Neuroprotective effects of a triple GLP-1/GIP/glucagon receptor agonist in the APP/PS1 transgenic mouse model of Alzheimer's disease

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is a risk factor for Alzheimer disease (AD). Previous studies have shown that the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulino tropic polypeptide (GIP) that have anti-diabetic properties show very promising effects in animal models of AD. Glucagon (Gcg) is a hormone and growth-factor, and the Gcg receptor is expressed in the brain. Here we test the effects of a triple receptor agonist (TA), which activates GIP-1, GIP and glucagon receptors at the same time. In the present study, the effects of the TA were evaluated in the APP/PS1 transgenic mouse model of AD. The TA was injected once-daily (10 nmol/kg i.p.) for two months. The results showed that treatment with TA significantly reversed the memory deficit in the APP/PS1 mice in a spatial water maze test. Moreover, the drug reduced levels of the mitochondrial pro-apoptotic signaling molecule BAX, increased the anti-apoptotic signaling molecule Bcl-2 and enhanced the levels of BDNF, a key growth factor that protects synaptic function. Levels of synaptophysin were enhanced, demonstrating protection from synaptic loss that is observed in AD. Neurogenesis in the dentate gyrus was furthermore enhanced as shown in the increase of doublecortin positive cells. Furthermore, TA treatment reduced the total amount of β-amyloid, reduced neuroinflammation (activated microglia and astrocytes), and oxidative stress in the cortex and hippocampus. Thus, these findings show that novel TAs are a promising lead for the design of future treatment strategies in AD.

1. Introduction

Alzheimer’s disease (AD), is a progressive neurodegenerative disease, characterized clinically by progressive memory loss, cognitive decline, and aberrant behavior [1]. Currently, there is no treatment can improve this condition. Epidemiological studies have shown that type 2 diabetes mellitus (T2DM) is a risk factor for Alzheimer disease (AD) ([Luchsinger et al., 2004; Ohara et al., 2011]). The underlying mechanism is most likely that insulin signaling is impaired in the brains of AD patients (Moloney et al., 2010; Talbot et al., 2012). This motivated research in drugs that have shown good effects in treating diabetes to investigate if they could be helpful in treating AD as well. Previous studies have shown that the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulino tropic polypeptide (GIP) can play a neuroprotective role in the brain and show very promising effects in animal models of AD (Duffy and Holscher, 2013; Fairey and Holscher, 2013b; Li et al., 2010; McClean et al., 2011; McClean and Holscher, 2014). Glucagon (Gcg) is a hormone and growth-factor, and the Gcg receptor is expressed in the brain (Hoosein and Gurd, 1984; Mayo et al., 2003). Activation of the receptor can activate neuronal activity and cellular Ca\(^{2+}\) signaling and may have neuroprotective properties (Ayush et al., 2015). Here we test the effects of a triple receptor agonist (TA), which activates GIP-1, GIP and glucagon receptors at the same time. This novel drug has been developed as a potential treatment for diabetes (Finan et al., 2015). No studies have been published that test the neuroprotective effects of this novel drug. We therefore tested this promising TA in the APP\(_{Swe}\)/PS1\(_{de9}\) mouse model of AD. This mouse model recapitulates some of the hallmarks of AD, such as memory loss, synaptic loss, reduction of synaptic plasticity, reduction of neurogenesis in the dentate gyrus, chronic inflammation in the brain, and formation of amyloid plaques in the brain (Duffy and Holscher, 2013; Goto et al., 2008; Hamilton et al., 2011; Hamilton and Holscher, 2012; McClean et al., 2011). We tested
the effects of TA in this transgenic mouse model and evaluated the neuroprotective effects. We analyzed memory formation, hippocampal neurogenesis, the expression levels of Brain Derived Neurotrophic Factor (BDNF) (Blurton-Jones et al., 2009), mitochondrial apoptosis signaling proteins, neuroinflammation and oxidative stress levels.

