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RESEARCH ARTICLE

Beta 2-adrenergic receptor activation enhances neurogenesis in Alzheimer's disease mice

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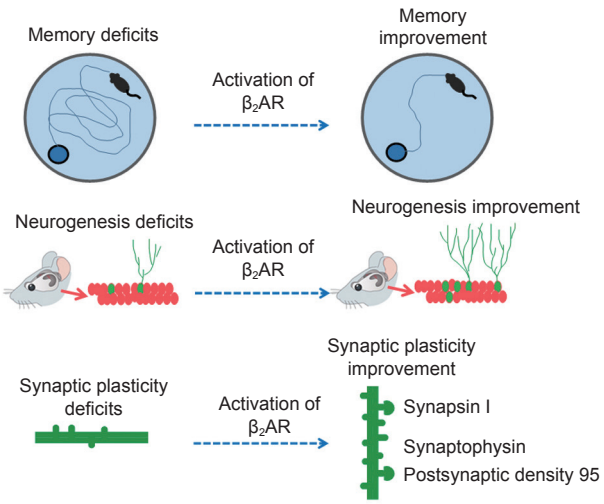
Graphical Abstract

Improvement in cognitive disorder after Alzheimer's disease: neurogenesis enhancement by activating β_2-adrenoceptors (β_2ARs)



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Abstract

Impaired hippocampal neurogenesis is one of the early pathological features of Alzheimer's disease. Enhancing adult hippocampal neurogenesis has been pursued as a potential therapeutic strategy for Alzheimer's disease. Recent studies have demonstrated that environmental novelty activates β_2-adrenergic signaling and prevents the memory impairment induced by amyloid-β oligomers. Here, we hypothesized that β_2-adrenoceptor activation would enhance neurogenesis and ameliorate memory deficits in Alzheimer's disease. To test this hypothesis, we investigated the effects and mechanisms of action of β_2-adrenoceptor activation on neurogenesis and memory in amyloid precursor protein/presenilin 1 (APP/PS1) mice using the agonist clenbuterol (intraperitoneal injection, 2 mg/kg). We found that β_2-adrenoceptor activation enhanced hippocampal neurogenesis, ameliorated memory deficits, and increased dendritic branching and the density of dendritic spines. These effects were associated with the upregulation of postsynaptic density 95, synapsin 1 and synaptophysin in APP/PS1 mice. Furthermore, β_2-adrenoceptor activation decreased cerebral amyloid plaques by decreasing APP phosphorylation at Thr668. These findings suggest that β_2-adrenoceptor activation enhances neurogenesis and ameliorates memory deficits in APP/PS1 mice.

Key Words: *nerve regeneration; Alzheimer's disease; β_2-adrenoceptors; amyloid β; neurogenesis; clenbuterol; APP/PS1 mice; memory; dendritic spine; synapsin 1; synaptophysin; postsynaptic density 95; neural regeneration*

Introduction

Alzheimer's disease (AD) is the most common form of senile dementia among older people. This disease is characterized by deposition of amyloid-β (Aβ) plaques, accumulation of intracellular neurofibrillary tangles, regionalized neuronal dysfunction and dendritic degeneration (Ramsden et al., 2005; Paulson et al., 2008). The clinical manifestations of AD include a progressive deterioration of memory, cognitive disorders and dementia (Mount and Downton, 2006; Iqbal et al., 2014).

β_2-adrenoceptors (β_2ARs) are G-protein-coupled receptors involved in a wide range of physiological functions ranging from control of vascular tone and cardiac performance to metabolism and memory formation (Gabilondo et al., 1997). The β_2AR is expressed in the hippocampus and the cortex (Russo-Neustadt and Cotman, 1997), and is required for learning and memory (Zhou et al., 2013). Accordingly, the role of the β_2AR in AD pathogenesis has garnered substantial interest. However, there is much controversy in the field. For example, it was reported that chronic treatment with β_2AR agonists increases Aβ load in transgenic mice, and that the use of β_2AR blockers decreases acute stress-induced Aβ production (Ni et al., 2006). However, activation of the β_2AR has been shown to enhance long-term potentiation (Gelinas and Nguyen, 2005) and overcome the inhibitory effects of Aβ on long-term potentiation (Wang et al., 2009; Li et al., 2013). Therefore, more studies are needed to clarify the role of the β_2AR in the pathogenesis of AD.

In the present study, we observed that activation of the β_2AR ameliorated memory deficits, enhanced hippocampal neurogenesis, and increased dendritic branches and the density of dendritic spines. These effects were associated with the upregulation of synaptic protein levels in amyloid precursor protein/presenilin 1 double transgenic (APP/PS1) mice. Furthermore, chronic treatment with a β_2AR agonist decreased cerebral amyloid plaques in APP/PS1 mice. These results suggest that the β_2AR is a potential therapeutic target for AD.

