Dimeric Tacrine (10)-Hupyridone Effectively Combats Alzheimer's Disease as A Multi-Target-Directed Ligand

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Abstract

Background

Alzheimer's disease (AD) is a neurodegenerative disorder with multiple pathological features. Therefore, multi-target-directed ligands (MTDLs) strategy has been developed to combat this disease. We have previously designed and synthesized dimeric tacrine (10)-hupyridone (A10E), a novel tacrine derivative with acetylcholinesterase (AChE) inhibition and brain-derived neurotrophic factor (BDNF) activation activity, by linking tacrine and a fragment of huperzine A. However, it was largely unknown whether A10E could act on other AD targets and produce cognition-enhancing ability in AD animal models.

Methods

Behavioral and biochemical methods were applied to evaluate multi-target cognitive-enhancing effects and mechanisms of A10E in APP/PS1 transgenic mice and β-amyloid (Aβ) oligomers-treated mice. The neuroprotective mechanisms of A10E were explored in SH-SY5Y cells. And the anti-aggregation effects of A10E on Aβ were directly investigated *in vitro*.

Results

A10E could prevent cognitive impairments in both APP/PS1 mice and Aβ oligomers-treated mice, with higher potency than tacrine and huperzine A. Moreover, A10E could effectively inhibit Aβ production and deposition, reduce neuroinflammation, enhance brain derived brain-derived neurotrophic factor (BDNF) expression, and elevate cholinergic neurotransmission *in vivo*. A10E, at nanomolar concentrations, could also inhibit Aβ oligomers-induced neurotoxicity via the activation of the TrkB/Akt pathway. Furthermore, Aβ oligomerization and fibrillization could be directly disrupted by A10E.

Conclusion

A10E could produce anti-AD neuroprotective effects via multi-target mechanisms, including the inhibition of Aβ aggregation, the activation of the BDNF/TrkB pathway, the reduction of neuroinflammation and the decrease of AChE activity. As MTDLs could produce additional benefits, such as overcoming the deficits of drug combination and enhancing the compliance of AD patients, our results suggested that A10E might be developed as a promising MTDL lead for the treatment of AD.

Background

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder in the world, and has became a leading cause of dementia among elderly. However, the pathogenesis of AD remains unclear, and no disease-modifying treatments has been discovered since the first case reported [1]. The formation of

senile plaques by β-amyloid (Aβ) is one of main pathological characteristics of AD. Therefore, the amyloid cascade hypothesis postulates that the neurodegeneration in AD might be caused by abnormal accumulation and aggregation of Aβ in the brain, particularly in the cortex and the hippocampus [2]. Based on this hypothesis, Aβ precursor protein with Swedish mutation (APPswe) / presenilin 1 (PS1) transgenic mice were developed to mimic AD. APP/PS1 transgenic mice could express human APP and PS1, with brain Aβ deposition at 3-month old, and detectable cognitive impairments at 6-month old [3]. Moreover, excessive Aβ could further aggregate into oligomers, fibrils and other aggregates, which have various degrees of toxicity. Among these aggregates, oligomers were regarded as the main substance to induce neurotoxicity and neuroinflammation in AD. Importantly, intrahippocampal injection of Aβ oligomers could induce acute neurotoxicity and neuroinflammation in mice, and result in cognitive impairments eventually [4]. Therefore, APP/PS1 transgenic mice and hippocampal Aβ oligomers-injected mice were widely used to screen potential cognitive-enhancing agents, and to investigate the neuroprotective mechanisms of these agents [3].

It is demonstrated that the dysregulation of cholinergic system, the death of functional neurons and the activation of neuroinflammation were existed, concurrently, during AD progress [5]. In the brain, acetylcholinesterase (AChE) is an essential enzyme that regulates the function of cholinergic neurotransmission in the brain, and choline acetyltransferase (ChAT) is a marker for cholinergic neurons [6]. The over-activation of AChE and the declined level of ChAT could be found in the brain of AD patients, leading to the low level of acetylcholine in the synapses [7]. Therefore, AChE inhibitors, such as donepezil, huperzine A and galantamine, which could increase acetylcholine levels, are currently used to alleviate the symptoms of AD but not able to halt or reverse AD progress. The loss of functional neurons could lead to cognitive impairments in AD. Therefore, it is important to treat AD via the inhibition of neuronal death. Brain-derived neurotrophic factor (BDNF), a neurotrophin with neuroprotective property, could bind to the receptor of tyrosine kinase receptor B (TrkB), activate downstream phosphatidylinositol 3-kinase (PI3-K) / protein kinase B (Akt) pathway, and produce anti-AD neuroprotective effects [8]. Many compounds, such as curcumin and cystamine, with BDNF-increasing properties have been tested to treat AD [9, 10]. Long-term neuroinflammation is also presented in AD, and regarded as one of main causes to induce neuronal loss [11]. Particularly, Aβ oligomers could activate microglia, promote the production of pro-inflammatory factors, and lead to cognitive impairments [12]. Moreover, overactivated astrocytes could contribute to neuroinflammation of AD [13]. Therefore, many anti-neuroinflammation drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs), have been tested in AD therapy [14]. Moreover, chemicals with Aβ aggregation-inhibition properties might have potential to treat AD [15]. Importantly, in order to combat multiple pathological features of AD, multi-target-directed ligands (MTDLs) strategy has been developed [16]. ASS234, a donepezil-based MTDL, could produce multiple anti-AD effects, including the inhibition of Aβ aggregation, the suppression of AChE activity, and the reduction of oxidative stress [17]. ARN14140, a galantamine-based MTDL, could improve cognitive impairments induced by Aβ oligomers through inhibiting neuroinflammation and oxidative stress, concurrently [18].

Tacrine was the first AChE inhibitor approved for the treatment of AD by Food and Drug Administration (FDA). However, the clinical use of tacrine was discontinued due to its hepatotoxicity [19]. Huperzine A is

an AChE inhibitor approved to treat AD in China [20]. Many tacrine and/or huperzine A derivatives, with low hepatotoxicity have been developed as MTDLs for AD treatment [19]. For example, tacrine-melatonin hybrids displayed AChE inhibition activity and antioxidant properties without notable hepatotoxicity [21]. Tacrine-bifendate conjugates showed potent AChE and Aβ aggregation inhibitoion with reduced hepatotoxicity [22].And Huprine X, a huperzine A derivative, could inhibit AChE activity and improve cognitive impairments in AD mice [23].

Dimeric Tacrine(10)-hupyridone (A10E), was designed and synthesized by linking tacrine with hupyridone, a fragment of huperzine A molecule using methylene groups [24]. Previously, we have reported that A10E could inhibit AChE, attenuate scopolamine-induced cognitive impairments in mice, and prevent post-operative cognitive impairments via the enhancement of BDNF expression [25, 26]. However, it is largely unknown whether A10E could act on other AD-related targets, and whether A10E could produce cognition-enhancing ability in AD animal models.

In this study, we have investigated whether A10E could produce cognitive-enhancing effects in APP/PS1 mice and Aβ oligomers-treated mice, respectively. Moreover, the multiple neuroprotective mechanisms of A10E, including the decreasing of Aβ production and aggregation, the elevation of TrkB/Akt pathway, the reduction of neuroinflammation, and the inhibition of AChE, were explored both *in vitro* and *in vivo*.

Materials And Methods

Chemicals and reagents

A10E was synthesized on the basis of our previous study [24]. Tacrine, huperzine A, dithiobisnitrobenzoic acid (DTNB), ethopropazine hydrochloride, and acetylthiocholine iodide (ATCI) were obtained from the company of Sigma-Aldrich (St. Louis, MO, USA). Aβ peptide was obtained from GL Biochem (Shanghai, China).

Preparation of Aβ oligomers

Aβ oligomer preparation was described in previous studies reported by us [27-29]. Briefly, Aβ peptide was dissolved in hexafluoroisopropanol (HFIP, Aladdin, Shanghai, China) to form Aβ monomers. Afterwards, 100 μL Aβ monomers were added to a tube and diluted with 900 μL Milli-Q water. HFIP in the solution was evaporated under nitrogen till a final concentration of Aβ monomers at 60 μM. After being persistent agitated for two days at room temperature, the Aβ solution was centrifuged at 14,000 g for 15 min at 4 °C. And the supernatant was collected and mainly contained Aβ oligomers.

Animals

Use and care of laboratory animals followed the National Institutes of Health (NIH) Guide (NIH Publications No. 8023, revised 1978) and were authorized by the Animal Care and Use Committee of Ningbo University. Male Institute of Cancer Research (ICR) mice (around 30g, 3 months old) were purchased from Zhejiang Academy of medical Sciences (Hangzhou, Zhejiang, China), wild-type (WT) C57BL/6 mice and APP/PS1 mice (around 25g, 1 month old) were purchased by Southern Model Biological Research Center (Nanjing, China). Mice were raised with a 12-h light/dark cycle (humidity: 50 ± 10 %, temperature: 22 ± 2 °C).

Intrahippocampal injection (*i.h.p.*) of Aβ oligomers

In advance of *i.h.p.*, mice were given sodium pentobarbital [50 mg/kg, intraperitoneal injection (*i.p.*)] to be anesthetized, and then using a stereotaxic instrument (RWD Life Science, Shenzhen, China) to fix their heads tightly. After the skull was exposed, two holes were drilled using the following coordinate: AP - 0.4 mm from bregma; ML ± 1.0 mm from the midline; and DV − 2.0 mm from pia mater [30]. Aβ oligomers (1 μL/side, 0.6 μg) were individually injected into both holes at a constant speed of 0.2 μL/min using an UltraMicroPump (RWD Life Science). Control mice were given an equal volume of 0.9% normal saline as vehicle (*i.p.*).