2. Results

2.1. TA-treatment improved the learning and memory impairment of APP/PS1 mice in the water maze

A two-way ANOVA found a significant difference between groups (p < 0.0001) and over time (p < 0.001). In the Morris water test, it was observed that the control mice, APP/PS1 mice and TA-treated APP/PS1 mice had a similar escape latency (P > 0.05). On day 5, the average escape distance in searching for the hidden platform in the APP/PS1 mice was significantly longer than that in the control mice (P < 0.05). Compared to the APP/PS1 group, the TA-treated APP/PS1 group’s average escape distance was decreased (P < 0.01). There was no difference in swimming speed among these three groups in training days (P > 0.05).

Then on day 6, the platform was removed and the probe trial test was conducted. A one way ANOVA analysis showed an overall difference between groups. The APP/PS1 mice spent less time in the target quadrant (P < 0.01) and had fewer times passing through the original position of the hidden platform (P < 0.01), compared with control mice. In the TA-treated APP/PS1 group, the time spent in target quadrant (P < 0.05) and the frequencies to cross the platform significantly increased (P < 0.05), compared to the APP/PS1 group Fig. 1.

2.2. TA-treatment ameliorated amyloid plaques in the brains of APP/PS1 mice

In the immunohistochemical analysis, we analyzed the amount of amyloid plaques present in the brains of APP-PS1 mice. A one-way ANOVA found an overall difference on the amyloid deposits in the cortex (F = 31.10, P < 0.0001) and the hippocampus (F = 30.38, P < 0.0001) of mice between groups. The Aβ1-42 depositions in the cortex and hippocampus of the APP/PS1 mice were significantly higher than that in the control mice (P < 0.001), while TA-treatment group significantly decreased the deposition (P < 0.001) compared to the APP/PS1 group. N = 6 per group. See Fig. 2.

Fig. 1. The novel TA improved the learning ability of APP/PS1 mice in the spatial water maze task. (A) The escape latency and swimming speed in the training trials. (B) The percentage of time in target quadrant and crossing platform times in the probe trials. The circle indicates the position of the platform during acquisition (C) Representative swimming tracks of the probe trial. * = P < 0.05, ** = P < 0.01 compared to the control group, ## = P < 0.01, # = P < 0.05 compared to the APP/PS1 group; Two-way ANOVA with Bonferroni repeated measures post hoc tests.
2.3. TA-treatment reduced the chronic inflammation response in the cortex and hippocampus of APP/PS1 mice

In the immunohistochemical analysis, an anti-GFAP antibody was used as an indication of activated astrocytes. A one-way ANOVA found an overall difference on the GFAP expression in the cortex (F = 77.03, \( P < 0.0001 \)) and the hippocampus (F = 86.51, \( P < 0.0001 \)) of mice between groups. The levels of GFAP in the cortex and hippocampus of the APP/PS1 mice were significantly higher than that in the control mice (\( P < 0.001 \)), while TA-treatment group significantly decreased (\( P < 0.001 \)) compared to the APP/PS1 group. N = 6 per group. See Fig. 3.

In the immunohistochemical analysis, an anti-IBA1 antibody was used as an indication of microglia activation. A one-way ANOVA found an overall difference on the IBA-1 expression in the cortex (F = 28.71, \( P < 0.0001 \)) and the hippocampus (F = 66.42, \( P < 0.0001 \)) of mice between groups. The levels of IBA-1 in the cortex and hippocampus of the APP/PS1 mice were significantly higher than that in the control mice (\( P < 0.001 \)), while TA-treatment group significantly decreased (\( P < 0.001 \), \( P < 0.01 \)) compared to the APP/PS1 group. N = 6 per group. See Fig. 4.