Materials and Methods

Animals and drug administration

APP/PS1 mice (specific-pathogen-free, male, 8 months, 30 ± 3 g, n = 20) and wild-type mice (specific-pathogen-free, male, 8 months, 30 ± 3 g, n = 10) were purchased from the Model Animal Center of Nanjing University of China (certificate No. 201501556; license No. SCXK (Su) 2015-001). The APP/PS1 mouse is a double transgenic hemizygote that expresses a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and mutant human presenilin 1 (PS1-dE9). APP/PS1 mice were intraperitoneally injected with 2 mg/kg clenbuterol (Sigma-Aldrich, St. Louis, MO, USA) or an equal volume of dimethyl sulfoxide in 0.9% saline, as vehicle control, once a day for 2 months, from 8 to 9 months (n = 10 in each), once a day, for 4 days. 5-Bromo-2-deoxyuridine (BrdU; 100 mg/kg; Sigma-Aldrich) was intraperitoneally administered before the Morris water maze test, once daily for 4 days. 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.

Morris water maze test

After treatment with clenbuterol, spatial memory was measured with the Morris water maze test (Morris, 1984). For spatial learning, mice were trained in the water maze to find a hidden platform for 5 consecutive days, four trials per day with a 10-minute interval from 14:00 to 18:00 p.m. If a mouse failed to find the platform within 60 seconds, it was guided to the platform by the researcher and kept there for 10 seconds. On day 6, the platform was removed, and the testing session was performed. The pathway and the latency/length that the mice passed through the previous platform quadrant were recorded by a video camera. The camera was connected to a digital-tracking device attached to an IBM computer loaded with the water maze software (Ethovision, Noldus Information Technology, Holland).

Immunohistochemistry

Mice were decapitated after the spatial memory retention test. Immunohistochemistry was performed as previously described (Chai et al., 2013). Briefly, the mouse brain sections were blocked in 1% bovine serum albumin/0.3% Triton X-100 for 30 minutes, followed by overnight incubation with primary antibodies, BrdU, NeuN and doublecortin (DCX), at 4°C. Synaptophysin (polyclonal, 1:200) and 4G8 (monoclonal, 1:200) were obtained from Millipore (Billerica, MA, USA). After washing with PBS, sections were incubated with biotin-labeled secondary antibodies for 1 hour at 37°C. The immunoreactions were detected by incubating with horseradish peroxidase-conjugated antibodies for 1 hour at 37°C, and visualized with the diaminobenzidine tetrachloride system (brown color). Rhodamine Red-X-conjugated goat anti-rabbit/mouse IgG was purchased from Molecular Probes (Eugene, OR, USA). The number of hippocampal dentate gyrus neurons was determined by probing for NeuN. The images were observed using a microscope (BX60, Olympus, Tokyo, Japan) or an epifluorescence microscope (Axioplan2, Zeiss, Oberkochen, Germany).

Golgi staining

Mice were decapitated after the spatial memory retention test. Golgi staining was performed according to a previous report (Chai et al., 2013). Briefly, the mouse brains were fixed *in situ* by perfusion with 400 mL Golgi fixative (0.9% NaCl, 5% formaldehyde, 5% potassium dichromate, 5% chloral hydrate) in the dark, and postfixed for 3 days in the same Golgi fixative. The brains were transferred into 2% silver nitrate solution and incubated for 3 days. Coronal brain sections of hippocampal tissue were cut at 35-μm thickness using a vibratome (VT1000S, Leica, Wetzlar, Germany). The images were observed on a microscope (BX60, Olympus), and a digital camera system and Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA) were used for morphometric analysis of digitized images. Using the center of the soma as the 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 analysis). For each animal, at least 36 neurons were analyzed. Spine quantification was carried out by observers who were unaware of the experimental conditions.

Western blot assay

Mice were decapitated after the spatial memory retention test. The hippocampi were rapidly removed and homoge-

nized at 4°C using a Teflon glass homogenizer in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 5 mM EDTA, 2 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride. The bicinchoninic acid protein detection kit was purchased from Pierce Chemical Company (Rockford, IL, USA). The extract was mixed with sample buffer (1:9; m/v) containing 200 mM Tris-HCl, pH 7.6, 8% sodium dodecyl sulfate, 40% glycerol and 40 mM dithiothreitol, and then boiled for 10 minutes. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk dissolved in Tris-buffered saline with Tween (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.2% Tween-20) for 1 hour and probed with primary antibody at 4°C overnight. The primary antibodies against synapsin-1 (polyclonal, 1:1,000) and postsynaptic density 95 (PSD95) (polyclonal, 1:1,000) were obtained from Abcam (Cambridge, UK). PS1 (monoclonal, 1:1,000), APP (monoclonal, 1:1,000) and synaptophysin (monoclonal, 1:1,000) were from Millipore. Phosphorylated APP (p-APP) (polyclonal, 1:1,000), DM1A (monoclonal, 1:1,000) and β-actin (monoclonal, 1:1,000) were obtained from Cell Signaling Technology (Boston, USA). The blots were incubated with anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (1:5,000; Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) for 1 hour at 37°C, and visualized with enhanced chemiluminescence. Proteins were visualized on X-ray film and quantified using Quantity One software (Bio-Rad, Hercules, CA, USA). Target protein expression was normalized to β-actin (a reference control).