Animal group and drug treatment

A10E were dissolved in 0.9% normal saline. 4-week-old APP/PS1 mice were randomly assigned into 3 groups (n = 7). Two A10E-treated groups were treated with A10E at the dose of 0.36 and 0.72 μmol/kg (*i.p.*), respectively, into APP/PS1 mice every third day for 28 weeks. APP/PS1 and WT mice were injected with equal volumes of 0.9% normal saline daily (*i.p.*).

For Aβ oligomers-injection experiments, mice were randomly assigned into 7 groups (n = 8): vehicle (0.9% normal saline, *i.h.p.*) plus vehicle (0.9% normal saline, *i.p.*), Aβ oligomers (*i.h.p.*) plus vehicle (0.9% normal saline, *i.p.*), Aβ oligomers (*i.h.p.*) plus A10E (0.18 μmol/kg, *i.p.*), Aβ oligomers (*i.h.p.*) plus A10E (0.54 μmol/kg, *i.p.*), Aβ oligomers (*i.h.p.*) plus A10E (1.1 μmol/kg, *i.p.*), Aβ oligomers (*i.h.p.*) plus tacrine (7.9 μmol/kg, *i.p.*) and Aβ oligomers (*i.h.p.*) plus huperzine A (4.1 μmol/kg, *i.p.*). Drugs was treated daily until the day of sacrifice.

Open field (OF) test

OF test is used to analyze the exploratory and locomotor activities of animals [31]. The test was conducted in an open plastic box (50×50×39 cm) in which the floor was divided into four equal quadrants (25×25 cm) by crossed black lines as a previous study described [32]. Mice were placed in the center of the floor and permitted to explore it for 5 min. The number of rearing and line crossing of mice was

recorded. The floor was cleaned between two individual tests using 10 % ethanol in order to avoid influence of the urine and odor on behavior.

Novel object recognition (NOR) test

NOR test is used to test the cognitive function of animals, and carried out in a black open plastic box (50×50×39 cm) described previously [33]. Briefly, the test consisted of training section and exploring session. In the training session, mice were placed in the center of the box and permitted to explore two identical stone cubes (5×5×5 cm) freely for 5 min. The exploring session was conducted after 24 h and one stone cube was replaced by a stone square pyramid (5×5×7 cm). Mice were placed in the center of the box and permitted to explore the two different stone cubes freely for 5 min. The behavior that Mice looked towards and sniffed the object was considered exploratory behavior while walking around the identified object or just moving around the recognized object was not considered exploratory behavior. The field was cleaned between two individual tests using 10% ethanol in order to avoid influence of the urine and odor on behavior. The cognitive function was evaluated by a recognition index, which was the exploration time involving either of the two objects (training session) or the novel object (exploring session) compared with the total exploration time. Because rodents have the nature to explore new things, the increase in cognitive index can be used to reflect an increase in cognitive function.

Morris water maze (MWM) test

MWM test is a classic method to evaluate the spatial learning and memory of mice described in a previous study [34]. The water maze was a circle poor (radius at 75 cm, height at 50 cm), and was filled with water (depth at 30 cm, temperature at 25 °C). Various objects were set outside the pool and could be observed by mice to identify the spatial orientation. The pool is divided into four identical quadrants, and the platform was located in one of the pool quadrants and submerged 1 cm below the water surface. The training session lasted for four consecutive days. Mice were allowed to find the platform up to 90 s, and permitted to remain on it for 10 s to be familiar with the surrounding. The time of mice required to enter the hidden platform called escape latency was recorded, while the mice who could not reach the platform within 90s would be guided to the platform gently and permitted to remain on it for 10s. In the probe session, the platform was removed and mice were allowed to explore freely in the pool for 90 s. Duration in the target quadrant and number of platform area crossing, and motion path of the mice were recorded for analysis.

Brain tissue preparation

The mice were sacrificed to prepare brain tissue the next day after the MWM test. Mice were anesthetized by injection of sodium pentobarbital (50 mg/kg, *i.p.*) and cardiac-perfused phosphate-buffered saline (PBS). The hippocampus and the whole brain tissue were dissected for biochemical analysis.

Furthermore, as for immunohistochemical (IHC) staining, after perfusion with saline, mice were fixed by perfusion with 4% paraformaldehyde. The brain tissues were carefully removed and placed in a centrifuge tube containing 4% paraformaldehyde overnight at 4 °C, then placed into a centrifuge tube containing 30% sucrose solution for dehydration.

Measurement of AChE and ChAT activity

The measurement of AChE activity was conducted according to the protocol described in a previous study [35]. Briefly, the whole brain was added with 10 times volume of lysis buffer [10 mM HEPES (pH 7.5), 1 mM EGTA, 1 mM EDTA, 150 mM Triton X-100 and 1 mM NaCl]. In order to obtain AChE, the mixture was homogenized on ice for 15 min and then centrifuged for 15 min at 3000 rpm at 4 °C, and the supernatant was collected. Afterwards, the brain lysate was incubated with 0.1 mM ethopropazine hydrochloride at 37 °C for 5 min to inhibit butyrocholinesterase (BuChE) activity. Then the test compound was added to the assay medium [0.1 M Na_2HPO4 (pH 7.5), 10 mM DTNB and 1 mM ATCl] and pre-incubated at 37 °C with the enzyme for 15 min. Then ATCI was added, and incubated at 37 °C for 30 min. AChE activity was determined by measuring the absorbance at 412 nm.

The measurement of ChAT activity was conducted by using ChAT assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). According to the manual, briefly, brain tissue homogenate was prepared by normal saline according to the ratio of weight to volume, afterwards, supernatant of homogenate was added after the prepared mixture was bathed at 37 °C for 5 min. After bathing at 37°C for 20 min, mixture was centrifuged for 10 min at 4000 rpm, the supernatant was collected and R7 reagent was added. Finally, the active unit of ChAT was measured with the absorbance at 324 nm.

Western blotting analysis

Western blotting assay was carried through on the basis of a protocol described previously [32]. The brain tissues were homogenized in lysis buffer containing RIPA, protease inhibitors and phosphatase inhibitors (Sangon Biotech, Shanghai, China). Afterwards, the mixture was centrifuged at 13200 rpm for 15 min, and the supernatant was taken. The concentration of protein was determined by using a BCA Protein Kit (Beyotime Biotechnology, Shanghai, China). Sample was added with appropriate amount of loading buffer and heated to denature the protein, and stored at -20 °C. According to the size of the target protein, the corresponding concentration of the separation gel and the concentrated gel were prepared, and the loading quantity of sample was determined on basis of the protein concentration. In electrophoresis process, firstly the concentrated gel was run with a constant pressure of 80 V, and the separator gel was run with a constant pressure of 120 V until target protein band was separated. The separated proteins in gel were then transferred to polyvinylidene fluoride membrane in a transfer conditions of 100 V for 1.5 h. After transfection, 5% BSA was used to block the membrane at room temperature for 2h, followed by incubating the membrane with primary antibodies ChAT (Santa Cruz, USA, 1:1000), BDNF, tau (Abcam,

UK, 1:1000), TrkB, p-TrkB, Akt, p-Akt, p-tau (Cell Signaling Technology, USA, 1:1000), and β-actin (Affinity bioscience, USA, 1:1000) respectively overnight at 4 °C.

The next day, the membrane was washed for 3 times (15 min for each time) using TBST buffer (2 mM NaCl, 10 mM Tris-HCl and 0.1 % Tween-20; Shanghai Aladdin Biochemical Technology). Then the secondary antibodies were applied to specifically bind to corresponding primary antibody at room temperature for 1 h. After washing the samples three times with TBST, the development of the protein bands was using ECL luminescent substrate. The amount of protein was evaluated by analyzing the intensity of each band using Image J software (NIH Image, Bethesda, MD, USA).

IHC staining

IHC staining was conducted according to a protocol described in a previous study [36]. Briefly, after blotting the dehydrated brain with absorbent paper, the brain tissue was fixed on the sample plate with an embedding agent at -20 °C. Brain tissue was cut into slices with 25 μm thick using a freezing microtome. Brain slices were incubated with antigen repair fluid at 60 °C for 30 min, followed by incubation with the immunized blocking solution at room temperature for 1 h. Afterwards, the brain slices were incubated with primary antibodies Iba-1 and GFAP (Cell Signaling Technology, 1:400) at 4 °C overnight. The next day, the brain slices were washed for 3 times (15 min for each time) using PBS, and then added with corresponding fluorescent secondary antibody, and incubated at room temperature for 1 h in the dark. Subsequently, the slices were washed for 3 times (15 min for each time) using PBS, and nuclei were stained with 4',6-dimidyl-2-phenylindole (DAPI). The slices were eventually sealed with anti-quenching seals. Fluorescent marks in brain slices were observed using a confocal fluorescence microscope.

As for staining of Aβ, brain slices were incubated with antigen repair fluid at 60 °C for 30 min, followed by incubation with the immunized blocking solution at room temperature for 1 h. Afterwards, the brain slices were incubated with primary antibodies Aβ (Sigma-Aldrich, 1:400) at 4°C overnight. The next day, the brain slices were washed for 3 times (15 min for each time) using PBS, and then added with secondary antibody working solution including Horseradish Peroxidase and Goat Anti-rabbit IgG in 1:200 solution, and incubated at 37°C for 30 min. Subsequently, the slices were washed for 3 times (15 min for each time) using PBS, and colored by using diaminobenzidine color development kit. The images were captured by the camera of the light microscope.