2.4. TA-treatment suppressed oxidative stress in the cortex and hippocampus of APP/PS1 mice

In the immunohistochemical analysis, 4-HNE was measured as an indicator of lipid peroxidation. A one-way ANOVA found an overall difference on the 4-HNE expression in the cortex (F = 60.60, \( P < 0.0001 \)) and the hippocampus (F = 35.22, \( P < 0.0001 \)) of mice between groups. The expressions of 4-HNE in the cortex and hippocampus of the APP/PS1 mice were significantly higher than that in the control mice (\( P < 0.001 \)), while TA-treatment group significantly decreased (\( P < 0.001 \), \( P < 0.01 \)) compared to the APP/PS1 group. N = 6 per group, see Fig. 5a.

In the immunohistochemical analysis, 8-OHdG was measured as an indicator of DNA oxidation. A one-way ANOVA found an overall difference on the 8-OHdG expression in the cortex (F = 38.72, \( P < 0.0001 \)) and the hippocampus (F = 56.60, \( P < 0.0001 \)) of mice between groups. The expressions of 8-OHdG in the cortex and hippocampus of the APP/PS1 mice were significantly higher than that in the control mice (\( P < 0.001 \)), while TA-treatment group significantly decreased (\( P < 0.01 \), \( P < 0.001 \)) compared to the APP/PS1 group. N = 6 per group, see Fig. 5b.

2.5. TA-treatment increased immature neurons in the dentate gyrus of APP/PS1 mice

In the immunohistochemical analysis, numbers of neurogenesis, assessed by doublecortin (DCX). A one-way ANOVA found an overall difference on the DCX positive cell in the dentate gyrus (F = 43.61, \( P < 0.0001 \)) of mice between groups. The numbers of DCX were much reduced in the APP/PS1 mice (\( P < 0.001 \)) compared to the control mice. While TA-treatment partially prevented the reduction (\( P < 0.001 \)) compared to the APP/PS1 mice. N = 6 per group. See Fig. 6.
2.6. TA-treatment reversed the decrease of BDNF levels in the hippocampus of APP/PS1 mice

BDNF, a key neurotrophic factor in the brain, was detected by Western blot analysis. A one-way ANOVA found an overall difference between groups (F = 20.01, P < 0.001). The expression of BDNF in hippocampus was reduced in APP/PS1 group (P < 0.001) compared to the control group. TA-treatment reversed the decreased (P < 0.05) compared to the APP/PS1 group. N = 6 per group. See Fig. 7a.

2.7. TA-treatment reversed the reduction of synaptophysin levels

Synaptophysin (SYN), a synaptic markers, was detected by Western blot analysis. A one-way ANOVA found an overall difference between groups (F = 20.46, P < 0.001). The level of SYN in hippocampus was decreased in APP/PS1 group (P < 0.001) compared to the control group. While TA-treatment prevent the reduction (P < 0.01) compared to the APP/PS1 group. N = 6 per group. See Fig. 7b.

2.8. TA-treatment showed potential anti-apoptotic effects by decreasing the BAX/Bcl-2 ratio in APP/PS1 mice

The expression of apoptotic proteins, including Bcl-2 (that suppress apoptosis) and BAX (that promotes apoptosis), was detected by Western blot analysis. A one-way ANOVA found an overall difference between groups (F = 77.64, P < 0.001). The ratio of BAX/Bcl-2 in hippocampus was increased (P < 0.001) compared to the control group. While TA-treatment partly decreased the ratio of BAX/Bcl-2 (P < 0.001) compared to the APP/PS1 group. N = 6 per group. See Fig. 7c.

