

# Protective effect of tetrahydroxy stilbene glucoside on learning and memory by regulating synaptic plasticity

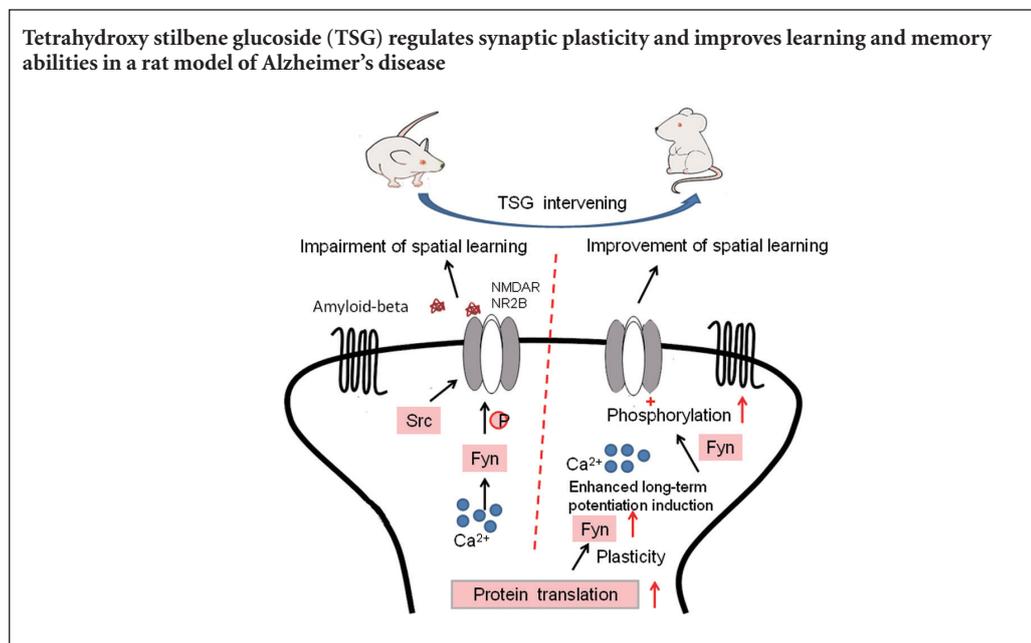
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## Graphical Abstract



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## Abstract

Damage to synaptic plasticity induced by neurotoxicity of amyloid-beta is regarded to be one of the pathological mechanisms of learning and memory disabilities in Alzheimer's disease patients. This study assumed that the damage of amyloid-beta to learning and memory abilities was strongly associated with the changes in the Fyn/N-methyl-D-aspartate receptor 2B (NR2B) expression. An APP695V7171 transgenic mouse model of Alzheimer's disease was used and treatment with tetrahydroxy-stilbene glucoside was administered intragastrically. Results showed that intragastric administration of tetrahydroxy-stilbene glucoside improved the learning and memory abilities of the transgenic mice through increasing NR2B receptors and Fyn expression. It also reversed parameters for synaptic interface structure of gray type I. These findings indicate that tetrahydroxy stilbene glucoside has protective effects on the brain, and has prospects for its clinical application to improve the learning and memory abilities and treat Alzheimer's disease.

**Key Words:** nerve regeneration; tetrahydroxy stilbene glucoside; Alzheimer's disease; amyloid-beta; cognitive impairment; learning and memory; synaptic plasticity; Fyn/N-methyl-D-aspartate receptor 2B signaling pathway; neuroprotection; neural regeneration

## Introduction

Alzheimer's disease (AD) is a common neurological disorder in which the death of brain cells causes gradual memory loss and cognitive decline (Robakis, 2014). AD affects the ability to perform everyday activities. With an unfavorable prognosis and a life expectancy of approximately 8–10 years post diagnosis, AD is becoming one of the most costly diseases for

families and society (Alzheimer's Association, 2015; Santana et al., 2015). Some studies show that incidence rates of AD may have declined in recent years (Matthews et al., 2013; Qiu et al., 2013; Langa, 2015), possibly because of the improved life-style that reduces AD risk, such as diabetes and smoking (Langa, 2015; Satizabal et al., 2016). However, no effective method or medicine is available currently (Chiang and Koo,

