Spatial training preserves associative memory capacity with augmentation of dendrite ramification and spine generation in Tg2576 mice

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Alzheimer’s disease (AD) is the most common neurodegenerative disorder characterized clinically by spatial memory loss in early stage of the onset. Accumulation of β-amyloid and hyperphosphorylated microtubule-associated protein tau are the major pathological hallmarks in the AD brains. The mechanisms underlying neurodegeneration are not fully elucidated, and there is currently no proven, effective disease-modifying therapy for this devastating disorder.

Low education increases risk for AD, and cognitive stimulating therapy improves the memory functions with the same efficacy as galantamine or tacrine1. Studies also show that participation in cognitively stimulating activities, such as reading, is associated with a reduced risk of dementia2,3 and a reduced risk of amnestic mild cognitive impairment (aMCI)4,5. Different types of cognitive training programs can also improve the cognitive function in elder population6,7. These studies strongly suggest that cognitive stimulation can preserve memory capacities in AD and aMCI patients. However, the neurobiological mechanisms underlying the training-induced memory improvement are largely unknown.

Morris water maze training was reported to reduce the amyloid load and tau hyperphosphorylation with improvement of memory in 3 × Tg-AD mice8. Although the amyloid plaques and tau hyperphosphorylation/neurofibrillary tangle formation are the primary characteristics of pathology in AD, these pathological changes are not strongly correlated with the cognitive decline of the patients9,10. On the other hand, the synaptic strength, which is influenced by dendrite ramification and spine generation/plasticity, play a crucial role in learning and memory11–13. Loss of synapse and spine in various brain areas strongly correlates with the clinical scores of dementia of AD14–15. The dendrite ramification and spine generation are regulated by the expression of post-
synaptic proteins, such as postsynaptic density protein 93 (PSD93), postsynaptic density protein 95 (PSD95), and calcium/calmodulin-dependent protein kinase II (CaMKII)16–19.

In the present study, we trained Tg2576 mice, a widely used AD-like model that shows memory deficits at 8 m old. The mice were received 6 consecutive days training in Morris water maze (MWM) given at 8 m, then the effects of spatial training on the memory capacity were measured using contextual fear conditioning. The freezing response during the fear training (B), the retrieval test of freezing response measured at 24 h after fear training (recent memory) (C), the retrieval test of freezing response measured at 28 days after fear training (remote memory) (D), quantitative analyses of average freezing response over the 3 min session (E). (f) The activity during the initial 3 min exposure to the conditioning chamber. Not trained wild type littermates (N-wt); not trained Tg2576 control mice (N-Tg2576); the trained Tg2576 mice (T-Tg2576). Data were expressed as mean ± SD (n = 10 ~ 12 each group). One-way ANOVA followed by post hoc tests (LSD) was used. *p < 0.05; **p < 0.01 vs N-wt; #p < 0.05; ##p < 0.01 vs N-Tg2576. (c) N-Tg2576 vs N-wt: p1 = 0.002, p2 = 0.004, p3 = 0.023; T-Tg2576 vs N-Tg2576: p1 = 0.006, p2 = 0.007, p3 = 0.030; (d) N-Tg2576 vs N-wt: p1 = 0.033, p2 = 0.006, p3 = 0.002; T-Tg2576 vs N-Tg2576: p1 = 0.012, p2 = 0.031, p3 = 0.001; (e) N-Tg2576 vs N-wt: n0 = 0.002, n1 = 0.010; T-Tg2576 vs N-Tg2576; n0 = 0.039, n1 = 0.017.

Figure 1 | Spatial training ameliorates hippocampus-dependent memory deficits in Tg2576 mice. (a) Schematics show experimental procedure: Tg2576 mice received 6 days Morris water maze (MWM) training at ~8 m. Then, the recent and remote memory was retrieved at 24 h or 28 days by contextual fear conditioning. (b–e) Contextual fear conditioning: The freezing response during the fear training (B), the retrieval test of freezing response measured at 24 h after fear training (recent memory) (C), the retrieval test of freezing response measured at 28 days after fear training (remote memory) (D), quantitative analyses of average freezing response over the 3 min session (E). (f) The activity during the initial 3 min exposure to the conditioning chamber. Not trained wild type littermates (N-wt); not trained Tg2576 control mice (N-Tg2576); the trained Tg2576 mice (T-Tg2576). Data were expressed as mean ± SD (n = 10 ~ 12 each group). One-way ANOVA followed by post hoc tests (LSD) was used. *p < 0.05; **p < 0.01 vs N-wt; #p < 0.05; ##p < 0.01 vs N-Tg2576. (c) N-Tg2576 vs N-wt: p1 = 0.002, p2 = 0.004, p3 = 0.023; T-Tg2576 vs N-Tg2576: p1 = 0.006, p2 = 0.007, p3 = 0.030; (d) N-Tg2576 vs N-wt: p1 = 0.033, p2 = 0.006, p3 = 0.002; T-Tg2576 vs N-Tg2576: p1 = 0.012, p2 = 0.031, p3 = 0.001; (e) N-Tg2576 vs N-wt: n0 = 0.002, n1 = 0.010; T-Tg2576 vs N-Tg2576; n0 = 0.039, n1 = 0.017.

