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Autophagy Enhancer Carbamazepine Alleviates Memory Deficits and Cerebral Amyloid-β Pathology in a Mouse Model of Alzheimer's Disease

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Abstract: Autophagy plays an important role in Alzheimer's disease (AD). It has been reported that autophagic flux is altered in patients with AD, and application of the autophagy enhancer rapamycin may alleviate the cognitive impairment and amyloid-β (Aβ) neuropathology in transgenic animal model of AD. Since rapamycin is also an immune suppressor, there is a concern that long-term use of rapamycin may bring severe unwanted side effects. The aim of this study is to test if carbamazepine (CBZ), an anti-epileptic drug that has a potent autophagy enhancement effect, has anti-AD effects in $APP^{swe}/PS1^{deltaE9}$ transgenic mice model of AD. We found that $APP^{swe}/PS1^{deltaE9}$ mice display increased autophagic activity accompanied by decreased mTOR activity. After three months treatment with CBZ in the $APP^{swe}/PS1^{deltaE9}$ mice, we demonstrated that the spatial learning and memory deficits in these mice are significantly alleviated. We also documented that the cerebral amyloid plaque burden and $A\beta_{42}$ levels in these mice are significantly reduced. Furthermore, we showed that CBZ significantly enhances the autophagic flux in the $APP^{swe}/PS1^{deltaE9}$ mice which is unlikely via mTOR-dependent autophagy pathway. These data suggest that long-term CBZ treatment may have a protective effect in AD mouse model possibly through enhancing the autophagic flux.

Keywords: Amyloid-β; Alzheimer's disease; Autophagy; Carbamazepine; mTOR pathway; spatial memory

INTRODUCTION

Alzheimer's disease (AD) is a common neurodegenerative disorder with progressive and gradual decline in cognitive function. It has been forecasted that there would be nearly 9.2 million patients with this disease in 2050 [1]. Amyloid plaque is one of the important pathological hallmarks in AD which is composed of Aβ generated from amyloid precursor protein (APP) processing by β-secretase and γ-secretase. The mice which express mutated genes associated with familiar AD could generate significant portions of amyloid plaques, and are thus believed to be good models for studying the mechanism and therapy of AD.

Autophagy is a major pathway involved in degradation of long-lived proteins, and autophagy consists of components: macroautophagy, microautophagy and chaperon-mediated autophagy [2]. The form of autophagy is the delivery of cytoplasmic cargo sequestered inside double-membrane vesicles to the lysosome. Previous reports have shown that loss of autophagy in the central nervous system (CNS) causes wild-spread neurodegeneration in mice [3], reflecting the important role of autophagy in CNS. Aggregated proteins formation is the common character in neurodegeneration disease. It is believed that activated autophagy could enhance the clearance of abnormal aggregation [4]. In AD, there are several reports to indicate a correlation between autophagy and AD pathology [5-8]. For instance, AD animal models and patients display macroautophagic induction and pathological autophagic vacuole accumulation [6, 8]. The expression of Beclin1, a key component in autophagy pathway involving in the recruitment of membranes to form autophagosomes, is reduced in early AD patients. Beclin1 may participate in the regulation of Aβ metabolism as seen in AD mice [7]. Meanwhile, presenilin1 (PS1) which is one of the γ-secretases to generate Aβ has an important role in lysosomal proteolysis. The mutation of PS1 would disrupt the lysosomal/autophagic function, which may cause accumulation of mis-folded proteins and neuronal cell death and eventually lead to AD [5]. Furthermore, it was reported that increasing the autophagic flux may ameliorate Aβ neuropathology and memory deficits in AD model [9-12]. Therefore, regulating autophagic level may be a potential therapeutic strategy for AD.

Autophagy is regulated by several signaling pathways in mammalian cells, including mammalian target of rapamycin (mTOR)-dependent pathway and mTOR-independent pathway [13]. The p70 S6 kinase (p70S6K) and translation initiation factor 4E binding protein-1 (4EBP1) are the downstream targets of mTOR and the phosphorylated state of these proteins are used to evaluate the mTOR activity [9]. Rapamycin is a commonly used autophagy inducer through mTOR-dependent pathway [11]. Apart from the classical mTOR-dependent pathway, mTOR-independent pathway may also participate in regulating autophagy in mammalian organisms. Phosphoinositol signaling pathway is one of the mTOR-independent autophagy pathways [13]. Autophagy is negatively regulated by intracellular inositol and inositol 1, 4, 5-trisphosphate (IP3) [14].

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CBZ is a commonly used anti-epileptic drug and is also a widely used mood stabilizer. Recently, it has been found that CBZ has a potent autophagy enhancement effect [14, 15]. CBZ could decrease the hepatic load of mutant α1-antitrypsin Z and hepatic fibrosis in a mouse model of α1-antitrypsin deficiency-associated liver disease by enhancing autophagic flux [15]. Furthermore, CBZ could significantly reduce abnormal protein aggregation and toxicity [14]. All these reports suggest that CBZ may have the ability to clear autophagic substrates by increasing autophagic flux.

To determine the effects of CBZ in AD neuropathology and memory ability, we treated the $APP^{swe}/PS1^{deltaE9}$ transgenic (Tg) mice and wild-type (Wt) littermates with CBZ. After three months treatment with CBZ, we examined the cognitive function, AD associated neuropathology, and autophagic level in these mice. We found that CBZ significantly alleviates the cognitive deficits and Aβ neuropathology, and enhances the autophagic flux in the Tg mice.

