1. Introduction

In children with neurodevelopmental problems, autism spectrum disorder (ASD) has a 1% [1] incidence rate. Recently, it has been a major topic of discussion because of its impact on family and social life. Social interaction difficulties, particularly difficulties in verbal communication and socializing, are among the hallmarks of ASD, a broad cognitive disorder [2]. Mental retardation (about 45%) and attention deficit hyperactivity disorder (ADHD) in children (28% to 44%), tic dyskinesia (14% to 38%), gastrointestinal issues (9%-70%), immune disorders (38%), anxiety (42% to 56%), depression (12% to 70%), and obsessive-compulsive symptoms (19% to 32%) are all common co-morbid conditions. ASD has a multifactorial etiology, encompassing both genetic and environmental influences. About a quarter of people are genetically predisposed to the condition. More than 102 genes that have been linked to autism have been identified so far [3]. For all that has been learned about autism over the past few decades, there is no obvious cohesive mechanism for how the disorder manifests itself.

Autophagy is a self-destruction process in cells and is responsible for degrading and recycling cellular components such as misfolded, stacked, or aggregated proteins and damaged organelles [4]. Changes in environmental circumstances like dietary deficit or stress might alter autophagy [5]. Along with component renewal, autophagy plays a role in the development, differentiation, and tissue remodeling of various biological organisms [6]. If the autophagy process cannot correctly destroy waste cell components, they will accumulate in cells, causing aberrant function and potential illness [7]. Numerous publications indicate that aberrant autophagy regulation plays a critical role in the pathophysiology of significant diseases [8-10]. Additionally, autophagy is required to regulate neuronal homeostasis and plasticity, which results in cognitive deterioration. Although the precise mechanism of autophagy in neurons is unknown, activating the phosphoinositol 3 kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway may be a critical element. The PI3K/Akt/mTOR signaling pathway belongs to one of the main cellular signaling pathways, which plays a crucial part in basic intracellular functions [11]. Dysregulation of the PI3K/Akt/mTOR pathway has become a convergence point in ASD. Downregulation of PI3K/Akt/mTOR pathway activity is a latent therapeutic mechanism with broad applicability in ASD-associated diseases [14]. Trigger receptor 2 (TREM2), expressed on myeloid cells, is an...
nate immune receptor found in microglia and has been linked to an elevated risk of early-onset dementia, progressive dementia, and Alzheimer’s disease [15, 16]. In addition, studies have discovered that the autophagy of microglia in mice with the TREM2 gene knockout is inhibited [17], which strongly suggests that there is a link between TREM2 and autophagy [18, 19]. In this context, we believe that TREM2 affects autophagy and the development of ASD through the PI3K/AKT/mTOR signaling pathway.

In this study, we established a model for ASD, and then investigated the effects of TREM2 on cognitive function during the development of ASD, and explored whether TREM2 regulated the PI3K/Akt/mTOR mediated autophagy in vivo and in vitro.

2. Materials and Methods

2.1. ASD model construction

Mice of the C57BL/6J strain (specific pathogen-free (SPF) grade, 8-10 weeks old, provided by Unilever Beijing, China) are mated with males of the C57BL/6J strain according to the timing of estrus. The successfully mating female mice were separated into two groups using a random number generator: the normal saline group and the valproic acid (VPA) group. Female mice were examined and recorded as having a vaginal embolism as early as 0.5 days of pregnancy, after which the pregnant female mice were maintained on their own. The VPA group received a subcutaneous injection of VPA (600 mg/kg) at 12.5 days of gestation to replicate VPA autistic spectrum disorder [20] in the laboratory. Female mice in the control group were given an intraperitoneal injection of normal saline in an equal volume, and the results were analyzed. After 3 weeks of weaning, 15 males were randomly picked from each litter and raised separately. It was decided that males born to mothers who received normal saline would be included in the control group, and males born to mothers who received VPA would be included in the autism spectrum disorder (ASD) group. Male mice were fed until they reached the age of 8 weeks. This study was approved by the Ethics Committee of our hospital before its implementation.