3. Discussion

The results demonstrate for the first time that the novel GLP-1/GIP/Gcg receptor agonist has clear neuroprotective effects in the APP/PS1 mouse model of AD. Memory formation in the spatial water maze task was improved by the drug, and the amyloid plaque load in the cortex and hippocampus was reduced. This result confirms our previous findings that single GLP-1 or GIP analogues can protect from memory loss in this mouse model of AD (Faivre and Holscher, 2013a,b; McClean et al., 2011; McClean and Holscher, 2014). The chronic inflammation response as seen in the activation of microglia and astrocytes is much reduced by the novel TA, similar to single GLP-1 or GIP receptor agonists (Duffy and Holscher, 2013; Ji et al., 2016a; McClean et al., 2011). Levels of the peroxidized lipid 4-HNE that also acts as a pro-apoptotic signal in mitochondria have been reduced by the TA. 4-HNE is one of the markers of membrane lipid peroxidation induced by cytotoxic radicals such as .OH. 4-HNE has cytotoxic, mutagenic and genotoxic properties and 4-HNE is reported to stimulate apoptosis via...
caspase 3 activation and by inducing mitochondrial damage as shown in cytochrome c release ( Ji et al., 2001 ). The levels of the mitochondrial apoptosis signal BAX ( Kim et al., 2005 ) were also reduced. Importantly, the anti-apoptotic signaling molecule B-cell lymphoma 2 ( Bcl-2 ) which is active in functioning mitochondria that have normal inner membrane voltage potentials ( Kavitha et al., 2013 ) is upregulated by the TA, adding to the neuroprotective effect. The level of chronic oxidative stress as measured by the levels of the oxidized nucleotide 8-Hydroxoguanosine ( 8-OHdG ) was also reduced by the drug. Neurogenesis in the dentate gyrus was normalized. Importantly, the levels of the key growth factor BDNF were normalized in the brain. BDNF has shown clear neuroprotective effects in a range of animal models of disease ( Allen et al., 2013 ; Benedetti et al., 2014 ; Blurton-Jones et al., 2009 ; Nagahara et al., 2013 ). An important factor of BDNF activity is that it maintains synaptic activity and protects synapses from stressors ( Kuipers and Bramham, 2006 ). The observation that the synaptic marker synaptophysin was expressed at normal levels after TA treatment also indicates that synaptic numbers and function was improved in the AD mouse. We have shown previously that GLP-1 and GIP analogues protect synapses from amyloid induced stress and keeps them functional ( Faiivre and Hölscher, 2013b ; Gault and Hölscher, 2008a ; Gault and Hölscher, 2008b ; McClean and Hölscher, 2014 ).

Recently, multiple receptor agonists have been developed to treat type II diabetes ( Finan et al., 2013, 2015 ). We have tested novel dual GLP-1/GIP receptor agonists that showed good neuroprotective effects that are superior to single GLP-1 analogues ( Cao et al., 2016 ; Jalewa et al., 2017 ; Ji et al., 2016b ; Yuan et al., 2017 ). The novel dual agonist DA3-CH was found to be superior to liraglutide in a mouse model of Parkinson's disease ( Yuan et al., 2017 ), demonstrating that the strategy of activating more than one incretin receptor type offers an advantage over single receptor agonists. Here, we show that a novel triple receptor agonist also shows promise as a potential treatment for AD, but further dose–response tests and direct comparisons with other drugs have to be conducted in order to evaluate if this new drug is superior to previous ones.

GLP-1, GIP and glucagon are members of the growth factor family ( Hölscher, 2016 ). Their respective receptors are classic 7-membrane spanning domain, G-protein coupled receptors that activate cAMP synthesis ( Campbell and Drucker, 2013 ; Doyle and
Fig. 5. Oxidative stress was reduced by the TA. 5A. TA-treatment reduced the increase of 4-HNE levels in the cortex and hippocampus in APP/PS1 mice. A, D: control group; B, E: APP/PS1 group; C, F: APP/PS1+TA group. Scale bar in image D: 100 μm. *** = P < .001 compared with controls; ### = P < .001 compared to the APP/PS1 group. 5B. TA treatment reduced the increase of 8-OHdG levels in the cortex and hippocampus of APP/PS1 mice. A, D: control group; B, E: APP/PS1 group; C, F: APP/PS1+TA group. Scale bar in image D: 100 μm. *** = P < .001 compared to the control group; ## = P < .01, ### = P < .001 compared with the APP/PS1 group. One-way ANOVA with Bonferroni repeated measures *** post hoc tests. N = 6 per group.
Egan, 2007; Holst et al., 2011). The activation of the second messenger cascade cAMP-PKA-CREB induces a number of cytoprotective processes, including the normalization of energy utilization, cell repair and growth-factor related gene expression (Holscher, 2014; Jalewa et al., 2016; Perry and Greig, 2004; Sharma et al., 2013). Importantly, GLP-1 and GIP also have anti-inflammatory properties. Astroglia and microglia express the receptors, and activating these reduce the inflammation response (Duffy and Holscher, 2013; Jalewa et al., 2017; Parthsarathy and Holscher, 2013; Spielman et al., 2017). Chronic inflammation is a main driver of neurodegenerative disorders, and a reduction of the inflammation in the brain shows protective effects (Akiyama et al., 2000; Clark et al., 2012; Lee et al., 2010).

