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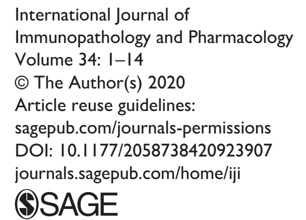
Original Research Article

Study on myelin injury of AD mice treated with Shenzhiling oral liquid in the PI3K/Akt–mTOR pathway



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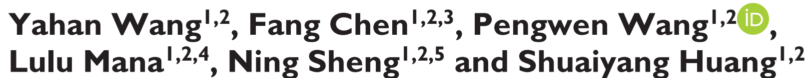

Yahan Wang[1,2], Fang Chen[1,2,3], Pengwen Wang[1,2], Lulu Mana[1,2,4], Ning Sheng[1,2,5] and Shuaiyang Huang[1,2]

Abstract

Shenzhiling oral liquid (SZL) is a Traditional Chinese Medicine (TCM) compound to be approved by the China Food and Drug Administration (CFDA) (Z20120010) for the treatment of mild-to-moderate Alzheimer's disease (AD). However, its mechanism in early AD is not clear. We studied its mechanism in protecting myelin. Three-month-old APPswe/PS1dE9double transgenic mice were used as AD model and wild-type C57BL/6 mice were used as control. After 3-month intervention, the Morris water maze was used to detect behavioural changes. Myelin mTOR pathway (PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR), myelin basic protein (MBP) and postsynaptic density protein 95 (PSD95) were detected by immunohistochemistry and western blot and reverse transcriptase polymerase chain reaction (RT-PCR). After 3 months of SZL treatment, compared with the model group (M), SZL medium-dose (SM) and SZL low-dose groups (SL) exhibited increased staying and crossing results in Morris water maze ($P < 0.05$). Compared with M, PI3K-positive cells in SM and SL groups were increased ($P < 0.01$), p-PI3K expression increased in the Donepezil group (D), SZL high-dose group (SH) and SM ($P < 0.05$); number of Akt-positive cells and Akt expression in D, SM and SL were increased ($P < 0.01$, $P < 0.05$); number of p-Akt- and mTOR-positive cells and mTOR expression in all drug-treated groups were significantly increased ($P < 0.01$); p-Akt and p-mTOR expression increased in all drug-treated groups ($P < 0.05$, $P < 0.01$); MBP expression in D and SH increased ($P < 0.05$), while in SM and SL it increased more significantly ($P < 0.01$); and PSD95 expression in D, SM and SL was increased ($P < 0.05$). RT-PCR results showed that compared with M, PI3K mRNA and Akt mRNA expression in all drug-treated groups increased, but there was no statistical difference ($P > 0.05$), mTOR mRNA expression in all the drug-treated groups increased significantly ($P < 0.01$) and MBP mRNA and PSD95 mRNA expression in D and SH increased ($P < 0.05$). SZL oral liquid could play a role in myelin protection in early AD.

Keywords

Alzheimer's disease, APPswe/PS1dE9 transgenic mice, myelin, myelin basic protein (MBP), PI3K/Akt/mTOR pathway, PSD95, Shenzhiling oral liquid

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[1]Key Laboratory of Chinese Internal Medicine of Ministry of Education and Beijing, Dongzhimen Hospital, Beijing University of Chinese Medicine (BUCM), Beijing, China
[2]Key Laboratory of Pharmacology of Dongzhimen Hospital (BUCM), State Administration of Traditional Chinese Medicine, Beijing, China
[3]Rehabilitation Department, The Hospital of Traditional Chinese Medicine Affiliated to Xinjiang Medical University, Ürümqi, China
[4]College of Chinese Medicine, Xinjiang Medical University, Ürümqi, China
[5]Oncology Department, Beijing University of Chinese Medicine (BUCM) Affiliated Zaozhuang Hospital, Zaozhuang, China

Corresponding author:
Pengwen Wang, Key Laboratory of Chinese Internal Medicine of Ministry of Education and Beijing, Dongzhimen Hospital, Beijing University of Chinese Medicine (BUCM), No. 5 Haiyuncang, Dongcheng District, Beijing 100700, China.
Email: pw_wang@163.com

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Introduction

The main characteristics of Alzheimer's disease (AD) are tau hyperphosphorylation and amyloid aggregation.[1] However, we still lack basic understanding of AD pathogenesis and have no effective medical methods to prevent or cure its deterioration. Most studies of AD have mainly focused on extracellular senile plaques and intracellular neurofibrillary tangles in the brain.[2] As research regarding myelin sheaths has progressed, loss of myelin integrity in the early stage of AD has become an important research direction.[3] In recent years, numerous studies of AD have shown that cerebral white matter damage occurs in every pathological progress period of AD.[4] Some imaging studies have demonstrated that AD patients suffer greater demyelination, and this pathology occurs before the onset of cognitive impairment.[5,6] Although Aβ and tau proteins are clearly associated with the pathogenic manifestations of AD, they also play a role as byproducts and exhibit interactive effects related to myelin integrity loss in the pathogenesis of AD.[7] Research has shown that the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway plays a vital role in cell growth, proliferation and metabolism.[8] Akt and mTOR, as important regulatory molecules of oligodendrocytes, play important roles in the formation and development of the myelin sheath.[9,10]

The double-transgenic mice used in this study co-expressed mutant PS1 and amyloid precursor protein (APP). This has always been associated with robust amyloid deposition and has been widely used to explain the pathological mechanism of AD synaptic dysfunction and memory loss.[11] Previous studies have shown that 6-month-old APPswe/PS1dE9 mice have corpus callosum atrophy, oligodendrocyte disorder and shrinkage of the corpus callosum.[12] Downregulated myelin basic protein (MBP) mRNA expression was observed in 3-month-old APPswe/PS1dE9 mice.[13] It can simulate pathological changes, such as the neurotic inflammatory plaques caused by glial cell proliferation and nutritional disorders, which comprise a subject of intense research in the field of AD.

Shenzhiling oral liquid (SZL) is a pure plant medicine, which is derived from ancient Chinese medicine, that is used to treat mild-to-moderate AD. It has yet to be determined whether SZL could improve cognitive function by providing 'upstream' therapeutic targets and restoring neural network function. Pharmacological research results may provide some treatment direction for AD, for example, ginsenoside Rb1, Rg1 and Re, the main extracts of SZL compound, could promote the formation of neuron nucleic acid and related proteins and increase the content of acetylcholine in the brain. Asarone in SZL has the effect of sedation, antioxidation and anti-inflammatory properties. Furthermore, the Tuckahoic acid in SZL can regulate immune function.[14–16] In particular, it has been demonstrated in vivo and in vitro that the SZL Traditional Chinese Medicine (TCM) formula has the effects of regulating various central neurotransmitters, inhibiting neuronal apoptosis and damage and restraining Aβ deposition and tau hyperphosphorylation.[17–20]

Our previous studies have shown that SZL can improve the spatial learning ability of APPswe/PS1dE9 transgenic mice, increase the number of synapses in the hippocampi and improve myelin structure. In OLN-93 cells damaged by streptozocin (STZ), it reversed the decrease of myelin-associated proteins. However, its effect and mechanism on the formation and repair of myelin, as well as the mTOR pathway in the APPswe/PS1dE9 transgenic mice, remain unclear.