Results
Spatial training preserves the recent and remote memory capacities in Tg2576 mice. Tg2576 mice were trained for 6 consecutive days (T-Tg2576), while the control Tg2576 (N-Tg2576) and the non-Tg littermates (N-wt) were handled with swimming activity but without training (Fig. 1a). The three groups of mice had experienced same swimming time in the maze, and no significant difference in swimming distances was detected (Fig. S1), suggesting

Figure 2 | Spatial training potentiates LTP. (a) Input-output curve was similar in the untrained and trained Tg2576 mice. (b, c) The normalized fEPSP slope at the medial perforant path to granule cells synapse in ~9 m old Tg2576 mice. Data were expressed as mean ± SD (n = 3 ~ 4 each group), Student’s t test was used. (c) $p = 0.0004$.

In the present study, we trained Tg2576 mice, a widely used AD-like model that show memory deficits at 8 m old. The mice were received 6 consecutive days training in Morris water maze (MWM) given at ~8 m, then the effects of spatial training on the memory capacity were measured using contextual fear conditioning. We found that spatial training significantly improved the subsequent memory acquisition and maintenance in the mice. This cognitive improvement was associated with the enhanced long term potentiation (LTP) and the CaMKII-associated remodeling of dendritic plasticity.
Spatial training enhances hippocampal LTP. By using electrophysiological recording in dentate gyrus (DG) granule cells in acute hippocampal slices, we found that spatial training did not change the baseline synaptic response evaluated by input/output (I/O) curves of field excitatory postsynaptic potentials (fEPSP) slope in Tg2576 mice (Fig. 2a). However, during the second half of baseline recordings co-terminating with the end of high frequency stimulation, a significantly augmented LTP was detected in the trained groups compared with the not-trained Tg2576 mice (Fig. 2b and c), suggesting an increased synaptic transmission induced by spatial training.

Spatial training remodels dendritic complexity with increased spine density and plasticity. To explore the mechanisms underlying the training-induced preservation of memory capacity, we measured the dendritic arbors in hippocampal DG and CA1 regions by using Golgi impregnation and concentric circle analysis. We found that the untrained Tg2576 mice had shorter dendrite length and fewer dendritic crossings in both DG and CA1 compared with the age-matched non-tg littermates (Fig. 3b, c, e–h). Spatial training significantly increased the length and the number of dendritic branches extending beyond 75–175 μm from the cell soma in DG (Fig. 3a–c). In CA1 pyramidal neurons, the increased dendritic branches extending beyond 50–100 μm (basal) or 75–125 μm (apical) from the cell soma were also detected in trained Tg2576 mice (Fig. 3d–h).

We further refined the analysis by investigating whether spatial training affects the formation of dendritic spines which indirectly reflect the presence of synaptic inputs. By Golgi staining and morphological analysis, we found that the spine density in DG and CA1 neurons was significantly reduced in ~9 m-old Tg2576 mice compared with the age-matched non-tg littermates (Fig. 4a–f). After spatial training, the spine number in primary and secondary branches of CA1 and DG neurons were significantly increased in Tg2576 mice (Fig. 4a–f). By dividing the spines into two categories based on the head and neck size, we observed that both the thin and mushroom spines were increased in DG neurons after training. More interestingly, the number of thin spines was respectively 2.5- and 2.3-fold of the mushroom spines in Tg2576 (Fig. 4g), which supports the conclusion of increased post-synaptic plasticity induced by spatial training.