Enzyme linked immunosorbent assay (ELISA)

The concentration of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in hippocampus was detected by ELISA. The sample including 10 mg of mouse hippocampus tissue and 90 μl of PBS was totally homogenized. After centrifugation at 4 °C for 20 min (2000–3000 rpm), the supernatant was carefully collected for ELISA. According to the ELISA kit (Meibiao Biology, Jiangsu, China) detection method, the OD value of each well was measured by adjusting the blank control hole at a wavelength of

450 nm on the microplate reader. A standard curve was prepared based on the concentration and OD value of the standard, and then the concentrations of IL-6 and TNF-α in the hippocampus of each group of mice were calculated according to the standard curve equation.

SH-SY5Y cells

SH-SY5Y cells (Chinese Academy of Sciences, Shanghai, China) were cultured in a mixed medium containing 90% high glucose modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), and 1% penicillin (100 U/mL)/ streptomycin (100 μg/mL). Culture medium was replaced every two days. The culturing condition was 37°C with 5% CO_2. The culture medium of SH-SY5Y was replaced with DMEM with 1% FBS and continue culture for 24 h before experiments.

Measurement of cell viability

3 (4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT) assays was applied to evaluate the cell viability as previous study described [27]. In short, 10 μL MTT solution (5 mg/mL) was added to 96-well plates and incubated at 37 °C for 4 h. Then 100 μL solvating solution (10 % SDS solution supplemented with 0.01 N HCl) was added, and the plate continued incubating tranquilly for 16-20 h. A spectrophotometrer (Thermo Fisher Scientific, Waltham, MA, USA) was applied to evaluate the cell viability according to the absorbance at 570 nm with 655 nm as a reference wavelength.

Dot blotting analysis

Dot blotting analysis was also carried out according to the methods we reported previously [37]. Firstly, the mixture of equal volume of different concentrations of the drugs and Aβ solution were prepared. The mixture was agitated to form the sample. Afterwards, 2 μl of the sample were spotted onto the nitrocellulose membrane, and were dried by air. After blocking the membrane 5% BSA diluted in 1% TBST solution overnight, the membrane was incubated with anti-oligomer antibody A11 (Thermo Fisher Scientific, 1:1000) or anti-$Aβ_{1-17}$ antibody 6E10 (Sigma-Aldrich, 1:1000) with gentle shaking lasting for 1 h. Afterwards, the membrane was washed by 1% TBST for 15 min before it was incubated with second antibody for 1 h. After 3 washes in 1% TBST, the membranes were visualized by using the ECL detection reagents (Tanon 5200 Automatic chemiluminescence imaging analysis system, Shanghai, China).

Preparation of Aβ fibril and thioflavin-T (ThT) assay

Aβ fibrils preparation was also described previously [38]. Briefly, Aβ monomer was dissolved in HFIP, and diluted with Milli-Q water. After fully evaporating HFIP with nitrogen, Aβ monomer was diluted with sodium hydroxide solution to form 1 mM Aβ solution. Next, 2 μL Aβ solution with chemicals or vehicle were added to PBS at 197 μM with 5 μM ThT (Sigma-Aldrich). The system was incubated in the dark for 3

days at 37°C. The quantity of Aβ fibrils was evaluated by fluorescence intensity at the excitation and emission wavelengths of 440 and 485 nm, respectively.

Immunoblotting analysis

The Immunoblotting analysis of Aβ oligomers formation was performed as previously described [34]. To begin, Aβ monomer was dissolved in HFIP to 2.5mg/ml, evaporated under nitrogen until the final concentration of Aβ1-42 becomes 60 μM, and mixed with an equal part of A10E sample. then the sample was shook for 48 h at 27 °C. Subsequently, samples were electrophoresed and transferred. Membranes were boiled for 10 min for 3 times, and blocked with 5% BSA for 1 hour at room temperature, incubated with rabbit anti-$A\beta_{1\text{-}42}$ antibody 6E10 (1:1000, Invitrogen, California, USA) followed by corresponding secondary antibody, and developed in an automatic chemiluminescence imaging system.

Data analysis and statistics

The results were exhibited as mean ± SEM. The data was analyzed by GraphPad Prism (version 6.0, GraphPad Software, Inc., San Diego, CA, USA). The difference between groups was evaluated by one-way analysis of variation (ANOVA) and statistical comparison was evaluated by Tukey's test. However, the group variance of the body weight of mice and recognition index were analyzed by two-way repeated measures ANOVA. Values of $p < 0.05$ were considered statistically significant. In figures, % or folds of control was calculated by separating each control value on the average of total control group values for a particular protein, and then the values of treated group were normalized to control (100% or 1.0).

Results

Long-term treatment of A10E effectively attenuates cognitive impairments in APP/PS1 mice

The schedule of whole experiments is shown in Fig. 1A. Briefly, at 8 weeks of age, WT mice were injected with vehicle (0.9% normal saline, *i.p.*), and APP/PS1 mice were injected with A10E (0.36 or 0.72 μmol/kg in vehicle, *i.p.*) every third day. The drug treatment lasted 28 weeks. NOR and MWM test were used to evaluate the cognitive level of mice at 36 weeks of age. To evaluate the influence of long-term treatment of A10E on body weight, we measured the body weight of mice every 4 weeks. There is no significant change for time effect (two-way ANOVA, $F(7, 192) = 1.346$, $p > 0.05$), treatment effect (two-way ANOVA, $F(3, 192) = 2.498$, $p > 0.05$) and treatment × time interaction (two-way ANOVA, $F(21, 192) = 0.156$, $p > 0.05$, Fig. 1B) on body weight, suggesting that long-term treatment of A10E could not produce significant influence on body weight. In the training session of NOR test, there was no significantly change on recognition index among all groups for two uniform objects in the training session of NOR test (one-way ANOVA, $F(3, 24) = 0.300$, $p > 0.05$, Fig. 1C). In the exploring session, the recognition index was

significantly altered among groups (one-way ANOVA, F (3, 24) = 5.295, $p < 0.01$, Fig. 1D). Particularly, the recognition index in the WT group was significantly higher than that in the APP/PS1 group (Tukey's test, $p < 0.01$, Fig. 1D). Moreover, A10E (0.36–0.72 μmol/kg) significantly increased recognition index of APP/PS1 mice (Tukey's test, $p < 0.05$, Fig. 1D). The above results showed that long-term treatment of A10E could attenuate recognitive impairments in APP/PS1 transgenic mice. In the training session of MWM test, escape latency was the indicator of learning ability, which equals to the time animals take to find a platform. At the last day of training session, the escape latency of APP/PS1 group significantly longer than the WT group, suggesting that learning ability of Aβ oligomers-treated mice to find the platform was impaired (Tukey's test, $p < 0.05$, Figs. 1E-F). At the last day of training session, the treatment of A10E at 0.72 μmol/kg significantly decreased escape latency when compared with the APP/PS1 group, indicating that long-term treatment of A10E could attenuate spatial cognitive impairment of APP/PS1 mice (Turkey's test, $p < 0.05$, Figs. 1E-F). In the probe trial, the platform was removed, and the duration in the target quadrant and the number of platform area crossings of each group within 90 s were recorded, which indicated learning ability. The duration in the target quadrant (Turkey's test, $p < 0.01$, Fig. 1H) and the number of platform area crossings (Turkey's test, $p < 0.05$, Figs. 1I-J) of APP/PS1 mice were significantly decreased compared with the WT group. Moreover, A10E at 0.72 μmol/kg significantly increased the duration in the target quadrant (Turkey's test, $p < 0.01$, Fig. 1H) and the number of platform area crossings (Turkey's test, $p < 0.01$, Figs. 1I-J) of APP/PS1 mice, indicating that long-term treatment of A10E could attenuate spatial cognitive impairment of APP/PS1 mice.

Long-term A10E treatment prevents Aβ deposition and neuroinflammation in APP/PS1 mice.

After behavioral tests, mice were sacrificed for biochemical study. Typical Aβ plaque were presented in the hippocampus of APP/PS1 mice (Fig. 2A). We applied immunohistochemical staining to detect whether long-term A10E treatment could reduce Aβ plaques in APP/PS1 mice (Fig. 2A). The quantitative analysis of percentage of area with positive Aβ staining showed that treatment with A10E (0.36 or 0.72 μmol/kg) significantly reduced Aβ deposition in the hippocampus of APP/PS1 mice (Turkey's test, $p < 0.01$, Fig. 2B). Some tacrine derivatives could reduce the level of Aβ in AD mice [39]. Therefore, we detected whether A10E could produce similar effects by using ELISA assays. A10E (0.36–0.72 μmol/kg) could reduce the level of $A\beta_{1-40}$ (Turkey's test, $p < 0.01$, Fig. 2C) and $A\beta_{1-42}$ (Turkey's test, $p < 0.01$, Fig. 2D) in the hippocampus of APP/PS1 mice. In addition, we found that interleukin-1β (IL-6) (Turkey's test, $p < 0.01$, Fig. 2E) and tumor necrosis factor-α (TNF-α) (Turkey's test, $p < 0.01$, Fig. 2F) levels were decreased in A10E-treated group compared to APP/PS1 group. These results indicated that A10E might alleviate neuroinflammation in the hippocampus of APP/PS1 mice.

A10E attenuates Aβ oligomers-induced cognitive impairments in mice.

We further established another AD animal model by hippocampal injection of Aβ oligomers in mice. The schedule of whole experiments was shown in Fig. 3A. Briefly, vehicle (0.9% normal saline, *i.p.*) or drug (A10E at 0.18, 0.54 and 1.1 μmol/kg, tacrine at 7.9 μmol/kg and huperzine A at 4.1 μmol/kg, *i.p.*) was daily injected into ICR mice. The OF test was conducted at the 8th day. NOR test was conducted at the 9th

-10th day. The MWM test was conducted at the 11th -16th day. And at the 16th day, mice were sacrificed for biochemical evaluation. The motor ability of each group of mice was detected by OF test. There was no significant difference in the number of rearing (one-way ANOVA, F (6, 49) = 0.568, $p > 0.05$, Fig. 3B) and line crossing (one-way ANOVA, F (6, 49) = 0.081, $p > 0.05$, Fig. 3C) among all groups, indicating that Aβ oligomers or drug did not significantly affect the motor capacity of mice. And similar to the effects of long term of treatment with A10E on APP/PS1 mice. Moreover, A10E (0.54–1.1 μmol/kg) could attenuate Aβ oligomers-induced recognition and spatial cognitive impairment in mice by applying NOR test and MWM test (Tukey's test, $p < 0.05$, **Figs. 5E-I**).