Enzyme-linked immunosorbent assay (ELISA) for $A\beta_{40}$ and $A\beta_{42}$

Mice were decapitated after the spatial memory retention test. After assessing Aβ accumulation in the cerebral cortex, the concentrations of $A\beta_{40}$ and $A\beta_{42}$ in the hippocampal homogenate were measured using ELISA kits (Thermo Fisher Scientific, Waltham, MA USA) according to the manufacturer's directions. Briefly, hippocampal tissue was weighed and homogenized at 4°C in the cell extraction buffer provided in the kit and supplemented with a protease inhibitor cocktail (Sigma-Aldrich). The homogenates in Eppendorf tubes were then centrifuged at 13,000 r/minute at 4°C for 10 minutes. The concentration of protein in the supernatant was measured using the microBCA protein assay (Thermo Fisher Scientific).

Statistical analysis

Data, expressed as the mean ± SD, were analyzed using SPSS 12.0 software (SPSS, Chicago, IL, USA). We used one-way analysis of variance, followed by least significant difference *post hoc* tests or Student's *t*-test. $P < 0.05$ was considered to indicate statistical significance.

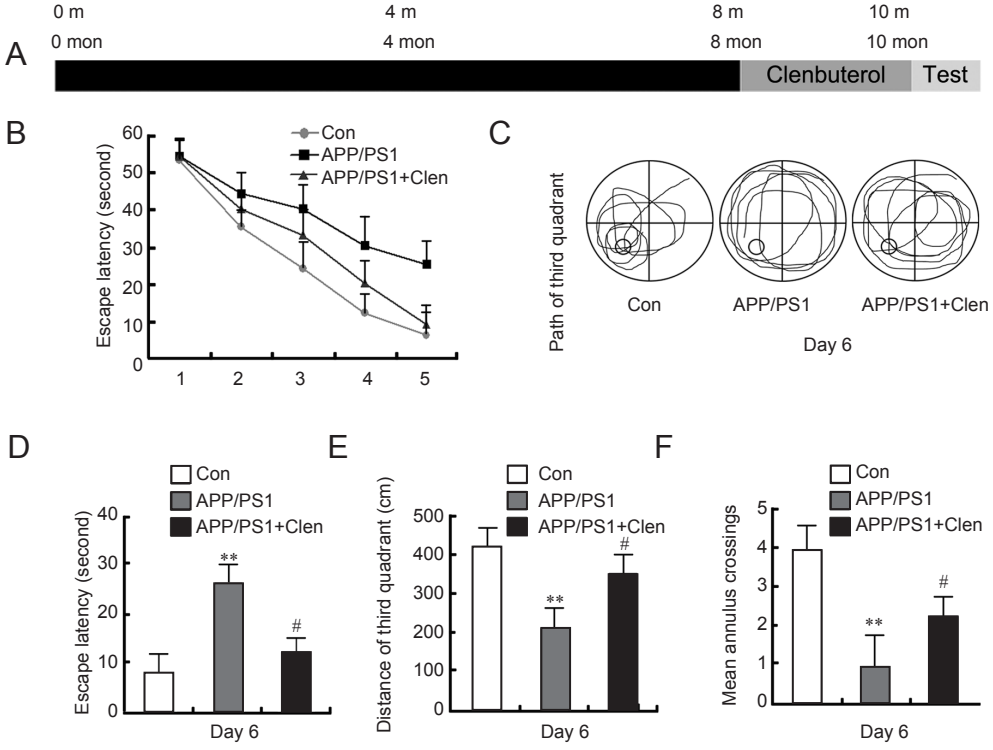

Figure 1 Activation of β_2AR ameliorated memory deficits in APP/PS1 mice.
(A) APP/PS1 mice were intraperitoneally injected with clenbuterol (2 mg/kg/d) or vehicle control (dimethyl sulfoxide) from 8 to 10 months. Spatial memory was subsequently assessed. (B) Escape latency to find the hidden platform from the first day to the fifth day. (C–F) The path to find the platform area, the escape latency to find the hidden platform, the distance swam in the third quadrant, and the number of crossings of the target quadrant within 1 minute after removal of the platform on day 6. Data are expressed as the mean ± SD ($n = 10$; one-way analysis of variance followed by least significant difference *post hoc* tests). $^{**}P < 0.01$, *vs.* Con; $^{\#}P < 0.05$, *vs.* APP/PS1. mon: Month(s); Con: control; β_2AR: β_2-adrenoceptor; APP/PS1: amyloid precursor protein/presenilin 1; Clen: clenbuterol.

