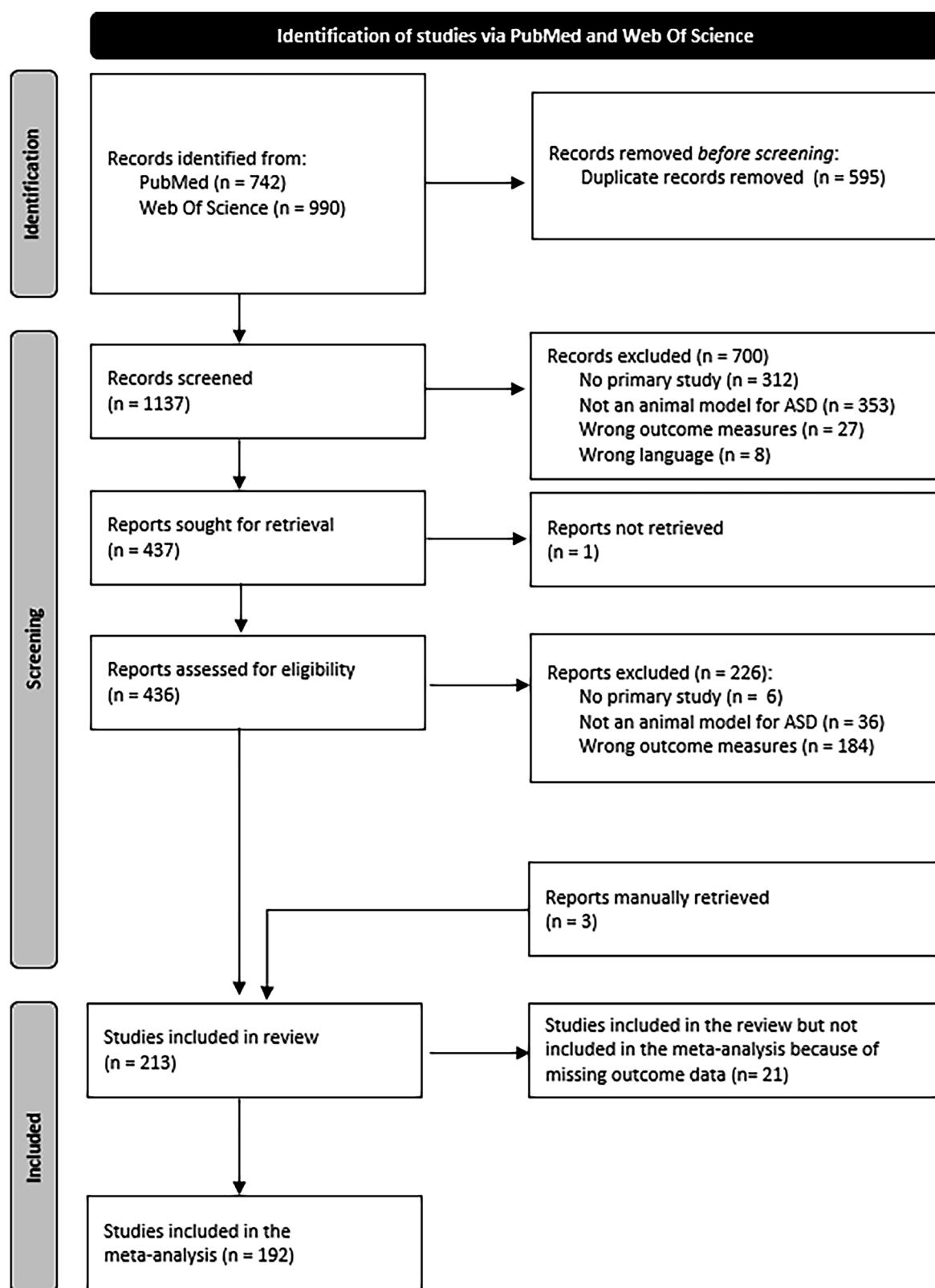


Fig. 2 PRISMA study flow chart. From the 213 studies included in this SR, 192 could be used for meta-analyses. The difference (n = 21) stems from incomplete outcome data that prevented us from including some papers into the meta-analyses.

$n = 31$, $I^2 = 65.46$) as well as stronger ($p < 0.001$) in rat models (Hedge's $G = -3.305$ [-4.177 ; -2.432], $n = 15$, $I^2 = 81.86$) when compared to mouse models (Hedge's $G = -0.764$ [-1.27 ; -0.257], $n = 37$, $I^2 = 70.36$) (Figs. 5, 6a).