A10E attenuates the dysfunctions of cholinergic system and the inhibition of BDNF/TrkB Pathway in Aβ oligomers-treated mice.

ChAT is a marker for cholinergic neurons, while AChE is an essential enzyme that regulates the function of cholinergic neurotransmission in the brain. The expression of ChAT in the whole brain was analyzed by Western blotting analysis. ChAT expression in Aβ oligomers-treated mice was significantly lower than that in the control group (Tukey's test, $p < 0.01$, Fig. 4A). Moreover, treatment with A10E (0.54–1.1 μmol/kg) significantly reversed Aβ oligomers-induced decrease of ChAT expression (Tukey's test, $p < 0.05$, Fig. 4A). ChAT activity was further detected. And treatment with A10E (1.1 μmol/kg) significantly reversed Aβ oligomers-induced decrease of ChAT activity (Tukey's test, $p < 0.01$, Fig. 4B). AChE activity in the whole brain were also analyzed. Treatment with A10E (0.54–1.1 μmol/kg) significantly prevented Aβ oligomers-induced increase of AChE activity (Tukey's test, $p < 0.05$, Fig. 4C). All these results indicated that A10E might attenuate the dysfunctions of cholinergic system induced by Aβ oligomers.

TrkB is a main receptor of BDNF. Previously, we reported that A10E could prevent surgery-induced decrease of BDNF expression [25]. Therefore, in this study, we applied Western blotting analysis to detect the effect of A10E on the BNDF-TrkB pathway. The expression of BDNF, p-TrkB, p-Akt was decreased, while the expression of p-tau was increased in the hippocampus of Aβ oligomers-treated group compared with the control group (Tukey's test, $p < 0.05$, Figs. 4D-G). In addition, A10E treatment significantly reversed the expression of these proteins compared with Aβ oligomers-treated group, suggesting that A10E might activate the BNDF-TrkB pathway (Tukey's test, $p < 0.05$, Figs. 4D-G).

A10E attenuates Aβ oligomers-induced glial activation in mice.

Glia, including microglia and astrocyte, can be over-activated by Aβ oligomers, and thereby leading to neuroinflammation and cognitive impairments eventually. $GFAP^+$ and $Iba\text{-}1^+$ cells were evaluated by IHC staining. Both the percentages of area with $GFAP^+$ staining and the number of $GFAP^+$ astrocyte significantly increased in Aβ oligomers-treated group compared with the control group (Tukey's test, $p < 0.01$, **Figs. 5A-C**). However, treatment with A10E reversed the activation of astrocytes induced by Aβ oligomers (Tukey's test, $p < 0.01$, **Figs. 5A-C**). Similarly, treatment with A10E significantly reversed the activation of microglia induced by Aβ oligomers (Tukey's test, $p < 0.05$, **Figs. 5D-F**). All these results suggested the anti-neuroinflammation effects of A10E.

A10E decreases Aβ oligomers-induced neurotoxicity in SH-SY5Y cells via the activation of the TrkB/Akt signaling pathway.

To further evaluate the neuroprotective mechanisms of A10E, we used *in vitro* neuronal cultures. SH-SY5Y cells are widely used to investigate anti-AD drugs. The cell viability of SH-SY5Y cells was significantly reduced by Aβ oligomers compared with control group (Tukey's test, $p < 0.01$, Fig. 6A). Moreover, A10E (1–3 nM) treated-group prevented the reduction of cell viability compared with Aβ oligomers-treated group (Tukey's test, $p < 0.05$, Fig. 6A). Besides, A10E significantly reversed Aβ oligomers-induced down-regulation of the expression of p-TrkB (Tukey's test, $p < 0.05$, Fig. 6B) and p-Akt (Tukey's test, $p < 0.01$, Fig. 6C). To further evaluate the effects of A10E on the activation of TrkB/Akt pathway, we used LY294002, a PI3-K inhibitor. LY294002 could abolish the neuroprotective effects of A10E against Aβ oligomers-induced neurotoxicity (Tukey's test, $p < 0.05$, Fig. 6D), suggesting that PI3-K was a key protein that contributed to the neuroprotective effects of A10E. In addition, ANA-3, a TrkB inhibitor, could abolish the neuroprotective effects of A10E against Aβ oligomers-induced neurotoxicity (Tukey's test, $p < 0.01$, Fig. 6E). Furthermore, Western blotting analysis showed that 30 μM ANA-3 could abolish the increased expression level of p-TrkB (Tukey's test, $p < 0.01$, Fig. 6F) and p-Akt (Tukey's test, $p < 0.01$, Fig. 6G) induced by A10E. All these results suggested that A10E might combat Aβ oligomers-induced neurotoxicity in SH-SY5Y cells via the activation of the TrkB/Akt signaling pathway.

A10E inhibits Aβ aggregationin vitro.

Many tacrine derivatives could produce anti-AD neuroprotective effects via directly acting on Aβ aggregation [34]. Therefore, we evaluated whether A10E participates in the pathological alteration of Aβ. Aβ peptide was dissolved in HFIP to form Aβ monomers, and then incubated with A10E in an oligomer formation condition. A10E (3–10 μM) could concentration-dependently inhibit the formation of Aβ oligomers (Tukey's test, $p < 0.01$, **Figs. 7A-B**). Assemblies ranging from dimers to medium-size oligomer were recognized as Aβ oligomers. A10E could largely reduce the amount of Aβ oligomers (**Fig. 7C**), providing a support that A10E could directly reduce Aβ oligomerization. ThT assay was used to detect Aβ fibrils. HFIP-pretreated Aβ monomer was incubated with various drugs in a fibril formation condition. A10E (3–10 nM) could significantly inhibit Aβ fibrillation (Tukey's test, $p < 0.05$, **Fig. 7D**). All these results suggested that A10E could significantly inhibit the pathological Aβ aggregation.

Discussions

In this study, we found that 28-week treatment of A10E could attenuate cognitive impairments, reduce neuroinflammation and lead to the robust reduction of $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in APP/PS1 transgenic mice. Moreover, A10E could not significantly alter body weight after 28-week treatment, indicating that long-term *i.p.* treatment of 0.36–0.72 μmol/kg A10E was quite safe to APP/PS1 mice. Tacrine was discontinued in the treatment of AD because of its hepatotoxicity. The hepatotoxicity induced by tacrine could be largely attributed to its free primary amine group [40]. For example, tacrine-melatonin hybrids and tacrine-bifendate conjugates, with the modification of their free primary amine group in tacrine moist,

did not show obvious signs of hepatotoxicity [21, 22]. The free primary anime group in A10E has also been modified, indicating that the hepatotoxicity of A10E might be low.

How A10E produces anti-AD effects in APP/PS1 transgenic mice? Aβ could be derived from the sequential enzymatic actions of β-secretase (BACE-1) and γ-secretase on APP [41, 42]. A10E could largely reduce Aβ levels, indicating that A10E might affect Aβ production from APP. Many tacrine derivative, such as bis(7)-tacrine and tacrine-benzofuran, could directly inhibit the activity of BACE-1 [39, 43]. Moreover, huperzine A could decrease mRNA expression of BACE-1 [44]. All these results suggested that A10E, with its chemical structure similar to bis(7)-tacrine and huperzine A, might affect the activity and/or the expression of BACE-1. However, the detailed interaction between A10E and BACE-1 was currently under investigation in our lab.

Interestingly, A10E was able to reduce the formation of Aβ plaques in APP/PS1 mice. As AChE could promote the aggregation of Aβ, we speculated that A10E, as a potent AChE inhibitor, might produce anti-Aβ aggregation effects via an AChE-dependent pathway [45]. Whether A10E could directly affect the formation of Aβ aggregation? Our results showed that A10E at 3–10 μM could inhibit Aβ oligomerization and fibrillization. At the same condition, tacrine and huperzine A could not produce anti-aggregation effects. Moreover, A10E could reduce the amount of Aβ oligomers after incubation with Aβ monomers. These results suggested that Aβ aggregation might be another anti-AD target of A10E, providing a support for the use of A10E in the treatment of AD. Many tacrine derivates showed anti-aggregation ability. For example, tacrine-bifendate conjugates could prevent Aβ aggregation at milimolar concentrations [22]. Bis(propyl)-cognitin greatly inhibited the formation of Aβ fibrils and disaggregate pre-formed Aβ fibrils [46]. Moreover, Bis(heptyl)-cognitin could directly inhibit the oligomerization and fibrillization of Aβ via the interaction between the hydrophobic pockets of Aβ and its tacrine moist, indicating the similar interaction might be present between Aβ and A10E [34].

APP/PS1 mice were used as a classic AD animal model to investigate AD-related neurotoxicity. To further elucidate the anti-AD mechanisms of A10E, we applied hippocampal injection of Aβ oligomers in ICR mice to establish another AD animal model. To date, the maximum concentration of A10E (1.1 μmol/kg) used in Aβ oligomers-treated model was higher than that used in the APP/PS1 mice (0.72 μmol/kg). It is because that long-term treatment of A10E might cause the accumulation of A10E in the brain of mice. A10E could elevate the expression of BDNF and activate the TrkB/Akt pathway, the downstream pathway of BDNF, which is consistent to our previous study [25]. BDNF was reported to produce neuroprotective effects against Aβ-induced neurotoxicity in AD. Moreover, BDNF could promotes non-amyloidogenic APP processing through the inhibition of BACE-1 [47]. Therefore, we speculated that A10E might produce anti-AD effects partially via activating BDNF and BNDF-related pathways. We also confirmed that A10E could inhibit Aβ oligomers-induced neurotoxicity via BDNF-related TrkB/PI3-K/Akt pathway due to the following reasons: 1) A10E could induce the activation of TrkB and Akt at a very low concentration in SH-SY5Y neuronal cells. And 2) the neuroprotective effects of A10E could be almost completely abolished by PI3-K and TrkB inhibitors.

