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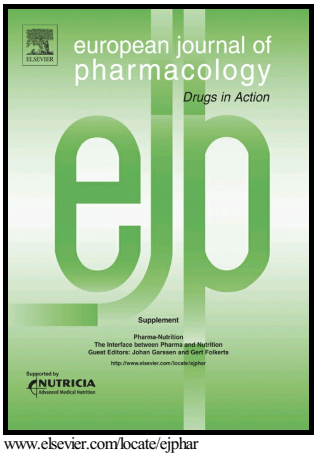

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DA5-CH, a novel GLP-1/GIP dual agonist, effectively ameliorates the cognitive impairments and pathology in the APP/PS1 mouse model of Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder for which there is no cure. The early primary symptom of AD is the decline of memory ability, which gradually develops into complete dementia. Type 2 diabetes mellitus (T2DM) is an important risk factor of AD; and mimetics of the incretin hormone GLP-1 developed to treat diabetes are being tested as a novel therapeutic strategy for AD. In the present study, we reported for the first time the neuroprotective effects of a novel GLP-1/GIP dual agonist DA5-CH that activates the incretin hormone GLP-1 and GIP receptors in the APP/PS1 transgenic AD mouse model. We found that: (1) DA5-CH administration effectively improved working-memory and long-term spatial memory of 9-month-old AD mice in Y-maze and Morris water maze tests; (2) DA5-CH also reduced hippocampal amyloid senile plaques and phosphorylated tau protein levels; (3) DA5-CH basically reversed the deficits in hippocampal late-phase long-term

potentiation; (4) DA5-CH up-regulated the levels of p-PI3K and p-AKT growth factor kinases and prevented excessive activation of p-GSK3β in the hippocampus of APP/PS1 mice. Therefore, the neuroprotection of DA5-CH in alleviating cognitive impairments and pathological damages might be associated with the improvement of hippocampal synaptic plasticity and activation of the PI3K/AKT signaling pathway. We propose that DA5-CH may be beneficial for the treatment of AD patients, especially those with T2DM or hyperglycemia.

Keywords: GLP-1/GIP dual agonist; APP/PS1 AD mice; Working memory; Long term memory; L-LTP; Amyloid-β protein; Phosphorylated tau protein; PI3K/AKT/GSK3β

1. Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder. The early primary symptom of AD is the decline of memory ability, which gradually develops into mental decline, and complete dementia in the end (Selkoe, 2001). As the population in the industrial nations reach higher life expectancies, the number of AD cases will increase continually to around 131 million by 2050 (Herrera et al., 2016). The typical neuropathological characteristics in the AD brain include high density of senile plaques, intracellular neurofibrillary tangles (NFTs) and neuronal death (Medina and Avila, 2013; Moreth et al., 2013). The main components of senile plaques are neurotoxic amyloid-β (Aβ) protein. NFTs are formed by excessively phosphorylated tau protein and the number of NFTs in the brain is closely associated to the severity of dementia and can result in impairment of hippocampal synaptic plasticity (Batool et al., 2016; Tong et al., 2015) and learning and memory in wild type rats and transgenic mice (Paul and Magda, 2009). Unfortunately, effective AD treatment is still lacking to this day.

According to recent reports, type 2 diabetes mellitus (T2DM), another degenerative disease, is an important risk factor for the development of AD (Craft, 2007; Hölscher,

2005; Hoyer, 2004; Luchsinger et al., 2004; Perry et al., 2007). A 1.5–2.5 fold increased risk of dementia has been reported in the T2DM (Strachan et al., 2011). Both AD and T2DM influence each other and share several common pathological characteristics including abnormalities in insulin level, insulin receptor and glucose metabolism. It is reported that Aβ can competitively bind to and inhibit neuronal insulin receptors in early-onset familial AD, while the neuronal insulin receptor can be desensitized in late-onset sporadic AD which is similar to those caused by T2DM. Further, insulin resistance has been linked to the increases of Aβ and inflammatory agents in the human brain (Craft, 2007; Hoyer, 2004; Li and Hölscher, 2007). These researches support the hypothesis that the disorder of insulin signaling in the brain is associated with the occurrence of AD and other neurodegenerative diseases. Thus, a probable new strategy for the AD treatment is to regulate and normalize the insulin signaling system in the brain by activation of neuronal growth factor receptors and insulin-like receptors. Interestingly, Glucagon-like protein 1 (GLP-1), an incretin hormone used for the treatment of T2DM (Lovshin and Drucker, 2009), and its mimetics also have protective effects in animal models of AD (Hölscher, 2016; McClean and Holscher, 2014a). And a first pilot study with GLP-1 analogue liraglutide has shown beneficial effects in AD patients (Gejl et al., 2016). Glucose-dependent insulinotropic polypeptide (GIP), another incretin hormone, also shows neuroprotective effects in modulating neurotransmitter release, LTP formation, and protecting synapses (Faivre et al., 2011; Faivre et al., 2012). Furthermore, GLP-1 analogues and GIP analogues have shown neuroprotective effects in AD mouse models, and this offers a new hope for the treatment of AD (Duffy and Hölscher, 2013; Faivre and Hölscher, 2013; Hölscher, 2014).

Recently, a GLP-1/GIP Dual-Agonist-5 (DA5-CH), a novel protease-resistant GLP-1/GIP dual agonist, has been designed to treat AD or Parkinson's disease. DA5-CH not only activates the GLP-1 receptor but also the sister incretin GIP receptor. Similar dual agonists have been developed to treat diabetes (Finan et al., 2013). DA5-CH can cross the blood–brain barrier much better which is important in

treating diseases of the CNS. Since separate application of GLP-1 analogue and GIP analogue has shown beneficial effects on the neuroprotection (Duffy and Hölscher, 2013; Faivre and Hölscher, 2013; Hölscher, 2014), the activation of both GLP-1 and GIP receptors with the unimolecular dual agonist may be a superior strategy for protecting neurons against AD-like insults.

2. Materials and Methods

2.1 Animals and drugs

A total of sixty 9-month-old APPswe /$PS1_{dE9}$ (APP/PS1) transgenic mice (half male and half female) and wild type (WT) C57BL/6J (C57) nest control mice (half male and half female) were ordered from Chinese Academy of Medical Sciences (Beijing, China). The mice were fed in an animal house with independent air supply system. The temperature of the animal room was maintained at 20℃-25℃, with 12 h light -12 h dark cycle and sufficient food and water supply. All animal handling and procedure were carried out according to the guidelines of the Shanxi Committee on Ethics of Animal Research. The dual agonist DA5-CH was gotten from Chinapeptides Ltd (Shanghai, China). The purity of the peptide (95%) was confirmed by reversed-phase HPLC and characterised using matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry. The drug was stored in -80˚C and dissolved in saline before the experiments.

All of the mice were divided into the following four groups: WT+Saline, WT+DA5-CH, APP/PS1+Saline and APP/PS1+DA5-CH. DA5-CH was dissolved in saline and injected intraperitoneally at a dose of 10 nmol/kg per day. In the control group, the same volume of saline was given. Behavioral tests were performed 28 days after drug application.

Antibodies against p-PI3Kp85, PI3Kp85, p-Akt (ser473), Akt, p-GSK3β (Y216), GSK3β were purchased from Abcam, Inc. (Cat #: ab182651, ab189403, ab81283, ab28422, ab75745, ab93926, Cambridge, UK). The antibody to Aβ (6E10) was

bought from Biolegend (Cat #:803015, USA). The antibody to p-tau (AT8) was purchased from Thermo Scientific (Cat #:QE217252, USA). Anti-Rabbit IgG, Anti-mouse IgG, Mouse Anti-β-actin, CY3- Anti-mouse IgG, RIPA Lysate, BCA Protein Assay Kit, DAPI staining solution and Anti-fluorescence decay agent were from Boster (Wuhan, China).

2.2 Behavioral tests

2.2.1 Open field test

To examine the locomotor activity and exploratory behavior of mice, open field test was used at first. Before the experiment, the animals had a 30 min of adaptation in the laboratory. The open field (40 ㎝×40 ㎝×40 ㎝) was divided into 16 equal size squares, in which 4 squares in the middle were defined as central area and the rest as peripheral area. Each mouse was placed individually in the apparatus from the center for 5 min and the total distance, as well as the percentage of time in center, was recorded by Smart 3.0 software system (Panlab, Spain) (Kovalenko et al., 2014). The apparatus was cleaned with 70% ethanol before each test.

2.2.2 Y-maze test

After open field test, the mice were subjected to a spontaneous alteration test in Y-maze to assess the spatial working memory of mice. The maze was made of three 30 cm long, 7 cm high, 15 cm wide arms, with a triangular joint region in the central area. In the experiment, each mouse was placed in the central triangle region, and the mouse was allowed to explore freely the open arms for 8 min. The total arm entries and arm order was recorded by Smart 3.0 software system. A correct spontaneous alternation was defined as continuously entering 3 different arms. The percentage of correct alteration was calculated (Hritcu et al., 2007; Yamada et al., 1996).