2.2. Stereotactic brain injection

The mice in the ASD group were sedated with isoflurane before an incision was made along the midline of the scalp. TREM2 overexpressing virus (ASD+TREM2 group), TREM2 silencing virus (ASD+siTREM2 group), and empty vector virus (NC group) were injected into the following coordinates of each hippocampus [21] (relative to the bregma): anterior-posterior -2 mm, medial-lateral -1.75 mm, and dorsal-ventral -1.75 mm. All viruses were purchased from GenePharma (Shanghai, China). The virions were administered in a volume of 0.3 mL. The injection rate was 0.5 liters per minute. After each injection, the needle remained in the brain for 5 minutes. Following the operation, the wound on the scalp was sutured. Place the mouse back in its cage after the anesthetic effect has gradually faded away. According to the manufacturer, the pain was controlled by kalofen (5 mg/kg, SQ) 48 hours after the operation.

2.3. Open-field test

When experimental animals were placed in a new habitat, field tests were utilized to analyze those animals’ neurological and autonomic behavior. The box measures 50 cm, 50 cm, and 40 cm in height, width, and depth. In the center of the room is a black inner wall, and the bottom is separated into two sections: the core region and the periphery area. The background noise in the laboratory is less than 65 decibels. Mice were placed in a central area during the experiment and allowed to roam freely. The behavior analysis system began recording the video and automatically recorded the activity of the experimental mice for 5 minutes. It then examined the amount of time the mice spent in the central region and the overall distance traveled by the mice during the experiment. After each experiment, wipe the box with 75 percent alcohol to avoid interference with unusual odors or excrement.

2.4. New object recognition test

Set up the observation boxes one day before the experiment and allow the mice to become acclimated to the boxes in a quiet setting for one hour before starting the experiment. Exclusion criteria were applied to mice exhibiting anomalous activity in the opening (e.g., too little activity, too much activity, circling, etc). The mice were placed in the laboratory for one hour on the experiment day to allow them to become acclimated to the surroundings before the formal experiment could begin. Two identical objects were placed in the center of the opening box in two different sections of the opening box, and the mice were placed separately in the opening box for a total of ten minutes in each area. After the exploration, the mice were removed from the cage and placed back into the feeding cage. Remove any strange odors from the observation boxes by cleaning them. After a 4-hour interval, one of the objects was swapped out for a different one, and the mice were placed in the opening box to investigate for a further 5-minute period. After the experiment is completed, carefully clean the observation box with rubbing alcohol.

2.5. Morris water maze test

Using the Morris waters maze experiment, data acquisition and analysis software were utilized to record pertinent data and photographs, then analyzed to determine the cognitive level of spatial learning and memory in mice. The following are the specific contents: Adaptability training: The hidden platform was removed, and the mice were permitted to swim in the pool at the same time every day to acclimate to the new environment. This stage will persist for three days. An experiment on positioning navigation was conducted in which mice were placed in the water facing the pool wall at a fixed time every morning, and the time it took for them to find the pool and climb onto the platform was recorded, i.e., the incubation period of escaping from the pool wall was determined. Unless the animal can locate the platform within 120 seconds, the escape incubation period is reported as 120 seconds. The mice were allowed to remain on the platform for ten seconds under the experimenter’s supervision. While the positional navigation experiment was carried out over four days, the space exploration experiment was conducted after a one-day break. Experimental procedure for space exploration: The experimenter removed the platform from the water's surface, designated the quadrant where the platform was located as the target quadrant, dropped the mouse into the pool from this quadrant, and recorded the mouse's movement track for 120 seconds, along with the time and dis-
tance of the target quadrant and the number of times the mouse had travelled past that quadrant previously.