2014; Kumar et al., 2015). Therefore, there is a great need for an efficient treatment to be developed for this disease. It is currently held that the neurotoxicity of amyloid-beta ( $A\beta$ ) is a key factor in the occurrence and progression of AD (Querfurth and LaFerla, 2010; Allen et al., 2014; Morris et al., 2014). Damage to synaptic plasticity induced by early extracellular aggregation of  $A\beta$  is regarded to be one of the pathological mechanisms of learning and memory disabilities of AD patients (Chin et al., 2005; Calabrese et al., 2007; Lacor et al., 2007; Shankar et al., 2007; Evans et al., 2008). Fyn is a major member in the non-receptor tyrosine kinase Src family that regulates the scaffolding proteins of the N-methyl-D-aspartate (NMDA) receptor 2B (NR2B) in the post-synaptic density of the cell membrane. It plays a significant role in inducing synaptic plasticity during the learning and memorizing processes (Mucke and Selkoe, 2012; Trepanier et al., 2012; Spiro-Jones and Hyman, 2014).

Our previous research shows that tetrahydroxy stilbene glucoside (2,3,5,4-tetrahydroxy-stilbene-2-glucoside, TSG), the main active ingredient from *Polygonum multiflorum*, can help to improve the learning and memorizing abilities of AD mice, and to reduce the expression level of amyloid precursor protein (APP), Beclin-1 and LC3-II (Luo et al., 2009, 2015). Additionally, TSG has a wide range of biological functions, including anti-oxidant and anti-inflammatory properties (Zeng et al., 2011; Zhang et al., 2012; Zhang et al., 2013).

In this experiment, APP695 V7171 transgenic mice were used as AD models. We planned to observe the effect of TSG on behaviors of the mice, synaptic plasticity and phosphorylation of Fyn and NR2B in the hippocampus. The results could help reveal the main mechanism of AD and TSG might be developed as a new drug of treatment for AD.

## Materials and Methods

### Ethics statement

All animal handling and procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study was approved by the Experimental Animal Ethics Committee of Lanzhou General Hospital, Lanzhou Military Area Command, China (approval No. 2014KYLL021). Precautions were taken to minimize suffering and the number of animals used in each experiment.

### Experimental mice and group assignment

Forty clean specific-pathogen-free APP695V7171 transgenic mice aged 10 months (20 females and 20 males), and 20 specific-pathogen-free C57BL/6J mice (10 females and 10 males) at the same age and with the same background as for the normal controls, were selected for this study. The mice were all purchased from Research Center for Laboratory Animal Science, Chinese Academy of Medical Sciences in China (certification No. SCKK 20080013). The transgenic mice were equally randomized into two groups by random number table method: TSG group ( $n = 20$ ) and AD group ( $n = 20$ ). The TSG group received intragastric administration of

TSG (100 mg/kg), once a day for 30 days. TSG was dry powder extracted and separated from *Polygonum Multiflorum* Thunb., with a content of 68% (supplied by Hunan Academy of Chinese Medicine, China), and TSG was dissolved in water at a concentration of 100 mg/mL. The normal control and AD groups were intragastrically administered physiological saline for 30 days (5 mL/kg per day).

### Y-maze test

Y-maze test was implemented before and after 30 days of TSG intervention. The Y-maze system was provided by TSE Systems China Ltd., Beijing, China. The number of electric shocks was a measure of the learning and memorizing abilities, and the number of each mouse escaping the electric-stimulus was recorded, with the maximum number of 30 (O'Keefe and Dostrovsky, 1971).

### Morris water maze

The Morris water maze test was performed after 30 days of TSG intervention. The water maze system was provided by TSE Systems China Ltd.

In the navigation test, the mice were put into water to swim freely for 2 minutes on the day before the test. At a fixed time after the formal test, with the mouse facing the pool wall and without selecting the quadrant of the platform as the place of entry, the mice were put into water in a clockwise direction. The escape latency and the swimming distance from being put into water to finding and climbing onto the platform were observed and recorded (D'Hooge and De Deyn, 2001).