While LC3-II showed a clearly significant overall downregulation (Hedge's $G = -1.026$ [-1.657 ; -0.396], $n = 53$, $I^2 = 87.11$), the subgroup analysis (Fig. 6b) revealed that only certain subgroups showed the same significant effect: While LC3-II downregulation

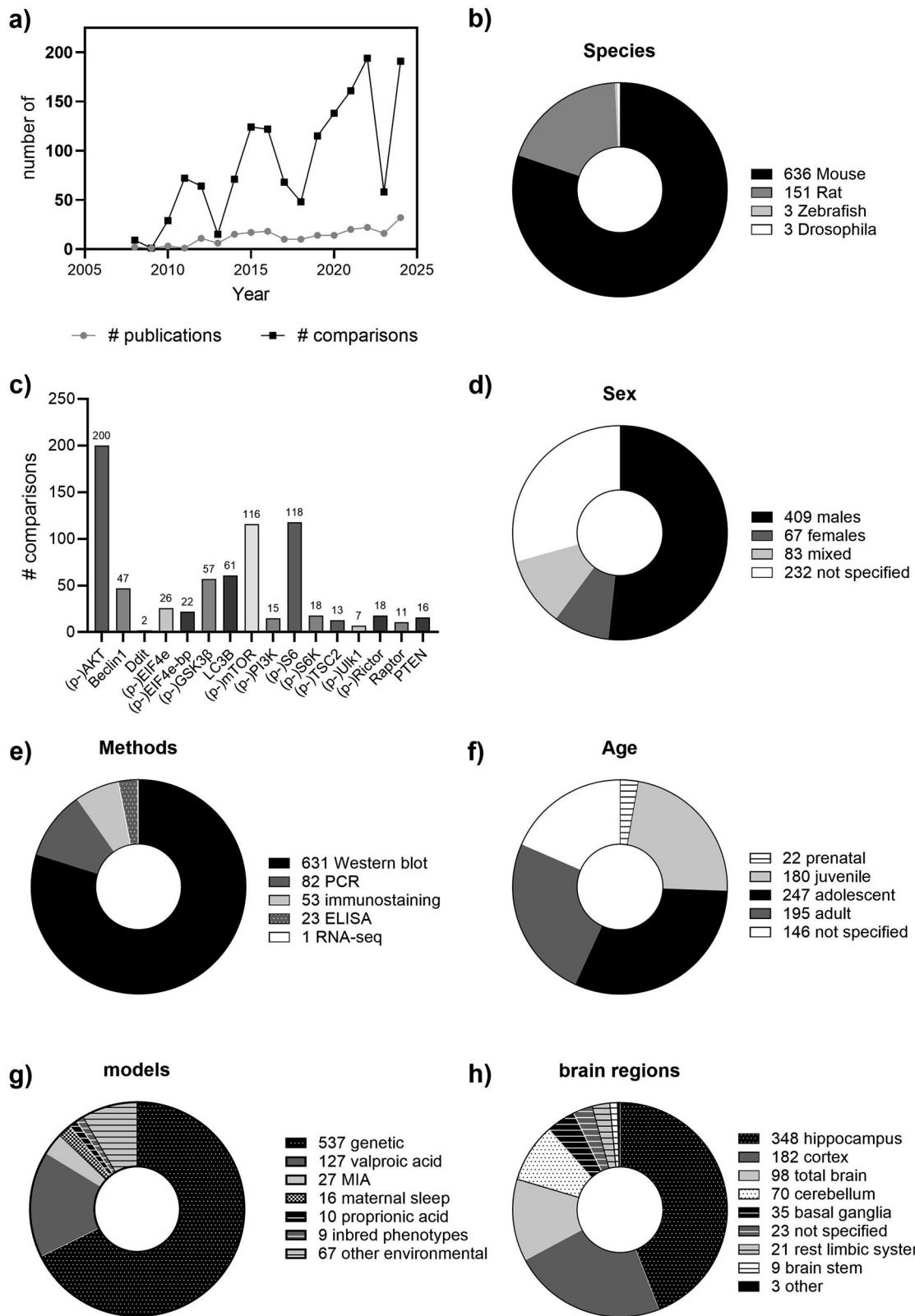


Fig. 3 Characteristics of comparisons included in the meta-analyses. **a)** Number of comparisons and studies per year. **b)** Use of species in the included comparisons. **c)** Number of comparisons per protein. **d)** Sex of the animals used in the included comparisons. **e)** Molecular methods used in the included comparisons. **f)** Age of the animals used in the included comparisons. **g)** Type of model used in the included comparisons. **h)** Brain regions analyzed in the included comparisons.