Materials and method

Materials

Animals and groups. A total of 75 male 3-month-old APPswe/PS1dE9 transgenic mice and 15 male C57BL/6 wild-type (WT) mice weighing 22–25 g and of the same age and background were purchased from Beijing Hua Fu Kang Experimental Animal Technology Co., LTD. (Beijing, China), licence (SCXK (Beijing) 2014-0004). After 3 days of adaptive feeding, the APPswe/PS1dE9 transgenic mice were randomly grouped as follows: (1) the carboxymethyl cellulose (CMC)-treated model group (M), (2) the Donepezil-treated group (D), (3) the SZL oral liquid high-dose-treated group (SH), (4) the SZL oral liquid medium-dose-treated group (SM) and (5) the SZL oral liquid low-dose-treated group (SL). Meanwhile, the 15 WT mice were regarded as the CMC-treated WT group. All the mice were kept in the barrier environment animal room of the Chinese Medicine Pharmacology Laboratory of Dongzhimen Hospital, licence (SYXK

Table 1. Primary antibodies.

Name	Dilution for IHC-P	Dilution for WB	Code
PI3K	1:100	1:1000	Abcam 74136
p-PI3K	/	1:1000	Abcam 182651
Akt	1:1000	1:500	Abcam 8805
p-Akt	1:50	1:2000	Cst 4060
mTOR	1:100	1:1000	Cst 2983
P-mTOR	/	1:1000	Cst 5536
MBP	/	1:1000	Cst 78896
PSD95	/	1:1000	Abcam 18258
β-actin	/	1:5000	Abcam 6276

MBP: myelin basic protein; PSD: postsynaptic density protein; mTOR: mammalian target of rapamycin; IHC-P: immunohistochemistry paraffin; WB: western blot.

(Beijing) 2015-0001) in a single cage. In the experiment, the mice were fed freely and provided with drinking water. The room temperature was maintained at 20°C–22°C and relative humidity was kept at 30%–60%. The light cycle was 12 h (light 7:00–19:00; 19:00–7:00 darkness).

Drug treatment. SZL was obtained from Shandong Wohua Pharmaceutical Co. (Shandong, China) (batch number: Z20120010), which was dissolved in distilled water at the doses of 12.5, 25 and 50 g/kg/d. Donepezil was obtained from Eisai Pharmaceutical Co., LTD. Production (Jiangsu, China) (batch number: 140635), which was dissolved in distilled water at a dose of 0.92 mg/kg/d. Mice were treated continuously at a dosage of 0.1 mL/10 g/d for 3 months. WT and M group was given an equal volume of distilled water. After the 3-month intervention and behavioural testing, all the mice were sacrificed after being anaesthetised for further tests.

Antibodies and chemicals. Primary antibodies are given in Table 1.

Streptavidin–biotin complex (SABC) immunohistochemical staining kit and diaminobenzidine (DAB) colour kit were purchased from BOSTER Biological Technology Co., Ltd. (Wuhan). Standard western blot reagents, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gel preparation kit, radioimmunoprecipitation assay (RIPA) organisation/cell lysis liquid, Tris base, sodium lauryl sulphate (SDS), sulphuric acid amine (ammonium persulfate (APS)), skim milk powder and glycine were bought from Beijing Huanyataike Biomedical Technology Co., Ltd. The enhanced chemiluminescent (ECL) supersensitive substrate chemiluminescence detection kit was purchased from the SunbioBiomedical Technology (Beijing) Co., Ltd. Polyvinylidene difluoride (PVDF) membranes were purchased from the Millipore company. Reverse transcription polymerase chain reaction (RT-PCR): Trizol kit (Invitrogen), Moloney Murine Leukaemia Virus (M-MLV) reverse transcription kit (Takara), Mix (the Swiss Roche).

Method

Morris water maze. The Morris water maze was used to measure the memory retention of experimental animals and the test procedure is consistent with previous studies.[8]

Fresh tissue specimen. The mice were euthanised quickly by breaking the cervical spine, and the hippocampus tissues were dissected under an ice bath. After stripping the hippocampus tissue, the left and right sides were separated, one side of the hippocampus was placed at −80°C, the other side was placed in a cryotube and placed in liquid nitrogen for RT-PCR experiments. A total of nine mice from each group were used for analysis.

Western blot. After placing the sample protein-side up on a clean plastic film, the experimental procedures, including protein extraction, protein electrophoresis, transmembrane, hybridisation and colouration, were conducted. First, the preconfigured light-emitting detection fluid was carefully transferred to the protein membrane, and the fluid was made to cover the protein membrane uniformly through a straw. Then, the protein membrane was incubated for 1–2 min at room temperature. It was ensured that in this phase, the protein membrane was bubble free. Next, the protein membrane was fixed in the X-ray film cassette (this process was conducted in a dark room, but low light was allowed). In a closed dark room, a piece of X-ray film was placed on the packed membrane, and then the film cassette was closed and exposed for 30 s to 1 min. The next step was to develop and fix the result and then shorten or prolong the exposure time of the next X-ray film according to its exposure intensity. The acquired images were scanned and stored in the TIF format using an HP Scanjet G 4050 scanner. The results were analysed through Quantity One software. The

final result was obtained by analysing the bands of different groups and comparing the integrated greyscale of these bands with that of internal reference (β-actin) and using the β-actin ID date to unify the data of different groups to obtain the result in the form of percentage (i.e. ID/internal reference ID × 100%).

Paraffin section. After the last behavioural test, the mice were euthanised, and paraformaldehyde was perfused. Then, the fixed mice were dehydrated, paraffin-embedded and sectioned. The CA1 region of the hippocampus of each mouse brain was consecutively sectioned into slices that were approximately 4 μm thick (six mice in each group).

Immunohistochemical detection and analysis. The brain slices of the adjacent parts were immunohistochemically stained according to the manufacturer's instructions. A total of five slices of brain were selected from each mouse and were observed under 20 times magnification using a physical microscope for counting the number of positive-stained neurons in the hippocampal CA1 region. After that, the Image-pro Plus image analysis system was used to collect and analyse the images, and the number of positive cells was recorded.

RT-PCR

Total RNA extraction. The hippocampus tissues were placed in the grinding bowl, and 1 mL of TRlzol was added. The samples were ground, put into 1.5-mL Eppendorf (EP) tubes, left to stand for 5 min, then centrifuged in a low-temperature centrifuge at 12,000 r/min for 5 min at 4°C. Chloroform was added according to the ratio of chloroform/TRlzol of 1/5, and the EP tubes were tightened and shaken vigorously for 15 s. Once the centrifuge tubes were milky white, it was left at room temperature for 10 min and then centrifuged at 12,000 r/min for 15 min at a low temperature of 4°C. The solution in the EP tubes was observed to be divided into three layers, from top to bottom, a clear aqueous phase, a white protein layer and a pale pink phenolic layer. RNA was in a clear aqueous phase (about 500 μL). Four hundred microlitres of the clear aqueous phase were taken, placed in new EP tubes; an equal volume of isopropanol was added and leave them at room temperature for 20 min. RNA precipitation was observed by centrifugation at 12,000 r/min for 15 min at a low temperature of 4°C. The supernatant was discarded, 1 mL of 75% ethanol was added, the mixture was left for 5 min and then centrifuged at 12,000 r/min for 15 min at 4°C. The supernatant was discarded, being careful not to pour out the RNA pellet and use a pipette to aspirate excess liquid. The super clean bench was dried at room temperature. Thirty to –50 μL of diethyl pyrocarbonate (DPEC) water was used to dissolve the RNA in DPEC water, left at room temperature for 5 min and blown with a pipette to promote uniform dissolution. The RNA samples were stored at −80°C. The optical density (OD) value and concentration of the diluted RNA extraction were measured using a nucleic acid ultraviolet spectrophotometer to calculate the concentration of RNA.