2.2.3 Spatial water maze and reversal water maze tests

The spatial learning and memory of mice was tested using Morris water maze (MWM). The maze was composed of a large circular swimming pool (diameter, 120

cm; high, 50 cm) and a small escape platform (diameter, 12 cm) in the pool. The tap water was injected into the pool, with a temperature of 22±2℃. The escape platform was submerged 1 cm beneath the water surface and placed in the center of one quadrant. The test includes two phases, acquisition trial and probe trial. The acquisition phase lasted for five training days (days 1–5) for the mice to search for the underwater platform located at the first quadrant according to the cues on the inner wall of the pool. When the mice climbed onto the platform or the time reached 1 min, the records stopped. The swimming traces of mice were monitored by a cameral right above the pool and the escape latencies were automatically recorded by Ethovision 3.0 software system (Noldus Information Technology, the Netherlands). In the probe trial on the day 6, the platform was removed from the pool, and animals were allowed to swim freely for 1 min. The frequency of platform crossing and the swimming time in the target quadrant (the first quadrant) were recorded. After the classical MWM test finished, reversal water maze test was performed to examine the cognitive flexibility of mice. The platform was moved to the opposite quadrant (the third quadrant), and mice were placed into the water for similar four days acquisition trials and one day probe trial as mentioned above (Shang et al., 2016). In visible platform test, the platform was elevated 1cm above the water level and the mice were placed into two randomly selected quadrants. Swimming time to target was recorded.

2.3 In vivo hippocampal L-LTP recording

Considering the close correlation between the long term spatial memory and the synaptic plasticity in the hippocampus, (Clements et al., 1993), we observed hippocampal long-lasting LTP by recording excitatory postsynaptic potentials (fEPSPs) in the CA1 region after finishing MWM tests. The mice, under the anesthetization with urethane (i.p. 1.5 g/kg, Sigma, UK), were placed in a stereotaxic apparatus (RWD Life Science, China). The skin was cut open to expose the skull, and a binding stimulating/recording electrode (Sequim, WA, USA) was inserted into the CA1 region of hippocampus through a small hole drilled on the skull. The two tips of the stimulating/recording electrode were located at the Schaffer collateral and stratum

radiatum of the CA1 region, respectively. Basic synaptic transmission was observed for 30 min, with stable fEPSPs under test stimulation. Then, short term synaptic plasticity was observed at first by recording paired-pulse facilitation (PPF) with two paired test stimuli. The percentage of PPF ratio (fEPSP2/fEPSP1) was calculated. To induce long lasting L-LTP, three sets of high-frequency stimulation (HFS) with an interval of 5 min were delivered to the Schaffer collateral after PPF recording. Then, the fEPSPs under test stimulation were recorded for at least 180 min after HFS. A biological signal amplifier (Chengdu Instruments Ltd, China) was applied to record and analyze the obtained data. The percentage change of fEPSP slop at different time points after HFS was compared.

2.4 Immunofluorescence staining

Immunofluorescence experiments were performed to examine the distribution of Aβ plaques and phosphorylated tau protein in the hippocampus. After *in vivo* L-LTP recording, the brain of the mice were removed and fixed with paraformaldehyde for 24 h, after that the tissue was transferred to 30％ sucrose solution. The brain was frozen to -80℃ and sectioned with freezing microtome (LEICA, CM1850, Germany) at a thickness of 30 μm. Sections were transferred to PBS, blocked with normal goat serum (Solarbio, Beijing, China), followed by adding the primary antibody, second antibody, and DAPI. The Aβ plaque and phosphorylated tau protein were observed by confocal laser scanning microscopy (OLYMPUS, FV1200, Japan). The area percentage of anti-Aβ (6E10) or anti-p-tau (AT8) immunopositive particles in at least three hippocampal slices per mouse was calculated with the software Image-Pro Plus 6.0.

2.5 Western blotting

The hippocampal tisue of mouse were isolated and homogenized in tissue protein extraction reagent, and a BCA Protein Assay Kit (Boster, Wuhan, China) was used to measure protein concentration. Taking out 50 μg of protein from each sample and separating the protein constituents on 12% SDS-polyacrylamide gels. Then, the

proteins on the gels were transferred onto PVDF membranes and the membranes were incubated with a primary antibody overnight at 4℃, followed by a secondary antibody reaction for 2 h. The optical density of the target strip (p-PI3K, t-PI3K, p-AKT, t-AKT, p-GSK3β and t-GSK3β) was analyzed by using a gel image processing system (Alpha View SA, USA).

2.6 Statistics

All data in the study were represented as mean ± standard error of mean (S.E.M.). Statistical packages including SPSS 16.0 and SigmaPlot 12.3 were used for statistical analyses. The data from acquisition trial phases in MWM tests and in vivo hippocampal L-LTP were analyzed using three-way repeated measure analysis of variance (ANOVA). Two-way ANOVA was used to analyze other data. A statistical value of $P < 0.05$ was considered as significant.

3. Results

3.1 DA5-CH did not affect the locomotor and exploratory behaviors of APP/PS1 and WT mice

In Open-field test, the spontaneous behavior of WT and APP/PS1 mice was similar to each other. The total running distances of the mice in WT+Saline, WT+DA5-CH, APP/PS1+Saline and APP/PS1+DA5-CH were 1282.85 ± 89.51 cm, 1114.40 ± 96.04 cm, 1158.11 ± 182.33 cm, and 1259.28 ± 125.81 cm, respectively. The ratio of time spent in the center were 1.99 ± 0.29%, 2.00 ± 0.27%, 1.79 ± 0.33%, and 1.88 ± 0.38%, respectively. The two-way ANOVA statistical analysis did not show any significant main effects in total distance covered by the animals (APP/PS1: $F_{(1, 43)} < 1$; DA5-CH treatment: $F_{(1, 43)} < 1$; APP/PS1 by DA5-CH interaction: $F_{(1, 43)} = 1.222$, $P > 0.05$; Fig. 1A) and the ratio of time spent in the center (APP/PS1: $F_{(1, 43)} < 1$; DA5-CH treatment: $F_{(1, 43)} < 1$; APP/PS1 by DA5-CH interaction: $F_{(1, 43)} < 1$; Fig. 1B). These results suggest that the spontaneous behavior in nine-month-old APP/PS1 AD

mice were not impaired, and DA5-CH did not affect the locomotor and exploratory performance of APP/PS1 and WT mice.

3.2 DA5-CH improved the working memory of APP/PS1 mice

In Y maze test, two-way ANOVA revealed that neither APP/PS1 nor DA5-CH treatment had significant effects on the total arm entries (APP/PS1: $F_{(1, 33)}$ =1.033, P >0.05; DA5-CH treatment: $F_{(1, 33)}$ <1; APP/PS1 by DA5-CH interaction: $F_{(1, 33)}$ <1; Fig. 2A). However, there were significant main effects of APP/PS1 and DA5-CH treatment in the percentage of right alternation (APP/PS1: $F_{(1, 33)}$ =15.704, P < 0.001; DA5-CH treatment: $F_{(1, 33)}$ =13.911, P < 0.001; APP/PS1 by DA5-CH interaction: $F_{(1, 33)}$ =13.644, P < 0.001; Fig. 2B). The percentage of right alternation in the mice of APP/PS1+Saline group (52.13 ± 2.47%, P < 0.001) had a significant decrease compared to that in WT+Saline group (69.92 ± 2.01%), while the percentage in DA5-CH treated APP/PS1 transgenic mice had a significant improvement (69.38 ± 2.47%, P < 0.001), very close to the value in the normal control group. These results indicate that the working memory, not general motor ability, of APP/PS1 transgenic mice in Y-maze was seriously impaired, which could be improved by DA5-CH treatment.

3.3 DA5-CH ameliorated the spatial cognition deficits of APP/PS1 transgenic mice in classical MWM test.

Fig. 3A and 3B show the results of the acquisition phase for each group of mice in the MWM for 5 consecutive days. The mean escape latency in the four groups was decreased after each training day ($F_{(4, 148)}$ =17.012, P < 0.001). Significant main effects existed in group WT vs. APP/PS1 ($F_{(1, 38)}$ =5.839, P < 0.05) and in group saline vs. DA5-CH treatment ($F_{(1, 38)}$ =6.643, P < 0.05) on escape latency. There was a significant interaction between APP/PS1 and DA5-CH treatment ($F_{(1, 38)}$ =7.573, P < 0.05). The escape latencies in the APP/PS1+Saline group were significantly higher on the training day 2 (48.62 ± 4.18 s, P < 0.05), day 3 (50.84 ± 4.00 s, P < 0.01), day 4

(48.77 ± 4.44 s, $P < 0.01$), and day 5 (45.46 ± 4.14 s , $P < 0.01$) compared to the values in the WT+Saline group on the corresponding training days 2-5 (37.48 ± 3.42 s, 35.40 ± 3.27 s, 31.62 ± 3.63 s and 26.89 ± 3.38 s). Importantly, DA5-CH treatment significantly decreased the values in APP/PS1 mice, being 33.44 ± 4.05 s ($P < 0.01$), 35.79 ± 4.39 s ($P < 0.05$) and 31.61 ± 4.11 s ($P < 0.05$) on day 3-5, respectively, significantly lower than that of the APP/PS1+Saline group. This indicates that chronic treatment with DA5-CH can significantly ameliorate spatial learning in APP/PS1 mice.