2.6. Three boxes of social experiments

The experimental contraption measures 120 centimeters in length, 20 centimeters in width, and 22 centimeters in height. It is necessary to insert an empty cage in the left and right cavities of the apparatus. Stage 1: The mice were allowed to run about freely for 10 minutes to get used to the environment. Stage 2: The scientist placed a tender in an empty cage in the left compartment, animal 1 and observed how the mice interacted (Stranger 1). The tested mice were replaced and monitored for a total of ten minutes. The length of time the mice spent in each room was measured and compared to the length of time Stranger 1 communicated with them and the length of time the mice spent in the empty cage (Object). The experimenter placed the empty cage in the right chamber of another odd mouse and observed his social preference (Stranger 2). The mice were replaced, and the social preferences of mice were measured within 10 minutes of the mice being replaced.

2.7. Immunofluorescence staining

The mice were sedated and then perfused with 0.9 percent normal saline and 4 percent paraformaldehyde, after which the brain tissue was removed and dried with a sucrose gradient, as previously described. After that, frozen sections with a thickness of 15 microns were created. PBS was rinsed three times, five minutes each time, 0.3 percent Triton X100 was broken for one hour, PBS was rinsed three times for five minutes each time, and sheep serum was blocked and incubated at 37 degrees Celsius for one hour. It was then treated at 4℃ overnight with the primary antibodies NeuN (Rabbit-anti-mouse 1:400, CST, USA) and LC3II (Mouse-anti-mouse 1:400, CST, USA) before being washed. The following day, PBS was rinsed three times for a total of five minutes each time, and fluorescent secondary antibodies goat anti-rabbit (Alexa Fluor 594, 1:300, Proteintech, USA) and goat anti-mouse (Alexa Fluor 488, 1:300, Proteintech, USA) were added and incubated at 37°C for 40 minutes before being rinsed three times for a total of five minutes each time. Following the addition of the DAPI (1:1000, MCE, China), the sample was incubated for 5 minutes at room temperature. It was viewed under a confocal laser microscope after the solution had been sealed against fluorescence attenuation.

2.8. Western blot

Protease inhibitors and RIPA lysate (from General Biosystems, China) were added to the excised tissue and then pulverized. The supernatant was collected and centrifuged, and the protein content was calculated as previously described. To prepare for the experiment, the protein sample concentration was normalized, thermally denatured, and frozen at -20°C. In this experiment, the protein samples were exposed to constant pressure electrophoresis, while the PVDF membrane was treated to constant current electrophoresis. Afterwards, the samples were sealed with 5 percent skim milk powder solution before being incubated at 4 degrees Celsius overnight with the appropriate antibodies (all of which were produced from CST in the United States, Rabbit, 1:1000). Following that, TBST was rinsed three times for 10 minutes each time. It was then incubated at 37 degrees Celsius for 1 hour with the secondary antibo-

2.9. Preparation of TEM sections

It was necessary to remove the fixed hippocampus tissue from a 4 percent paraformaldehyde solution before treating it with 1 M phosphate buffer (5 minutes), followed by washing and fixing in 1 percent osmic acid for 30 minutes. Afterwards, the tissue was washed twice with 0.1 M phosphate buffer (5 minutes each time) and then embedded in 3-4% agarose, which was then cut, and the agarose solidified. The implanted tissue was sliced into portions 1-3 mm thick. After that, the parts were immersed in 50 percent solution, 70 percent and 90 percent ethanol, 90 percent and 10 percent acetone, and then dehydrated in TURS, followed by transit with propylene oxide, immersion, embedding, polymerization in ethoxy resin, and finally slicing with a super slicer to achieve the desired thickness. After drying, the sections were doubly stained with uranyl acetate and lead citrate and then examined under a transmission electron microscope to determine their composition (Fei TECNAI G2 12, USA). It was decided to examine five different areas, and the number of autophagosomes in each area was counted under a scanning electron microscope and averaged out.