Neuroinflammation, with the representatives of the increased expression of pro-inflammatory factors, and the activation of microglia and astrocytes, could be found in AD mice [48]. A10E could reduce the expression of IL-6 and TNF-α in the hippocampus of APP/PS1 mice, and inhibit the aviation of glia in Aβ oligomers-treated mice. Besides neurons, TrkB was reported to be expressed in microglia. The activation of TrkB could inhibit aging-related activation of microglia and neuroinflammation [49]. In addition, BDNF supplement could attenuate ischemic stroke-induced activation of astrocytes, and reduce the expression of the pro-inflammation factors [50]. Therefore, we speculated that A10E, with the ability to activate BDNF and TrkB, might inhibit the activation of gila, and reduce neuroinflammation in AD surroundings via acting on the BDNF pathway.

Aβ abnormal aggregation is considered as the initiating factor in AD pathological process, which happens years before the occurrence of symptoms [51]. The inhibition of AChE activity can significantly increase the central acetylcholine content, so that acetylcholine can accumulate at the synapse and alleviate the symptoms of AD [20]. Moreover, BDNF deficits could lead to neuronal death during AD progress, and is tightly associated with the severity of AD [52]. As a MTDL, A10E might be able to act on key pathophysiological targets, simultaneously (Fig. 8). However, what is the leading target of A10E to produce anti-AD effects? A10E could inhibit AChE with IC_{50} at nanomolar level *in vitro* [26]. In this study, A10E could inhibit Aβ aggregation with EC_{50} at around 3 μM, and prevent Aβ oligomers-induced neurotoxicity with EC_{50} at around 3 nM. Therefore, we speculated that A10E produced anti-AD effects mainly from acting on AChE and neuroprotection, possibly via the activation of the TrkB/Akt pathway. To date, the potency of A10E against AD is largely greater than tacrine and huperzine A, A10E monomers. More importantly, among more than 200 tacrine derivatives published currently, A10E is the minority to inhibit AChE and to produce neuroprotective effects at very low concentrations. Considering the free primary anime group in tacrine moist, the main group leading to hepatotoxicity, has been modified in A10E, and MTDLs might overcome the deficits of drug combination and enhance the compliance of AD patients, our results indicated that A10E as a promising MTDL, might be developed as an anti-AD lead.

Conclusion

We have found that A10E, at very low concentrations, could produce cognitive-enhancing effects in two AD animal models. Moreover, A10E could produce anti-AD neuroprotective effects via multi-target mechanisms, including the inhibition of Aβ aggregation, the activation of the BDNF/TrkB pathway, the reduction of neuroinflammation and the decrease of AChE activity. As MTDLs could overcome the deficits of drug combination and enhance the compliance of AD patients, our results suggested that A10E might be developed as a promising lead in the treatment of AD.

List Of Abbreviations

Aβ, β-amyloid; AD, Alzheimer's disease; MTDL, multi-target-directed ligand; A10E, tacrine(10)-hupyridone; Tac, tacrine; HupA, huperzine A; NSAID, nonsteroidal anti-inflammatory drug; AChE, acetylcholinesterase; BDNF, brain-derived neurotrophic factor; ChAT, choline acetyltransferase; TrkB, tyrosine kinase receptor B;

PI3-K, phosphatidylinositol 3-kinase; Akt, protein kinase B; BACE-1, β-secretase; *i.p.*, intraperitoneal injection; *i.h.p.*, OF, open field; NOR, novel object recognition; MWM, Morris water maze; intrahippocampal injection; IHC, Immunohistochemistry; ELISA, Enzyme linked immunosorbent assay; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide; ThT, thioflavin-T.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Wei Cui, Yifan Han, Dongsheng Zhou and Karl Tsim designed the study. Zhenquan Xuan, Wei Cui and Yifan Han drafted the manuscript; Paul R. Carlier synthesized A10E; Zhenquan Xuan, Xinmei Gu, Yiying Zhou, Hui Zhang, Yushan Yan, Shengquan Hu, Marvin Mak and Xinghan Huang performed the animal studies and biochemical tests; Xie Yanfei performed the *in vitro* test of Aβ aggregation inhibition; Sicheng Yan perform Aβ oligomers-induced neuronal death model. All authors read and approved the final manuscript.

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Figures

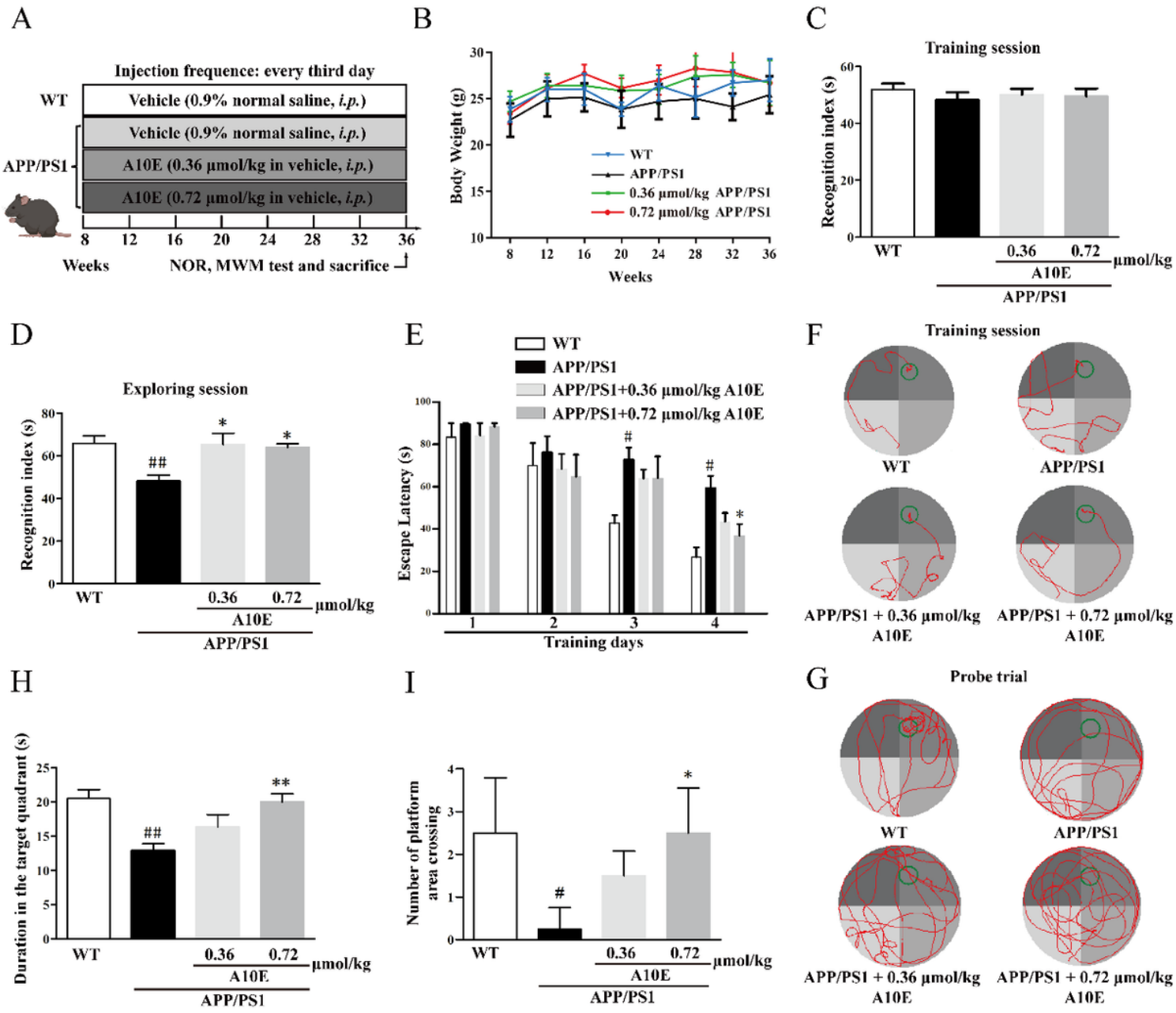

Figure 1

Long-term treatment of A10E effectively attenuates cognitive impairments in APP/PS1 mice. (A) Schedule of whole experiments. (B) A10E treatments did not significantly affect the body weight of APP/PS1 mice during the whole experiments. (C) There was no significantly change on recognition index among all these groups for two uniform objects in the training session of NOR test. (D) A10E significantly increased recognition index of APP/PS1 mice in the exploring session of NOR test. (E) A10E treatment decreased the escape latency of APP/PS1 mice to find the platform in the training session of MWM test. (F) Representative paths of mice in the training session of MWM test. A10E treatment significantly increased (H) the time spent in the target quadrant, and (I) the number of crossings of APP/PS1 mice in the platform area in the probe trial of MWM test. (G) Representative paths of mice in the probe trial of MWM test. i.p.: intraperitoneal injection. Data are expressed as the mean ± SEM (n = 7); #p < 0.05 and

##p < 0.01 versus WT group, *p < 0.05 and **p < 0.01 versus APP/PS1 group (one-way ANOVA and Tukey's test).