In the spatial probe test, the platform was removed after the last place navigation test for each group of mice. The mouse was put into water from the last place of entry, facing the pool tank, and kept swimming in the water maze. The number of times each mouse crossed the former platform within 120 seconds was recorded (D'Hooge and De Deyn, 2001).

### Ultrastructure detection

After TSG intervention for 30 days and when the behavior tests were finished, each mouse was decapitated after intraperitoneal anesthesia with 2.5 mL/kg chloral hydrate. The brain tissue was taken out. Half of each mouse's hippocampus was separated ( $-2$  mm at the anterior/posterior axis,  $\pm 1.8$  mm at the lateral/medial axis and  $-1.5$  mm at the dorsal/ventral axis) (Paxinos and Franklin, 2001), fixed with 4% glutaraldehyde, and cut into pieces after cooling. Samples were then fixed with 2% osmium tetroxide, dehydrated, soaked and embedded through a graded acetone series, and finally dual stained with uranyl acetate-lead citrate.

Observation was made in a transmission electron microscope (Hitachi Ltd., Tokyo, Japan) with three copper screens for each case and then five random shot photos magnified to 40,000 times for each copper screen. The width of synaptic cleft, thickness of postsynaptic density, chord length and arc length of the postsynaptic membrane and length of the synaptic active zone were measured with an image analyzer (Hitachi Ltd.). The synapse number, length of active zone,

curvature of synaptic interface and thickness of the postsynaptic density were also measured by a previously published method (Güldner and Ingham, 1980). The curvature of the synaptic interface was measured in accordance with an established method (Jones and Devon, 1978). A multi-point averaging method was used to measure the width of the synaptic cleft (Jones and Devon, 1978). A comparison of the percentage of concave synapses, flat synapses, convex synapses and perforated synapses between the three groups was analyzed statistically. The double-blind method was adopted in observation and measurement in the study.

### Western blot assay

The sample was sequentially centrifuged for 10 minutes at  $14,000 \times g$ , and then centrifuged for 15 minutes at  $12,000 \times g$ . After the supernatant was taken, an additional centrifugation for 30 minutes at  $12,000 \times g$  was performed. Lysate and protease inhibitor were added, and protein concentration was measured with bicinchoninic acid protein assay kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sample was mixed with buffer solution, boiled for denaturation, and separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein was transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk, and incubated with primary antibodies, including rat anti-mouse Fyn monoclonal antibody (1:1,000; Stressgen, Victoria, Canada), rat anti-NR2B polyclonal antibody (1:1,000; Stressgen), rat anti-phospho-NR2B polyclonal antibody (1:1,000, Thermo Fisher Scientific Inc., St. Louis, MO, USA) and  $\beta$ -actin (1:1,000; Abcam, Cambridge, UK). The proteins were detected using horseradish peroxidase conjugated goat anti-rat secondary antibodies (1:1,000; Abcam) and visualized using chemiluminescence reagents provided with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The intensity of blots was quantified using NIH image J software (NIH, Bethesda, MD, USA).

### Statistical analysis

The data, expressed as the mean  $\pm$  SEM, were analyzed using SPSS 12.0 software (SPSS, Chicago, IL, USA). All data were compared by one-way analysis of variance followed by *post-hoc* Scheffe's test. A value of  $P < 0.05$  was considered statistically significant.

## Results

### Effects of TSG on learning and memory abilities of AD model mice

#### *Y-maze test*

Compared with the normal control group, after 30 days, the number of electric-stimulus escapes was significantly greater in the AD and TSG groups ( $t = 10.0884$ ,  $P < 0.01$ ;  $t = 2.7781$ ,  $P < 0.05$ ). Compared with the AD group, the number of electric-stimulus escapes was significantly less in the TSG group ( $t = 11.1755$ ,  $P < 0.01$ ; **Table 1**).