The spatial memory retrieval of mice was assessed by the probe trial. Two-way ANOVA showed that APP/PS1 and DA5-CH treatments had significant main effects on the percentage of swimming time in target quadrant (APP/PS1: $F_{(1, 38)} =12.561$, $P < 0.01$; DA5-CH treatment: $F_{(1, 38)} =9.940$, $P < 0.01$; APP/PS1 by DA5-CH interaction: $F_{(1, 38)} =12.417$, $P < 0.01$) and the number of platform crossings (APP/PS1: $F_{(1, 38)} =6.955$, $P < 0.05$; DA5-CH treatment: $F_{(1, 38)} =4.618$, $P < 0.05$; APP/PS1 by DA5-CH interaction: $F_{(1, 38)} =4.720$, $P < 0.05$). As shown in Fig. 3C, the percentage of swimming time in target quadrant in the APP/PS1 mice (27.30 ± 2.40%, $P < 0.01$) was far lower compared to the WT mice (34.10 ± 1.10%), but not in the DA5 treated APP/PS1 mice (33.60 ± 2.00%, $P < 0.05$). We also counted the number of platform crossings (Fig. 3D) in the four groups of mice and found similar results. The frequency of platform crossings in APP/PS1+Saline group (1.00 ± 0.28) was significantly lower than that in WT+Saline group (3.00 ± 0.34, $P < 0.01$), while the frequency in DA5-CH treated APP/PS1 transgenic mice was higher (2.00 ± 0.29, $P < 0.01$), indicating that the spatial memory disorder in the AD mice could be effectively ameliorated by the chronic treatment with DA5-CH.

To exclude the impact of motor ability and vision impairments on the above tests, we performed a visible platform test after the probe trial. We found that there was no significant difference in the escape latency between all groups (20.77 ± 2.63 s, 20.13 ± 2.67 s, 22.21 ± 3.10 s, 25.26 ± 2.47 s, respectively, APP/PS1: $F_{(1, 38)} =1.297$, $P > 0.05$; DA5-CH treatment: $F_{(1, 38)} <1$; APP/PS1 by DA5-CH interaction: $F_{(1, 38)} <1$) in

the visible test, suggesting that the prolongation of the escape latency, decrease of the percentage of time in target quadrant and frequency of platform crossing in APP/PS1 mice is not due to the differences in vision and swimming ability, but is due to the impairments in spatial learning and memory function.

3.4 DA5-CH protected the relearning ability and cognitive flexibility of APP/PS1 transgenic mice in a reversal MWM test

In the reversal MWM test, as shown in the Fig. 4A and 4B, the times (escape latency) to find the hidden platform was decreased following the 4-d training sessions ($F_{(3, 87)}$ =6.455, $P < 0.01$). Significant main effects existed in group WT vs. APP/PS1 ($F_{(1, 33)}$ =5.912, $P < 0.05$) and in group saline vs. DA5-CH treatment ($F_{(1, 33)}$ =4.833, $P < 0.05$) on escape latency. There was a significant interaction between APP/PS1 and DA5-CH treatment ($F_{(1, 33)}$ =5.213, $P < 0.05$). The mean escape latency of mice in the APP/PS1 model group on the training day 9 (40.87 ± 3.61 s, $P < 0.01$), and day 10 (36.19 ± 3.63 s, $P < 0.05$) was significantly higher than that in the normal WT group (26.86 ± 3.38 s and 23.37 ± 3.40 s). However, DA5-CH decreased the escape latency in APP/PS1 mice on the day 8 (30.66 ± 4.52 s, $P < 0.05$), day 9 (28.03 ± 3.62 s, $P < 0.05$), and day 10 (25.14 ± 3.64 s, $P < 0.05$), compared with the APP/PS1+Saline group. In the probe trials, two-way ANOVA showed that APP/PS1 and DA5-CH treatments had significant main effects on the percentage of swimming time in target quadrant (APP/PS1: $F_{(1, 33)}$ =11.154, $P < 0.01$; DA5-CH treatment: $F_{(1, 33)}$ =4.898, $P < 0.05$; APP/PS1 by DA5-CH interaction: $F_{(1, 33)}$ =5.386, $P < 0.05$) and the number of platform crossings (APP/PS1: $F_{(1, 33)}$ =4.049, $P < 0.05$; DA5-CH treatment: $F_{(1,33)}$ =4.731, $P < 0.05$; APP/PS1 by DA5-CH interaction: $F_{(1, 33)}$ =6.466, $P < 0.05$). As shown in Fig. 4C, the significant decrease in the percentage of swimming time in target quadrant in the APP/PS1 mice (14.40 ± 1.60%, $P < 0.001$) compared with the WT mice (32.50 ± 4.40%) had an obvious recovery in the DA5 treated APP/PS1 mice (26.30 ± 2.30%, $P < 0.05$), compared with that in the APP/PS1+Saline group. Similarly, the decreased frequency of platform crossing (Fig. 4D) in APP/PS1+Saline group (1.00 ± 0.24, $P < 0.05$) had a significant increase in DA5-CH treated APP/PS1

mice (2.00 ± 0.45, $P < 0.05$). This indicates that chronic treatment with DA5-CH can alleviate the impairment in the relearning ability and cognitive flexibility of APP/PS1 transgenic mice.

3.5 DA5-CH effectively reversed the impairment of hippocampal synaptic plasticity in APP/PS1 transgenic mice.

We further analyzed the effects of DA5-CH on the *in vivo* hippocampal L-LTP after behavioral tests. As shown in Fig. 5A, the average fEPSP slopes during 30 min of baseline recording under low frequency of test stimulation were stable in all groups. After delivering three sets of HFS to the hippocampal Schaffer collateral, the induction of LTP was successfully induced in all groups and there was no significant difference between groups. However, the 180-min recording of L-LTP showed that the hippocampal L-LTP of APP/PS1 transgenic mice was impaired and the treatment with DA5-CH could reverse this impairment effectively ($F_{(3, 60)}$ =13.104, $P < 0.001$). There were a significant main effect of APP/PS1 ($F_{(1, 20)}$ =6.733, $P < 0.05$) and DA5-CH treatment ($F_{(1, 20)}$ =4.951, $P < 0.05$) on 180-min recording of L-LTP and a significant interaction between APP/PS1 and DA5-CH treatment ($F_{(1, 20)}$ =5.102, $P < 0.05$). Fig. 5B showed the percentage changes in fEPSP slopes at three time points after HFS. There was a significant decline in the maintenance of L-LTP at 60 min (119.10 ± 11.90%, $P < 0.05$), 120 min (112.80 ± 10.80%, $P < 0.05$) and 180 min (112.30 ± 11.50%, $P < 0.05$) after HFS in the APP/PS1 model group, compared with that in the control group (155.20 ± 12.00%, 155.30 ± 10.90% and 150.90 ± 11.60%, respectively). In contrast, in DA5-CH injected APP/PS1 transgenic mice, L-LTP was maintained at higher levels, being 153.30 ± 11.30%, 158.00 ± 10.20% and 150.70 ± 11.30% ($P < 0.05$ for each one) at 60 min, 120 min and 180 min after HFS, respectively. The results indicate that DA5-CH could effectively reverse the impairment of hippocampal L-LTP in the APP/PS1 AD mice.

In order to clarify the presynaptic or postsynaptic mechanism of DA5-CH affecting L-LTP, two paired test stimuli were delivered to the Schaffer before HFS to induce

paired-pulse facilitation (PPF), a short term synaptic plasticity reflecting presynaptic neurotransmitter release. No significant difference in the facilitation of fEPSP was found between all groups (APP/PS1: $F_{(1, 20)}$ <1; DA5-CH treatment: $F_{(1, 20)}$ =1.055, P > 0.05; APP/PS1 by DA5-CH interaction: $F_{(1, 20)}$ <1; Fig. 5D). The PPF ratio values were 149.85 ± 8.13%, 167.52 ± 13.81%, 151.56 ± 9.03%, and 154.44 ±7.85% in WT+Saline, WT+DA5-CH, APP/PS1+Saline, and APP/PS1+DA5-CH mice, respectively. This suggests that neither DA5-CH nor APP/PS1 gene expression had any effect on neurotransmitter release from presynaptic terminals, and the effects of DA5-CH and APP/PS1 genes on hippocampal L-LTP are mainly associated with postsynaptic changes.

3.6 DA5-CH reduced hippocampal Aβ plaque load and tau phosphorylation in APP/PS1 mouse brains

In immunofluorescence staining studies, we aimed to evaluate the histopathologic changes in hippocampus of mice. We observed and compared the Aβ plaque and neurofibrillary tangles of hippocampus among all groups. Two-way ANOVA showed that APP/PS1 and DA5-CH treatments had significant main effects on the Aβ plaque (APP/PS1: $F_{(1, 12)}$ =66.471, P < 0.001; DA5-CH treatment: $F_{(1, 12)}$ =60.085, P < 0.001; APP/PS1 by DA5-CH interaction: $F_{(1, 12)}$ =61.892, P < 0.001) and the neurofibrillary tangles of hippocampus (APP/PS1: $F_{(1, 12)}$ =45.710, P < 0.001; DA5-CH treatment: $F_{(1, 12)}$ =18.175, P < 0.01; APP/PS1 by DA5-CH interaction: $F_{(1, 12)}$ =9.868, P < 0.01). As shown in Fig. 6, there were few Aβ plaques in the hippocampal slices in the normal control group and DA5-CH alone group. In contrary, a large number of Aβ positive plaques was observed in the hippocampal slices of APP/PS1+Saline group (483.08 ± 65.03%, P < 0.001), significantly more than in the WT+Saline (100 ± 9.45%). Importantly, the numbers of Aβ plaques had a significant reduction in the hippocampus of APP/PS1+DA5-CH group mice (109.83 ± 15.71%, P < 0.001) compared to that in the APP/PS1 model group. The Fig. 7 shows representative phosphorylated tau-positive cells in the hippocampus of mice. The positive staining of phosphorylated tau in the hippocampus of APP/PS1+Saline group mice (327.83 ±

26.34%, $P < 0.001$) had a significantly increase compared to that in the normal WT mice (100 ± 16.78%), while it was strongly suppressed by the DA5-CH pretreatment in the APP/PS1+DA5-CH group (157.25 ± 35.25%, $P < 0.001$). These results demonstrate that nine-month-old APP/PS1 transgenic mice have shown distinct Aβ plaques and neurofibrillary tangles in the hippocampus and the treatment with DA5-CH can effectively reduce these pathological biomarkers.