Spatial training increases the expression of GluA1, PSD93 and PSD95 with a significant upregulation of phosphorylated CaMKII. To explore the molecular mechanisms underlying the spatial training-induced enhancement of synaptic structure and function, we measured the levels of several synaptic proteins by Western blotting. The results showed that spatial training induced upregulation of postsynaptic proteins glutamate AMPA receptor GluA1 subunit (GluA1), PSD93 and PSD95 with no obvious effects on the levels of vesicle associated membrane protein 2 (Vamp2), synaptophysin (Syn), N-Methyl-D-aspartate receptor 2A and 2B subunit (GluN2A, GluN2B) in Tg2576 mice (Fig. 5a and b).
To further explore the mechanisms underlying the training-induced upregulation of postsynaptic proteins, we measured the level and the activity-dependent phosphorylation of CaMKII, cAMP-dependent protein kinase (PKA) or extracellular signal-related kinase 1/2 (ERK1/2), which can regulate synaptic plasticity25–27. The results showed that level of the phosphorylated CaMKII at Thr286 (pCaMKII) was remarkably increased in hippocampus of Tg2576 mice after training, whereas the levels of total CaMKII, total ERK1/2 and pERK1/2, PKA catalytic subunit (PKA -cat), and PKA regulatory subunit (PKA -reg) were not changed (Fig. 5c and d). These data suggest that CaMKII activation may play a role in spatial training-induced dendrite remodeling.

Spatial training decreases the levels of Aβ and tau phosphorylation. We also observed that spatial training decreased Aβ level (Fig. 6a–c) with reduced tau phosphorylation at several AD-related sites in the hippocampus of Tg2576 mice (Fig. 6f and g). These changes in pathogenic Aβ and/or tau levels could also contribute to the improved memory by spatial training.

Discussion

In the present study, we found in Tg2576 mice that spatial training could improve recent and remote memory with enhancement of dendritic complexity and spine generation. Previous studies have shown that environmental enrichment or learning can delay the development of neuropathology or memory decline in 3×Tg-AD mice8,28, our current findings give new insights for the synaptic mechanisms underlying the spatial training-induced improvement of the cognitive functions.

Tg2576, htau and 3×Tg-AD transgenic mice have been widely used as AD models. The htau (human full length tau) transgenic mice show visuospatial learning impairment at 6 m29, abnormal neuronal morphology at 13 m and cognitive deficits in MWM at 12 m old30,31. The accumulation of the hyperphosphorylated tau in hippocampus was detected at 3 m, and paired helical filaments were observed at 9 m in htau mice. The 3×Tg-AD mice (homozygous for the PS1 mutation, APPSwe and tauP301L transgenes) show learning and memory deficits in MWM or contextual fear conditioning, formation of the extracellular Aβ plaques in the cerebral cortex and tau pathology at 6 m old32, and tangle pathology was advanced by 20 m33. The Tg2576 mice show dendrite impairments at 6 m, mild cognitive impairment at 8 m, and Aβ and tau pathologies with severe learning and memory deficits at 12 m34,35. These mice models have been widely employed for studying tau or/and Aβ pathologies. In the current study, we used 8 month old Tg2576 mice to study whether...
Spatial training could attenuate Aβ-induced neuronal dendrite and spine impairments and thus improve the cognitive ability of the mice. We found that spatial training significantly improved the memory acquisition (48 h after the last MWM training) and the maintenance (28 days after the last MWM training).

A previous study in 3 × Tg-AD mice showed that learning can significantly reduce the plaque loads in the brain. In the current study, we also observed that spatial training could reduce amyloid and tau pathologies. Although both abnormal tau and amyloid are implicated in memory deficits of AD, the direct link between these pathologies and the machineries related to cognition is still lacking. In AD patients, loss of neuronal processes is a major cause of cognitive impairment. The shape of dendritic arbor determines the total synaptic input a neuron can receive, and influences the types and distribution of these inputs. In the present study, we observed severe impairments of apical and basal dendritic arbors in hippocampus of ~9 m old Tg2576 mice, and spatial training significantly enhanced the dendrite complexity with enhanced LTP. These morphological and functional enhancements can facilitate learning and memory.

The postsynaptic spine remodeling is fundamental for memory formation. The recent and remote memory is associated with time-dependent formation of dendritic spines in the hippocampus and anterior cingulated cortex. One of the most remarkable features of dendritic spines is their morphological diversity that endows the distribution of these inputs. In the present study, we observed severe spine impairments and thus improve the cognitive ability of the mice. We found that spatial training significantly improved the memory acquisition (48 h after the last MWM training) and the maintenance (28 days after the last MWM training).