MATERIAL AND METHODS

Animals and Drug Administration

$APP^{swe}/PS1^{deltaE9}$ Tg AD mice and Wt littermates used in this study (The Jackson Laboratory, NO. 004462) were genotyped by PCR analysis of genomic DNA from tail tissues. When these mice were at 6-month old, we gave them with CBZ (Sigma, USA) through oral feeding at 100 mg/kg per day [15-17]. CBZ was dissolved in dimethylsulfoxide (DMSO) for stock in -80°C freezer, and further diluted with distilled water to final concentration of 10 mg/ml in 1% DMSO. The vehicle treated mouse was given the same final concentration of the DMSO vehicle. The mice were divided into four groups: CBZ treated Tg mice (Tg+C), vehicle treated Tg mice (Tg+D), CBZ treated Wt mice (Wt+C) and vehicle treated Wt mice (Wt+D). Animal care and procedures were performed in accordance with the Laboratory Animal Care Guidelines approved by Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences.

Morris Water Maze Tests

We used the modified Morris water maze tests to evaluate the spatial learning and memory ability of the mice. The procedures were previously described in detail [18, 19]. The diameter of the pool is 1.2 meter. Briefly, the procedure consisted of 5 days hidden platform tests, plus a spatial probe test and visible platform test. In the hidden platform tests, the mice were trained to find the hidden platform for 5 consecutive days with four trails per day. For each trail, the mouse was allowed to swim a maximum of 90 s in order to find the hidden platform. The time when the mouse climbed the platform was recorded as latency. On the 6th day, probe trails were performed by removing the platform. The percentage of total distance in the previous target quadrant and the crossing counts in the previous target platform were measured. Two hours later, the visible platform test was performed. The latency to get the platform was recorded. All the tests were monitored with the DigBehv-MM tracker system (Jiliang Software Technology Co. Ltd, China).

Immunostaining

After the learning and memory test, the mice were sacrificed. The mouse was anesthetized with 10% chloral hydrate, and then was transcardially perfused with ice-cold phosphate buffered saline (PBS). The left brain was used for examination of protein expression by immunoblot and Aβ quantification by ELISA. The right hemisphere was fixed in 4% paraformaldehyde overnight, and then the brain tissue was cryoprotected in 15% sucrose for 24 h and in 30% sucrose for 48 h before sectioning with a Leica cryostat (Leica CM1850, Germany) to 12 or 30 μm thickness. The 30 μm slice was used for Bielschowsky's silver staining with 20% silver nitrate, ammoniacal silver nitrate and 0.1% gold chlorid [20]. The 12 μm slice was used for immunohistochemical staining and immunofluorescent staining. The amyloid plaque was detected by anti-Aβ monoclonal antibody 6E10 (Covance, USA). The second antibody was biotinylated anti-mouse IgG, followed by ABC elite kit (Vector, USA) and diaminobenzydine-H_2O_2 reaction (Vector, USA). The slices were visualized and photographed by an inverted microscope (Olympus IX81, Japan) equipped with a DP70 CCD digital camera (Olympus, Japan). The rabbit anti-LC3B antibody (Cell Signaling, USA) was used for immunofluorescent staining. The secondary antibody was Alexa Fluor 488 Goat Anti-rabbit IgG (Invitrogen, USA). The immunostaining was visualized under confocal microscope (LCM510, Germany). The method to calculate the plaque number, plaque area and the number of cells containing massive LC3 positive puncta, was in accordance with our previous reports [19, 21]. Briefly, we randomly selected three microscopic fields in each slice with the same reference position to count the plaque number and the cells containing massive LC3 puncta per field. The plaque area was measured by Image-Pro Plus software and recorded as the average plaque area per field.

Immunoblot

The left hemisphere tissue was sonicated in ice-cold RIPA lysis buffer (1×PBS, 1%NP40, 0.1%SDS, 5mM EDTA, 0.5% sodium deoxycholate, 1mM sodium orthovanadate, and protease inhibitor PMSF) for 2 min. Half of the lysate was centrifuged at 13 000×g for 20 min at 4°C. The supernatant fraction was used for Western blot analysis. Another half of the lysate was used for ELISA analysis. The following primary antibodies were used in Western blot: rabbit anti-APP C-terminal monoclonal antibody (Sigma, USA), rabbit anti-BACE1 polyclonal antibody (Abcam, UK), rabbit anti-Aph1α polyclonal antibody (Zymed, USA), rabbit anti-neprilysin polyclonal antibody (Millipore, USA), mouse anti β-actin monoclonal antibody (Sigma, USA), rabbit anti-LC3B polyclonal antibody (Cell Signaling, USA), rabbit anti-P62 polyclonal antibody (Cell Signaling, USA), rabbit anti-mTOR polyclonal antibody (Cell Signaling, USA), rabbit anti-p-mTOR (S2448) polyclonal antibody (Cell Signaling, USA), rabbit anti-p70S6k polyclonal antibody (Cell Signaling, USA), rabbit anti-p-p70S6k (Thr389) polyclonal antibody (Cell Signaling, USA), rabbit anti-4EBP1 polyclonal antibody (Cell Signaling, USA), rabbit anti-p-4EBP1 (Thr70) polyclonal antibody (Cell Signaling, USA), rabbit anti-Akt polyclonal antibody (Cell Signaling, USA), rabbit anti-p-Akt (Thr308) polyclonal antibody (Cell Signaling, USA). The secondary antibodies were conjugated with horseradish peroxidase (Cell Signaling, USA). The result of the interested blot was quantified by Quantity One software.