Reverse transcription and PCR. The following reagents were added to the EP tube: RNA 1 μg, Dnasel 1 μL, 10× Dnasel buffer 1 μL. Then, ddH_2O. Diethyl pyrocarbonate (DEPC) was added to a total of 8 μL and applied at 37°C for 30 min. Then, 1 μL of ethylenediaminetetraacetic acid (EDTA) and 2 μL of random primer were added, and the cells were incubated for 10 min at 70°C for 1 min. The following reagents were added: 5× buffer 5 μL, M-MLV RT 0.5 μL, 10 nm dNTP 1 μL. A total of 25 μL of ddH_2O was added, incubated for 60 min at 42°C and incubated for 10 min at 70°C. The product cDNA was obtained and stored in a refrigerator at −20°C.

RT-PCR. The following reagents were added to the product cDNA as an RT-PCR template: ddH_2O 75 μL, cDNA 2 μL, upstream primer 0.5 μL, downstream primer 0.5 μL, SYBR mix 100 μL, and a total of 25 μL of ddH_2O was added. After mixing uniformly, the cells were briefly centrifuged below and predenatured at 94°C for 2 min. Forty cycles of amplification were performed as follows: denaturation at 94°C for 15 s, annealing at 60°C for 1 min, extension at 72°C 10 min.

Statistical analysis

All the results are presented as the mean ± SD and were analysed using SPSS 19.0 (SPSS, Inc., Chicago, IL, USA). Data were analysed statistically by one-way analysis of variance (ANOVA). Since no heterogeneity of variance was observed

for any of the parameters tested, differences among the groups were tested by the least significant difference (LSD) test. A level of $P < 0.05$ was accepted as statistically significant.

Results

SZL arrests (APPswe/PS1dE9) AD model mice cognitive deficits

To observe the effects of SZL on the memory retention of the APPswe/PS1dE9 transgenic mice, the platform was removed on the sixth day in the Morris water maze test, and the stationary time and crossing times of the mice were recorded (Figure 1). The results indicate that the mice in the M group showed typical AD-like memory ability decline. Namely, for the 120-s test session, the M group exhibited less time staying and fewer times crossing the location where the platform had been placed compared to the WT group ($P < 0.01$). Compared with the M group, the D, SM and SL groups exhibited increased staying and crossing results ($P < 0.05$). The experimental results show that SZL oral liquid repairs the cognitive impairment associated with AD.

SZL upregulated the PI3K expression

The immunohistochemical staining results showed that compared with the WT group, the number of PI3K-positive cells in the hippocampi of the M group decreased significantly ($P < 0.01$). Compared with the M group, PI3K-positive cells in the hippocampi of all these drug-treated groups were greatly increased. Specifically, the SM and SL groups were more obviously increased ($P < 0.01$).

Western blot results showed that compared with the WT group, the expression of PI3K protein in the M group was significantly reduced, but the difference was not statistically significant ($P > 0.05$). Compared with the M group, the expression of PI3K protein was increased in all the drug-treated groups (D, SH, SM, SL), but the difference was not statistically significant ($P > 0.05$).

RT-PCR results showed that compared with the WT group, the expression of PI3K mRNA in the M group was reduced, but there was no significant difference ($P > 0.05$). Compared with the M group, the expression of all the drug-treated groups increased, but there was no statistical difference ($P > 0.05$) (Figure 2).

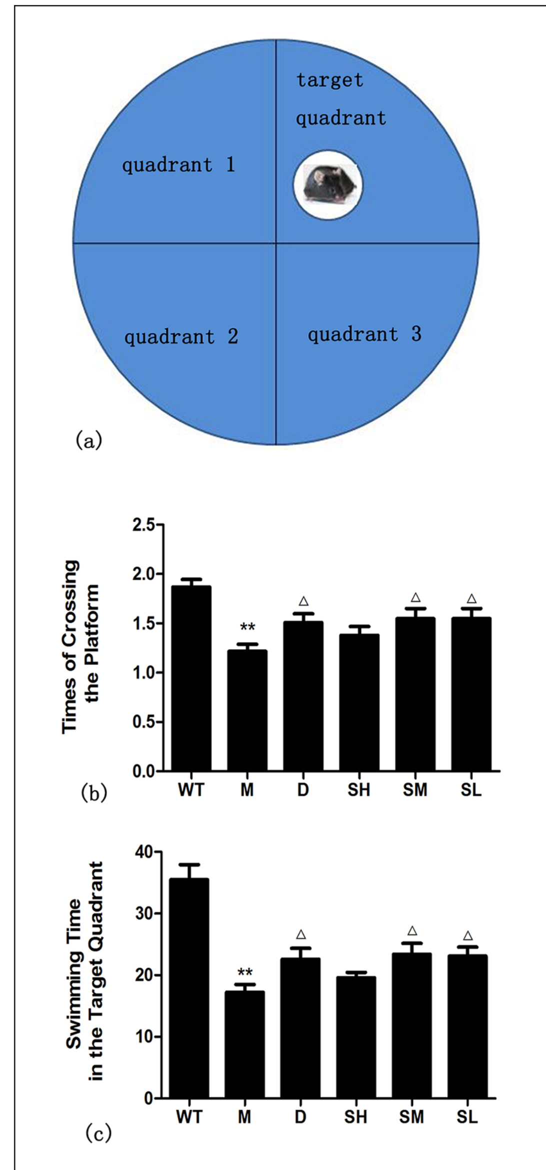

Figure 1. The effect of SZL on the spatial memory retention of APP/PS1 mice (n = 90): (a) the Morris water maze is divided into four quadrants, and the white circle is the platform, (b) recording the times of crossing platform in all experimental mice and (c) recording the swimming time in the target quadrant.
$^*P < 0.05$, $^{**}P < 0.01$ versus wild-type (WT) group; $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ versus model (M) group.

SZL oral liquid upregulated the Akt expression

Immunohistochemical staining results showed that the number of Akt-positive cells decreased significantly in the M group compared with the WT group ($P < 0.01$). Compared with the M group, the number of hippocampal Akt-positive cells in the mice of the D, SM and SL groups were increased ($P < 0.01$).