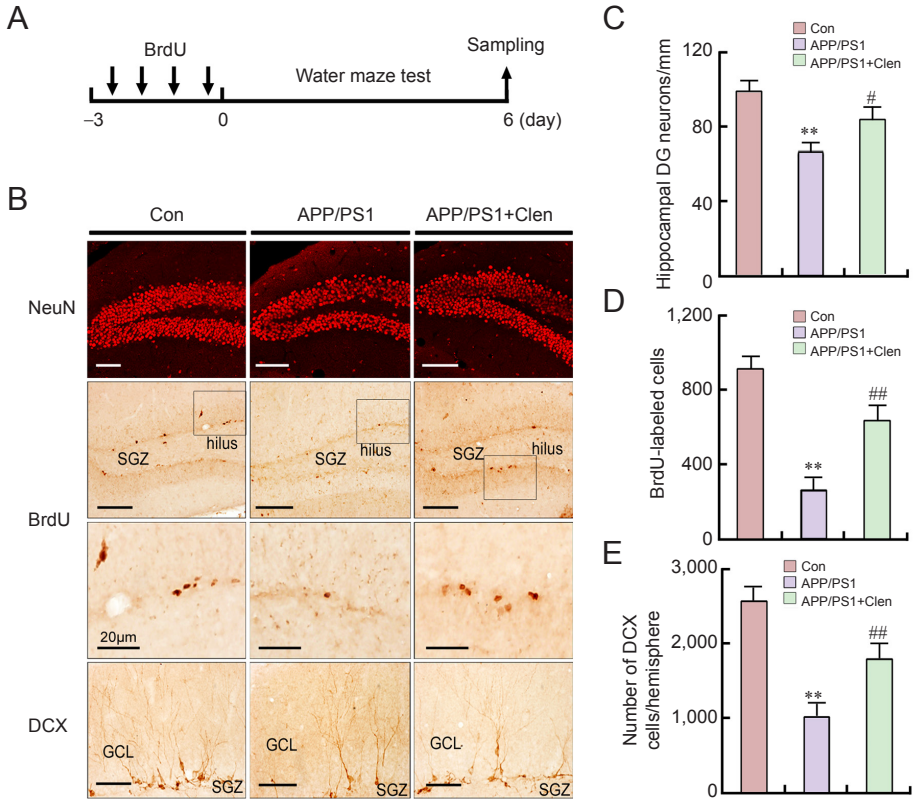

Figure 2 Activation of β_2AR enhances neurogenesis in the dentate gyrus in APP/PS1 mice.
(A) Mice were intraperitoneally injected with BrdU (100 mg/kg/d) for 4 consecutive days. Spatial memory retention was measured after BrdU injection. (B, C) The number of hippocampal DG neurons (NeuN-positive cells); scale bars: 50 μm. (B, D) BrdU-labeled cells in the DG; scale bars: 50 μm. (B, E) DCX-labeled cells in the DG; scale bars: 50 μm. Data are expressed as the mean ± SD (n = 3; one-way analysis of variance followed by least significant difference *post hoc* tests). **P < 0.01, *vs.* Con; #P < 0.05, ##P < 0.01, *vs.* APP/PS1. Con: Control; β_2AR: β_2-adrenoceptor; APP/PS1: amyloid precursor protein/presenilin 1; BrdU: 5-bromo-2-deoxyuridine; DCX: doublecortin; DG: dentate gyrus; Clen: clenbuterol; SGZ: subgranular zone; GCL: granule cell layer.