Brain $A\beta_{42}$ ELISA

Detergent soluble $A\beta_{42}$ was directly measured in the supernatant fraction of homogenates. After the centrifuge, the pellet was used to detect insoluble cerebral $A\beta_{42}$ following the previous protocol [19, 22]. Briefly, the pellet was suspended in 70% formic acid with sonication until clear, and then spun at 13 000×g for 20 min. The formic acid sample was neutralized in 1 M FA neutralization solution (1M Tris base, 0.5M Na_2HPO_4, 0.05%NaN_3). The levels of human $A\beta_{42}$ ($hA\beta_{42}$) were quantified using the Human $A\beta_{42}$ ELISA High sensitivity kit (Millipore, USA). The capture antibody of the human $A\beta_{42}$ ELISA kit recognizes the C-terminal end of human $A\beta_{1\text{-}42}$.

Statistical Analysis

Data were expressed as means ± SEM. The results were analyzed by Student's *t* test, one-way ANOVA and two-way repeated measures ANOVA. GraphPad Prism 5 software was used for all statistical analyses. $p<0.05$ was considered statistically significant.

RESULTS

Long-Term CBZ Treatment Alleviates the Spatial Learning and Memory Deficits in AD Mice

To investigate whether long-term CBZ treatment affects the cognitive function in AD mice, we treated the 6-months old Tg mice and Wt littermates with CBZ at a dose of 100 mg/kg daily. After three months treatment with CBZ, the mice were examined for the spatial learning and memory ability by the Morris water maze. The day before the test, CBZ was stopped to avoid the drug's acute influence. We did not find any weight loss and sedation in the mice during the long-term treatment with CBZ. In hidden platform test, the Tg mice had significant learning deficit, showing that Tg mice had longer latency than wild-type mice at day 2-5 test (Fig. **1A**; $p<0.01$). We found that CBZ treated Tg mice showed a shorter escape latency than vehicle treated Tg mice at day 5 escape latency test (46.2 ± 6.69 vs. 57.2 ± 6.69 s; Fig. **1B**; $p<0.05$). Subsequently, the probe trial was performed on the day immediately following the last day of the hidden platform test. Compared with Wt mice, Tg mice showed a deficit in spatial memory (Fig. **1E** and **F**; $p<0.01$). We found that the percentage of total distance and the number of passing times in CBZ treated Tg mice were significantly greater than vehicle treated Tg mice (Fig. **1E** and **F**; $p<0.05$), indicating CBZ treatment significantly improves the spatial leaning ability in Tg mice.

Two hours later, the visible platform version of the test was performed and all mice had similar visual acuity (Fig. **1G**; $p>0.05$). These data reflected that Tg mice had cognitive deficits, and long-term CBZ treatment would alleviate the cognitive deterioration. In order to see whether CBZ affects the motor ability in AD mice, we measured the swim speed at the water maze test and we found that CBZ treatment did not affect the swim speed either in Tg mice or in Wt mice (Fig. **1H**; $p>0.05$).

Long-Term CBZ Treatment Reduces the Number of Cerebral Amyloid Plaques and the Levels of $hA\beta_{42}$ in AD Mice

To see whether CBZ affects the Aβ neuropathology, we examined the number of amyloid plaques and the levels of $A\beta_{42}$ *in vivo*. 6E10 immunostaining and silver staining were used to detect the cerebral amyloid plaque. We found that the 9-months Tg mice had a large portion of amyloid plaques in the cortex and hippocampus, interestingly, the CBZ treated Tg mice showed fewer plaque number than vehicle treated