#### *Morris water maze*

In the Morris water maze test, the mean swimming latency

of mice was prolonged ( $t = 14.4648$ ,  $P < 0.01$ ), the distance increased ( $t = 5.6550$ ,  $P < 0.01$ ), the time of crossing the former platform significantly decreased ( $t = 15.5895$ ,  $P < 0.01$ ) in the AD group compared with the normal control group. The latency shortened ( $t = 5.5023$ ,  $P < 0.01$ ), the swimming distance decreased ( $t = 2.2341$ ,  $P < 0.05$ ), and the time of crossing the former platform increased ( $t = 4.8163$ ,  $P < 0.01$ ) in the TSG group compared with the AD group (**Table 2**).

### Effect of TSG on synaptic ultrastructure in CA1 region of the hippocampus of AD model mice

In the normal control group, there were distinct and complete synaptic structures. The presynaptic mitochondria were normal and had distinct cristae without swelling. Typical asymmetric interfaces for synapses of gray type I (synaptic cleft approximately 20 nanometers) were visible with significantly thicker postsynaptic densities than those presynaptic. Small synaptic clefts, the existence of concave synapses, convex synapses, flat synapses and perforated synapses, with more concave synapses, were all observed.

In the AD group, unlike the normal control group, we observed unclear presynaptic mitochondrial cristae, significantly increased width of synaptic clefts, shorter synaptic active zones, thinner postsynaptic densities, significantly increased flat synapses, but significantly reduced concave synapses and perforated synapses.

The TSG group showed less damage than in the AD group. We observed distinct presynaptic mitochondrial cristae, smaller width of synaptic cleft, increased postsynaptic densities, reduced flat synapses, but significantly increased concave synapses and perforated synapses. Compared with the normal control group, we observed larger synaptic cleft width, thinner postsynaptic densities, slightly increased flat synapses, but significantly reduced concave synapses and perforated synapses in the TSG group (**Figure 1**, **Table 3**).

### Effects of TSG on protein expression of Fyn and phosphorylation of NR2B in CA1 region of the hippocampus of AD model mice

Results of western blot assay showed that, compared with the normal control group, Fyn and phosphorylation of NR2B subunit expression significantly decreased in the AD group ( $P < 0.05$ ). Compared with the AD group, the Fyn and phosphorylation of NR2B subunit expression significantly increased in the TSG group ( $P < 0.05$ ; **Figure 2**).

## Discussion

Neurotoxicity of A $\beta$  is currently considered to be a key factor in the progression of AD (Holtzman et al., 2011; Li et al., 2012; Bass et al., 2015). Therefore, many principal targets and clinical trials of the compounds have been aimed at reducing A $\beta$  formation and plaques (Castello and Soriano, 2014; Chiang and Koo, 2014; Folch et al., 2016). However, no effective medicine has been found to decrease A $\beta$  (Chiang and Koo, 2014; Kumar et al., 2015). In this study, we have shown that TSG can change the plasticity of synaptic structures and functions and Fyn-mediated NR2B phosphorylation pathway, by

increasing the expressions of NR2B receptors and Fyn and by reversing the changes associated with AD to the synaptic interface structure of type Gray I to some extent.

In this study, behavioral experiments show that mice in the AD group had significantly decreased learning, spatial orientation and working memory abilities. After TSG intervention, escape latency shortened, swimming distance decreased, and times of crossing the former platform increased in the transgenic mice. The above-described results showed that TSG relieved the impairment of learning memory, spatial memory and working memory abilities caused by A $\beta$ , and had protective effects on the brain.