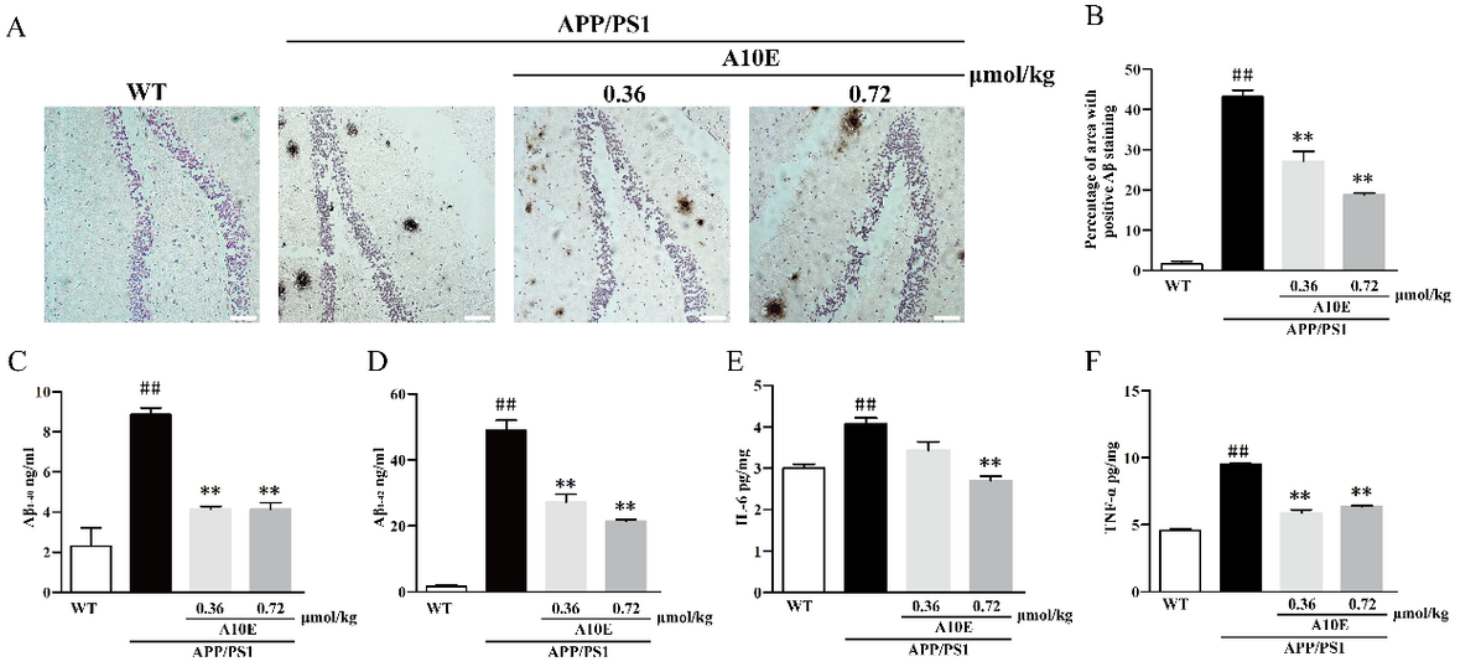

Figure 2

Long-term A10E treatment reduces Aβ deposition and inhibits neuroinflammation in APP/PS1 mice. (A) Representative images of immunohistochemical staining of Aβ plaques in the hippocampus of mice in each group (scale bar = 25 μm). (B) The quantitative analysis of percentage of area with positive Aβ staining showed that A10E significantly reduced Aβ deposition in the hippocampus of APP/PS1 mice. (C-F) A10E treatment significantly decreased the levels of (C) Aβ1-40, (D) Aβ1-42, (E) IL-6 and (F) TNF-α in the hippocampus of APP/PS1 mice. Data are expressed as the mean ± SEM (n = 3); #p < 0.05 and ##p < 0.01 versus WT group, *p <0.05 and **p < 0.01 versus APP/PS1 group (one-way ANOVA and Tukey's test).

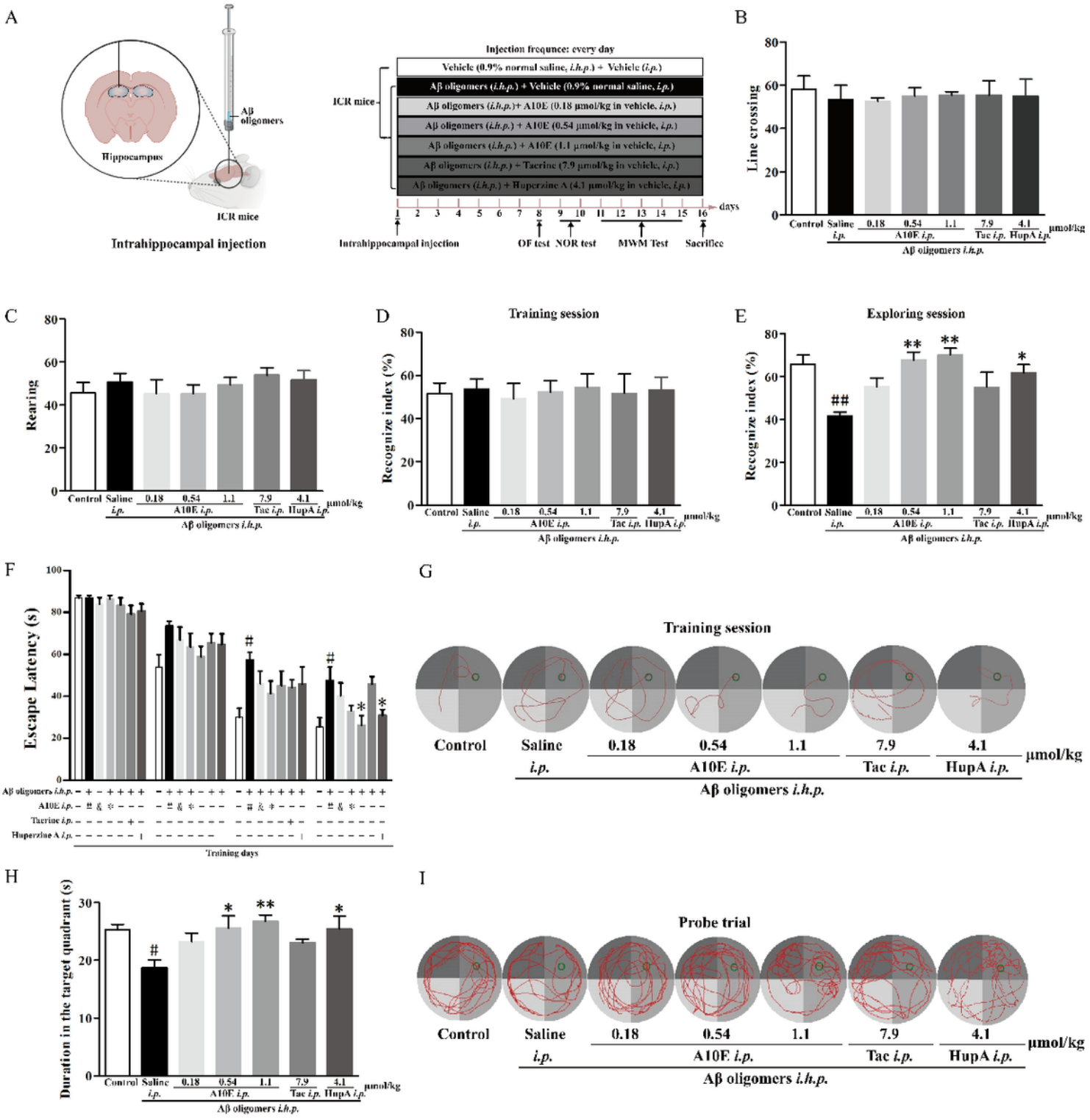

Figure 3

A10E attenuates Aβ oligomers-induced cognitive impairment in mice. (A) Schedule of whole experiments. Aβ oligomer was injected to the hippocampus of ICR mice to establish AD animal model. (B-C) In the OF test, there was no significantly change on (B) the number of line crossing and (C) the number of rearing among all groups. (D) There was no significantly change on recognition index for two uniform objects among all groups in the training session in NOR test. (E) A10E treatment significantly increased

recognition index of Aβ oligomers-treated mice in the exploring session in NOR test. (F) A10E treatment decreased the escape latency of Aβ oligomers-treated mice to find the platform in the training session of MWM test. (G) Representative paths of mice in the training session of MWM test. (H) A10E treatment significantly increased the time of Aβ oligomers-treated mice spent in the target quadrant in probe trial of MWM test in Aβ oligomers-treated mice. (I) Representative paths of mice in the probe trial of MWM test. Tac: tacrine, HupA: huperzine A, #: A10E at 0.18 μmol/kg, &: A10E at 0.54 μmol/kg, *: A10E at 1.1 μmol/kg. i.p.: intraperitoneal injection; i.h.p.: intrahippocampal injection. Data are expressed as the mean ± SEM (n = 8); #p <0.05 and ##p <0.01 versus WT group, *p <0.05 and **p < 0.01 versus Aβ oligomers-treated group (one-way ANOVA and Tukey's test).