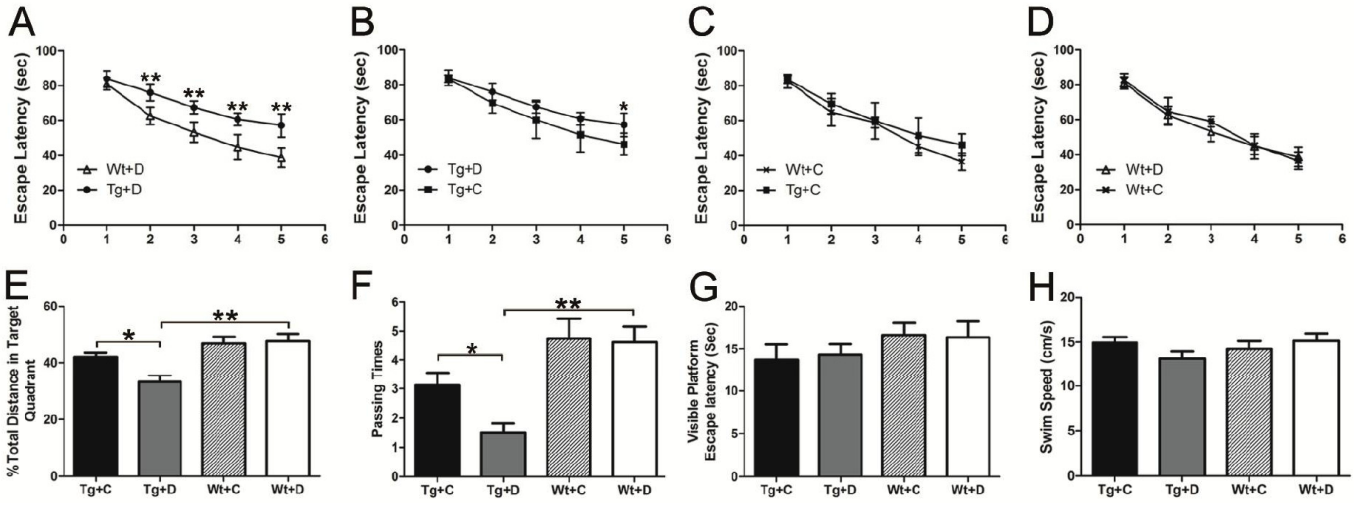

Fig. (1). CBZ treatment rescues spatial learning and memory deficits in the $APP^{swe}/PS1^{deltaE9}$ mice. The memory of Tg mice and Wt mice treated with CBZ or vehicle were evaluated in the Morris water maze. A-D) The performance of hidden platform test. The latency in seconds to find the hidden platform over five days testing is presented for each group. E-F) The percentage of total distance in the previous target quadrant and the passing times were measured in the probe trails. The spatial learning ability was significantly improved in CBZ treated Tg mice compared with vehicle treated Tg mice. G) Time to the visible platform is calculated to assess the visual acuity, not significantly different is found in the four groups. H) Swimming speed of day1 training trail is not significant across the four groups of mice. Data are presented as mean± SEM, n=8 in each group. *$p<0.05$, **$p<0.01$ by two-way repeated measures ANOVA and one-way ANOVA.

Tg mice (Fig. **2A** and **B**). Quantification analysis showed that the CBZ treatment reduced the number of amyloid plaque by approximately 2.5 fold (Fig. **2C**; $p<0.01$). In addition, the average of plaque area of CBZ treated Tg mice was smaller, about 2.0 fold decreased as compared with vehicle treated Tg mice (Fig. **2C**; $p<0.01$).

To further investigate how CBZ decreased the amyloid plaque burden, the ELISA analysis was performed to measure the levels of $hA\beta_{42}$ in the brain. The levels of detergent soluble $hA\beta_{42}$ and formic acid soluble $hA\beta_{42}$ in the brain of CBZ treated Tg mice were reduced to 71.2% (Fig. **2D**; $p<0.05$) and 61.0% (Fig. **2D**; $p<0.05$), respectively, as compared to vehicle treated Tg mice. These data indicate that the reduced number of amyloid plaque is correlated with the decreased levels of $hA\beta_{42}$ in the brain. CBZ treatment could reduce the number of amyloid plaques and the levels of $hA\beta_{42}$ in AD mice.

Long-Term CBZ Treatment Does Not Alter Aβ-Generated Secretases or Aβ-Degraded Enzyme Expression in AD Mice

Aβ was generated from APP processing by β-secretase and γ-secretase. Here we showed that 9-month Tg mice expressed higher levels of APP, BACE1 and Aph1a as compared to Wt mice (Fig. **3A**). To elucidate the mechanism responsible for the reduction in Aβ following CBZ administration, we examined these secretases levels by Western blot in the brain homogenates, showing that CBZ did not influence APP, BACE1 or Aph1a expression either in Tg mice or in Wt mice (Fig. **3A** and **C**-**E**; $p>0.05$). This is further supported by our other finding that the levels of β-secretase-generated C99 and α-secretase-generated C83 fragment are not different between CBZ treated and vehicle treated Tg mice (Fig. **3B** and **F**; $p>0.05$). These data suggest that the decreased $hA\beta_{42}$ is unlikely the result of the change in $hA\beta_{42}$ generation. To determine whether CBZ treatment affects Aβ