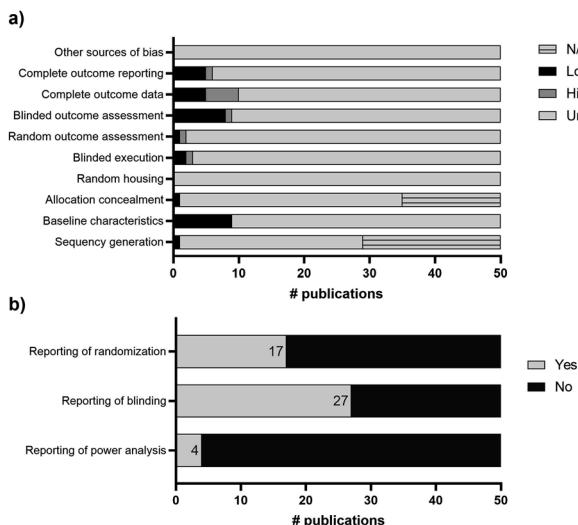


Fig. 4 Risk of bias assessment. The analysis was performed on a random sample of 50 included studies by two independent assessors. **a)** The assessment of 10 items was performed with SYRCLE's risk of bias assessment tool. **b)** Results of the three reporting questions regarding randomization, blinding and power analysis.

was significant in the rat models (Hedge's $G = -2.685 [-3.885; -1.486]$, $n = 15$, $I^2 = 88.08$), it was not in mouse models (Hedge's $G = -0.342 [-1.109; 0.426]$, $n = 37$, $I^2 = 86.85$). Environmentally-induced ASD models (Hedge's $G = -1.222 [-2.164; -0.279]$, $n = 24$, $I^2 = 88.67$) also reflected the clear overall effect.

The subgroup analysis of p-Akt (Fig. 6c) also revealed that only some subgroups showed significant effects. While only the brain area subgroup 'hippocampus' (Hedge's $G = 0.744 [0.443; 1.046]$, $n = 97$, $I^2 = 83.66$) showed a significant downregulation, the result for total brain tissue (Hedge's $G = 0.521 [-0.062; 1.086]$, $n = 25$, $I^2 = 74.98$) and 'cortex' (Hedge's $G = 0.299 [-0.0787; 0.678]$, $n = 62$, $I^2 = 80.53$) were not significant. For the age groups, only adolescent animals (Hedge's $G = 0.657 [0.201; 1.114]$, $n = 50$, $I^2 = 83.43$) and adult animals (Hedge's $G = 0.541 [0.028; 1.054]$, $n = 39$, $I^2 = 77.91$) showed a significant upregulation, while both induction methods 'environmental' (Hedge's $G = 0.647 [0.166; 1.128]$, $n = 45$, $I^2 = 83.69$) and 'genetic' (Hedge's $G = 0.527 [0.237; 0.815]$, $n = 122$, $I^2 = 81.09$) showed significant effects. In contrast, the subgroup analysis of rat studies did not show any significant effects (Hedge's $G = 0.592 [-0.063; 1.248]$, $n = 24$, $I^2 = 86.53$).

The significant downregulation of PTEN was also visible in almost all the analyzed subgroups (Fig. 6d) except the brain region 'total brain', where the effect was not significant (Hedge's $G = -0.367 [-1.434; 0.701]$, $n = 12$, $I^2 = 84.25$).

While none of the GSK3 β molecules showed significant overall effects, the subgroup analysis revealed a significant upregulation of p-GSK3 β (Ser9) when only looking at the cortex (Hedge's $G = 0.699 [0.107; 1.293]$, $n = 17$, $I^2 = 46.97$).

p-mTOR expression differences were highly dependent on age and brain region. While adolescent animals (Hedge's $G = 1.341 [0.729; 1.953]$, $n = 23$, $I^2 = 75.283$) and adult animals (Hedge's $G = 0.589 [0.008; 1.172]$, $n = 24$, $I^2 = 81.17$) showed a significant upregulation, the effect was not significant in juvenile animals (Hedge's $G = 0.198 [-0.504; 0.899]$, $n = 17$, $I^2 = 82.7$). Moreover, the analysis of p-mTOR showed that the upregulation is only significant in hippocampal tissue (Hedge's $G = 1.296 [0.901; 1.691]$, $n = 41$, $I^2 = 77.01$), but not in the cortex (Hedge's $G = 0.234 [-0.204; 0.672]$, $n = 32$, $I^2 = 74.19$) or total brain (Hedge's $G = 0.452 [-0.25; 1.155]$, $n = 12$, $I^2 = 79.62$) analyses (Fig. 6e). Moreover, while mTOR did not show a significant overall effect, some subgroups did show a significant upregulation: rat

models (Hedge's $G = 1.171 [0.319; 2.023]$, $n = 12$, $I^2 = 92.4$), environmental models (Hedge's $G = 0.968 [0.249; 1.687]$, $n = 16$, $I^2 = 91.10$) and hippocampus (Hedge's $G = 0.504 [0.015; 0.992]$, $n = 21$, $I^2 = 72.43$) (Supplementary File 2).