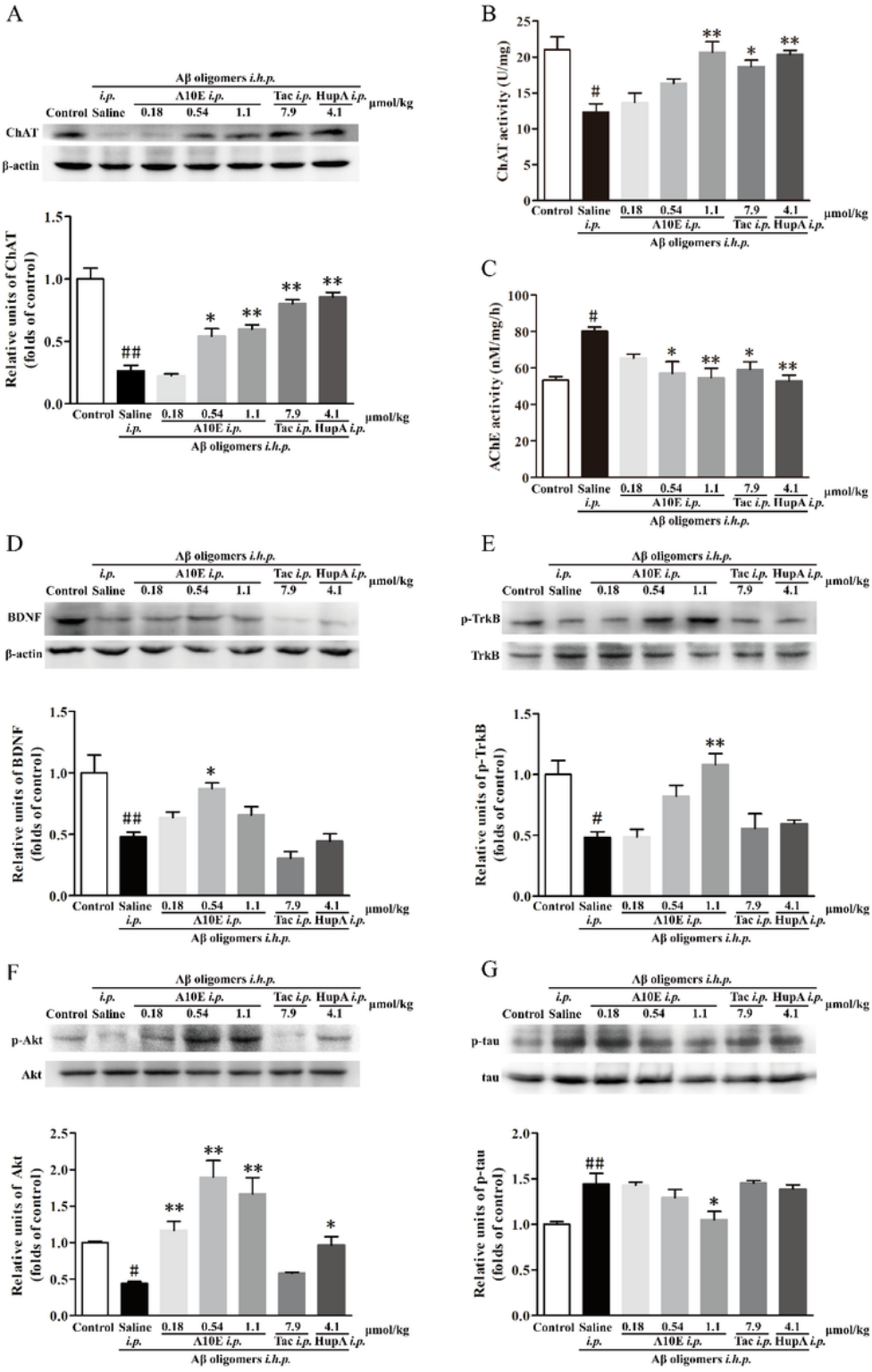

Figure 4

A10E attenuates the dysfunctions of cholinergic system and the inhibition of BDNF/TrkB Pathway in Aβ oligomers-treated mice. (A) Western blot analysis of ChAT in whole brain. The quantitative results demonstrated that A10E significantly increased the ChAT levels in the whole brain of Aβ oligomers-treated mice. (B) A10E treatment significantly increased ChAT activity in whole brain of Aβ oligomers-treated mice as evaluated by ChAT activity assay. (C) A10E treatment significantly inhibited AChE activity

in whole brain of Aβ oligomers-treated mice as evaluated by AChE activity assay. (D-G) Western blot analysis of (D) BDNF, (E) p-TrkB, (F) p-Akt and (G) p-tau in the hippocampus of mice. The quantitative results demonstrated that A10E significantly increased the expression of BDNF, p-TrkB, p-Akt, and decreased the expression of p-tau in the hippocampus of Aβ oligomers-treated mice. Tac: tacrine, HupA: huperzine A, i.p.: intraperitoneal injection, i.h.p.: intrahippocampal injection. Data are expressed as the mean ± SEM (n = 3); #p < 0.05 and ##p < 0.01 versus the control group, *p <0.05 and **p < 0.01 versus Aβ oligomers-treated group (one-way ANOVA and Tukey's test).

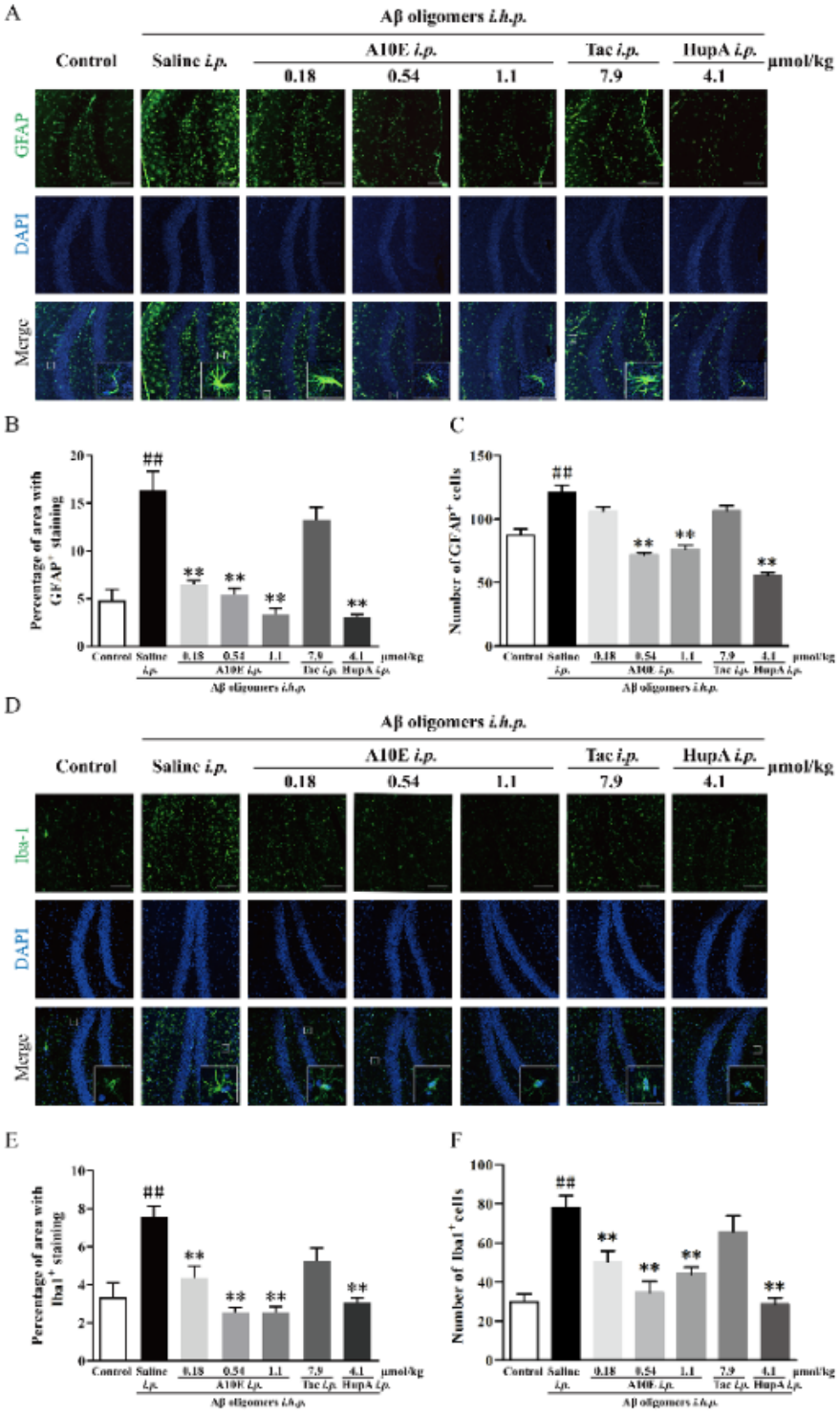

Figure 5

A10E attenuates Aβ oligomers-induced glial activation in mice. (A) Representative images of immunofluorescence staining of GFAP in the hippocampus of mice (scale bar = 25 μm). (B-C) A10E significantly reduced (B) the percentage of area with GFAP+ staining, and (C) the number of GFAP+ cells in the hippocampus of Aβ oligomers-treated mice. (D) Representative images of immunofluorescence staining of Iba-1 in the hippocampus of mice (scale bar = 25 μm). (B-C) A10E significantly reduced (B) the percentage of area with Iba-1+ staining, and (C) the number of Iba-1+ cells in the hippocampus of Aβ oligomers-treated mice. Tac: tacrine, HupA: huperzine A, i.p.: intraperitoneal injection, i.h.p.: intrahippocampal injection. Data are expressed as the mean ± SEM (n = 3); #p < 0.05 and ##p < 0.01 versus the control group, *p <0.05 and **p < 0.01 versus Aβ oligomers-treated group (one-way ANOVA and Tukey's test).