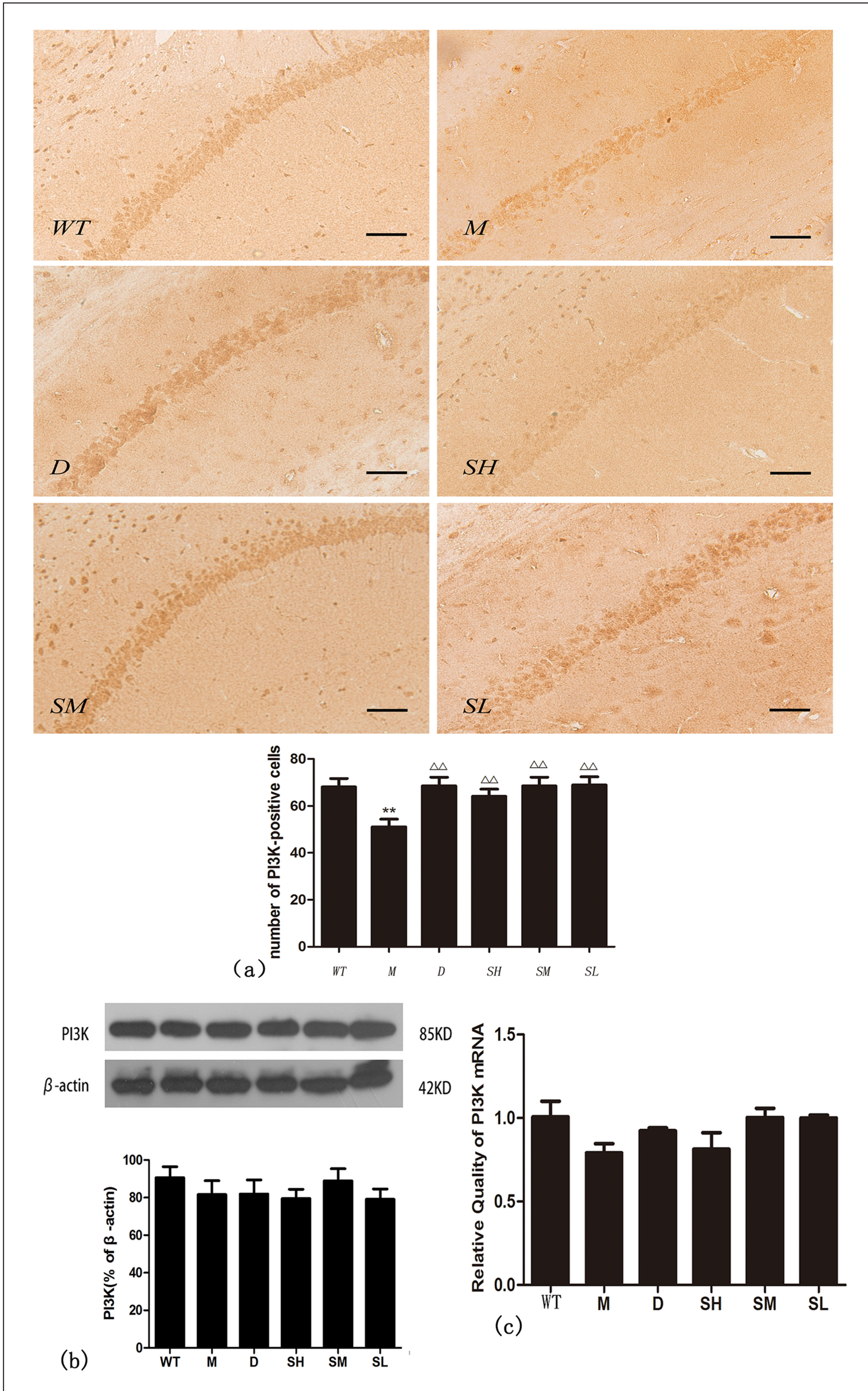

Figure 2. *(Continued)*

Figure 2. Immunohistochemistry, western blot and RT-PCR results of PI3K mRNA in 6-month-old mice (scale bar = 100 μm) (n = 6): (a) compared with the wild-type (WT) group, the number of PI3K-positive cells in the hippocampi of the model (M) group decreased significantly (**$P < 0.01$). Compared with model (M) group, PI3K-positive cells in the hippocampi of all these drug-treated groups were greatly increased (△△$P < 0.01$). Among them, the SM and SL groups were more obviously increased, (b) as western blot shows, there was no significant difference in PI3K expression level between these six groups, but it was still obvious that compared with the wild-type (WT) group, the PI3K expression in the model (M) group was reduced. Compared with the model (M) group, the PI3K expression was increased in all these drug-treated groups ($P > 0.05$) and (c) compared with the wild-type (WT) group, the expression of PI3K mRNA in the model (M) group was reduced, but there was no significant difference ($P > 0.05$). Compared with the model (M) group, the expression of all the drug-treated groups increased, but there was no statistical difference ($P > 0.05$).

Western blot results showed that Akt protein expression was significantly decreased in the M group compared with the WT group ($P < 0.05$). Compared with the M group, Akt protein expression of all the drug-treated groups was increased, of which the D, SM and SL groups were more significant ($P < 0.05$).

RT-PCR results showed that compared with the WT group, the Akt mRNA expression in the M group was significantly reduced, but there was no significant difference ($P > 0.05$). Compared with the M group, the expression of Akt mRNA in all the drug-treated groups increased, but there was no statistical difference ($P > 0.05$) (Figure 3).

SZL oral liquid upregulated mTOR expression

The immunohistochemical staining results showed that the mTOR-positive cells in the hippocampus of the M group were significantly decreased compared with the WT group ($P < 0.01$). Compared with the M group, the number of mTOR-positive cells in the hippocampi of all the drug-treated groups was significantly increased ($P < 0.01$).

Western blot results showed that mTOR protein expression was significantly reduced in the M group compared with the WT group ($P < 0.01$). Compared with the M group, the expression of mTOR was significantly increased in all the drug-treated groups ($P < 0.01$).

RT-PCR results showed that compared with the WT group, the expression of mTOR mRNA in the M group was significantly decreased ($P < 0.01$). Compared with the M group, the mTOR mRNA expression in all the drug-treated groups increased significantly ($P < 0.01$) (Figure 4).

SZL oral liquid improved the expression of p-PI3K, p-Akt and p-mTOR

Western blot results showed that compared with the WT group, the p-PI3K, p-Akt and p-mTOR expression in the hippocampi of the M group was decreased ($P < 0.05$, $P < 0.01$); compared with M group, p-PI3K increased in the D, SH and SM groups ($P < 0.05$); the expression of p-Akt in D, SH, SM and SL groups increased significantly ($P < 0.01$); p-mTOR expression in all the drug-treated groups increased significantly ($P < 0.01$) except for the SH group ($P < 0.05$) (Figure 5).

The immunohistochemical staining results showed that the p-Akt-positive cells in the hippocampi of the M group were significantly decreased compared with the WT group ($P < 0.01$). Compared with the M group, the number of p-Akt-positive cells in the hippocampi of all the drug-treated groups was significantly increased ($P < 0.01$) (Figure 6).

SZL oral liquid improved MBP expression

Western blot results showed that compared with the WT group, the MBP expression in the hippocampi of the M group was decreased significantly ($P < 0.01$). Meanwhile, compared with the M group, MBP expression in the D and SH groups was increased ($P < 0.05$), while in the SM and SL groups, it increased more significantly ($P < 0.01$).