3.7 DA5-CH normalized the PI3K/AKT/GSK3β signaling activity in the hippocampus of APP/PS1 mice

To further explore the probable molecular mechanism of DA5-CH in ameliorating the behavioral damage and pathological features in the APP/PS1 mice, we detected the expression levels of p-PI3K, p-AKT, and p-GSK3β in the hippocampus by Western-blot (Fig.8). We found that the relative protein expression of total PI3K/β-actin, total AKT/β-actin and total GSK3β/β-actin did not change in all groups (Fig.8 C, E, G). However, APP/PS1 and DA5-CH treatments had significant main effects on the levels of p-PI3K (APP/PS1: $F_{(1, 20)} = 4.781$, $P < 0.05$; DA5-CH treatment: $F_{(1, 20)} = 6.339$, $P < 0.05$; APP/PS1 by DA5-CH interaction: $F_{(1, 20)} = 4.922$, $P < 0.05$) and the levels of p-AKT (APP/PS1: $F_{(1, 20)} = 7.564$, $P < 0.05$; DA5-CH treatment: $F_{(1, 20)} = 4.251$, $P < 0.05$; APP/PS1 by DA5-CH interaction: $F_{(1, 20)} = 5.284$, $P < 0.05$) and the level of p-GSK3β (APP/PS1: $F_{(1, 20)} = 10.528$, $P < 0.01$; DA5-CH treatment: $F_{(1, 20)} = 8.407$, $P < 0.05$; APP/PS1 by DA5-CH interaction: $F_{(1, 20)} = 9.820$, $P < 0.01$). The levels of p-PI3K, p-AKT in APP/PS1+Saline group mice showed down-regulation (43.70 ± 8.40%, $P < 0.05$; 37.40 ± 9.00%, $P < 0.05$, respectively) , while the level of p-GSK3β (185.87 ± 14.86%, $P < 0.05$) showed up-regulation, compared to the values in the WT+Saline group. Importantly, compared with the APP/PS1+Saline group, the levels of the p-PI3K and p-AKT were increased (94.30 ± 18.90%, $P < 0.05$; 96.40 ± 16.40%, $P < 0.05$, respectively), and the levels of p-GSK3β had been decreased (105.11 ± 10.01%, $P < 0.05$) in the group of APP/PS1 mice injected with DA5-CH, suggesting that DA5-CH may plays a neuroprotective

role via normalizing the PI3K/AKT/GSK3β signaling pathway in the hippocampus of APP/PS1 mice.

4. Discussion

The earliest manifestation of AD is the deficit of working memory, which gradually develops into impairments in reference memory and general cognitive function, and eventually severe dementia (Selkoe, 2001). At present, the standard treatment for AD is the use of cholinesterase inhibitors or NMDA receptor blockers to improve the cognitive behavior of patients. However, apart from their side effects, these drugs can not fundamentally stop the disease progression of AD. We therefore tested the GLP-1/GIP dual agonist DA5-CH in nine-month-old APP/PS1 AD mice.

The results of behavioral tests showed that the APP/PS1 mice had a significant decrease in the percentage of spontaneous alternation in Y maze, a increase in the escape latency in underwater platform searching, and a significant decrease in the swimming time or platform crossing in the target quadrant; while the total running distance, total arm entries and average swimming speed in the AD mice were basically normal. These results indicated that the APP/PS1 mice displayed excellent behavioral phenotypes including working memory and long term spatial memory impairments. Moreover, the APP/PS1 mice also exhibited a decline in the cognitive flexibility in reversal MWM test. Importantly, we confirmed for the first time that chronic intraperitoneal injection of GLP-1/GIP dual agonist DA5-CH effectively improved the working memory and long-term memory in the APP/PS1 mice. This is in line with the results we have reported before, that the GLP-1 analogue Liraglutide prevents the decline of spatial memory, the deficits of hippocampal LTP, chronic inflammation and loss of synapses in APP/PS1 mice (McClean et al., 2011; McClean and Holscher, 2014a; McClean et al., 2015) or amyloid-infused rats (Qi et al., 2016), and that the GLP-1 analog Lixisenatide also reduces amyloid plaque load, memory and LTP impairments, and synapse loss and chronic inflammation in the APP/PS1 mouse model (McClean and Holscher, 2014b), and effectively antagonizes Aβ

induced neuronal impairment and improves spatial memory (Cai et al., 2017). Besides, Velmurugan et al. found the neuroprotection of GLP-1 analogues in differentiated human neuroprogenitor cells (Velmurugan et al., 2012). When analyzing GIP analogues, D-Ala^2-GIP also protects memory formation, LTP induction, and increases the number of synapses in the brain and reduces Aβ plaque deposition while reducing chronic inflammation in APP/PS1 mice (Duffy and Holscher, 2013; Faivre and Holscher, 2013a, b). As for the role of GLP-1/GIP dual agonist on memory disorders, Shi et al. have shown the potential neuroprotective effect of dual GLP-1/GIP receptor agonist (DA-JC4) in the i.c.v. streptozotocin (STZ)-induced AD rat model, and they found that treatment with DA-JC4 significantly prevented working memory and long term spatial memory impairments in Y-maze test and Morris water maze tests (Shi et al., 2017a). This is in line with the results in the present study that chronic intraperitoneal injection of GLP-1/GIP dual agonist DA5-CH effectively improved the working memory and long-term memory in the APP/PS1 mice. These results suggest that a dual GLP-1/GIP receptor agonist such as DA5-CH may have disease modifying properties in AD patients. The probability is still to be confirmed by further clinical trials in the future.

It has been certified that the spatial learning and memory in animals are encoded by alteration or modulation of synaptic strength in the hippocampus (Clements et al., 1993). The hippocampal LTP, a persistent increase in postsynaptic reaction induced by HFS, has been thought as a primary cellular mechanisms determining learning and memory (Clements et al., 1993; Cooke and Bliss, 2006). It is well known that different types of stimulation can induce different strength of hippocampal LTP. In the present study, three sets of HFSs were applied to induce the long-lasting late phase of LTP, in which the enhanced synaptic transmission was kept at least for 180 min. The long-lasting LTP is dependent on cAMP-dependent protein kinase activity (PKA) and de novo gene expression, and the synthesis of new synaptic protein (Impey S et al., 1996). Thus, L-LTP may be the electrophysiological basis affecting long term memory in behavior (Abraham et al., 1993). Indeed, we found that DA5-CH alone did

not affect the basic synaptic transmission, but treatment with DA5-CH could significantly prevent the hippocampal L-LTP deficit in the APP/PS1 AD mice. The protective effect of DA5-CH on L-LTP might well explain the behavioral improvements of APP/PS1 transgenic mice in the successive 5 days of MWM test. It is reported that AD mouse model has significant decrease in dendritic spines density in hippocampal neurons (Chen et al., 2014); Aβ overproduction also affected dendritic spine morphology in the hippocampus (Knafo et al., 2009; Merino-Serrais et al., 2011); GLP-1 improved synaptic plasticity and structural plasticity (i.e., dynamics of dendritic spines) (Mainardi et al., 2015). Further, over excitation of NMDA receptors led to excessive Ca^{2+} influx through receptor-associated ion channels, resulting in neuronal cell injury or death (Folch et al., 2017); our previous study also showed that Exendin-4, a GLP-1 analog used for T2DM treatment, significantly antagonized Aβ1-42-induced LTP suppression and $[Ca^{2+}]_i$ elevation through VDCCs and NMDARs (Wang et al., 2015). Therefore, we suppose that the alteration of hippocampal dendritic spines and NMDARs might be implicated in the impairments of LTP and cognitive behavior in the AD mice in the present study, while the improvement of cognitive behavior and synaptic plasticity by DA5-CH could be associated with the modification of synaptic morphology and the regulation of NMDARs.