The pathogenesis of AD has been investigated extensively (Liang et al., 2015). Much research shows that expressions of synapse-related proteins were high in the molecular layer of the dentate gyrus of the hippocampus and low in other areas of the hippocampus and in the neocortex during early phases of AD, which will cause abnormal synaptic structure and transmission (Mota et al., 2014). Several studies have also revealed that soluble A $\beta$  was highly correlated with memory-related synaptic dysfunctions (Parodi et al., 2010; Sivanesan et al., 2013). Some findings concluded that A $\beta$  interacts with phosphorylated tau and calcium channel (Lau et al., 2009; Hermann et al., 2013), so that A $\beta$  may damage neuronal structure and function, particularly synapses, leading to cognitive decline in AD (Manczak and Reddy, 2013). Therefore, abnormal synaptic transmission and synaptic damage at the early phase may be major causes for the progressive decline in learning and memory abilities of AD patients. The learning and memory process requires structural and functional plasticity of synaptic connections (Nabavi et al., 2014). Structural plasticity mainly includes changes in the form of the presynaptic terminal, the curvature of the synaptic interface and postsynaptic densities. Functional plasticity includes changes in synthesis and release of neurotransmitters, phosphorylation and dephosphorylation of the second messenger G protein and regulatory protein after activation of the receptor (Colón-Ramos, 2009). Synaptic plasticity is crucial in the formation of the learning and memory process (Zovkic et al., 2013; Poo et al., 2016). Our results in this study show that in the AD group compared with the normal control group, presynaptic mitochondria cristae were unclear; the postsynaptic densities were significantly thinner, the widths of synaptic clefts were significantly larger, the synaptic active zones were significantly shorter and the curvature of synaptic interface in CA1 area of the hippocampus was reduced. The percentage of flat synapses increased, but the concave and perforated synapses in CA1 area of the hippocampus were significantly reduced in the AD group. The above effects may greatly affect the synaptic transmission efficiency, causing significant learning and memory impairment to mice in the AD group. Compared with the AD group, the TSG group had clearer presynaptic mitochondria crista, significantly thicker postsynaptic density, significantly smaller width of synaptic cleft, significantly increased synaptic active zone and increased curvature of synaptic interface. The percentage of flat synapses was less,

but the distribution of concave synapses and perforated synapses in CA1 area of the hippocampus was greater than in the AD group.

The NMDA receptor 2B (NR2B) is strongly associated with synaptic plasticity (Köhr, 2006). Müller et al. (2009) considered that both NR2A and NR2B subunits, NMDA receptor, are critical for long-term potentiation. Bliss called NR2B “a smart gene” and regards it a memory gene (Bliss and Collingridge, 1993; Corlew et al., 2008).

Tyrosine phosphorylation of NR2B requires the mediation of Src protein tyrosine kinases (PTKs), which is a family of membrane-bound non-receptor protein tyrosine kinases composed of 10 types with individual gene codes (Berg et al., 2013). From current studies on non-receptor PTKs, only Fyn is closely related to learning and memory functions. Mice with Fyn inhibition show a large number of neurological defects, including lack of long-term potentiation, impaired spatial memory, synaptic loss and uncoordinated hippocampal structures (Grant et al., 1992; Kojima et al., 1997; Minami et al., 2012). Mizuno et al. (2003) confirmed that mice with behavioral training had an increased activity of Fyn in the hippocampus. Fyn can also induce long-term potentiation in CA1 region through NMDA receptors (Lu et al., 1999; Trepanier et al., 2012). Therefore, Fyn and NR2B pathways are a key link in the learning and memory impairment process. Results of this study showed that compared with the AD group, NR2B and Fyn expressions were significantly higher in the TSG group, though not at normal levels. As a result, postsynaptic density became obviously thicker, which would promote synaptic transmission and therefore the signaling complex of NMDA receptors and change the permeability of ionic NMDA receptors to monovalent cation and calcium. These would enable the formation of long-term potentiation. Thus, TSG would improve the acquisition of learning and memory in the AD transgenic mice.

Change in the phosphorylation of some enzymes and substrate proteins (such as NMDA receptor and Src) will induce change in the molecular configuration, resulting in thicker or thinner postsynaptic densities (Li and Ju, 2012). Thicker postsynaptic density and smaller synaptic cleft will allow easier transmission of nerve impulse (Petzoldt and Sigrist, 2014). Conversely, if the synaptic cleft becomes wider, and the postsynaptic density becomes thinner, transmission of nerve impulses becomes more difficult. This is consistent with the significantly thinner postsynaptic density and wider synaptic cleft in the CA1 region of the hippocampus in mice in the AD group. Jones and Calverley (1991) have observed and demonstrated under different conditions that the curvature of synaptic interface is associated with different functionality of the synapses. Increased curvature of the synaptic interface indicates better synaptic transmission, while the reduced curvature of synaptic interface indicates poorer synaptic transmission and long-term potentiation is less probable. The change in percentage of flat synapses and concave synapses among the three types of synapses is also a key factor affecting the synaptic transmission efficiency. Concave