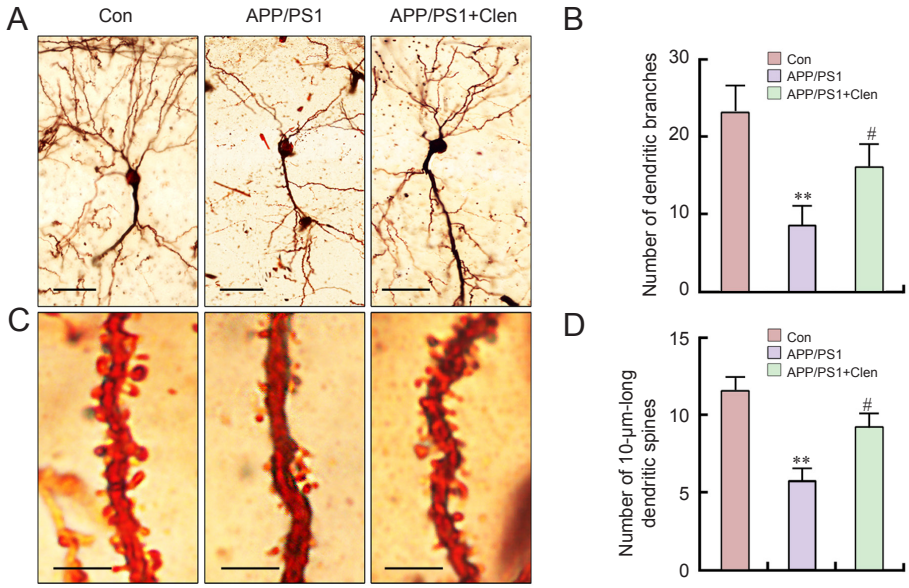

Figure 3 Activation of β_2AR restores dendritic branches and spine density in the hippocampus.
The mice were sacrificed, and Golgi staining was used to detect the dendrites and spines in the hippocampus. (A) Golgi-stained hippocampal pyramidal neurons; scale bars: 50 μm. (B) The number of dendritic branches was quantified from randomly selected neurons (Golgi staining). (C) Dendritic spines of different hippocampal pyramidal neurons; scale bars: 10 μm. (D) The number of dendritic spines was quantified from randomly selected dendritic segments of neurons (Golgi staining). Data are expressed as the mean ± SD (n = 3; one-way analysis of variance followed by least significant difference *post hoc* tests). **P < 0.01, *vs.* Con; #P < 0.05, *vs.* APP/PS1. Con: Control; β_2AR: β_2-adrenoceptor; APP/PS1: amyloid precursor protein/presenilin 1; Clen: clenbuterol.

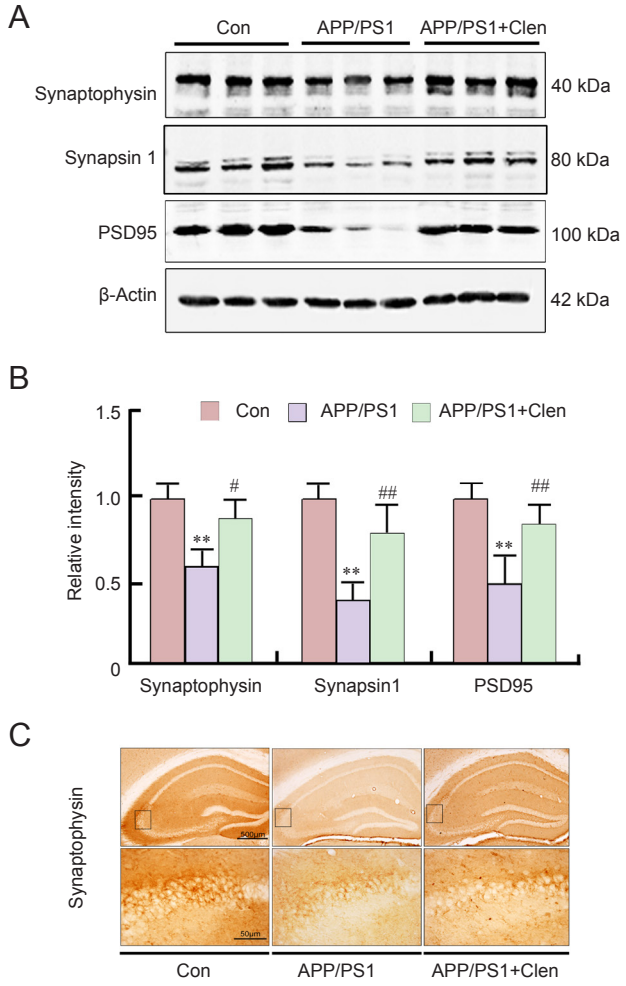

Figure 4 Activation of β_2AR increased the levels of synaptic proteins.
(A, B) The levels of synaptophysin, synapsin 1 and PSD95 in the hippocampus were assessed by western blot assay and quantitative analysis. (C) Synaptophysin level was also measured by immunohistochemistry. Scale bar: top: 500 μm; bottom: 50 μm. Data are expressed as the mean ± SD (n = 3; one-way analysis of variance followed by least significant difference *post hoc* tests). **P < 0.01, *vs.* Con; #P < 0.05, ##P < 0.01, *vs.* APP/PS1. Con: Control; β_2AR: β_2-adrenoceptor; APP/PS1: amyloid precursor protein/presenilin 1; PSD95: postsynaptic density 95; Clen: clenbuterol.