RT-PCR results showed that compared with the WT group, the relative quantity of MBP mRNA in the M group decreased ($P < 0.05$), while in the D and SH groups, it increased ($P < 0.05$) (Figure 7).

SZL oral liquid improved PSD95 expression

Western blot results showed that compared with the WT group, the PSD95 expression in the hippocampi of the M group was decreased ($P < 0.05$). Meanwhile, compared with the M group, PSD95 expression in the D, SM and SL groups was increased ($P < 0.05$).

RT-PCR results showed that compared with the WT group, the relative quantity of MBP mRNA in

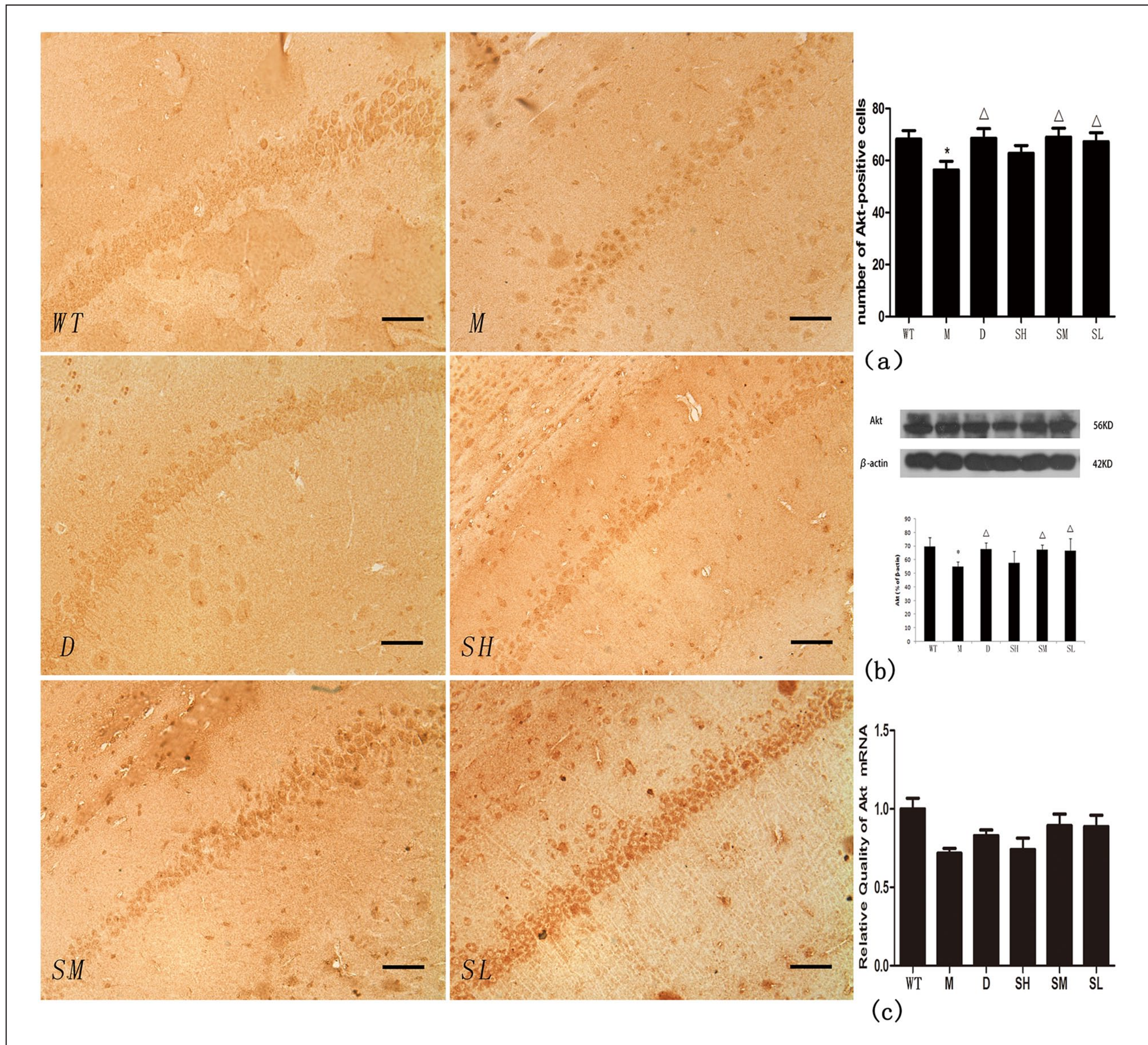

Figure 3. Immunohistochemistry, western blot and RT-PCR results of Akt mRNA in 6-month-old mice (ruler = 100 µm) (n = 6): (a) the number of Akt-positive cells decreased significantly in the model (M) group compared with the wild-type (WT) group (*$P < 0.05$). Compared with the model (M) mice, the number of hippocampal Akt-positive cells in the Donepezil (D), SZL medium-dose and SZL low-dose groups (SM, SL) was increased (△$P < 0.05$), (b) western blot results were consistent with the tendency in immunohistochemistry and (c) compared with the wild-type (WT) group, the Akt mRNA expression in the model (M) group was significantly reduced, but there was no significant difference ($P > 0.05$). Compared with the model (M) group, the expression of Akt mRNA in all the drug-treated groups increased, but there was no statistical difference ($P > 0.05$).

the M group decreased ($P < 0.05$), while in the D and SH groups, it increased ($P < 0.05$) (Figure 7).

Discussions

Our research demonstrated a significantly decreased cognitive function in the 6-month-old APPswe/PS1dE9 transgenic mice through the Morris water maze test, which was opposite to that observed in the WT group. Meanwhile, the expression of PI3K, Akt, mTOR, p-PI3K, p-Akt and p-mTOR in 6-month-old APPswe/PS1dE9 transgenic mice showed a decreasing trend, highlighting the relationship between the PI3K/Akt–mTOR pathway and cognitive disorder. Myelin injury may play a key role in early cognitive impairment of AD. Myelin is the basis for maintaining rapid electrical signalling along the axons and normal functioning