Based on the impressive preclinical data that demonstrate neuroprotection of such drugs, clinical trials have started that investigate the neuroprotective effects of the GLP-1 receptor agonists exendin-4 (Byetta, Bydureon®) and liraglutide (Victoza®) in patients with AD or with Parkinson disease (PD). A pilot study testing the GLP-1 analogue liraglutide in AD patients showed promising results. In a double blind, placebo controlled trial, AD patients showed no deterioration in brain activity as measured in the 18F-FDG-PET scan after 12 months of drug treatment. In contrast, the placebo group showed clear reductions in cortical activity as expected for this progressive neurodegenerative disease (Gejl et al., 2016). A phase II clinical trial is currently ongoing (Holscher, 2016). A pilot trial testing exendin-4 in PD patients has shown good effects (NCT01174810). This open-label study tested the effects of exendin-4 in 45 patients. There were clear improvements in the motor coordination as shown in the MDS-UPDRS part 3 assessment, and cognition was improved as measured by the Mattis DRS-2 test battery that had been designed to evaluate cognitive impairments in PD patients (Aviles-Olmos et al., 2013). Patients were re-tested 12 months later, and the differences in motor performance and cognitive scores were still visible (Aviles-Olmos et al., 2014). A phase II double-blind, placebo controlled clinical trial that tested the once-weekly formulation of exendin-4, Bydureon®, confirmed the results of the pilot study. The drug group did not deteriorate in the MDS-UPDRS part 3 assessment after 48 weeks of treatment, and after 12 weeks of a wash-out period, the disease-modifying effect was still visible. In contrast, the placebo group deteriorated over time as would be expected in this disease (Athauda et al., 2017). In addition, a phase II trial testing liraglutide in PD patients has started, testing patients in a double blind, placebo controlled trial for one year (NCT02953665). A further clinical trial testing the GLP-1 receptor agonist Lixisenatide in PD patients is in planning.

These very promising preclinical and the first clinical outcomes demonstrate the efficacy of these novel multiple receptor agonists that originally were developed to treat type II diabetes, but have shown consistent neuroprotective effects in several studies. In the present study, the novel TA improved learning and memory, reduced amyloid plaque load, chronic inflammation and oxidative stress, as well as mitochondrial stress signaling, and improved BDNF release and synaptic markers while normalising neurogenesis in the dentate gyrus. The novel triple GLP-1/GIP/Gcg receptor agonist holds clear promise of being developed into a new treatment for chronic neurodegenerative disorders such as Alzheimer’s disease.