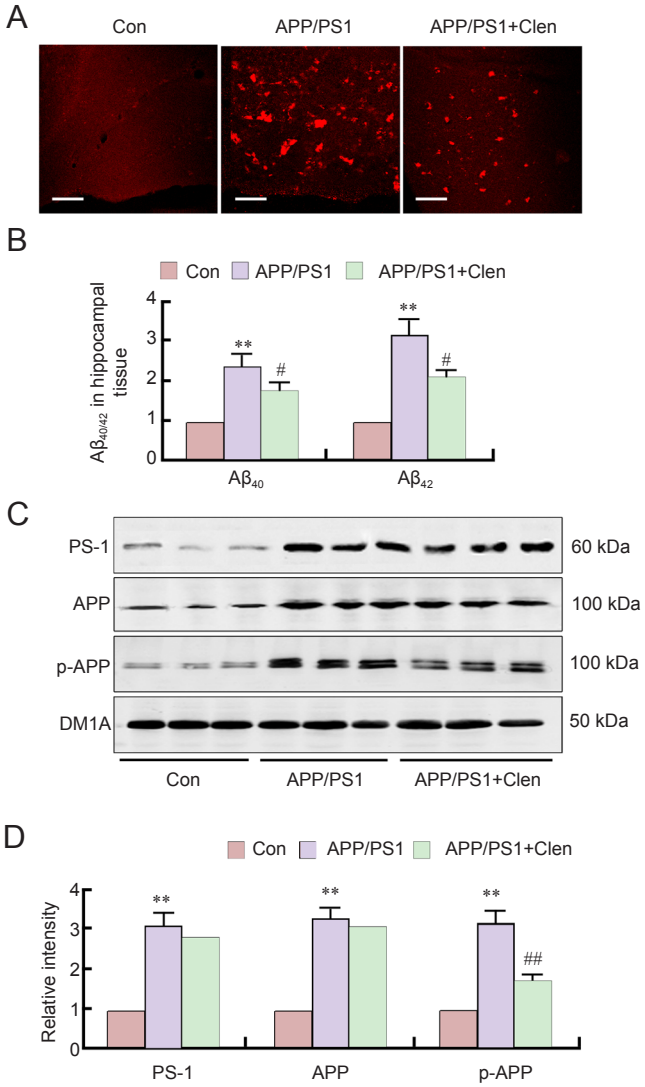

Figure 5 Activation of β_2AR reduced Aβ accumulation in APP/PS1 mice.
(A) Plaques in the cortex, detected by immunostaining with Aβ antibody; scale bars: 100 μm. (B) ELISA data showing increased levels of $A\beta_{40}$ and $A\beta_{42}$ in hippocampal extracts. (C, D) PS1, APP and Thr668-p-APP levels were evaluated by western blot assay and quantitative analysis. Data are expressed as the mean ± SD (n = 3; one-way analysis of variance followed by least significant difference *post hoc* tests). **P < 0.01, *vs.* Con (control); #P < 0.05, ##P < 0.01, *vs.* APP/PS1. β_2AR: β_2-Adrenoceptor; APP/PS1: amyloid precursor protein/presenilin 1; Aβ: amyloid beta; p-APP: phosphorylated APP; DM1A: α-tubulin; Con: control; Clen: clenbuterol.

Results

Activation of β_2AR attenuated memory deficits in the APP/PS1 mice

Previous studies have shown that APP/PS1 mice exhibit Aβ accumulation, synaptic deficits and memory impairment at 8 months (Sadowski et al., 2004). Therefore, we started to inject the mice with clenbuterol at 8 months of age, and we examined changes in memory function at 10 months of age (**Figure 1A**). The Morris water maze test was performed to explore the effects of clenbuterol on spatial memory deficits in APP/PS1 mice. The latency to finding the hidden platform was significantly increased in APP/PS1 mice compared with the control mice (**Figure 1B**; P < 0.01). Treatment with clenbuterol attenuated memory deficits in the APP/PS1 mice (**Figure 1B**; P < 0.01). The memory deficits in APP/PS1 mice were also observed after removing the platform on day 6 (**Figure 1C–F**; P < 0.01). Greater latency to the target quadrant, reduced distance traveled in the target quadrant, and fewer crossings of the target quadrant were observed in APP/PS1 mice, compared with the controls (**Figure 1C–F**; P < 0.01). β_2AR activation by clenbuterol effectively attenuated the memory deficits in these mice (P < 0.05).

Activation of β_2AR enhanced neurogenesis in the dentate gyrus in APP/PS1 mice

To explore the involvement of neurogenesis in the β_2AR

activation-induced memory improvement, the mice were injected with BrdU for 4 days before the Morris water maze test (**Figure 2A**). Neuronal density was assessed using the neuronal marker NeuN. Neuronal loss was apparent in the APP/PS1 mice (**Figure 2B–C**; $P < 0.01$). However, neuronal density in the clenbuterol-treated APP/PS1 mice was similar to that in control mice (**Figure 2B–C**; $P < 0.05$).

The number of BrdU-positive cells in the hippocampal dentate gyrus was greatly reduced in APP/PS1 mice compared with wild-type (**Figure 2B**, **D**; $P < 0.01$). However, significantly increased immunoreactivity to BrdU was detected in clenbuterol-treated mice (**Figure 2B**, **D**; $P < 0.01$).

DCX is a marker of immature neurons and neurite extension (Kempermann et al., 2004). By analyzing the number of DCX-positive cells in the dentate gyrus, we found that β_2AR activation significantly increased the number of immature neurons and/or dendritic arborization in APP/PS1 mice (**Figure 2B**, **E**; $P < 0.01$). These data confirm the key role of neurogenesis in spatial memory and suggest that β_2AR activation enhances neurogenesis in the adult hippocampus.