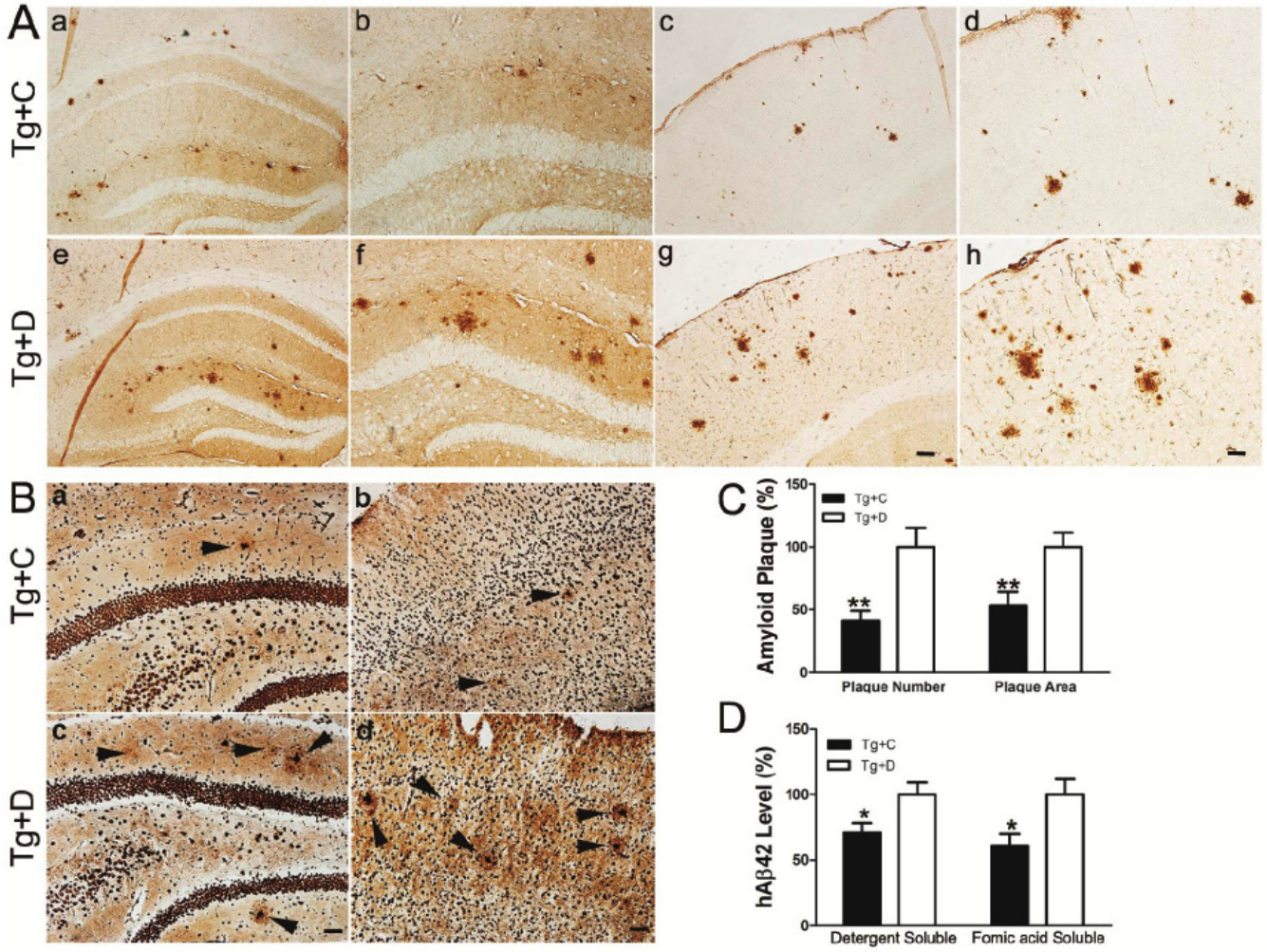

Fig. (2). CBZ treatment reduces the number of amyloid plaque and the level of $hA\beta_{42}$ in the $APP^{swe}/PS1^{deltaE9}$ mice. A) Representative images of Aβ-immunostained brain sections. The 6E10 was used to detect the amyloid plaques in the hippocampus (a, b, e, f) and cortex (c, d, g, h). B) Amyloid plaque was stained by Silver Staining in the hippocampus (a, c) and cortex (b, d). Arrows point to plaque. C) The number of plaque and the average of plaque area are quantified. D) ELISA was performed to measure levels of detergent soluble $hA\beta_{42}$ and formic acid soluble $hA\beta_{42}$ in cerebral homogenate of CBZ treated and vehicle treated Tg mice. Scale bar, Ag, 100 μm; Ah, Bc, Bd, 50 μm. Data are presented as mean± SEM, n=5 in each group. *$p<0.05$, **$p<0.01$ by Student's *t*-test.

clearance, we measured the levels of neprilysin (NEP), a major Aβ-degrading enzyme [23, 24]. Western blot showed that the brain homogenates of Tg mice had less NEP expression than Wt mice (Fig. **3B** and **G**; $p<0.01$), whereas CBZ treatment increased the NEP levels by about 23% in Tg mice, although it is not statistically significant (Fig. **3B** and **G**; $p>0.05$). These data suggest that CBZ-mediated increase in Aβ-degrading enzyme is unlikely the major factor to reduce Aβ levels.

Long-Term CBZ Treatment Enhances the Autophagic Flux in AD MICE

Autophagic-lysosomal system is one of the proteolytic pathways to clear abnormal cellular proteins. In order to determine whether CBZ treatment affects the autophagic activity in Tg mice and Wt mice, we examined the LC3 levels in the brain homogenates. LC3-II is regarded as an autophagic marker localized in autophagosomes and autolysosomes, and the intra-autophagosomal LC3-II is quickly degraded by lysosome [25]. We found that Tg mice had higher levels of LC3-II than Wt mice, and CBZ treatment significantly increased the LC3-II levels in Tg mice (Fig. **4A** and **D**; $p<0.01$). We further examined the levels of P62 protein, an indicator of autophagic flux which is degraded by autophagy pathway [26, 27]. Western blot and further quantitative analysis showed that P62 levels were decreased by 1.7 fold in CBZ treated Tg mice (Fig. **4B** and **E**; $p<0.01$).

When autophagy is induced, LC3 redistributes to autophagosomes, which can be visualized as puncta in individual cell [11, 25, 28]. Using immunofluorescence, we found that LC3 was redistributed to bright puncta in the cortex, and the number of LC3 puncta was significantly increased in CBZ treated Tg mice (Fig. **5A**) which is consistent with the increased levels of LC3-II. The cells containing massive LC3 puncta in the cortex of CBZ treated Tg mice was increased to 1.56 fold as compare to vehicle treated Tg mice (Fig. **5B**; $p<0.05$).

These data suggest that Tg mice has increased autophagic activity compared to Wt mice. Three months CBZ treatment can significantly enhance the autophagic flux in Tg mice.

Long-Term CBZ Does Not Affect the Activity of mTOR-Dependent Autophagy Pathway in AD Mice

To further determine whether CBZ alter the mTOR signaling pathway in Tg mice, we measured the associated protein in the brain homogenates. Using Western blot analysis, we found that levels of total and phosphorylated mTOR were not significantly different between CBZ treated and vehicle treated Tg mice (Fig. **4G** and **H**; $p>0.05$). Because mTOR phosphorylation does not always correlate with its activity, we further examined mTOR downstream targets. Quantification showed that neither p-p70S6K/p70S6K nor p-4EBP1/4EBP1 display a significant change in CBZ treated Tg mice (Fig. **4G**, **I** and **J**; $p>0.05$). Furthermore, CBZ did not change the ratio of p-Akt/Akt (Fig. **4C** and **F**; $p>0.05$). Akt is believed to be an upstream regulator of the mTOR pathway [29]. These findings suggest that CBZ-induced autophagic flux enhancement in Tg mice is unlikely to result from the activation of mTOR-dependent autophagy pathway. Interestingly, we found that the 9-months vehicle treated Tg mice had a significant lower ratio of p-p70S6K/p70S6K and p-4EBP1/4EBP1 as compared to vehicle treated Wt mice (Fig. **4G**, **I** and **J**; $p<0.01$), although they had similar levels of the total mTOR and the phosphorylated mTOR, indicating that the mTOR activity is decreased in 9-months Tg mice.