For p-eIF4e (Fig. 6f), only two subgroups had enough comparisons to be analyzed. Both subgroups (type of model and brain region) showed a significant upregulation (genetic: Hedge's $G = 1.353 [0.536; 2.169]$, $n = 13$, $I^2 = 66.27$; hippocampus: Hedge's $G = 1.296 [0.4; 2.194]$, $n = 12$, $I^2 = 72.18$).

The subgroup analysis of p-S6K (Fig. 6g) provided another example of results that vary by brain region, as studies focused on cortical tissue did not show a significant effect (Hedge's $G = 0.194 [-0.463; 0.85]$, $n = 16$, $I^2 = 81.17$). The subgroup of 'adolescent animals' also did not show an overall effect (Hedge's $G = 0.498 [-0.454; 1.45]$, $n = 11$, $I^2 = 89.26$). All other analyzed subgroups did uncover a significant upregulation.

Phosphorylated S6 (at 235/236) also showed an overall significant upregulation (Hedge's $G = 0.519 [0.093; 0.946]$, $n = 44$, $I^2 = 77.97$). However, subgroup analyses showed that only the subgroups 'mouse model' (Hedge's $G = 0.452 [0.019; 0.887]$, $n = 42$, $I^2 = 77.03$) and 'genetic model' (Hedge's $G = 0.524 [0.053; 0.996]$, $n = 37$, $I^2 = 79.28$) confirmed this overall effect (see Fig. 6h). In contrast, phosphorylated S6 (at 240/244/245) did not show a significant effect across all included comparisons, while subgroup analyses revealed a significant upregulation only in the hippocampus (Hedge's $G = 0.712 [0.143; 1.281]$, $n = 26$, $I^2 = 82.19$) (Supplementary File 2).

Publication bias

Publication bias analysis was only assessed for outcomes with at least 15 independent studies. Analysis of the funnel plots suggested some asymmetry due to an underrepresentation of studies. Egger's regression test revealed a significance for p-AKT ($p = 0.0117$), which indicates a potential publication bias in this body of studies. Duval and Tweedie's trim and fill analysis resulted in extra data points for six of the analyzed proteins, namely LC3-II, p-eIF4e-bp, p-mTOR, p-S6, p-S6K and PTEN, indicating the presence of publication bias and a small overestimation of the summary effect size for these proteins (see Table 1).

DISCUSSION

The aim of this study was to investigate mTOR dysregulation as a common mechanism in ASD. Therefore, we analyzed and compared regulation of proteins of the mTOR pathway across a wide variety of genetic and environmental animal models, taking into account species, age, sex, brain region and the way of inducing an ASD-like phenotype.

Our systematic review and meta-analysis indicate that dysregulation of the mTOR pathway may be a common mechanism associated with ASD etiology, as evidenced by altered expression of mTOR-related proteins across various animal models. Specifically, Beclin-1 expression showed a consistent downregulation both as an overall effect and across all subgroup analyses that could be assessed, pointing to autophagy dysregulation as a possible common underlying mechanism in ASD.

Involvement of the mTOR pathway in ASD has received increasing attention over the last years. Several studies have found increased mTOR activity in individuals with ASD [37, 38]. Animal studies have identified several mechanisms through which mTOR dysregulation could contribute to ASD etiology [16]. For instance, increased activity of mTORC1 can, through phosphorylation of 4E-BP1 and S6K1, increase protein synthesis and thereby alter synaptic protein production, which is thought to contribute to ASD-like phenotypes. In addition, increased mTORC1 activity inhibits autophagy by preventing the formation of autophagosomes. Autophagy impairment has been connected to disruptions in cellular homeostasis, another factor implicated in ASD [16].