β_2AR activation restored dendritic branches and spine density in the hippocampus

To investigate the mechanisms underlying the β_2AR activation-mediated attenuation of memory deficits in APP/PS1 mice, the average number of dendritic branches and dendritic spine density in pyramidal neurons in the hippocampus were analyzed by Golgi staining. The average number of dendritic branches was significantly reduced in APP/PS1 mice (**Figure 3A**, **B**; $P < 0.01$). β_2AR activation restored the number of dendritic branches in these mice (**Figure 3A**, **B**; $P < 0.05$). We further investigated whether β_2AR activation affects the formation of dendritic spines, which indirectly reflects the presence of synapses (Tronel et al., 2010). Consistently, spine density in CA1 neurons was significantly reduced in APP/PS1 mice compared with the control mice (**Figure 3C**, **D**; $P < 0.01$). This reduction in dendritic spine density was effectively prevented by clenbuterol in APP/PS1 mice (**Figure 3C**, **D**; $P < 0.05$). Thus, these changes in dendritic branching and spine density might account for the improved memory in the clenbuterol-treated APP/PS1 mice.

Activation of β_2AR increased the levels of synapse-associated proteins

Synaptic loss is an early event in AD, and it is also observed in APP/PS1 mice (Bigio et al., 2002). To elucidate the molecular mechanisms underlying the β_2AR-mediated improvement of the memory deficit, the expression levels of several synaptic proteins were assessed by western blot assay. Clenbuterol treatment prevented the decrease in synaptophysin, synapsin 1 and PSD95 levels in the brain (**Figure 4A**, **B**; synaptophysin, $P < 0.05$; synapsin 1, $P < 0.01$; PSD95, $P < 0.01$). The significantly elevated immunohistochemical staining for synaptophysin in APP/PS1 mice also demonstrates that β_2AR activation prevents the synaptic loss in APP/PS1 mice (**Figure 4C**). These results suggest that β_2AR activation protects synapses, and that changes in memory-associated proteins might partially underlie the improved learning and memory in the mice.

Activation of β_2AR reduced Aβ accumulation in APP/PS1 mice

Previous studies have demonstrated that APP/PS1 mice show Aβ accumulation at 8 months (Kiyota et al., 2009; Liao et al., 2014). Therefore, we started to treat the mice with clenbuterol at 8 months of age, and we measured the changes in Aβ levels at 10 months of age. Clenbuterol treatment for 2 months decreased Aβ accumulation in APP/PS1 mice (**Figure 5A**). ELISA showed that the levels of $A\beta_{40}$ and $A\beta_{42}$ were significantly increased in APP/PS1 mice (**Figure 5B**; Aβ40, $P < 0.01$; $A\beta_{42}$, $P < 0.01$), and that β_2AR activation reduced $A\beta_{40/42}$ levels (**Figure 5B**; $A\beta_{40}$, $P < 0.05$; $A\beta_{42}$, $P < 0.05$).

To clarify the mechanisms underlying the inhibition of Aβ production, we assessed changes in PS-1 and APP in APP/PS1 mice by western blot assay. However, we did not observe any significant difference between the two groups (**Figure 5C**, **D**; $P > 0.05$). We then measured the levels of p-APP (pThr668), because phosphorylation at this site has been reported to facilitate Aβ production (Shin et al., 2007). Compared with the control mice, a significant increase in APP phosphorylation was observed in APP/PS1 mice. This change was largely suppressed by β_2AR activation (**Figure 5C**, **D**; $P < 0.01$). Taken together, these findings suggest that β_2AR activation attenuates Aβ production in APP/PS1 mice by reducing APP phosphorylation at Thr668.

Discussion

In the present study, we found that β_2AR activation enabled the recovery of memory deficits in APP/PS1 mice. Activation of β_2AR in APP/PS1 mice substantially enhanced neurogenesis in the dentate gyrus, rescued the loss of neuronal dendritic branches and spines, and upregulated synaptic protein levels. Notably, β_2AR activation decreased Aβ accumulation by reducing APP phosphorylation. Our findings suggest that β_2AR might be a novel target for AD treatment.

Thomas et al. (1996) showed that β_2AR activation was required for normal learning and memory, which was confirmed *in vitro*. Our present results are consistent with previous studies in animal models, where β_2AR activation positively modulated memory formation and consolidation (Gibbs et al., 2010; Zhou et al., 2013). Furthermore, Li et al. (2013) reported that β_2AR activation prevented the Aβ-induced impairment of hippocampal long-term potentiation. Our data are consistent with these observations, as we found that β_2AR activation alleviated the memory impairment in the APP/PS1 mice (Roozendaal et al., 2008).