**Table 1 Effects of TSG on learning and memory abilities of AD model mice in Y-maze test**

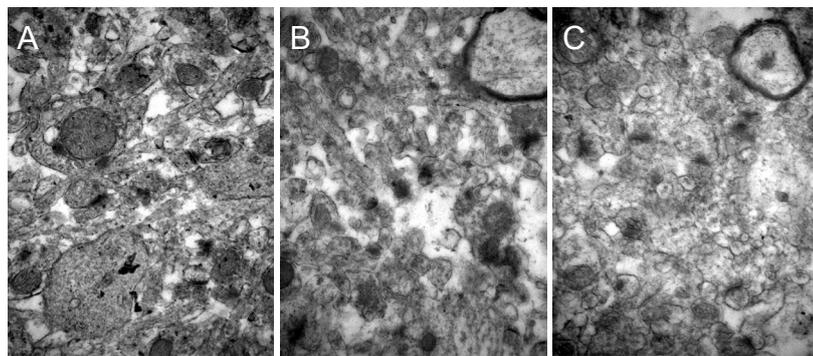
Group	Before modeling	30 days after TSG intervention
Normal control	16.94±2.56	17.03±2.18
AD	16.09±4.35	28.16±3.46*
TSG	16.01±2.03	20.08±2.24*#

The results were expressed as number of shocks. Data are expressed as the mean ± SD (n = 20). All data were compared by one-way analysis of variance followed by *post-hoc* Scheffe's test. \*P < 0.05, vs. normal control group; #P < 0.05, vs. AD group. TSG: Tetrahydroxy stilbene glucoside; AD: Alzheimer's disease.

**Table 2 Effects of TSG on learning and memory abilities of AD model mice in the Morris water maze test**

Group	Latency (second)	Distance (m)	Platform crossing (times within 120 seconds)
Normal control	18.19±1.23	3.45±1.12	17.93±2.13
AD	31.20±3.49*	7.80±2.91*	3.13±1.04*
TSG	24.37±2.86*#	6.25±2.14*#	4.71±1.12*#

Data are expressed as the mean ± SD (n = 20). All data are compared by one-way analysis of variance followed by *post-hoc* Scheffe's test. \*P < 0.05, vs. normal control group; #P < 0.05, vs. AD group. TSG: Tetrahydroxy stilbene glucoside; AD: Alzheimer's disease.



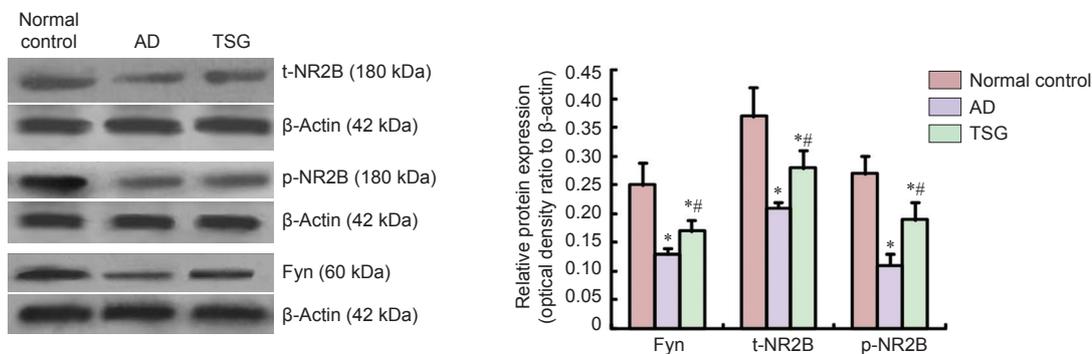
**Figure 1 Effect of TSG on synaptic ultrastructure in CA1 region of the hippocampus of AD model mice (× 40,000).**

(A) Normal control group: Normal presynaptic mitochondria, postsynaptic densities, synaptic cleft and concave synapses. (B) AD group: Unclear presynaptic mitochondria, thin postsynaptic density, reduced concave synapses, increased synaptic cleft and flat synapses. (C) TSG group: Compared with the AD group, synaptic parameters have improved. TSG: Tetrahydroxy stilbene glucoside; AD: Alzheimer's disease.