4. Materials and methods

4.1. Peptide and chemicals

The triple GLP-1/GIP/Gcg used in this study was synthesized by China Peptides Co, Ltd. (Shanghai, China) to 95% purity. The identity and purity of the peptide was confirmed by reversed-phase HPLC and characterized using matrix assisted laser resorption/ionization time of flight (MALDI–TOF) mass spectrometry. The peptide was stored in dry form and dissolved in double-distilled water containing 0.9% NaCl2 before experiments. A Bi-cinchoninic acid (BCA) protein assay kit was purchased from Apply gen Technologies Inc. (Beijing, China). 3, 3-diaminobenzidine (DAB) was purchased from ZSBG-BIO Co. (Beijing, China). The primary antibodies recognizing Aβ1-42, IBA-1, BDNF, 4-Hydroxyynonenal (4-HNE), 8-Hydroxyguanosine (8-OHdG), synaptophysin (SYN) and β-actin were obtained from Abcam (Cambridge, UK). The primary antibody GFAP, Bcl-2, and BAX were obtained from Boster Biotechnology (Wuhan, China). The primary antibody doublecortin (DCX) buy from Santa Cruz Biotechnology. HRP-labeled goat anti-rabbit immunoglobulin was obtained from Abcam (Cambridge, UK). The biotinylated goat anti-rabbit IgG, biotinylated rabbit anti-goat IgG, avidinbiotin peroxidase complex reagent was purchased from Boster Biotechnology (Wuhan, China). All other reagents used in the research were of analytical grade and of the highest quality commercially available. The peptide sequence was as follows (Fian et al., 2015):

\[
\text{H}XQ\text{GTTFSDKSKYLDERAAQDVFQVWLLDGPPSSGAPPPS}
\]

\[X = \text{aminoisobutyric acid, } K = \gamma\text{-C16 acylation (with a C16 fatty acid)}\]

4.2. Animals and treatments

APP/PS1 transgenic mice were purchased from the Academy of Military Medical Sciences (AMMS, China), were maintained in a constant temperature (22 ± 1 °C) and humidity (relative 30%) under a 12 h light/dark cycle in animal house. As well as age-matched non-AD mice, which are wild-type animals as control. When animals were 6 months of age, TA was dissolved in saline. The dose was a once-daily injection of 10 nmol/kg body weight ip. for two months. Injections were given in the morning (8:00 am). Control mice received equal volumes of saline solution. All experimental procedures involving animals were approved by the Institutional Animal Care Committee of Shaxi Medical University and conform to the guidelines of National Institute of Health (NIH) guideline (NIH publication No. 85-23. Revised 1985). They were divided into three different groups (n = 12): (i): control group treated with saline only; (ii): APP/PS1 group treated with saline only; (iii): APP/PS1 group treated with TA. At the end of drug treatments, measurements of behavioral change, neuronal damage, oxidative stress and inflammatory markers were conducted.

4.3. Morris water maze test

The water maze test is a standard method for assessing learning and memory in animals. The water maze (WM) task was performed 5 days after two months intraperitoneal injection. The apparatus was a circular, white painted pool (150 cm in diameter and 60 cm in height) which was filled to a depth of 40 cm with warm water at a temperature 23 ± 2 °C to avoid hypothermia, and was surrounded by a white curtain around the pool. The pool was divided into four quadrants with four starting locations, north (N), east (E), south (S) and west (W), the starting quadrant was randomly chosen. A perspex platform (diameter, 10 cm) was located in a specific location during the acquisition trials and submerged approximately 1.0 cm below the surface of the water. A video camera was mounted directly above the pool to record the movement and the trail of the mice. During the acquisition phase, each mouse was subjected to four consecutive training trials on each day. The mouse was gently placed in the water between the quadrants, and allowed 60 s to search the hidden platform. If it failed to find the platform within 60 s, it was guided to the platform and allowed
to remain there for 10 s. Training continued for 5 days. On the sixth day, the hidden platform was removed and each mouse was allowed to swim in the pool for 60 s. The mean time spent in all four quadrants and the target area for searching the hidden platform was recorded.

4.4. Brain tissue preparation

Half of the animals were decapitated after anesthetization with ethyl carbamate, hippocampus tissues were immediately isolated and stored at −80 °C for further investigations. The second half of each group was used for immunohistochemistry analysis. Animals were perfused intracardially with saline and cold 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 24 h.