2.10. Transfection of cells

The mouse BV2 microglia used in this study were obtained from the Type Culture Bank of the Chinese Academy of Sciences Cell Bank (Shanghai, China). Storage of the cells was accomplished using Dulbecco-modified Eagle medium (High glucose, GIBCO), which contained 10% heat-inactivated fetal bovine serum (FBS, GIBCO), and the cells were maintained at 37°C in a humidified incubator with 5% CO2. They were employed in the experiment once they had grown to a density of around 80% confluence.

The adherent cells were inserted in a 24-well plate with 1×104 cells per well 24 hours before lentivirus transfection by the experimenter. The number of cells transfected by the lentivirus was around 2×104 cells per well. A suitable amount of virus suspension was added on the second day, and the original media was replaced with a 2ml fresh medium containing 6 g/ml polybrene and a feasible amount of virus suspension. The culture was continued for another 24 hours, after which the virus-containing culture medium was replaced with a brand-new culture medium. The passage was conducted 72 hours later, and the subsequent studies were carried out after that. In our investigation, the TREM2 virus was transected into BV2 cells, and the results were separated into four groups: the control group (Con), the negative control group (NC), the overexpression TREM2 group (overexpression TREM2), and the low expression of TREM2 group (low expression TREM2).

2.11. Statistical analyses

The statistical analysis of the restudy was carried out using GraphPad Prism 8.0 (San Diego, CA, USA) and IBM SPSS Statistics 22.0 (Aramonk, NY, USA) software. The LSD and Tukey’s post hoc test assessed all data reported as means and standard deviations. The variation in the control group, the ASD group, and the ASD + TREM2 and ASD + siTREM2 groups were all analyzed using the
t-test. A two-way ANOVA was performed to determine whether there was an interaction between autism spectrum disorder and overexpression of TREM2 (two independent variables) on mice's cognitive behaviour. In both the in vivo and in vitro experiments, a p-value of less than 0.05 was considered statistically significant (n=5 per group in the vivo experiment and n=3 per group in the vitro experiment, respectively).

3. Result

3.1. TREM2 restores cognition in mice with ASD

According to the results of open field tests, mice in the ASD group travelled less distance and spent less time in central area than mice in the control group. Mice in the ASD + TREM2 group had significantly far traveling distances and more time in central area than mice in the ASD group (Figure 1A). These findings indicated that TREM2 could improve neurological and autonomic behavior in ASD mice.

When the ASD group mice were compared to the control group, the recognition index of the ASD group mice declined dramatically (P<0.001) however, when the ASD + TREM2 group was compared to the ASD group, the recognition index of the ASD + TREM2 group increased significantly (P<0.001). This finding implied that the learning and memory abilities of ASD mice were impaired but that TREM2 could help increase the learning and memory abilities of ASD mice, which was a promising development (Figure 1B).

The Morris water maze test findings revealed that the number of mice crossing the platform in the ASD group was substantially lower than that of the control group (P<0.01) when compared to that of the control group. Compared to the ASD group, the time and time spent crossing the platform by the ASD + TREM2 group mice increased (P<0.01, P<0.05, respectively). This demonstrated that overexpression of TREM2 could improve individuals’ learning and memory abilities with autism spectrum disorder (ASD) (Figure 1C).

The three-box social interaction study revealed that the time required to identify unusual mice in the control group was much longer than in the empty cage. Compared to the control group, the contact time of ASD mice with strange mice was reduced, suggesting that the social ability of ASD mice had decreased significantly (P<0.001), which is also one of the hallmark behaviors of autism spectrum disorder. TREM2 overexpression resulted in the restoration of social abilities in ASD mice. This demonstrated that TREM2 could also help restore social competence and minimize the symptoms of autism (Figure 1D).

In conclusion, the cognitive function and social ability of ASD mice were significantly reduced, but mice’s cognitive function and social ability were significantly improved after overexpression of TREM2.