**Table 3 Parameters for synaptic interface structure and average percentage of three types of synapse in the CA1 region of the mouse hippocampus**

Group	Synaptic number	Length of active zone (nm)	Curvature of synaptic interface (D)	Width of synaptic cleft (nm)	Thickness of postsynaptic density (nm)	Concave (%)	Flat (%)	Convex (%)	Perforated (%)
Normal control	99	369.23±14.09	1.2831±0.0579	10.08±1.79	93.24±6.23	78.6±2.07	16.3±0.83	4.53±0.16	7.01±0.06
AD	96	267.04±8.03*	1.0043±0.0228*	22.13±2.10*	48.28±4.26*	61.4±2.19*	35.2±1.82*	2.48±0.15*	2.08±0.12*
TSG	98	312.09±7.14*#	1.1405±0.0207*#	17.32±1.96*#	69.20±5.13*#	70.3±2.36*#	24.6±0.91*#	3.50±0.13*#	5.02±0.08*#

Data are expressed as the mean ± SD (n = 20). All data were compared by one-way analysis of variance followed by *post-hoc* Scheffe's test. \*P < 0.05, vs. normal control group; #P < 0.05, vs. AD group. TSG: Tetrahydroxy stilbene glucoside; AD: Alzheimer's disease.



**Figure 2 Effect of TSG on protein expression of Fyn and phosphorylation of NR2B in CA1 region of the hippocampus of AD model mice.**

Fyn and p-NR2B mediated amyloid-beta-induced synaptic plasticity damage in parallel. Amyloid-beta can inhibit Fyn kinase-mediated phosphorylation pathway of NR2B, while TSG can improve learning and memory abilities of the transgenic rats through increasing NR2B receptors and Fyn expression. Data are expressed as the mean ± SD (n = 20). All data were compared by one-way analysis of variance followed by *post-hoc* Scheffe's test. \*P < 0.05, vs. normal control group; #P < 0.05, vs. AD group. TSG: Tetrahydroxy stilbene glucoside; AD: Alzheimer's disease.

synapses, with their more concentrated interface, can allow the released neurotransmitters to arrive at the target point and improve the effectiveness of information transmission with neurotransmitters. Synaptic perforation also plays a significant role in synaptic plasticity and hippocampal synapse formation (Sorra et al., 1998). The loss of synapses in AD may include a loss of perforated synapses (Jones, 1993; Jones and Harris, 1995). It is held that synaptic perforation can increase the contact area between neurotransmitters and postsynaptic density to improve the efficiency of synaptic transmission.

Our study demonstrated that the mechanism of TSG action may be the increased expressions of NR2B and Fyn proteins, which are of great significance to long-term potentiation. Fyn can cause the phosphorylation of NR2B, which is a major functional unit of the NMDA receptors and one of the main components of postsynaptic membranes. NR2B can bind other protein molecules and enzymes. The increased expressions of Fyn and NR2B can not only be good for the formation and functions of the NMDA receptor complex, but also it can cause thicker postsynaptic density, narrower synaptic cleft and so improve the effectiveness of synaptic transmission and long-term potentiation induction. With increased NR2B expression, more enzymes will be bound and anchored on the membrane, which could improve the activation of such enzymes and synaptic transmission.

TSG can reverse the distribution of structural parameters for concave synapses and perforated synapses in AD transgenic mice. That improves the information transmission *via* neurotransmitters resulting in significant relief from the learning and memory impairment caused by A $\beta$ . These anatomical and behavioral benefits of TSG suggest it is a promising medicine for the future treatment of AD.

**Author contributions:** HBL and XQS conceived and designed the experiments. HBL, YL, ZQZ, LC, YW, XYZ and ZL performed the experiments. HBL, ZJL and XQS analyzed the data. HBL wrote the paper. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Plagiarism check:** This paper was screened twice using Cross-Check to verify originality before publication.

**Peer review:** This paper was double-blinded and stringently reviewed by international expert reviewers.

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