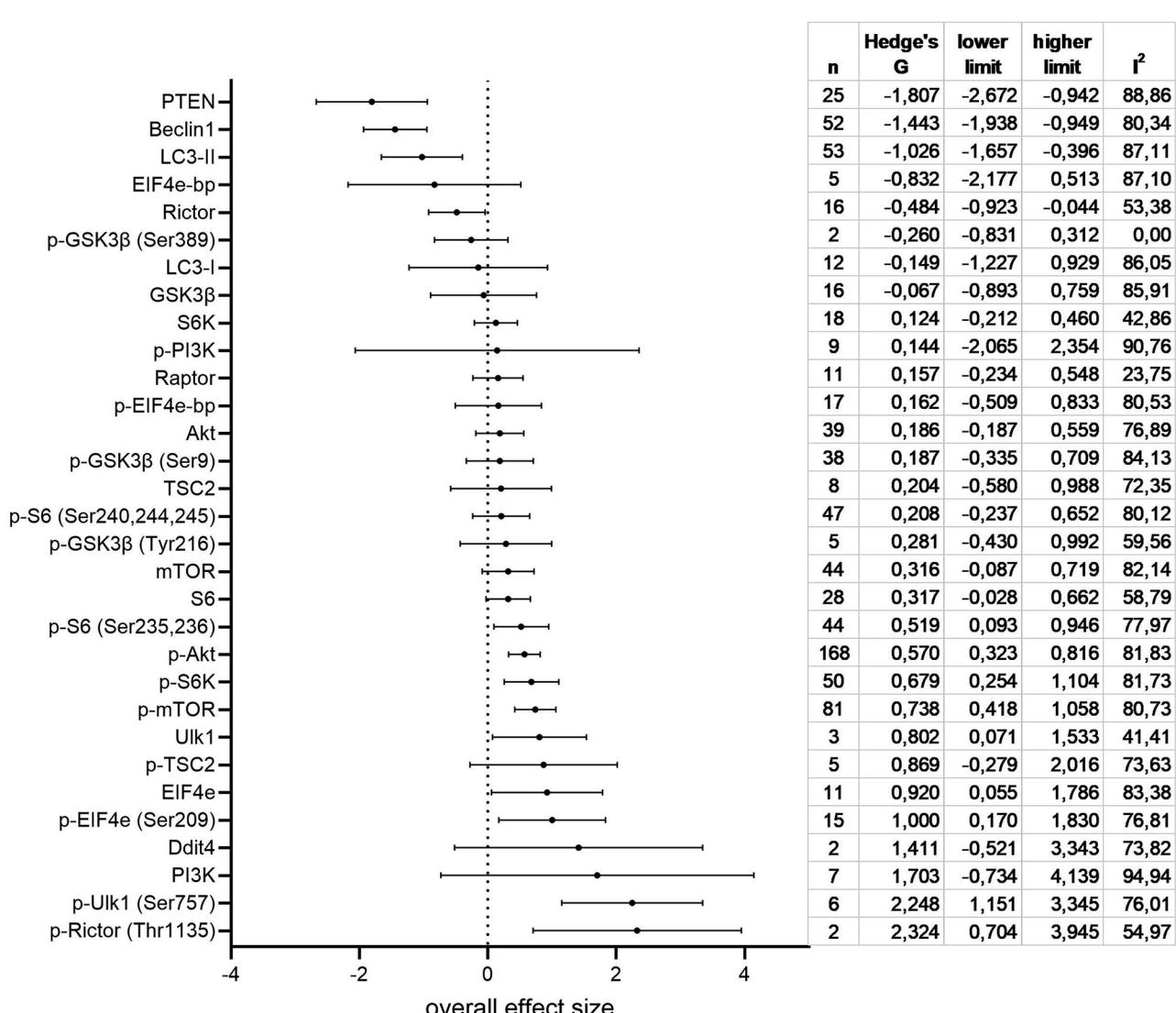


Fig. 5 Results of the meta-analyses per protein. The forest plot indicates the estimated effect sizes (Hedge's G) with 95% confidence intervals for each protein. The number of comparisons (n), the descriptive statistics as well as I^2 as heterogeneity measure can be found on the right side for every analysis. When possible, phosphorylation sites are indicated. For other phospho-proteins, phosphorylation sites that were not functionally different were pooled.

The results from our meta-analysis, showing an overall decrease in the expression of PTEN, Beclin1, LC3-II, and Rictor, and an increase in p-S6, p-Ulk1, p-Rictor, p-EIF4e, p-S6K, Ulk1, p-Akt, and p-mTOR (Fig. 5), point towards an overall increase in mTOR activity in ASD animal models. While the increase of p-Akt and p-Rictor are indicative of mTORC2 activity, the other proteins point towards an increase in mTORC1 activity. mTOR itself did not show an overall effect in our meta-analysis, but subgroup analysis showed a significant upregulation in rats, environmental models and hippocampus. These results are in line with several syndromic forms of autism resulting from mutations in genes such as PTEN, TSC1 and TSC2, that lead to mTOR hyperactivation [12]. Moreover, our results show that mTOR hyperactivation is also present across environmental animal models for ASD. The increased expression of EIF4e and p-EIF4e indicates upregulation of the translation pathway downstream of mTORC1. Elevated levels of p-EIF4e have also been found in ASD patients; especially in severe, compared to mild cases [38].