3.2. Autophagosomes in the hippocampus of mice

Under the transmission electron microscope, autophagy hippocampal neurons were found in the control group (1.30±0.65) and the ASD+TREM2 group (2.12±1.42) but not in the ASD group (Figure 2). First, the bilayer separation membrane is induced, and then it continues to expand and wrap until autophagy is formed. Once autophagosomes are formed, some of them continue to fuse with lysosomes to produce autolysosomes, resulting in lysosomal degrada-

3.3. Location of LC3 II/NeuN by double immunofluorescence staining

Following staining, we used a laser scanning confocal microscope to evaluate the expression of LC3 II (green fluorescence) and NeuN (red fluorescence) (Figure 3). The green fluorescence of hippocampal CA1 neurons was not detectable in the ASD group, and the percentage of autophagy-positive cells was lower (2.46±0.43%). In comparison to the ASD group, the control group (6.95±1.09%) and the ASD+TREM2 group (7.18±1.12%) had significantly higher levels of green fluorescence in hippocampal CA1 (P<0.05).
3.4. Expression of PI3K/Akt/P-S6 pathway protein in the mouse hippocampus

The autophagy process was characterized by several essential proteins, including PI3K, p-Akt, Akt, and P-S6. Increased P-S6 expression is associated with decreased autophagy activity, attributed to PI3K and Akt activation [22]. According to the results of Western blot analysis, when compared to the control group, the expression of p-PI3K, p-Akt, p-S6 and P-S6 in the ASD group was significantly increased (P<0.05), while the expression of TREM2 and BCL-2 was significantly decreased (P<0.01). However, there was no statistically significant difference in PI3K and Akt (P>0.05). p-PI3K, p-Akt, p-S6 and P-S6 expression were considerably lower in the ASD+TREM2 group than in the ASD group (P<0.05), the expression of TREM2 and BCL-2 was significantly increased (P<0.05), although the expression of PI3K and Akt was like that in the ASD group (P>0.05) (Figure 4).

3.5. Expression of PI3K/Akt/P-S6 pathway protein in BV2 cells

Based on the results of the Western blot analysis, the levels of p-PI3K, p-Akt, and P-S6 in the overexpression of the TREM2 group were significantly lower than those in the control group (P<0.001), while the levels of p-PI3K, p-Akt, and P-S6 in the low expression of TREM2 group were significantly higher than those in the control group (P<0.05) (Figure 5).

4. Discussion

Autism spectrum disorder (ASD) is a severe and widespread social interaction, genetic, and diverse neuro-developmental disorder characterized by impaired communication skills, cognitive impairment, and stereotyped behaviours, interests, and activities. It is also referred to as autism spectrum disorder (ASD) in some circles [23].

The social interaction and cognitive function of the ASD group were significantly worse than those of the control group, which suggested that our ASD models were successfully generated in the experiment, according to the results of the behavioural studies conducted on the included mice. To test the hypothesis that TREM2 may be implicated in the onset and progression of autism spectrum disorder (ASD) through the PI3K/Akt/mTOR pathway-driven autophagy, we developed ASD overexpression TREM2 and ASD knockdown TREM2 groups. In the beginning, TREM2 was discovered on the surface of dendritic cells, osteoclasts, and microglia [15]. TREM2 was highly expressed only in microglia found in the brain. It has been demonstrated that activation of the TREM2 receptor on microglia can activate two essential functions: enhancing the phagocytic activity of microglia and raising the anti-inflammatory response to microglia [16]. According to the most recent research, a decrease in TREM2 can result in neurodegenerative diseases, whereas activation of the TREM2 receptor can inhibit the overactivation of microglia and reduce the secretion of proinflammatory factors and enhance the phagocytosis of microglia [24-28]. TREM2 is also closely associated with autophagy. During the early stages of brain development, it has been discovered that the transcription factor TREM2 is required for microglia-mediated synaptic refinement. Specifically, the loss of TREM2 results in poor synaptic clearance, accompanied by increased excitatory neurotransmission and a reduction in far functional connections. Additionally, the levels of TREM2 protein were found to be adversely linked with the severity of symptoms in people with autism [29]. TREM2 effectively improved social interaction and cognitive function in ASD mice. Interestingly, when compared to the ASD group, ASD mice with TREM2 deficiency showed no social and cognitive function differences.