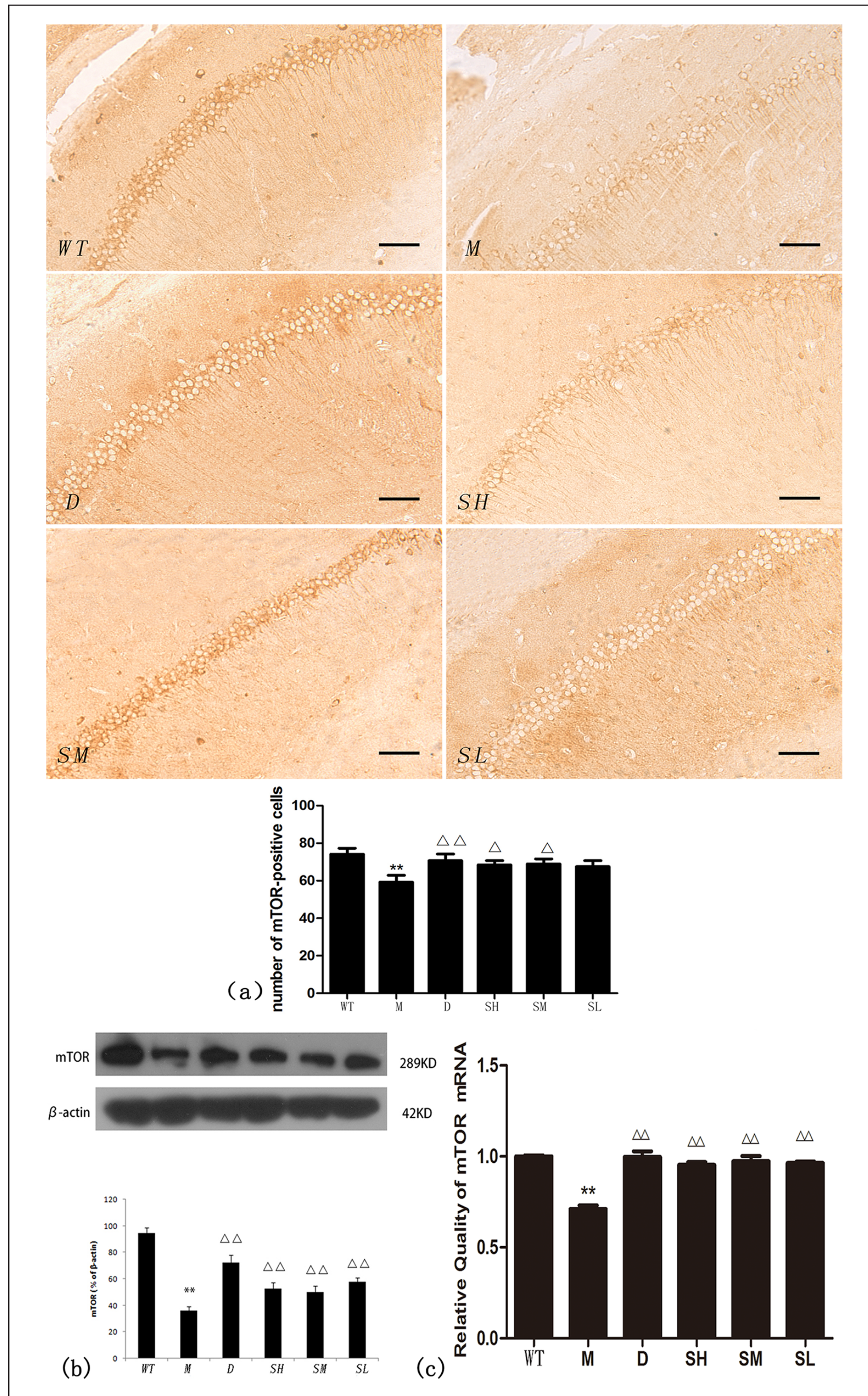

Figure 4. Immunohistochemistry, western blot and RT-PCR results of mTOR mRNA in 6-month-old mice (ruler = 100 μm) (n = 6): (a) mTOR-positive cells in the hippocampi of the model (M) group were significantly decreased compared with the wild-type (WT) (**$P < 0.01$). Compared with the model (M) mice, the number of mTOR-positive cells in Donepezil (D), SZL high-dose and SZL medium-dose (SH and SM) groups were significantly increased (△△$P < 0.01$, △$P < 0.05$), (b) in the western blot results, compared with the wild-type (WT) group, the expression of mTOR mRNA in the model (M) group was significantly decreased (**$P < 0.01$). Compared with the model (M) group, the expression of mTOR in all these drug-treated groups was increased significantly (△△$P < 0.01$) and (c) compared with the wild-type (WT) group, the expression of mTOR mRNA in the model (M) group was significantly decreased (**$P < 0.01$). Compared with the model (M) group, the mTOR mRNA expression in all the drug-treated groups increased significantly (△△$P < 0.01$).

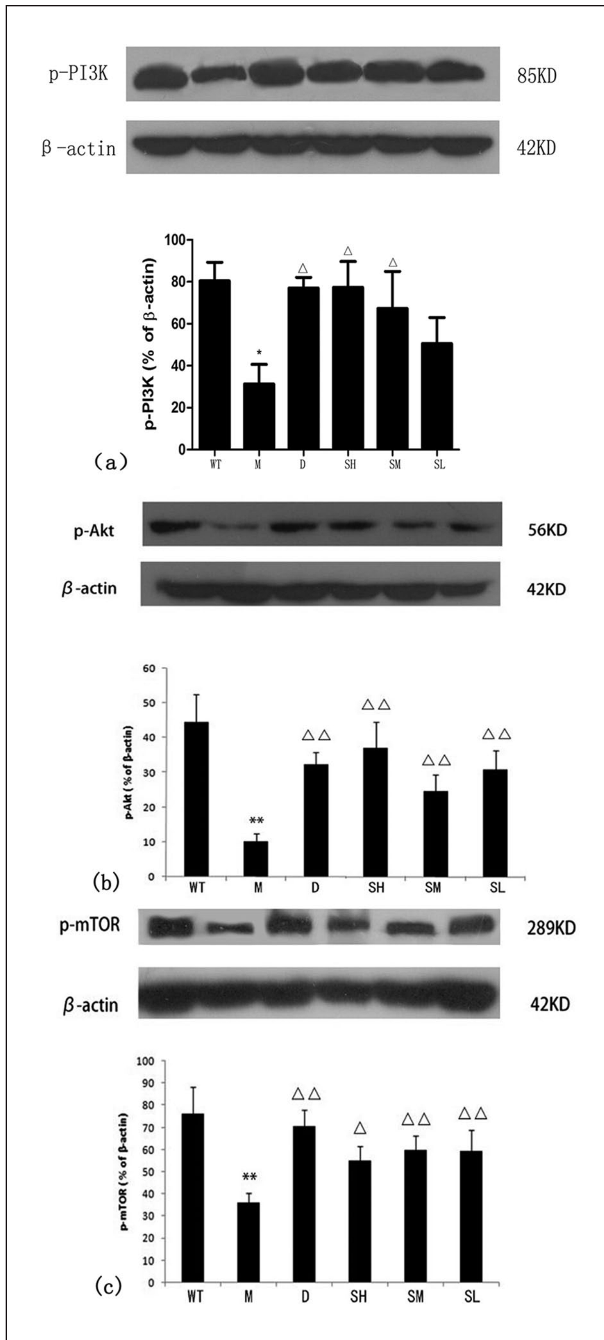

Figure 5. Western blot results of p-PI3K, p-Akt and p-mTOR in 6-month-old mice (n = 6): (a) western blot results of p-PI3K, (b) western blot results of p-Akt and (c) western blot results of p-mTOR.
** denotes statistical significance compared with wild-type (WT) group ($P < 0.01$); * denotes statistical significance compared with wild-type (WT) group ($P < 0.05$); △△ denotes statistical significance compared with the model (M) group ($P < 0.01$); △ denotes statistical significance compared with the model (M) group ($P < 0.05$).