Most notably, our results at the protein level point towards reduced autophagy as a common mechanism in ASD, which is in line with the effects of increased mTORC1 activity. Autophagy, a

cellular process essential for degrading damaged proteins and maintaining neuronal homeostasis, has recently been associated with intellectual ability in children with ASD [39]. Our results revealed an overall downregulation of the autophagy pathway downstream of mTORC1: Beclin-1 was decreased in all analyzed subgroups and in addition, we found an overall decrease in LC3-II expression. Beclin-1 converts LC3-I to LC3-II, which triggers autophagosome formation, and this pathway starts with activation of Beclin-1 by Ulk1 [40]. mTORC1 is known to inhibit this pathway, by inactivating Ulk1 through phosphorylation at Ser757 [41, 42]. Indeed, our meta-analysis showed increased expression of p-Ulk1 and Ulk1 as well. During development, this impairment can affect network organization and synaptic function, potentially contributing to the behavioral and cognitive symptoms seen in ASD [43]. Studies also suggest that reduced Beclin-1 and autophagy-related proteins like LC3 can lead to neuroinflammation, further exacerbating ASD symptoms by promoting neuroimmune dysregulation [44]. Moreover, interventions aimed at restoring Beclin-1 activity, such as modulating the mTOR pathway or using histamine H3 receptor antagonists, have shown promise in alleviating some autism-related behaviors in preclinical models [44, 45]. Recently,