In addition to the gathering of Aβ, another typical pathological features of AD is neurofibrillary tangles formed by excessive phosphorylated tau (p-tau) protein aggregation (Rönicke et al., 2011). Our experimental results demonstrated that the immunopositive staining of Aβ and phosphorylated tau in the hippocampus of APP/PS1 transgenic mice was significantly increased compared to WT mice, while DA5-CH treatment distinctively ameliorated the aggregation of Aβ plaques and excessive phosphorylation of tau protein. A previous study also showed a reduction of tau phosphorylation in a transgenic mouse model by treatment with liraglutide (Hansen et al., 2016). These changes may also account for the improvement of cognitive behavior in APP/PS1 AD mice. In addition, inflammation has been thought

as a critical event in AD pathology (Lim et al., 2015). Reactive microglia and astrocytes adjacent to Aβ core consist of inflammatory plaques with Aβ in the AD brain (Itagaki et al., 1989). Chronic inflammation leads to the production of several cytokines that have been demonstrated to exacerbate other AD pathologies (Kitazawa et al., 2004). Aβ-induced down-regulation of PI3K/AKT lead to excessive activation of GSK3β, which accelerating the gene expression of the inflammatory factors such as TNF-α，triggering the inflammatory reaction in the brain and exacerbating the AD's condition (Phoon et al., 2016). Previously, we have shown that the GLP-1 analogue Liraglutide prevents the chronic inflammation and loss of synapses in APP/PS1 mice (McClean et al., 2011; McClean and Holscher, 2014a; McClean et al., 2015) or amyloid-infused rats (Qi et al., 2016), and that the GLP-1 analogue Lixisenatide also reduces amyloid plaque load and chronic inflammation in the APP/PS1 mouse model (McClean and Holscher, 2014b). Further, the neuroprotection of DA-JC1, a GLP-1/GIP dual agonist similar to DA5-CH, has been reported by reducing chronic inflammation in the brain (Cao et al., 2016b). Thus, considering the close association between inflammation and Aβ or PI3K/AKT/GSK3β signals, it is reasonable to assume that anti-inflammation effect might contribute positively to the neuroprotection of DA5-CH.

The alterations in cerebral pathological features and cognitive behaviors in the AD model mice may reflect changes in the signaling pathways in the brain. GSK3β is one of the kinases that phosphorylates tau protein. This kinase is involved in the regulation of cellular processes (Farris et al., 2003; Gasparini et al., 2001). Over-expression of GSK3β in transgenic drosophila brains significantly elevated the phosphorylation level of tau protein (Leissring, 2003). GSK3β is activated by the phosphorylation of tyrosine at 216. PI3K/AKT signaling is an important pathway for regulating GSK3β. When activated by the cell surface receptor, PI3K phosphorylates downstream AKT. The activated AKT inhibits the phosphorylation of 216 tyrosine of GSK3β, thereby inhibiting the activity of GSK3β protein (Kitagishi et al., 2012). In our study, there were high levels of senile plaques and phosphorylated tau, as well as

unbalanced PI3K/AKT/ GSK3β activity in the APP/PS1 mice. DA5-CH treatment not only decreased the pathological biomarks, but also normalized the unbalanced PI3K/AKT/GSK3β levels. It's noteworthy that the mice used in the signal pathway experiment were just the animals after experiencing behavioral and electrophysiologic experiments. The changes in different physiological indexes on the same mice may reflect their close correlation and contribution in the study. In fact, the molecular mechanism of AD involves multiple signaling pathways such as PI3K/AKT/GSK3β, Ras/MAPK-ERK, PKA/CREB etc (Cuellar and Isokawa, 2011; Frago et al., 2011; Mainardi et al., 2015). We speculate that Aβ-induced down-regulation of PI3K/AKT lead to excessive activation of GSK3β, by which accelerating the phosphorylation of tau protein, the degradation of microtubule in neurons, and the impairment of synaptic plasticity and memory formation. In contrast, DA5-CH can prevent the excessive activation of GSK3β by up-regulating the PI3K/AKT signaling, thus protecting memory and improving the pathological changes in the AD transgenic mice. A previous study showed that the dual agonist DA4-JC improved insulin signaling in the i.c.v. STZ rat model of AD (Shi et al., 2017b). APP/PS1 mice show insulin desensitization that can be reversed by GLP-1 analogues (Long-Smith et al., 2013), and the normalization of insulin signaling could be the reason for the normalization of PI3K/AKT signaling. Previously, we have shown the such dual agonists have protective effects in animal models of Parkinson (Cao et al., 2016a; Hölscher, 2016; Jalewa et al., 2017; Ji et al., 2016; Yuan et al., 2017), and stroke (Han et al., 2016), demonstrating the great potential that such dual receptor agonists show. It is interesting that the neuroprotection of DA5-CH treatment achieves almost-complete reverse effect on the behaviors or the pathologies, while it can only partially ameliorate the PI3K/AKT/GSK3β signaling pathway. This result may suggest that not only PI3K/AKT/GSK3β but also other signaling pathways such as Ras/MAPK-ERK, PKA/CREB are involved in the neuroprotection mechanism of DA5-CH (Cuellar and Isokawa, 2011; Frago et al., 2011; Mainardi et al., 2015). Therefore, to be sure, there are many mechanisms involved in the neuroprotective effect of DA5-CH.

In summary, the present study confirmed for the first time the neuroprotective roles of the novel GLP-1/GIP dual agonist DA5-CH on the cognitive impairments and neuropathological processes in APP/PS1 transgenic AD mice. The neuroprotection of DA5-CH was associated with the improvement of hippocampal synaptic plasticity and PI3K/AKT/GSK3β signaling pathway. Therefore, this study provides a novel and promising strategy to treat neurodegenerative disease such as AD.

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Conflict of interest: CH is a named inventor on a patent application covering dual agonist peptides as treatments for neurodegenerative disorders. The patent is owned by Lancaster University, UK.

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Fig. 1. DA5-CH did not affect the locomotor and exploratory performance of APP/PS1 and WT mice. (A and B), Histograms showing the total running distance in the open field (A) and running time spent in the central area (B) of mice in 5 min, without any significant difference between the four groups (P>0.05, n＝8-14). Each column represents the mean ± S.E.M.

Fig. 2. DA5-CH improved working memory of APP/PS1 mice in the Y-maze. (A), Total arm entries of mice in 8 min, without any significant difference between the four groups (P>0.05, n＝8-14). (B), The percentage of correct spontaneous alternation of mice, with a decrease only in the mice of APP/PS1+Saline group (*** P<0.001 , n＝8-14). Each column represents the mean ± S.E.M.

Fig. 3. DA5-CH ameliorated the deficits in spatial learning and memory of APP/PS1 mice in the WM test. (A and B), Average escape latencies of mice in acquisition trial phase from training day 1 to day 5. *P<0.05, **P<0.01. (C and D), Percentage of swimming time in target quadrant (C) and frequency of platform crossing (D) in the probe trial phase on the sixth day. *P<0.05, **P<0.01. All values were displayed as mean ± S.E.M (n＝8-14). (E), Representative swimming tracks of mice in different groups in the probe trials. The black dots indicate the starting points placing the mice into the pool. The small open circles indicate the previous location of platform.

Fig. 4. DA5-CH improved relearning and the cognitive flexibility of APP/PS1 mice. (A and B), Average escape latencies of mice in acquisition trial phase from training day 7 to 10. *P<0.05, **P<0.01. (C and D), Percentage of time in target quadrant (C) and frequency of platform crossing (D) in the probe trial phase on the training day 11. *P<0.05, ***P<0.001. All values were displayed as mean ± S.E.M (n＝8-14). (E), Representative swimming tracks of mice in the probe trials. Note that the platform (small open circles) had been shifted to the opposite quadrant.

Fig. 5. DA5-CH effectively reversed the impairment of hippocampal L-LTP in APP/PS1 transgenic mice. (A), Scatter plots showing the changes of fEPSP slope in different groups. After application of three sets of HFS, LTP was successfully induced in all groups, but the maintenance of LTP, especially the L-LTP at 180 min after HFS, is different, with a lowest percentage in the APP/PS1+Saline mice. (B), Average

fEPSPs slope values in four groups at different time points before and after HFSs. Each column indicates the mean ± S.E.M. of fEPSPs slope. *P<0.05 (n＝6). (C), Representative fEPSP traces before (black) and 180 min after (red) HFS. (D), Histograms showing the PPF change percentage in all groups, without any significant differences between groups. Inserts: a representative PPF trace recorded before HFS.

Fig. 6. DA5-CH reduced hippocampal Aβ plaque loads in the brain of APP/PS1 mice. (A), The immunofluorescence micrographs showing the hippocampal Aβ plaques in four groups of mice. The immunofluorescent red stains are Aβ-positive plaques recognized by the 6E10 antibody. The blue shows nuclei (DAPI). Scale bar = 100 μm (top) and 50 μm (bottom). (B), Aβ plaque levels. All data are expressed as the percentage of control values. ***P<0.001 (n＝4)

Fig. 7. DA5-CH reduced hippocampal p-tau levels in APP/PS1 mouse brains. (A), The immunofluorescence micrographs showing hippocampal p-tau (red) recognized by the AT8 antibody, and nuclei in blue (DAPI). Scale bar = 100 μm (top) and 50 μm (bottom). (B), Quantitative analysis of p-tau levels. All data are expressed as the percentage of control values. ***P<0.001 (n＝4).