DISCUSSION

The presence of a large number of amyloid plaque is an important characteristic pathology in AD [30]. Amyloid plaque primarily consists of Aβ which is derived from the processing of APP. In this study, we used the AD mouse model which over-expresses two mutant human genes at a single locus, the Swedish mutations of human APP and the

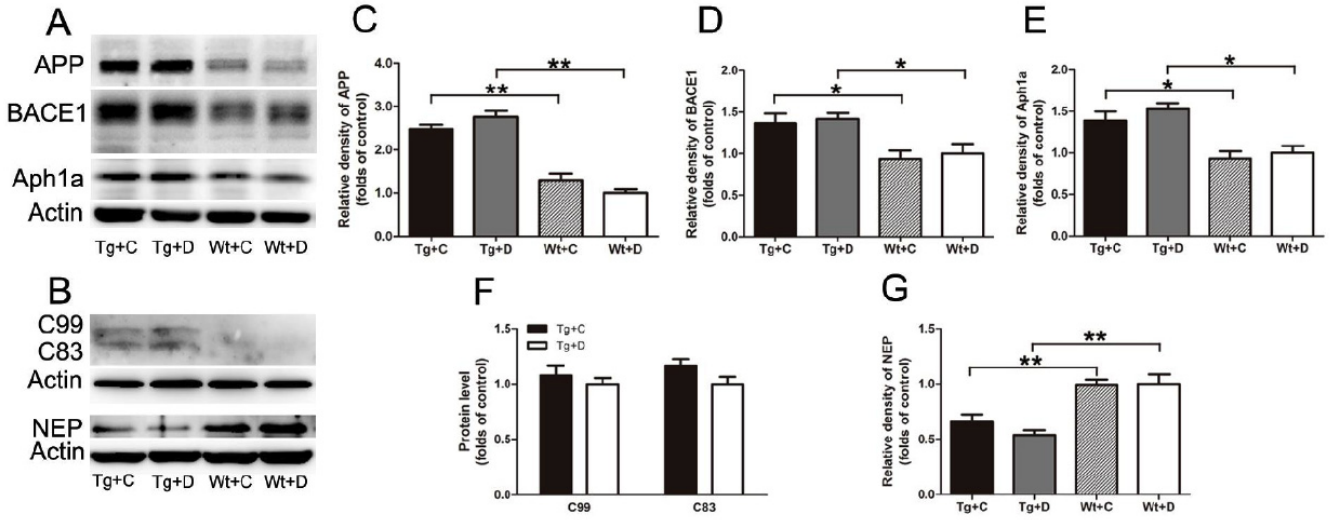

Fig. (3). CBZ does not affect the levels of Aβ-generated secretases or Aβ-degraded enzyme. A, B) representative Western blots of proteins extracted from the brain of four groups. C, D, E, G) Quantification of the levels of APP, BACE1, Aph1a and NEP proteins in the brain of four groups. F) Quantitative analysis of C99 and C83 levels in CBZ treated and vehicle treated Tg mice. Data are presented as mean± SEM, n=5 in each group. *$p<0.05$, **$p<0.01$ by Student's *t*-test (F) and one-way ANOVA (C, D, E, G).