Figure 6 | Spatial training decreases Aβ level and attenuates tau phosphorylation. (a) The representative silver staining in hippocampus and the cortex of ~9 m old Tg2576 mice with and without spatial training. (b) Aβ level in hippocampal CA3 probed by 4G8 antibody. (c–e) Aβ levels in hippocampus (Hip) (c) and cortex (Cor) (d) of Tg2576 mice measured by Western blotting and quantitative analysis (e). Data were expressed as mean ± SD (n = 4 each group). Bar = 50 μm. (f, g) The representative Western blots and quantitative analyses show tau phosphorylation levels at multiple AD-related sites in ~9 m old Tg2576 mice with or without spatial training. Blot images were cropped for comparison. DM1A was used as a loading control in each sample. The optical density of bands was quantified by Gel-Pro Analyzer 4 (Toyobo, Osaka, Japan) software. Data were expressed as mean ± SD (n = 4 each group). Student’s t test was used. (e) P_{Hip} = 0.032, P_{Cor} = 0.006. (g) P_{205} < 0.001, P_{96} = 0.036, P_{231} = 0.031, P_{262} < 0.001.

Conclusion. We conclude that spatial training can improve both recent and remote memory in Tg2576 mice, and remodeling of dendrite complexity and spine generation may underlie the cognitive training-induced memory improvement.
Methods

Mice. Tg2576 mice harboring the human amyloid precursor protein 695 with Swedish double mutation (hAPP) (HuAPP695; K670N/M671L) were purchased from Jackson Laboratory and bred in the Experimental Animal Central of Tongji Medical College, Huazhong University of Science and Technology. After weaning, the mice were housed (4 to 6 mice per cage) with free access to food and water under a 12:12 hr reversed light-dark cycle, with light on at 8:00 pm. A total of 72 tg2576 mice (46 male, 26 female) and 40 wild type C57 mice were included at the beginning of the study. 2 mice died over the course of the study. All animal experiments were performed according to the “Policies on the Use of Animals and Humans in Neuroscience Research” revised and approved by the Society for Neuroscience in 1995, and the animal study was approved by the Academic Review Board of Tongji Medical College.

Spatial training paradigm. The standard Morris water maze (MWM) procedure with minor modifications was used for the spatial training50. Briefly, the mice were trained in MWM to find the hidden platform for six consecutive days and four trials per day with a 30 min interval. During the training, each mouse was placed into the water by hand, so that it faced the wall of the pool, at one of four starting positions. The animals were not allowed to search the platform for more than 60 s, after which they were guided to the platform and placed on the platform for 30 s. In each trial, the swimming path and the latency to locate the hidden platform were recorded using Noldus video tracking system (Ethovision). The control groups were handled with swimming activity but without training (i.e., the same swimming time as the training mice in the maze without the platform). At 24 hr after spatial training in MWM, one part of mice were sacrificed to execute the biochemistry experiment, electrophysiological analysis and Golgi staining, the others were used to detect memory abilities by contextual fear conditioning. All experiments were conducted and analyzed by the experimenters blind to the grouping of the animals.

Contextual fear conditioning. To test the effect of spatial training on the hippocampus-dependent associative memory, contextual fear conditioning51 was used.

During the training phase of the fear conditioning, the animals were placed in the conditioning chamber for 3 min, then were subjected to 3 min unconditioned footshocks (one shock at the first min, three shocks at the second min and 8 shocks at the third min; 0.5 mA, 2-sec duration, and 1 min apart). After the last shock, mice were remained in the chamber for 1 min, and then returned to their home cages. During the testing phase, the mice were placed back into the conditioning chamber for 3 min. At the end of each session, the mice were returned to their home cages and the chambers were cleaned with water after 70% ethanol wiping. The conditioning chamber had a plexiglass front and gray side- and back-walls (width × depth × height; 175 × 165 × 300 mm), and the chamber floors were consisted of 26 stainless steel rods with a diameter of 2 mm diameter placed 5 mm apart. The rods were connected to a shock generator via a cable harness. All experiments were conducted using a video tracking system to measure the activity and freezing behavior of the animals. Freezing was defined as a complete absence of movement, and the duration of the freezing response was scored at 1 sec after the sustained freezing behavior. For each testing session, freezing (%) was then averaged among mice within same group.