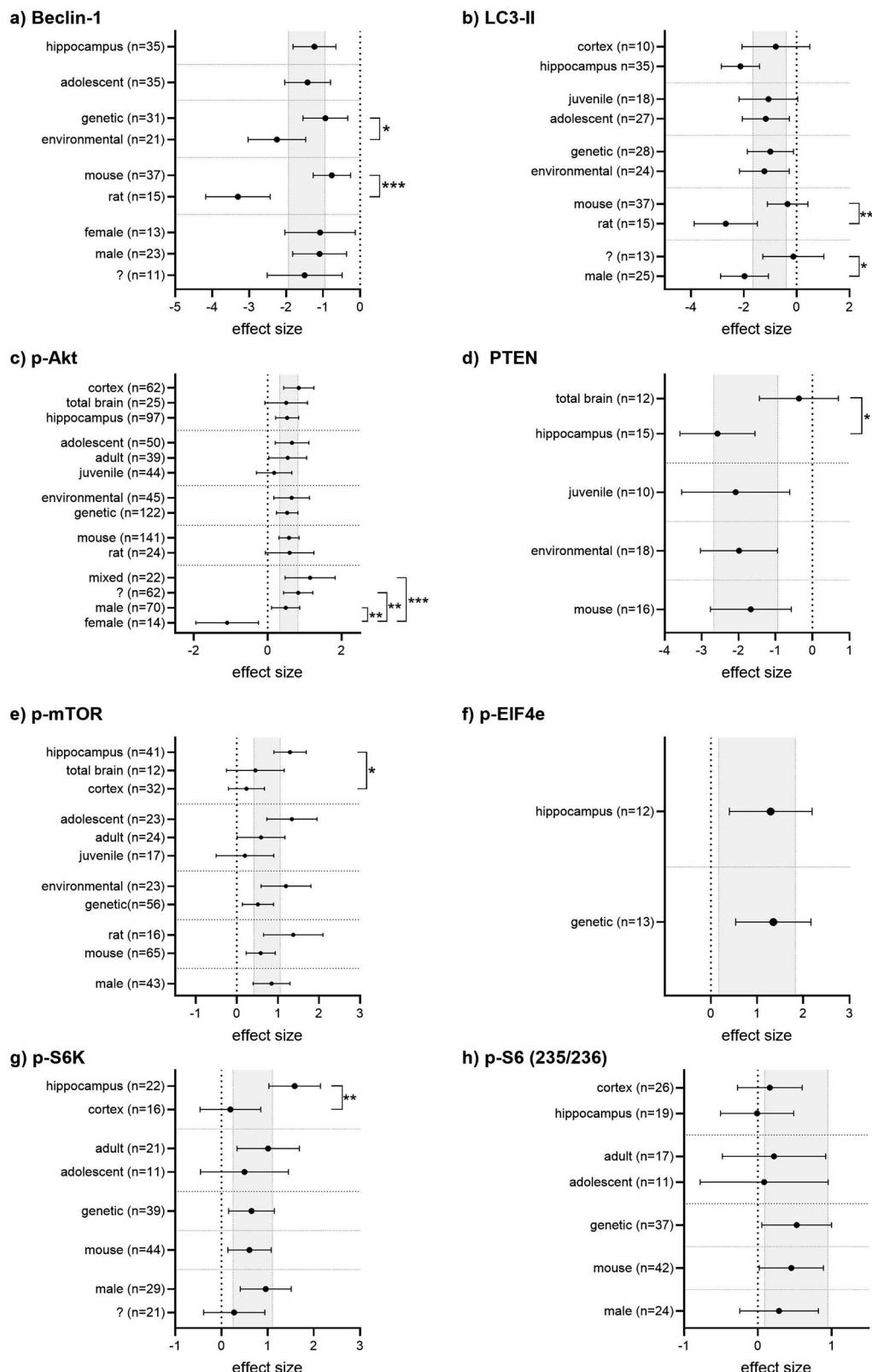


Fig. 6 Subgroup analyses of overall significantly dysregulated proteins. The gray area represents the 95% confidence interval of the overall effect estimate (Hedge's G). The data points indicate the effect estimate (Hedge's G) with the 95% confidence interval of the subgroups. Subgroup analyses were only considered when subgroups contained data of at least 10 comparisons. Subgroup analyses for **a) Beclin-1**, **b) LC3-II**, **c) p-Akt**, **d) PTEN**, **e) p-mTOR**, **f) p-EIF4e**, **g) p-S6K**, **h) p-S6 (235/236)**.

Table 1. Summary of Publication bias analyses per protein with more than 15 studies.

Protein	k	Egger's regression	Trim and Fill
Akt	30	$p = 0.8864$	imputed studies: 0
LC3-II	69	$p = 0.3033$	imputed studies: 16
mTOR	44	$p = 0.3124$	imputed studies: 0
p-Akt	164	$p = 0.0117^*$	imputed studies: 0
p-EIF4e-bp	20	$p = 0.2209$	imputed studies: 4
p-GSK3 β (Ser9)	44	$p = 0.9740$	imputed studies: 0
p-mTOR	108	$p = 0.9698$	imputed studies: 27
p-S6 (235/236)	65	$p = 0.0075$	imputed studies: 21
p-S6 (240/244/245)	66	$p = 0.4698$	imputed studies: 19
p-S6K	68	$p = 0.9472$	imputed studies: 18
PTEN	34	$p = 0.7551$	imputed studies: 9
S6	28	$p = 0.5003$	imputed studies: 0

k = number of included comparisons plus imputed studies.

* indicates asymmetry of the funnel plot, suggesting the presence of potential publication bias or small-study effects.

downregulation of the autophagy pathway has been shown in individuals with ASD [39, 46].

Taking into account that ASD exhibits clinical heterogeneity with varied social, cognitive, and behavioral symptoms among individuals, finding a shared underlying mechanism is valuable. In this systematic review, the clinical heterogeneity in ASD is reflected in the variety of animal models included. Since downregulation of Beclin-1 was found across genetically- and environmentally-induced models, including various genetic models such as Cc2d1a KO, ADNP KO, Mhtfr KO, GluD1 KO, and environmental models such as VPA, DEHP or Phenanthrene treatment, and in all other subgroups, the autophagy pathway emerges as a shared mechanism and might represent a potential target for further research.

Through subgroup analyses we were able to identify differences in mTOR regulation across species, age, sex, brain region and the way of inducing an ASD-like phenotype ('model type'). For Beclin-1, while the effect was present in all subgroups, the downregulation was even more pronounced in rats and environmentally-induced models compared to mice and genetic models (Fig. 6a). This result could reflect that most studies where ASD is induced environmentally use rats. Likewise, the decreased expression of LC3-II was primarily driven by rats (Fig. 6b).

For several other proteins, an increased expression was brain area-specific: p-S6K, p-S6 (240/244/245), mTOR and p-mTOR was upregulated in the hippocampus, but not in the cortex subgroups. The same is true for the downregulation of PTEN, which is only significant in the hippocampus. This highlights the role of the hippocampus in ASD, consistent with recent work [45, 47]. Alternatively, it could reflect a dominant representation of the hippocampus within the included comparisons (Fig. 3). Next, subgroup analyses revealed age-dependent differences. For p-mTOR and p-Akt, the increased expression was found in subgroups 'adults' and 'adolescents' but not in 'juvenile', indicating that mTOR hyperactivity may become apparent later in development. Conversely, p-S6K and p-S6 (235/236) were not upregulated when studying adolescents only. These puzzling results could reflect the differences in mTOR regulation, and the effects of brain plasticity, during different stages of neurodevelopment [48]. Finally, the upregulation of p-Akt seems sex-specific: in females, p-Akt was downregulated whereas in males, it was upregulated (Fig. 6c). Although by far most studies used only males or did not specify sex, leading to an underrepresentation of females, this result warrants further research, especially since sex differences in ASD are known and mTOR regulation could be influenced by sex hormones such as estrogen [49, 50].