Impaired neurogenesis strongly correlates with memory impairment in AD (Wen et al., 2004; Faure et al., 2011; Yan et al., 2014). Adult neurogenesis in the hippocampal dentate gyrus contributes to the formation of memories (Deng et al., 2010; Murai et al., 2014). The enhancement of hip-

pocampal neurogenesis has been associated with learning and memory (Deng et al., 2010). Moreover, the promotion of hippocampal neurogenesis facilitates spatial memory formation (Lepousez et al., 2015). Morphological analyses indicate that newly-generated neurons extend axonal and dendritic projections and functionally integrate into existing circuits by forming excitatory synapses with their targets (van Praag et al., 2002; Deshpande et al., 2013). Adult neurogenesis partially compensates for the neuronal loss in neurological disorders, including AD, and is important for memory (Davies and Maloney, 1976). Faure et al. (2011) found that Aβ accumulation hinders neuronal proliferation in the adult hippocampus, and impaired neurogenesis strongly correlates with cognitive impairments in AD transgenic mice and AD patients.

To explore the effect of clenbuterol on neurogenesis in APP/PS1 mouse, we intraperitoneally injected BrdU for 4 days to label the cells undergoing mitosis. We observed enhanced proliferation and survival of newborn neurons in clenbuterol-treated APP/PS1 mice. This may at least partially contribute to the attenuation of memory impairment in the APP/PS1 mice treated with the β_2AR agonist.

Synaptic plasticity, which can be assessed by examining changes in dendritic morphology, is a prerequisite for learning and memory (Matsuo et al., 2008). In the hippocampus, synapses in the basal CA1 mainly receive inputs from the CA3, and the CA3–CA1 projection has been widely studied because of its key role in memory (Attardo et al., 2015). Prior work has illustrated that inactivation of the CA1 region impairs retrieval of spatial memory in the Morris water maze (Stackman et al., 2016). By assessing changes in branches and spines of dendrites in the CA1 in the dorsal hippocampus, we found that β_2AR activation suppressed the reduction in dendritic branches and spine density in APP/PS1 mice.

PSD95 is a key regulator of the structure and function of dendritic spines, and is associated with hippocampal synaptic plasticity in the brain (Engert and Bonhoeffer, 1999). We observed that β_2AR activation increased PSD95 levels in APP/PS1 mice. Presynaptic release of neurotransmitters involves synaptic vesicle-associated proteins, including synaptophysin and synapsin I, which are considered markers of synaptic plasticity in neural networks (Pieribone et al., 1995; Slutsky et al., 2010). We observed that β_2AR activation attenuated the decrease in expression of synapse-associated proteins. Therefore, these morphological and molecular changes might underlie the improvement of memory deficits in APP/PS1 mice produced by β_2AR activation.

Aβ, a key pathogenic factor in AD, aggregates to form neurotoxic oligomers or insoluble deposits of plaques in the brains of affected individuals, and contributes to the loss of synaptic function as well as cognitive impairment (Li et al., 2014). Aβ is generated by the consecutive cleavage of APP by two proteases, β-secretase and γ-secretase (presenilin, PS1/PS2) (Dingwall, 2001; Nishitomi et al., 2006). Overexpression of APP and PS1 increases Aβ production in APP/PS1 mice. In the present study, although the levels of $A\beta_{40}$ and $A\beta_{42}$ were decreased in APP/PS1 mice treated with clenbuterol, the total levels of APP and PS1 were not changed. This suggests that other changes, such as in APP phosphorylation, might be involved. Because APP phosphorylation at Thr668 promotes Aβ production (Lee et al., 2003), we measured changes in pThr668-APP. We found that β_2AR activation by clenbuterol significantly reduced APP phosphorylation in the transgenic mice. Accordingly, we speculate that activation of β_2AR with clenbuterol decreases Aβ production by reducing APP phosphorylation at Thr668.

Recent studies have found that Aβ can bind to β_2AR, directly enhancing β-secretase activity, and further increasing Aβ production (Ni et al., 2006; Branca et al., 2014). It is likely that β_2AR activation by clenbuterol might interrupt the Aβ–β_2AR pathway, thereby reducing Aβ production and neuronal excitotoxicity in APP/PS1 mice. The harmful effects of β_2AR dysregulation by Aβ are therefore inhibited, decreasing PS1 activity and further lowering Aβ production.

In summary, β_2AR activation enhances hippocampal neurogenesis, ameliorates cognitive deficits, and increases dendritic branches and spine density, along with the upregulation of synaptic protein levels. β_2AR activation may decrease Aβ accumulation by decreasing APP phosphorylation at Thr668 in APP/PS1 mice. Based on the critical roles of β_2AR in hippocampal neurogenesis, memory and AD pathogenesis, pharmacologically targeting this receptor might have therapeutic potential for AD.

Author contributions: *GSC and PZ designed the study and wrote the paper. YYW and AY performed experiments. GSC and YYW analyzed data. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*
Plagiarism check: *This paper was screened twice using CrossCheck to verify originality before publication.*
Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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