Electrophysiological analysis. Animals were anesthetized with 6% chloral hydrate, then the brain slices were cut (300 μm) in ice cold artificial cerebrospinal fluid (aCSF) after removed from skull. The composition of aCSF was (in mM): NaCl 124; KCl 3.0; MgCl2 1.0; CaCl2 2.0; NaH2PO4 1.25; NaHCO3 26; glucose 10; saturated with 95% O2, 5% CO2 (pH 7.4). Individual slices were laid down over an 8 × 8 array of planar microelectrodes, each 50 × 50 μm in size, with an interelectrode distance of 450 μm (MED-P5455; Alpha MED Sciences, Kadoma, Japan) and kept submerged in aCSF (4 ml/min; 30°C) with a nylon mesh glued to a platinum ring. Voltage signals were acquired using the MED64 System (Alpha MED Sciences). fEPSPs were obtained by stimulating the Schaeffer collateral-commissural fibers. Stimulation intensity was adjusted to evoke fEPSP amplitudes that were 80% of maximal size. Long term potentiation (LTP) was induced by applying one train of high-frequency stimulation (HFS; 100 Hz, 1 s duration at test strength).

Golgi impregnation. Golgi-Cox impregnation was performed by GD Rapid Golgi Stain Kit (FD Neurotechnologies). Briefly, at 24 hr after spatial training in MWM, the animals (n = 4 – 6 per group) were sacrificed by overdose of 6% chloral hydrate, and perfused for 5 min with PBS, followed by 4% PFA in PBS for 15 min. Brains were immersed in impregnation solution (equal volumes of Solutions A and B, containing mercuric chloride, potassium dichromate, and potassium chromate), and stored at room temperature. Impregnation solution was replaced after 24 hr. After 2 weeks, brains were transferred to Solution C and stored at 4°C for 48 hr, with the solution replaced after 24 hr. The brain was sectioned sagittally (100 μm) using a vibratome (VT 1000 s, Leica) and sections were mounted on gelatin-coated microscope slides with Solution C. Slides were rinsed twice in distilled water (2 min each), and then placed in a mixture of Solution D: E: distilled water (1:1:2) for 10 min. After rinsing with distilled water, sections were dehydrated in 50%, 75%, 95% and 100% ethanol for 4 times (4 min each). Sections were cleared in xylene three times (4 min each) and coverslipped with Permount solution.

Dendrite and spine analyses. The dendrites selected for quantitative analyses must meet the following criteria: (i) the cells must have a universal and even impregnation from the cell body to the tertiary branches; (ii) the dendrite must not be at an angle with the image plane because these tilted dendrites would introduce errors in measuring the length of the dendrite; and (iii) the dendrite must be well separated from other dendrites to avoid confusion in which spines that actually belong to other dendrites are counted52. A digital camera system and Image software were used for morphometric analysis of digitized images. Using the center of the soma as reference point, dendritic length and branch points were measured as a function of radial distance from the soma by adding up all values in each successive concentric segment (Sholl’s analysis)53. For each animal, at least 36 neurons were analyzed. Total dendritic length, number of branch points, and the number of primary dendrites were analyzed for every neuron. In addition, spine density was determined in two segments of dendrites at a distance of 90–110 μm (proximal) and 190–210 μm (distal) from the soma. When counting different types of spines, we counted mushroom-shaped spines with well formed head and neck structures vs. thin spines and filopodia-like structures54. Spines with a head diameter of at least ~0.3 μm were categorized as mushroom types54. Spine quantification was carried out by observers who were unaware of the experimental conditions.