This review analyzed over 1500 references and ultimately included 213 in the final review, and 192 in the final meta-analysis. The substantial volume of evidence led to high precision and enhanced the reliability of the overall findings for most proteins of interest. For some proteins of interest (Beclin-2, mTOR kinase, p-PTEN, p-Raptor, Rheb, TSC1) however, not enough studies could be included in our meta-analysis.

Several limitations related to our inclusion- and exclusion criteria can be identified. First, only reports measuring direct protein or RNA levels were included, and reports in a different language than English were excluded. The latter led to exclusion of 8 reports in the title and abstract screening. Second, our search strategy included all mTOR-related proteins known today. This means that future studies could identify other new relevant proteins not included in this analysis.

The definition of ASD models might be considered as another limitation: genetic ASD models were considered an ASD model if the gene met the SFARI-criteria specified in the methods. As a consequence, genetic models that are employed to study related disorders such as Fragile X are included when used to investigate ASD characteristics, whereas the search strategy was restricted to pervasive developmental disorders. Extending the search strategy to include all disorders with an overlap/comorbidity with ASD may have led to a more expansive dataset; yet, for this review we chose a more focused approach accepting this limitation. Future studies could address involvement of the mTOR pathway in related disorders in more detail.

Other limitations concern the risk of bias and indirectness. The risk of bias analysis highlighted that essential details about the design and execution of the included studies were often poorly reported, making it difficult to estimate the risk of bias for most studies. This lack of reporting may suggest that certain methods to reduce bias were not adequately used, potentially skewing the results and limiting the reliability of conclusions drawn from these animal studies. Concerning indirectness, a limitation is that animal models may not be able to reflect such a complex and heterogeneous disorder as ASD. However, the large variety of animal models included in this review is in line with the large variety in ASD etiology, and provides insights into shared mechanisms underlying ASD etiology.

An important note here is that most studies included in this review utilized male animals, resulting in a significant underrepresentation of females. While this is a common issue in many research fields, it does affect the external validity of the findings. Future studies should aim to include both sexes to enhance the generalizability of the results, especially considering the ongoing debate about sex differences in ASD [49].

Lastly, an analysis of between-study heterogeneity indicated moderate to high levels of between-study variation, which is common in animal research due to variability in study designs, models and small sample sizes. To address this anticipated heterogeneity, a random-effects model was employed, alongside sensitivity analyses and subgroup analyses to explore possible sources of heterogeneity. Investigating such heterogeneity is a valuable aspect of meta-analyses in animal studies, as it can help inform the design of future research.

In conclusion, our results point towards the Beclin-1 autophagy pathway downstream of mTOR as a relevant common underlying mechanism in ASD. These results do warrant future research. First, the role of this pathway should be investigated by performing intervention studies in various ASD models. Additionally, our results warrant a systematic review of the proteins identified by this meta-analysis in human individuals with ASD. An initial search for human studies into the mTOR pathway resulted in 1019 studies, of which nine human studies analyzed mTOR pathway proteins in brain tissue of ASD patients. One of these studies also confirmed our finding of a downregulated Beclin-1 [51]. Future research could expand to peripheral measurement and in-vitro experiments with patient-derived tissue, which was outside the scope of our current meta-analysis. Next, the role of this pathway should be addressed in clinical research, investigating potential biomarkers related to Beclin-1 in autistic individuals. Ultimately, this could lead to investigating this pathway as a potential therapeutic target.

Our findings reveal that mTOR pathway dysregulation is a common feature across ASD animal models. While the specifics vary by age, sex, brain region, and ASD model type, the Beclin-1 downregulation and related autophagy impairment emerges as a key mechanism shared in ASD etiology.

DATA AVAILABILITY

Supplementary information is available at MP's website. Extracted and used data from the included studies, analyses, R scripts and CMA files are available from the corresponding authors, CMD or MJHK, upon reasonable request.

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AUTHOR CONTRIBUTIONS

AA, CMD and MJHK conceptualized the project. CRH consulted on methodology and statistical analysis. AA, CM and CMD conducted the data collection and evaluation. AA handled statistical analysis and visualization. AA and CMD wrote the original draft. AA, CMD, MJHK, CM and CH reviewed the manuscript and AA, CMD and MJHK edited the manuscript accordingly. MJHK obtained funding through the Aims-2-trials project. Securing ZonMW funding was a joint effort by AA, CMD and MJHK.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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