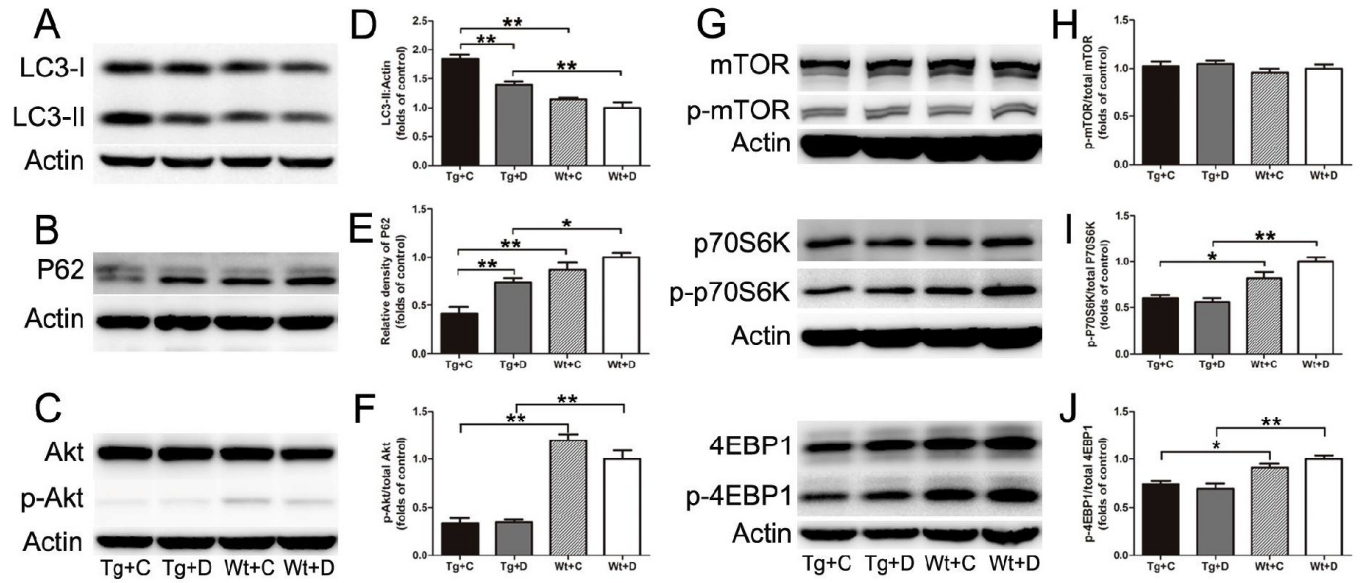

Fig. (4). CBZ treatment increases the autophagic flux unlikely via mTOR-dependent autophagy pathway. A-F) Representative protein levels of LC3-II, P62, Akt and p-Akt across the four groups. G-J) Representative protein levels of the mTOR-dependent autophagy pathway. The Tg mice show significantly lower ratios of p-p70S6K (Thr389)/total p70S6K and p-4EBP1 (Thr70)/total 4EBP1 than Wt mice. The CBZ treatment does not affect these ratios. Data are presented as mean± SEM, n=5 in each group. *p<0.05, **p<0.01 by one-way ANOVA.

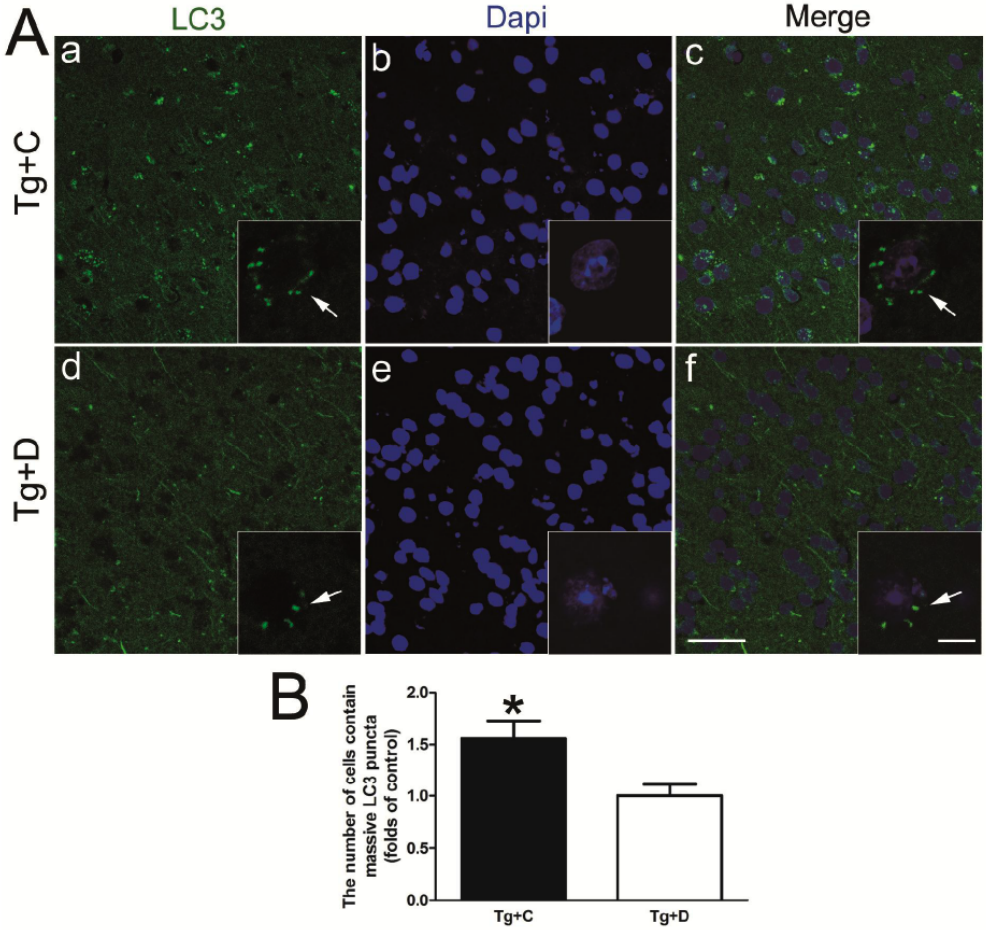

Fig. (5). CBZ increases the number of LC3 puncta in the brain of $APP^{swe}/PS1^{deltaE9}$ mice. A) Representative LC3-immunostained images of the cortex. An increased LC3-immunoreactive puncta is observed in the cortex of Tg mice following CBZ administration. Arrows point to the LC3-positive puncta. B) Quantification of the number of cells containing massive LC3 puncta in cortex. Scale bar, Af, 50 μm; the inset 10 μm. Data are presented as mean± SEM, n=5 in each group. *p<0.05 by Student's *t*-test.

DeltaE9 mutation of human PS1. Occasional plaques can be found in this Tg mouse as young as 6 months of age [31]. The previous report showed that Tg mice started to have cognitive deficits at 6 months of age [18], and continues to decline in learning and memory with aging [32]. In our experiment, we found that the 9-month Tg mice displayed significantly cognitive deficits and had obvious amyloid plaque formation in the cortex and hippocampus.