of the nervous system. Studies have found that myelin injury initiates a signal transduction pathway for corresponding myelin repair. If the repair fails, the axons continue to swell, the rapid bidirectional axonal transport stops and the axon terminals and synapses cannot obtain the neurotransmitters and other substances required for metabolism. The neuronal cell bodies cannot obtain the neurotrophic factors to maintain the survival of the neurons, resulting in synaptic loss, axonal degeneration and neuronal loss. The ability of the brain circuit to support high-frequency action potentials is reduced, impeding the triggering and formation of synaptic long-term potentiation (LTP). Therefore, myelin theory suggests that myelin maintenance and repair of the intrinsic phenotype is the upstream mechanism that initiates the pathophysiology of AD, which triggers the production of Aβ and highly phosphorylated tau, both of which are by-products of the myelin repair process. The PI3K pathway is a downstream pathway for many growth factors that regulate the development of oligodendrocyte cells (OCs). The central nervous system myelination requires proliferation and differentiation of OC precursor cells and mature OCs surrounding axons. Akt and mTOR as downstream of the PI3K pathway are important regulators of OCs and myelination. The PI3K/Akt–mTOR signalling pathway plays an important role in the myelination of the central nervous system, while in our observation of the relationship between SZL and the PI3K/Akt/mTOR pathway, the expression level of PI3K, Akt, mTOR and their phosphorylation forms in the SZL-treated APPswe/PS1dE9 transgenic mice was significantly higher than that in the M group. This result suggests that the effect of SZL oral liquid on repairing the cognitive impairment of AD may be partly or completely realised through the PI3K/Akt/mTOR pathway.

As a member of the phosphatidylinositol-3-OH kinase (PI3K) family, mTOR is one of the important target proteins downstream of PI3K/Akt. There are two different multiprotein complexes, mTORCl and mTORC2 in intracellular mTOR. mTORC1 is an important regulator of protein synthesis, which regulates translation initiation and ribosome biosynthesis by phosphorylating p70S6K and 4E-BP1.[21] mTOR regulates the expression of myelin proteins in the central nervous system.[22] In the Plp-AKT-DD transgenic mouse OCs, the activation of mTOR was observed, and the expression of myelin-associated protein was increased. When the mTOR signalling pathway was blocked, the expression of myelin-related protein was decreased and sheath formation was also significantly reduced.[21]

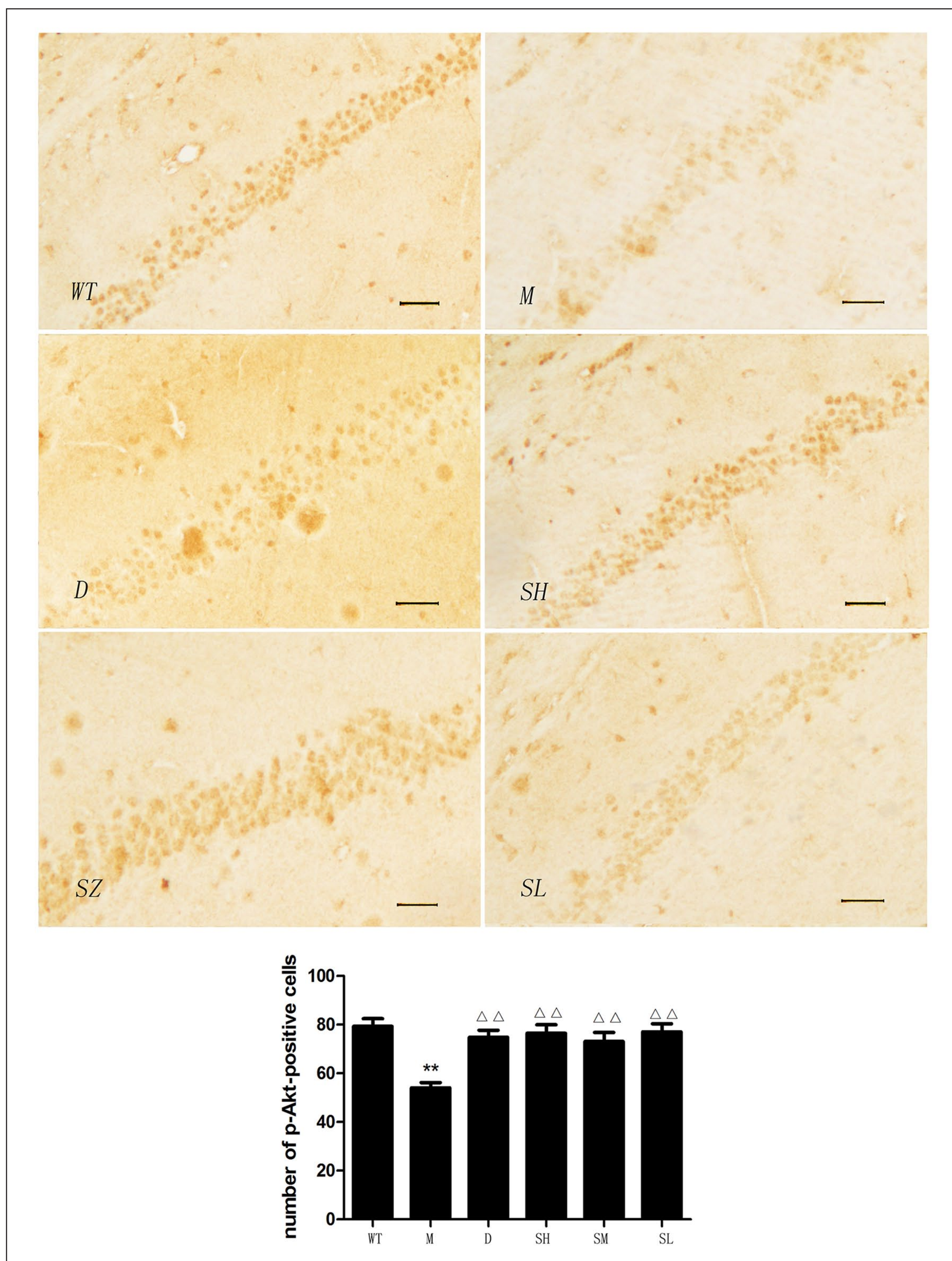

Figure 6. Immunohistochemical staining results of p-Akt-positive cells in 6-month-old mice (ruler = 100 μm) (n = 6).
** denotes statistical significance compared with wild-type (WT) group ($P < 0.01$); △△ denotes statistical significance compared with the model (M) group ($P < 0.01$).