Fig. 8. Western blot quantitative analysis for the PI3K-AKT-GSK3β signaling pathway in the hippocampus of all groups. (A), Representative Western blot scan showing the expression levels of p-PI3K, t-PI3K, p-AKT, t-AKT, p-GSK3β and t-GSK3β in different groups. (B, C, D, E, F and G), Quantitative analysis of levels. All data are expressed as the percentage of control values. *P<0.05 (n＝6)

Fig.1

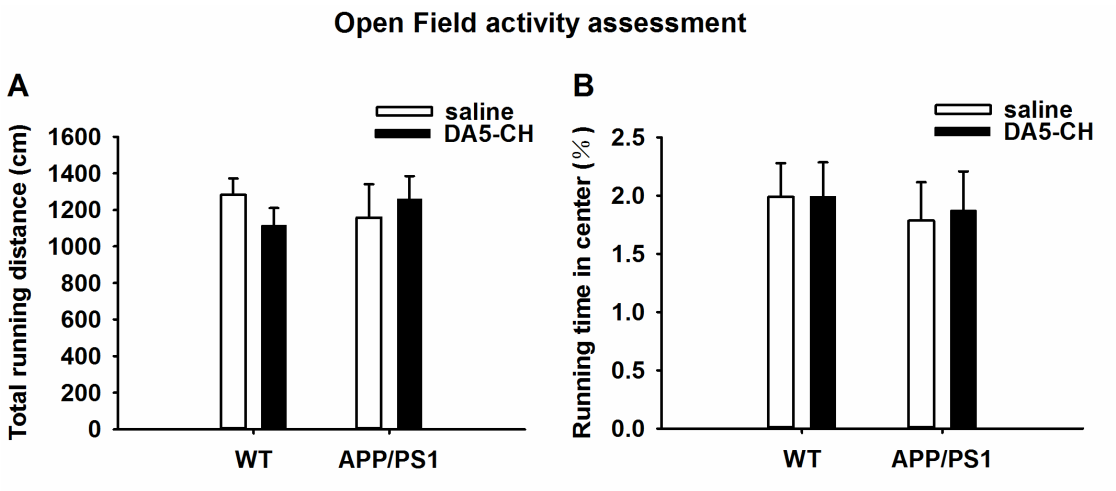
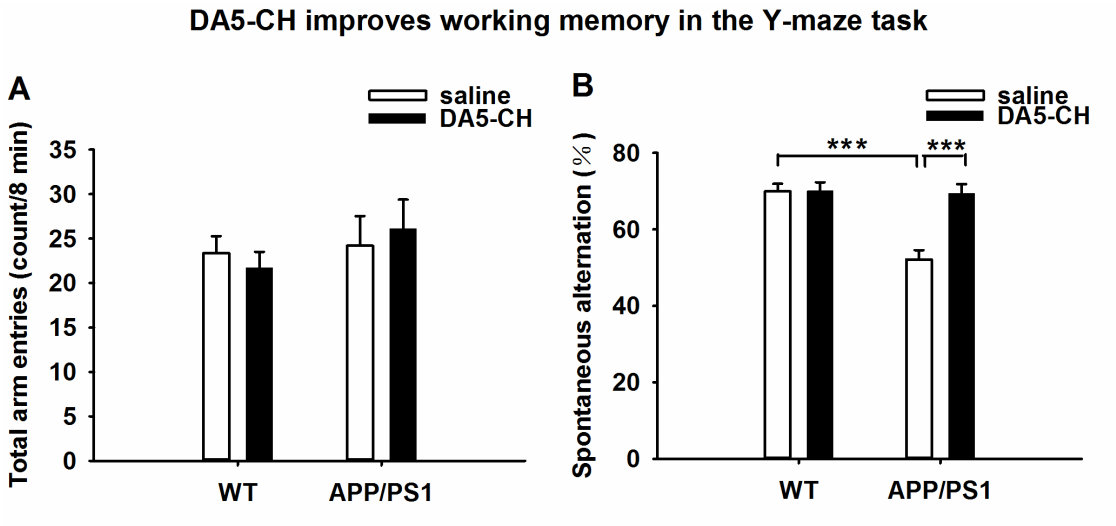


Fig.2



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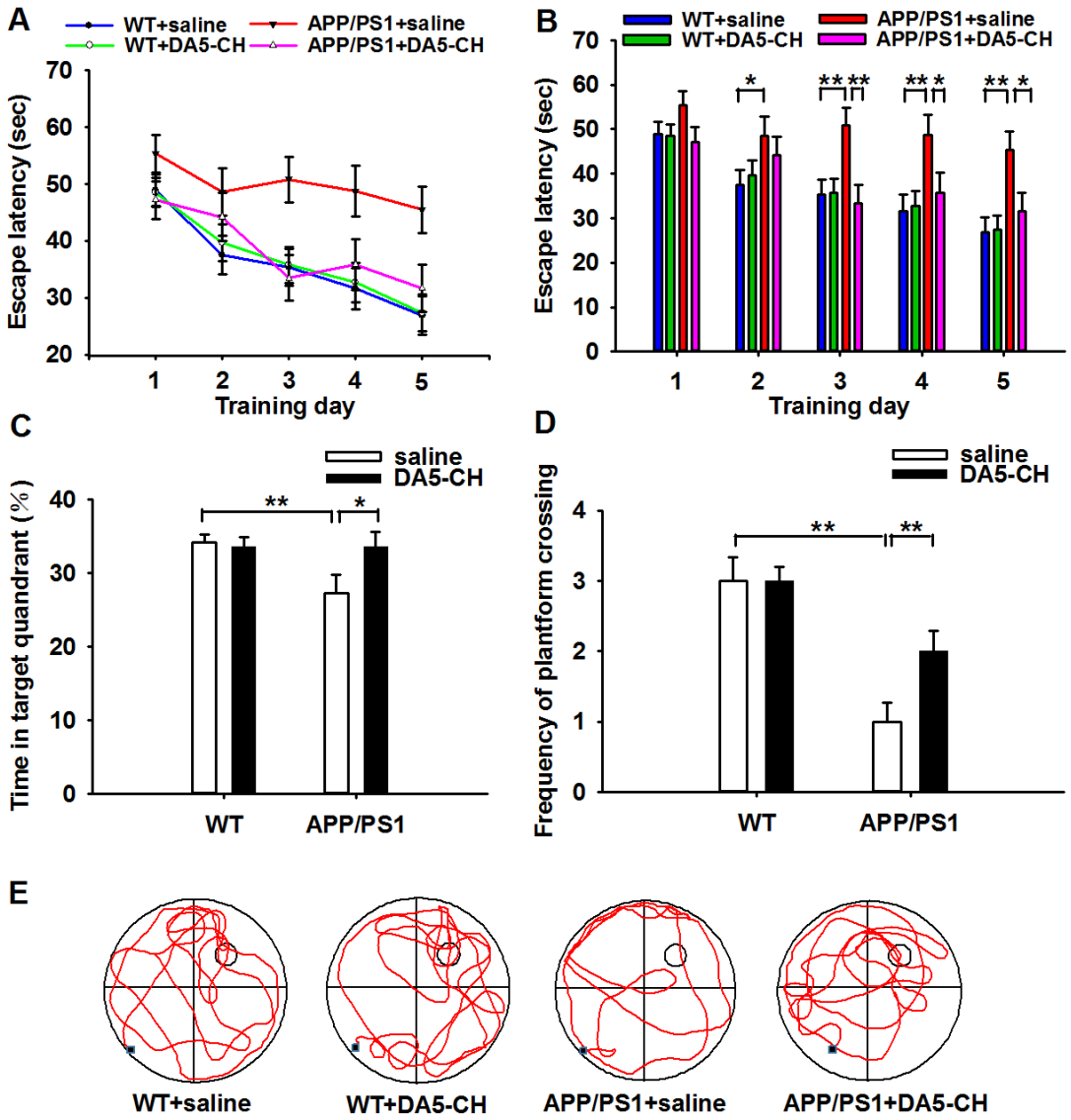