CBZ is wildly used an anti-epileptic drug and mood stabilizer probably through blocking sodium channels [17]. The previous reports demonstrated that CBZ can improve memory function in epileptic animal models [33-35]. The repeated administration of CBZ could protect against impairment of learning rate caused by chronic application of an electroconvulsive shock or ethanol in rats [35], and prolonged CBZ treatment could enhance discriminative memory in a rat model of epilepsy [33]. Furthermore, in the T-maze, rat with CBZ treatment learned much better than controls [34]. In our experiment, we found that three months CBZ treatment significantly improved the spatial learning and memory ability in Tg mice, and the improvement may be associated with the decreased cerebral amyloid plaque burden, suggesting CBZ may has beneficial effects on cognitive function in AD.

Autophagy is a pathway to degrade long-lived protein including Aβ [12, 36]. In our study, we found that the 9-months Tg mice had greater LC3-II levels than Wt littermates. Because the amount of LC3-II is closely correlated with the number of autophagosomes, the increased LC3-II levels may be the result of an augmentation in autophagosomal synthesis or impairment in autophagosome-lysosome fusion [25, 28]. We further detected other autophagic flux markers. It is known that P62 can bind LC3 to serve as a selective substrate of autophagy [26, 27]. We found that the Tg mice had lower levels of P62 compared to Wt mice, indicating the autophagy is induced. This is consistent with the previous report that the autophagy is induced in AD mice [8]. The increased autophagic activity is likely associated with the accumulation of cerebral Aβ. An induction of autophagy in AD is regarded to accelerate the protein turnover and Aβ clearance, and protect the neurons from Aβ-induced cytotoxicity [11, 36]. For example, autophagy inducer rapamycin could abolish cognitive deficits and reduce Aβ neuropathology in AD model by enhancing autophagic flux [9-11], suggesting that autophagy may have a protective role in AD pathology.

Rapamycin has been reported to possess beneficial effects by improving AD cognition and pathology in animal models. However, since rapamycin is an immune suppressor, long-term treatment could cause side-effects of thrombocytopenia and hyperlipidaemia [37]. Compared to rapamycin, CBZ is widely used in clinic. It has been shown that CBZ can significantly reduce EGFP-HDQ74 aggregates, attenuate polyglutamine toxicity, and enhance the clearance of A30P α-synuclein [14]. In our study, we found that CBZ significantly increase the levels of LC3-II and the number of LC3 puncta, and decrease P62 levels in Tg mice. All these data indicate that the autophagic flux is enhanced after the long-term CBZ treatment in Tg mice. Such increase in autophagic flux may result in the decrease of Aβ neuropathology [7, 10-12].

We treated the AD mice with CBZ at the age of 6 month. At that time the mice have mild cognitive deficits and occasional cerebral amyloid plaque; whereas in the later stage, the AD mice display large amount of amyloid plaques [10]. At the later stage, application of autophagic enhancer has no benefic effects on cognitive deficits and Aβ neuropathology [10]. Our finding indicates that the CBZ treatment in the early stage of AD could attenuate the AD progression. We showed that CBZ significantly decreased the levels of soluble and insoluble $A\beta_{42}$, and reduced the number of amyloid plaque deposition. The accumulation of extracellular Aβ may be at least partially dependent on the accumulation of the intracellular Aβ [38]. CBZ treatment enhanced the autophagic activity to degrade the intracellular Aβ, then further affects the dynamic relationship between intracellular and extracellular pool of Aβ.

It is clear that the role of mTOR in AD is very complicated, and it is possible that different stressors in various cells may have opposite effects on mTOR [9]. The previous reports have shown that mTOR signaling pathway is altered in AD [9, 40-42]. In some reports, it has been shown that phosphorylated p70S6K levels are increased in the brain of AD patient [39], and the mTOR activity is increased in 3xTg AD mouse model [9]. But in others, it was found opposite [40, 41]. In our Tg mice, we found that the mTOR activity is reduced. The phosphorylated Akt is shown to be decreased in the Tg mice [42]. However, CBZ treatment did not affect the mTOR activity neither in Tg mice nor in Wt mice, suggesting the mTOR-dependent autophagy pathway may not be regulated by CBZ. Inositol depletion is a common mechanism for mood stabilizer [43], and autophagy is negatively regulated by intracellular inositol and IP3 level, which is regarded as an mTOR-independent autophagy pathway [13, 14]. The previous reports have shown that the IP3 signal pathway is abnormal in AD [44-46]. Therefore, it is possible that CBZ is able to alter the intracellular inositol level, and then change the autophagic activity in Tg mice. In addition, sodium valproate (VPA) and lithium, also used as mood stabilizers, have been reported to inhibit inositol synthesis and thereby decrease intracellular IP3 level [43]. A recent study has shown a protective effect of lithium in increasing survival in ALS patients and mouse models, suggesting that it is at least partially related to autophagy induction [14, 47]. Furthermore, VPA could protect against neurodegeneration in mammalian cell, drosophila and zebrafish models of Huntington's disease via an mTOR-independent autophagy pathway [48].

CONCLUSION

In summary, our study reveals that the 9-months APP^{swe}/PS1^{deltaE9} Tg mice of AD model display cognitive deficits, and three months CBZ treatment significantly improves the spatial learning and memory ability. We found that long-term CBZ administration significantly decreases the number of cerebral amyloid plaques and the levels of $A\beta_{42}$ in the Tg mice. The reduced $A\beta_{42}$ level seems unlikely the result of the alteration of Aβ-generated secretases or Aβ-degraded enzyme expression. We further showed that the autophagy is induced in Tg mice and the CBZ treatment enhances the autophagic flux in Tg mice. It seems that CBZ increasing the autophagic flux is unlikely via the mTOR-dependent auto-

phagy pathway. Our findings support the notion that enhancement in autophagic flux may be a potential therapeutic approach in AD.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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