To conduct further research regarding the relationship between the cognition protective effects of SZL oral liquid and myelin damage, we observed the amount of myelin basic protein (MBP) and post-synaptic density protein 95 (PSD95). Myelin sheaths are composed of MBP and acidic lipid structure,

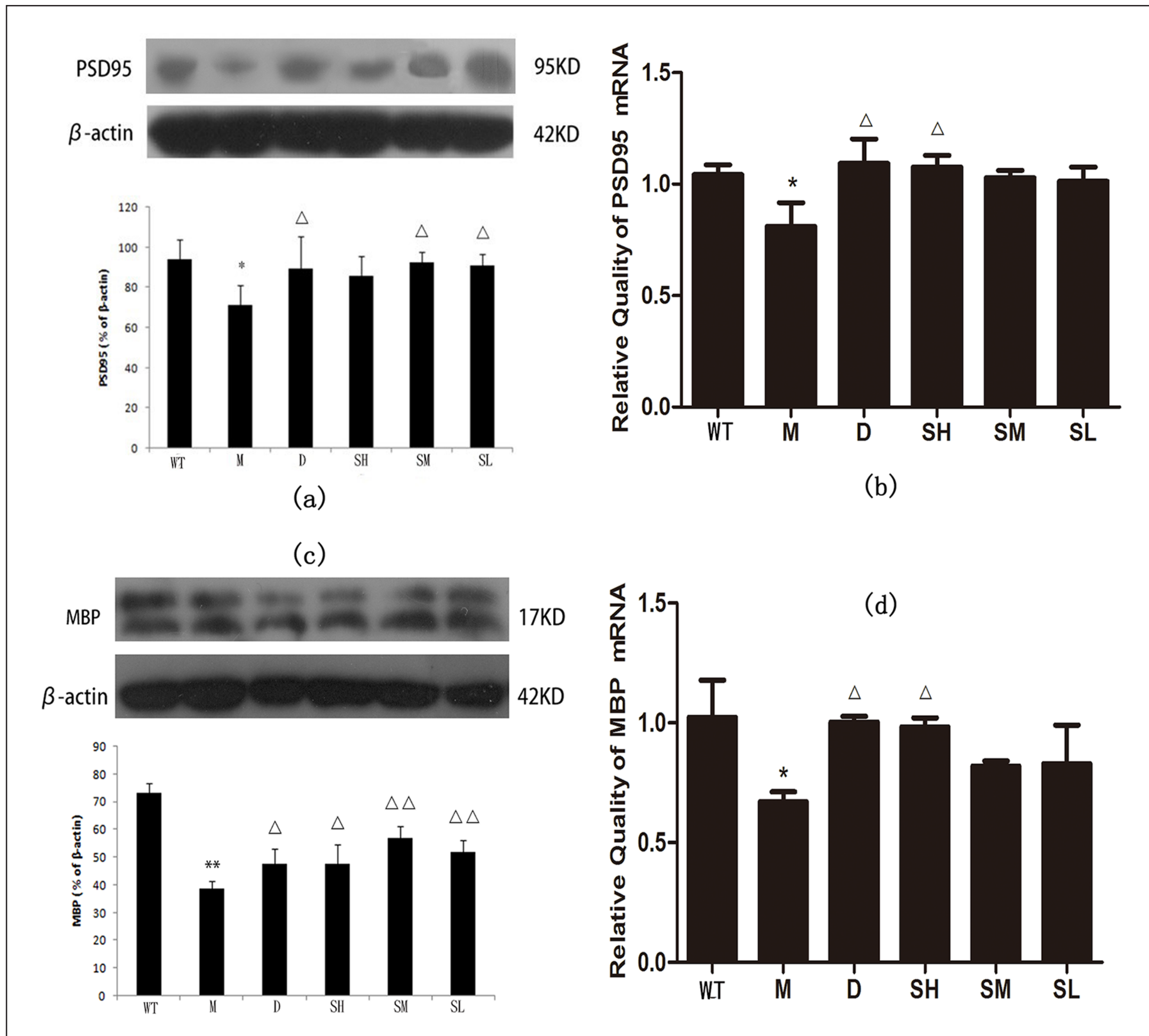

Figure 7. Western blot and RT-PCR results of MBP and PSD95 in 6-month-old mice (n = 6): (a) western blot result of PSD95 in 6-month-old mice. Compared with the wild-type (WT) group, the expression of PSD95 in the model (M) group decreased (**P* < 0.05). Compared with the model (M) group, the expression of PSD95 in all these drug-treated groups were increased except SZL high-dose (SH) group (Δ*P* < 0.05), (b) RT-PCR result of PSD95 mRNA showed that compared with the wild-type (WT) group, the expression of PSD95 mRNA in the model (M) group decreased (**P* < 0.05). Compared with the model (M) group, the expression of PSD95 mRNA in the Donepezil (D) and SZL high-dose (SH) group were increased (Δ*P* < 0.05), (c) western blot results of MBP showed that compared with the wild-type (WT) group, the expression of MBP in the model (M) group decreased significantly (***P* < 0.01). Compared with the model (M) group, the expression of MBP in all drug-treated groups increased (ΔΔ*P* < 0.01, Δ*P* < 0.05) and (d) RT-PCR result of MBP mRNA showed that compared with the wild-type (WT) group, the expression of MBP mRNA in the model (M) group decreased (**P* < 0.05). Compared with the model (M) group, the expression of MBP mRNA in the Donepezil (D) and SZL high-dose (SH) group increased (Δ*P* < 0.05).

which are secreted by mature OCs. Myelin sheaths make a type of steady membranous lamellar structure that wraps the axon and insulates nerve fibres so that proper and fast nerve signal transmission is guaranteed. MBP, the critical component of the central nerve medullary sheath membrane, is synthesised and secreted by OCs. Due to its characteristic of neural tissue specificity, MBP is regarded as an important indicator of the degree of damage of the myelin sheath in cerebral white matter. Destruction of the mTORC1 function affects the levels of various myelin proteins. In particular, MBP was significantly reduced, but there was no significant change in MBP mRNA levels.[23] However, in our study, the expression of both MBP and MBP mRNA decreased in the APPswe/PS1dE9 transgenic mice. After 3-month treatment of Donepezil and SZL, their expression levels increased in different degrees.

PSD95 is an important regulatory protein of synaptic structure and synaptic plasticity, which

promotes the formation of synaptic and polycranial spines and is involved in the regulation of *N*-methyl-D-aspartate (NMDA) receptor expression.[24] NMDA receptors play an important role in LTP, synapse formation and memory formation.[25] The expression of PSD95 in the brain of APP transgenic mice and AD and mild cognitive impairment (MCI) patients is reduced, which reduces the motility of the spine and leads to dysfunction of glutamate receptors, which is closely related to the pathological changes of AD.[26] Studies have also found that PSD95 can act on K+ channels and plays a role in myelin compaction.[27] A series of studies have found that there is a common working mechanism of myelination and synaptic plasticity. PSD95 as a synapse-related factor can affect the repair after myelin injury. This study showed that the MBP and PSD95 content of the M group decreased with the down-regulated progress of the PI3K/Akt/mTOR pathway, which was consistent with the results of the behaviour test, which showed that cognitive function in the M group was significantly decreased. After 3 months of treatment using SZL, all of the MBP, PSD95 content and the PI3K/Akt/mTOR pathway-related proteins of mice showed an increasing trend, consistent with that of the D group. One study suggested that SZL reduced Aβ amyloidosis by affecting oxidative stress to play a therapeutic role in AD,[28] while in this study, SZL oral liquid could repair cognitive impairment, improve MBP and PSD95 levels and promote the repair of demyelination. These neuroprotective effects may be closely related to the PI3K/Akt/mTOR pathway.

Authors' note

Yahan Wang is also affiliated with Neurology Department, Shandong Hospital of Traditional Chinese Medicine, Jinan, Shandong, China.

Declaration of conflicting interests

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Standard protocol on approvals, registrations, animal protection

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ORCID iD

Pengwen Wang https://orcid.org/0000-0003-0132-3955

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