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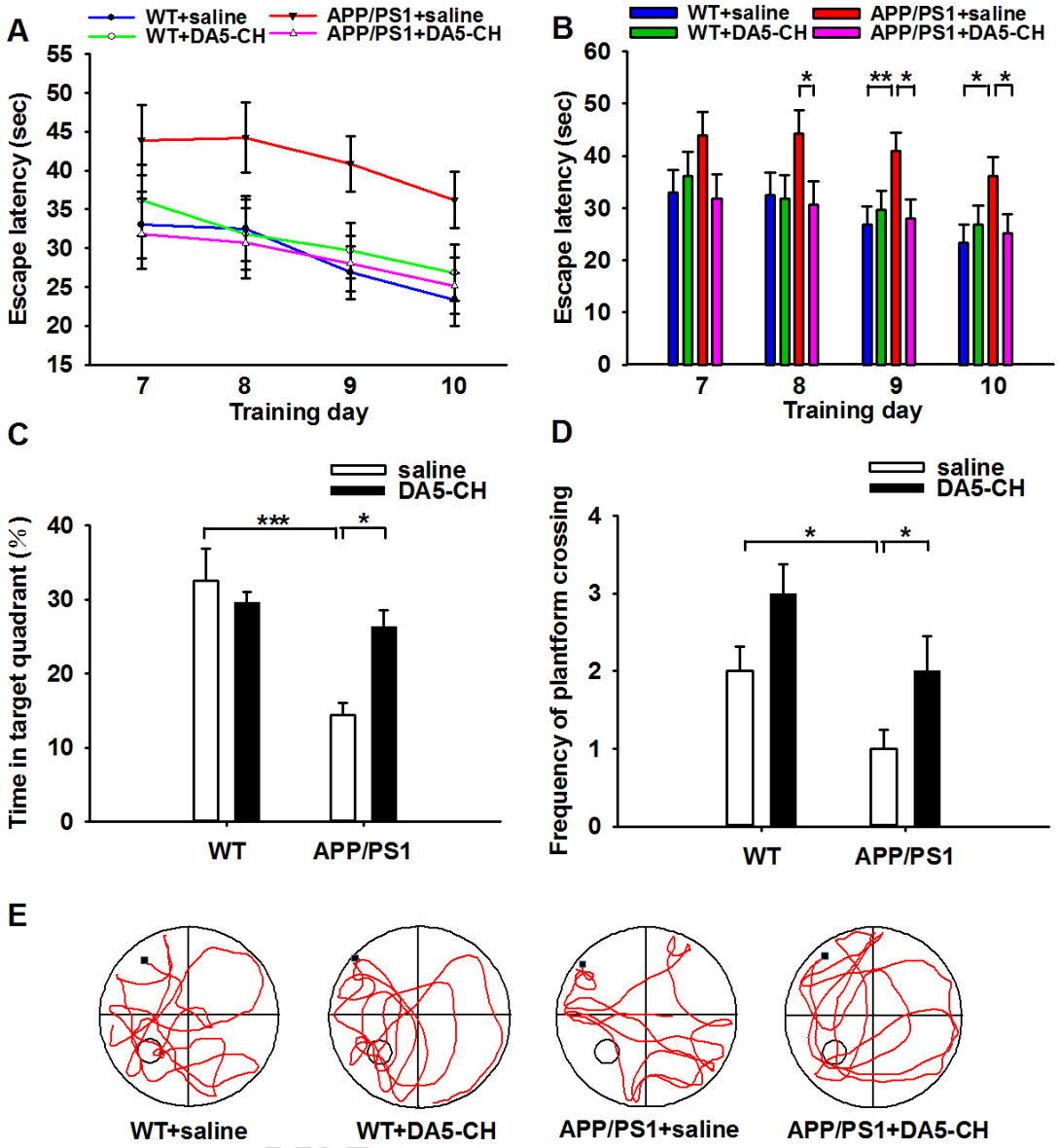

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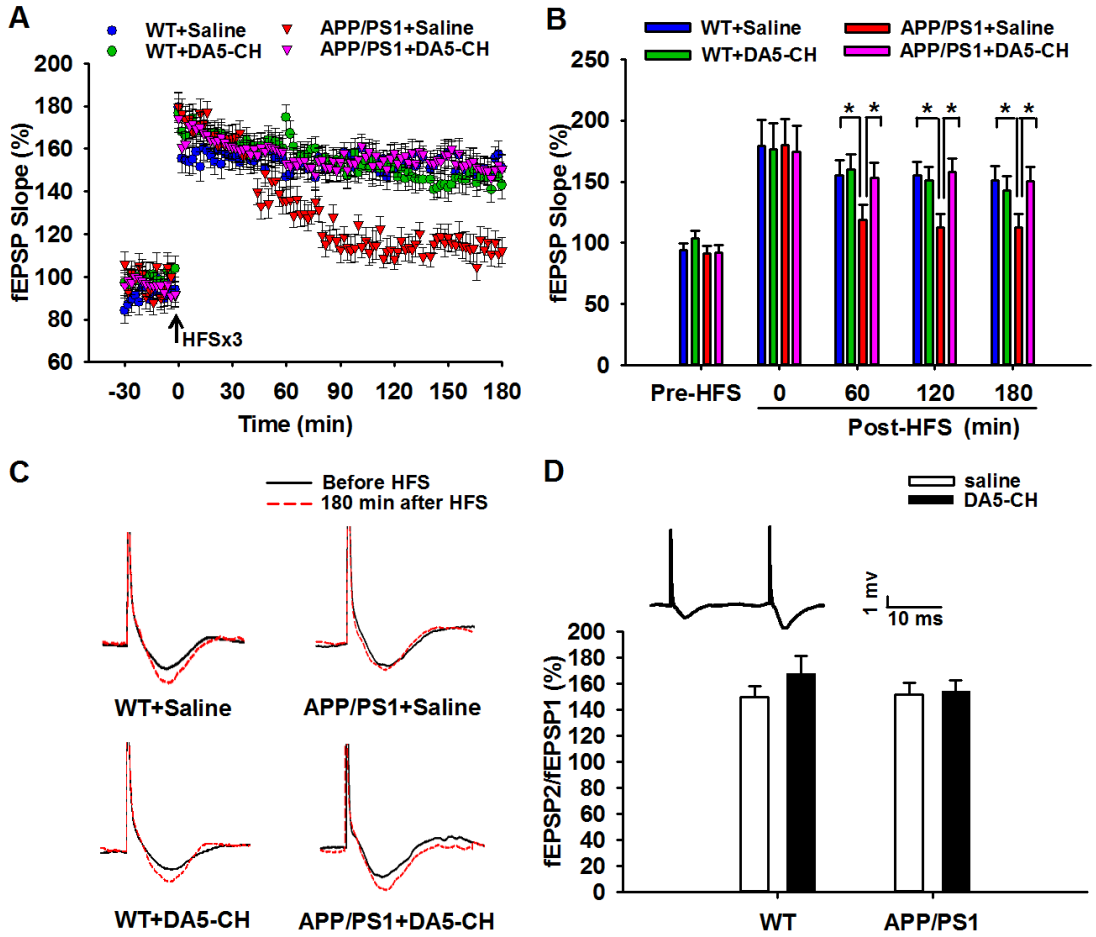


Fig.6

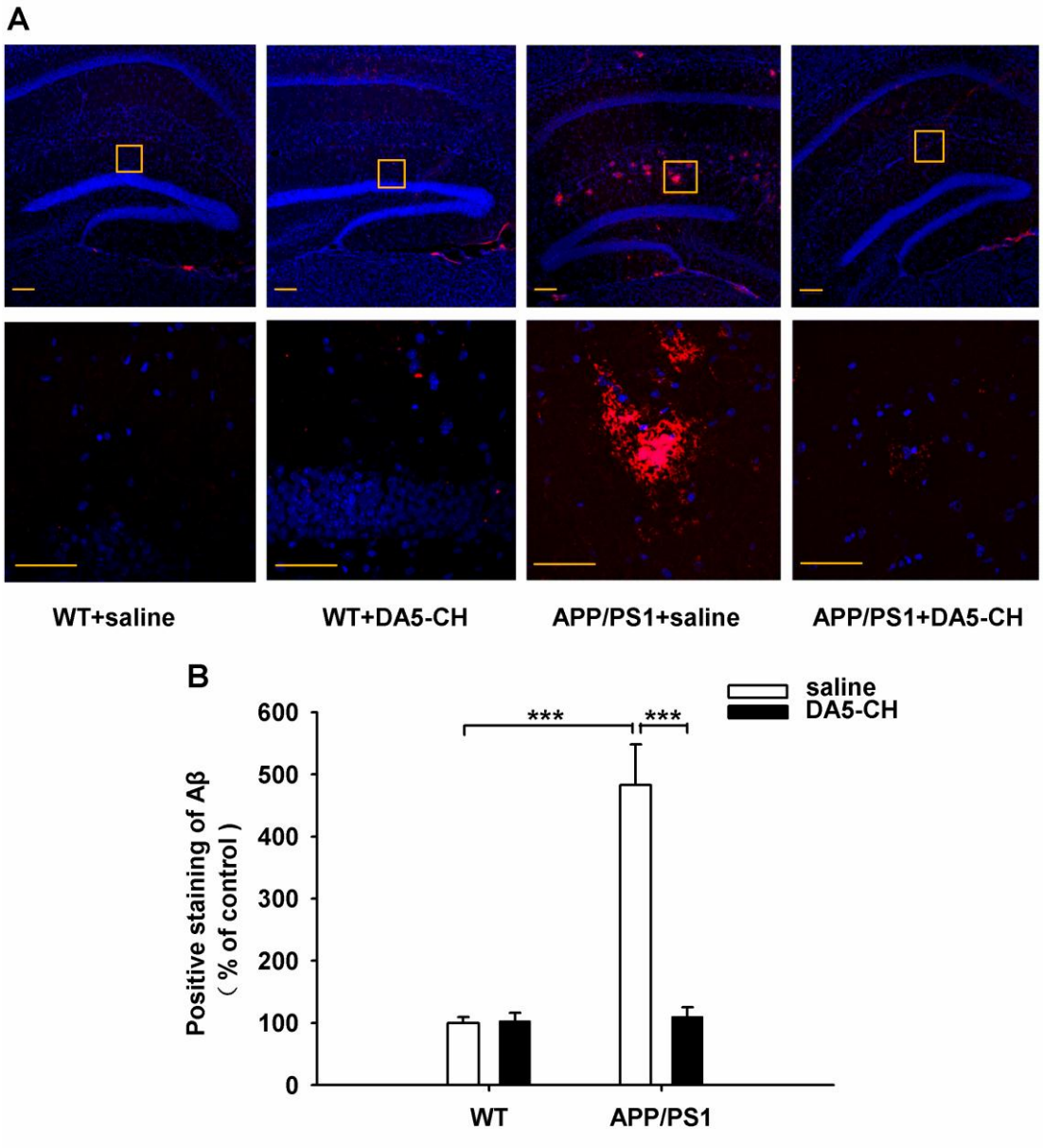

A
WT+saline
WT+DA5-CH
APP/PS1+saline
APP/PS1+DA5-CH
B
saline
DA5-CH
***
***
Positive staining of Aβ
（% of control）
600
500
400
300
200
100
0
WT
APP/PS1