Table 1 | Antibodies employed in the study

| Antibody | Specific | Type | WB | IH | Source |
|----------|----------|------|----|----|--------|
| pT205    | Phosphorylated tau at Thr205 | pAb | 1 : 1000 | SAB |
| pT231    | Phosphorylated tau at Thr231 | pAb | 1 : 1000 | SAB |
| pS262    | Phosphorylated tau at Ser262 | pAb | 1 : 1000 | SAB |
| pS396    | Phosphorylated tau at Ser396 | pAb | 1 : 1000 | BIOSOURCE |
| tau-5    | Total tau | pAb | 1 : 1000 | MILLIPORE |
| Vamp2    | Vesicle associated membrane protein 2 | pAb | 1 : 1000 | MILLIPORE |
| Syt1     | Total synaptophysin | mAb | 1 : 1000 | CELL SIGNALING |
| PSD95    | Total postsynaptic protein 95 | mAb | 1 : 1000 | MILLIPORE |
| PSD93    | Total postsynaptic protein 93 | mAb | 1 : 1000 | ABCAM |
| GluA1    | Total GluA1 | pAb | 1 : 1000 | MILLIPORE |
| GluN2A   | Total GluN2A | pAb | 1 : 1000 | MILLIPORE |
| GluN2B   | Total GluN2B | pAb | 1 : 1000 | MILLIPORE |
| PKA2     | PKA catalytic subunit | pAb | 1 : 1000 | SANTA CRUZ |
| PKAβ     | PKA regulatory subunit | pAb | 1 : 1000 | SANTA CRUZ |
| ERK1/2   | Total p44/42 mitogen-activated protein kinase | mAb | 1 : 1000 | CELL SIGNALING |
| pERK1/2  | Phosphorylated ERK | mAb | 1 : 1000 | CELL SIGNALING |
| CaMKII   | Total CaMKII | mAb | 1 : 1000 | CELL SIGNALING |
| pCaMKII  | Phosphorylated CaMKII at Thr286 | mAb | 1 : 1000 | SIENTET |
| 6E10     | A1–1.6 | mAb | 1 : 1000 | SIGMA |
| 4G8      | A1–17.24 | mAb | 1 : 1000 | SIGMA |
| DM1A     | tubulin | mAb | 1 : 1000 | SIGMA |

WB: Western blotting; IH: immunohistochemistry.
Western blotting. Western blotting was carried out as described previously. Mice were decapitated after the spatial memory retention test. The hippocampus was rapidly removed and homogenized at 4°C using a Teflon glass homogenizer with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM NaF, 1 mM Na3VO4, 5 mM EDTA, 2 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. The extract was mixed with sample buffer (3:1, v/v) 200 mM Tris-HCl (pH 7.6), 8% SDS, 40% glycerol, and 40 mM dithiothreitol (dithio) for 10 min, and then the mixture was stored at –80°C. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in TBS (100 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.2% Tween-20) for 1 h and probed with primary antibody at 4°C overnight. Finally, the blots were incubated with anti-rabbit or anti-mouse IgG conjugated to IRDye™ 800CW for 1 h and visualized using the Odyssey Infrared Imaging System (Licor biosciences, Lincoln, NE, USA). The antibodies used in the current study are listed in Table 1. Densitometry analysis was performed by measuring the optical densities of the targeted protein bands relative to the DMAP1 level from the same brain sample. The analysis was performed using Gel-Pro Analyzer 4 (ToyoBio, Osaka, Japan) software.

Immunohistochemistry. Animals were anesthetized with 6% Chloral hydrate, fixed in situ, and perfused through the aorta with 100 ml 0.9% NaCl, followed by 400 ml phosphate buffer containing 4% paraformaldehyde. The brain was postfixed in situ, and perfused through the aorta with 100 ml 0.9% NaCl, followed by 400 ml 4% paraformaldehyde solution. The sections were subsequently incubated with biotin-labeled horseradish peroxidase-labeled antibodies at 37°C overnight. Finally, the sections were incubated with anti-rabbit or anti-mouse IgG conjugated to IRDye™ 800CW for 1 h and visualized using the Odyssey Infrared Imaging System (Licor Biosciences, Lincoln, NE, USA). The antibodies used in the current study are listed in Table 1. Immunohistochemistry was performed by measuring the optical densities of the targeted protein bands relative to the DMAP1 level from the same brain sample. The analysis was performed using the Odyssey Infrared Imaging System (Licor Biosciences, Lincoln, NE, USA). The antibodies used in the current study are listed in Table 1. Densitometry analysis was performed by measuring the optical densities of the targeted protein bands relative to the DMAP1 level from the same brain sample. The analysis was performed using Gel-Pro Analyzer 4 (ToyoBio, Osaka, Japan) software.

Statistical analysis. The data were expressed as mean ± SD and analyzed by the one-way analysis of variance procedure followed by least significant difference post hoc tests or student’s t tests using SPSS 12.0 statistical software (SPSS Inc., Chicago, Illinois). A p value of <0.05 was considered as statistically significant in all experiments.
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Author contributions

Author contributions: G.P.L. and J.Z.W. designed research; X.J., S.G.C., Z.H.W., Y.H., X.C.L., Z.W.M. and Q.W. performed research; X.J., G.P.L. and J.Z.W. analyzed data, G.P.L. and J.Z.W. wrote the paper.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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