A recent study has reported ASD-related exon copy number variation mutations in autophagy-associated genes, which suggested that autophagy dysfunction is one of the triggers of ASD [30]. It was discovered that autophagy bodies were present in the CA1 region of the hippocampus of the control group. TREM2 was associated with significantly increased autophagy bodies in the ASD + TREM2 group, indicating that TREM2 may be associated with increased autophagy activity in the ASD group. On the other hand, autophagy is a dynamic process that consists of two main steps: autophagic lysosomal fusion and lysosomal degradation. Because of this, autophagic activity can be seen not only in the aggregation of lysosomes but, perhaps more importantly, in both the formation of autophagic fluxes and the degradation of lysosomes [31, 32]. As a result, LC3 II is an autophagy marker for monitoring autophagy flux. As a result, we detected the co-localization expression of LC3 II and NeuN, which further demonstrated the relationship between autophagy and neurons while also clarifying the effect of autophagy.
on cognitive function. However, we discovered that compared to the ASD group, the expression of LC3 II/NeuN increased and decreased compared to the ASD + TREM2 group, indicating that the autophagy of ASD mice was significant. Based on these findings, we proposed that TREM2 may be beneficial in treating ASD by regulating autophagy.

By modulating the PI3K/Akt/FOXO3a signaling pathway, TREM2 has improved neuroinflammatory response and cognitive impairment in Alzheimer’s disease mice [33]. Additionally, TREM2 has been shown to inhibit the proinflammatory response by activating the PI3K/NF-B signaling pathway [34]. More importantly, PI3K/Akt/mTOR signaling pathway is the main pathway implicated in the initiation and regulation of autophagy. Targeting PI3K/Akt/mTOR-mediated autophagy is a vital therapeutic strategy for various diseases [35]. We hypothesized that TREM2 may be involved in autophagy via the PI3K/Akt/mTOR pathway based on these findings. Our findings showed that the expression of PI3K/Akt/mTOR pathway-related proteins in TREM2 overexpressed ASD mice was significantly lower than that in the ASD group, which was consistent with this inference. Overexpression of TREM2 resulted in significantly decreased expression of proteins associated with the PI3K/AKT/mTOR pathway in microglia, whereas TREM2 knockdown resulted in significantly increased expression of proteins associated with the PI3K/AKT/mTOR pathway in microglia when compared with the control group. In summary, we believed that TREM2 mediated autophagy through the PI3K/Akt/mTOR pathway, thereby contributing to the occurrence and development of autism spectrum disorder. However, due to time and financial constraints, further research into the biological characteristics of TREM2 in ASD is required.

There are several limitations in our study. First, the samples of our study were relatively small. In addition, the biological characteristics of PI3K/Akt/mTOR signaling pathway in ASD were not explored.

This study overexpressed and knocked down TREM2 in mice with autism spectrum disorder (ASD). Using in vitro experiments, we discovered that TREM2 could inhibit the PI3K/Akt/mTOR signaling pathway, increase autophagy, and improve social communication ability and cognitive function in ASD mice. We demonstrated that TREM2 could reduce phosphorylated protein levels of the PI3K/Akt/mTOR signaling pathway in ASD mice, which provided a new target and direction for the treatment of ASD.

Conflict of interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
We have received approval from the Ethics Committee of Guizhou Medical University, The Affiliated Hospital of Guizhou Medical University.

Informed consent
The authors declare not used any patients in this research.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors’ contributions
TY: Conceptualization, Methodology, Writing original draft. AR: Methodology, Investigation, Formal analysis, Validation. XX: Investigation, Formal analysis, Data curation. LW and CS: Methodology, Validation, Writing review & editing, Supervision. ZX: Writing review & editing, Supervision.

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