Fig.7

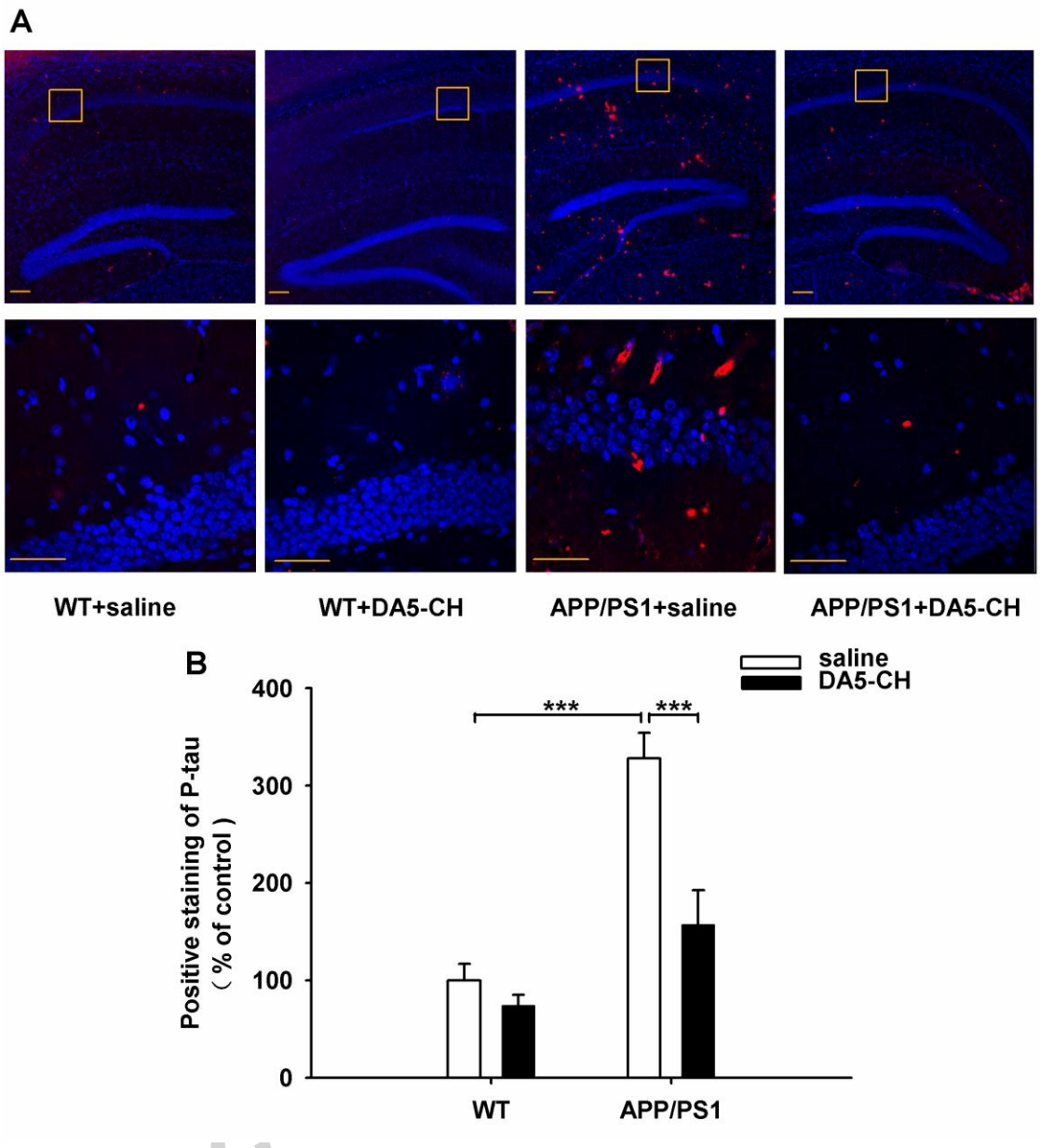
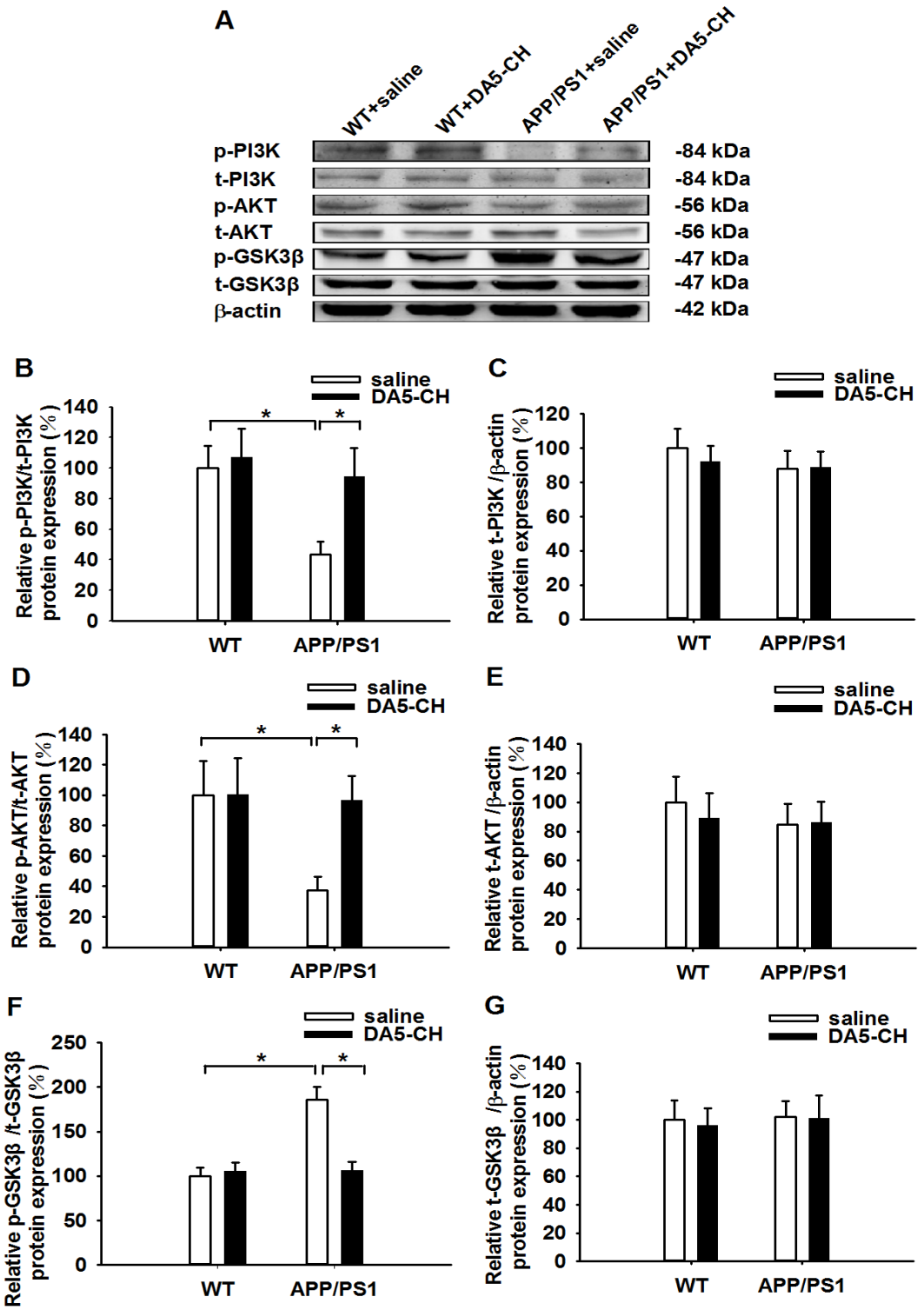

A
WT+saline
WT+DA5-CH
APP/PS1+saline
APP/PS1+DA5-CH
B
saline
DA5-CH
Positive staining of P-tau
（% of control）
400
300
200
100
0
***
***
WT
APP/PS1

Fig.8


A
WT+saline
WT+DA5-CH
APP/PS1+saline
APP/PS1+DA5-CH
p-PI3K
t-PI3K
p-AKT
t-AKT
p-GSK3β
t-GSK3β
β-actin
-84 kDa
-84 kDa
-56 kDa
-56 kDa
-47 kDa
-47 kDa
-42 kDa
B
Relative p-PI3K/t-PI3K protein expression (%)
saline
DA5-CH
WT
APP/PS1
C
Relative t-PI3K /β-actin protein expression (%)
D
Relative p-AKT/t-AKT protein expression (%)
E
Relative t-AKT /β-actin protein expression (%)
F
Relative p-GSK3β /t-GSK3β protein expression (%)
G
Relative t-GSK3β /β-actin protein expression (%)