4.5. Immunohistochemistry

For DAB staining, fixed tissues were embedded in paraffin. Coronal sections (5 μm thick) of the brains were cut on a Leica microtome for immunohistochemistry. Paraffin was removed from the tissue sections with xylene, and the sections were rehydrated in graded ethanol solutions. Endogenous peroxidase activity was blocked with 3% H2O2 for 10 min at room temperature. Antigen retrieval was performed by heating in 10 mmol/L citrate buffer (pH 6.0) for 10 min. After blocked with 5% BSA, sections were incubated with the primary antibody for doublecortin (goat anti-DCX; 1:50), GFAP (rabbit anti-GFAP; 1:100), IBA-1 (goat anti-IBA1; 1:200), 4-Hydroxynonenal antibody (goat anti-4 HNE; 1:200) and anti-8 Hydroxylguanosine antibody (goat anti-8 OHdG; 1:200) at 37 °C for 2 h. For the Anti-8 OHdG histology, enzymatic antigen retrieval with 10 μm/ml proteinase K for 40 min at 37 °C was performed. To detect amyloid deposits, the primary antibody was Ab1-42 (rabbit anti-Ab1-42; 1:100), brain sections were pre-incubated with 70% formic acid and incubated at 4 °C for 24 h. They were rinsed in PBS and incubated with biotinylated goat anti-rabbit IgG or biotinylated rabbit anti-goat IgG at 37 °C for 0.5 h and incubated with the avidin-biotin peroxidase complex reagent at 37 °C for 0.5 h. Samples were stained in DAB solution in the presence of H2O2. All stained sections were viewed and photographed under a Zeiss light microscope, and images were captured by Motic Images Plus2.0ML software. The numbers of DCX positive neurons, the areas of Ab1-42, GFAP, IBA-1, 4-HNE and 8-OHdG positive cells were determined using Image-pro plus6.0 software. The software counts objects that are defined in size and staining threshold by the user. This automated process avoided user bias.

4.6. Western blot

The hippocampal tissues from mice was homogenized in ice cold RIPA buffer (containing 1% Triton X-100, 0.1% SDS, 1% deoxycholate) and PMSF (phenyl-methylsulfonyl fluoride). The homogenate was centrifuged 12,000 rpm/min for 5 min at 4 °C before taking the supernatant. Tissue containing 60 μg of protein was separated on 10–12% gradient Bis–Tris gel with molecular marker and electrophoresed in running buffer at 120 mV for 90 min followed by transfer to the PVDF membrane. Following protein transfer, the membrane was washed in TBST (tris buffered saline with 0.05% Tween-20, pH 8) and blocked in 5% skim milk for 1 h. Then incubated overnight at 4 °C with rabbit anti-synaptophysin (SYN) (1:10,000), rabbit anti-Bcl-2 (1:400), rabbit anti-BAX (1:400), rabbit anti-BDNF (1:5000), after three washes in TBS further incubation for 2 h at room temperature with the HRP-labeled goat anti-rabbit immunoglobulin (1:5000). In some cases, the blots were stripped and reprobed with a rabbit anti-β-actin (1:5000) to ensure equal sample loading. The bound antibodies were then visualized by ECL-enhanced chemiluminescence (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer’s instructions. Western blot images were captured with a chemiluminescent imaging system (Sage Creation, Beijing, China).

Fig. 6. TA-treatment enhanced neurogenesis and increased numbers of DCX positive cell numbers in the dentate gyrus of APP/PS1 mice. A: control group; B: APP/PS1 group; C: APP/PS1+TA group. Scale bar in image A: 100 μm. *** = P < .001 compared with the control group; ### = P < .001 compared with the APP/PS1 group. One-way ANOVA with Bonferroni repeated measures post hoc tests. N = 6 per group.
All bands were quantified using the image system of Alpha View SA.

4.7. Statistical analysis

The data were presented as the mean ± standard error of mean (S.E.M.) and analyzed by one-way or two way ANOVA followed by Bonferroni’s multiple comparison test using the software Prism 5 (Graph Pad Software Inc, La Jolla, CA, USA). Statistical significance is defined as $P < 0.05$.

Contributors

JT, WL and YL conducted the experiments, LL and CH conceived the experiment and wrote the manuscript.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.brainres.2017.10.012

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