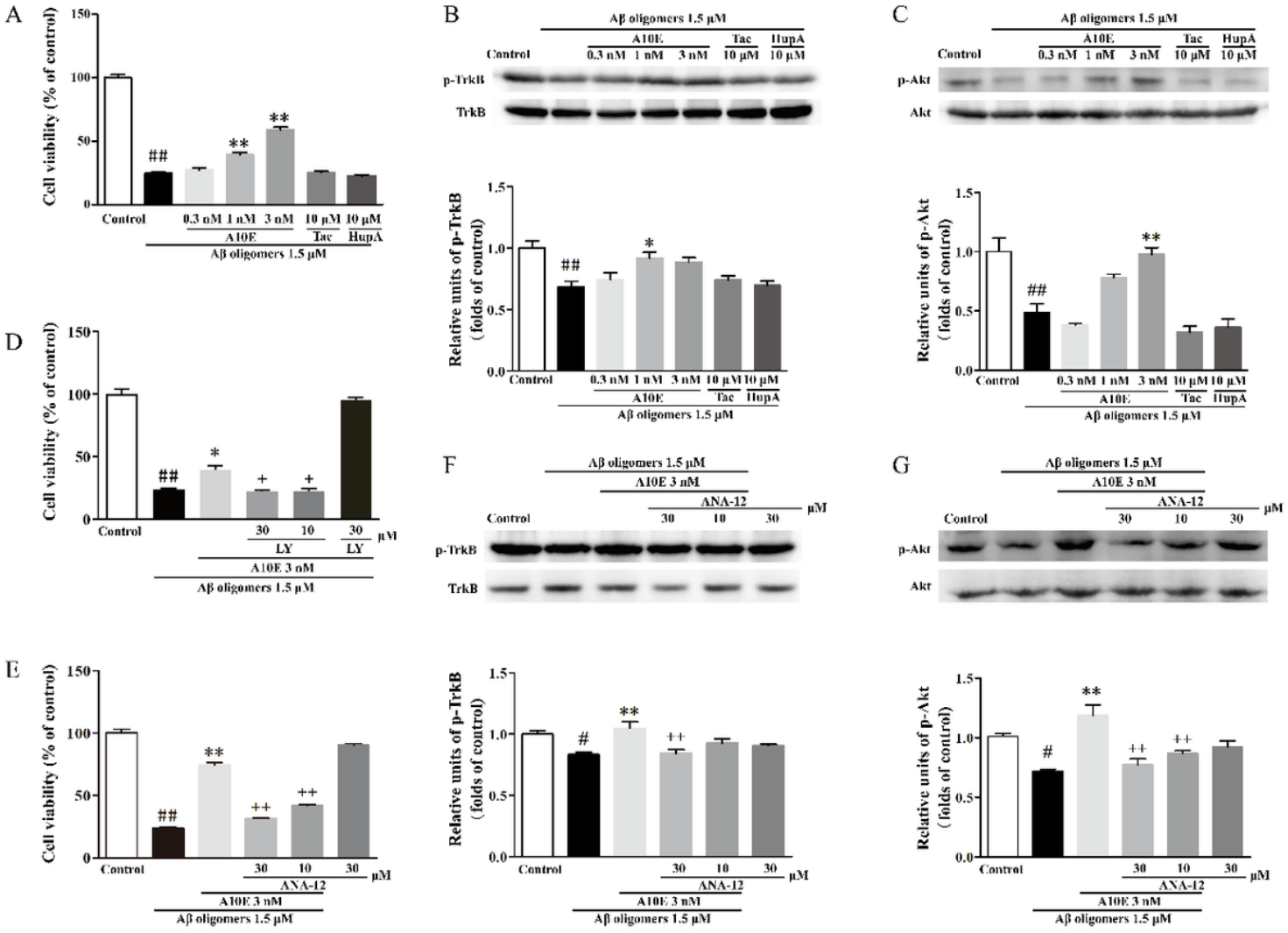

Figure 6

A10E treatment decreases Aβ oligomers-induced neurotoxicity in SH-SY5Y cells via the activation of the TrkB/Akt signaling pathway. (A) A10E significantly protected SH-SY5Y cells against Aβ oligomers-induced neurotoxicity. (B-C) The quantitative results demonstrated that A10E treatment significantly

increased the expression of p-TrkB and p-Akt. Representative images of Western blotting analysis of (B) p-TrkB and (C) p-Akt were shown. (D) LY294002, a PI3-K inhibitor, significantly abolished the neuroprotective effects of A10E against Aβ oligomers-induced neurotoxicity. SH-SY5Y cells were treated with LY294002 at various concentrations as indicated. After 1 h, 3 nM A10E was added. And then Aβ oligomers were added 24 h before the MTT assay. (E) TrkB inhibitor ANA-3 significantly abolished the neuroprotective effects of A10E against Aβ oligomers-induced neurotoxicity. (F-G) The quantitative results demonstrated that ANA-12 significantly abolished A10E-induced activation of TrkB/Akt pathway in Aβ oligomers-treated SH-SY5Y cells. Representative images of Western blotting analysis of (F) p-TrkB and (G) p-Akt were shown. Tac: tacrine, HupA: huperzine A, LY: LY294002. Data are expressed as the mean ± SEM; #p < 0.05 and ##p < 0.01 versus the control group, *p <0.05, **p < 0.01 versus the Aβ oligomers-treated group, and +p < 0.05, ++p < 0.01 versus the A10E plus Aβ oligomers group (one-way ANOVA and Tukey's test).

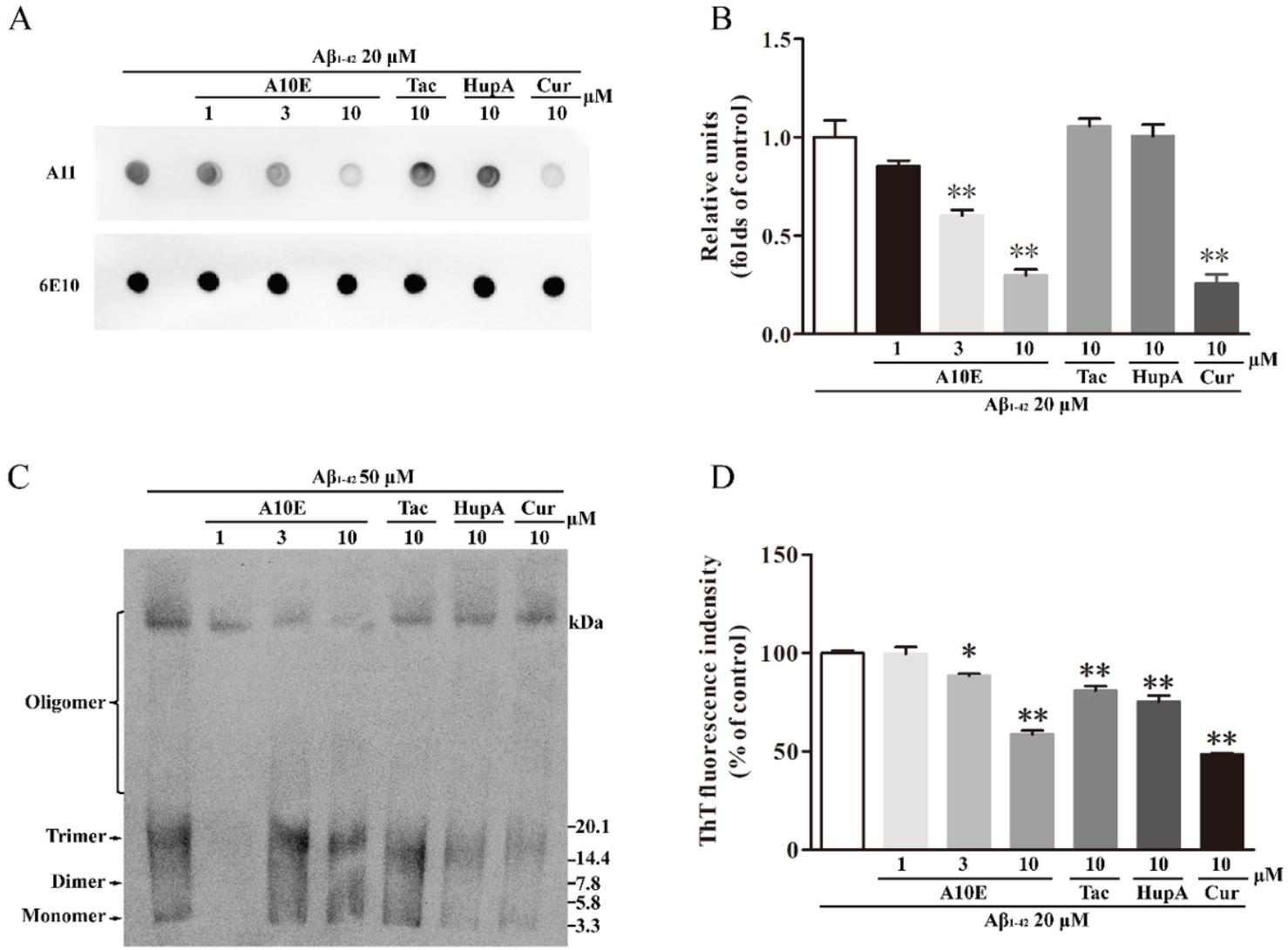

Figure 7

A10E inhibits Aβ aggregation in vitro. (A) Representative images of dot blotting with anti-oligomer antibody (A11) or anti-Aβ antibody (6E10) were shown. (B) Quantitative results demonstrated that A10E

inhibited Aβ oligomers formation. (C) A10E reduced the amount of Aβ oligomers. Representative images were demonstrated. Assemblies ranging from dimers to medium-size oligomer were recognized as Aβ oligomers. (D) A10E inhibited Aβ fibrils formation as demonstrated by ThT assay. Tac: tacrine, HupA: huperzine A; Cur: curcumin. Data are expressed as the mean ± SEM (n = 3); #p < 0.05 and ##p < 0.01 versus the control group, *p < 0.05 and **p < 0.01 versus Aβ oligomers group (one-way ANOVA and Tukey's test).

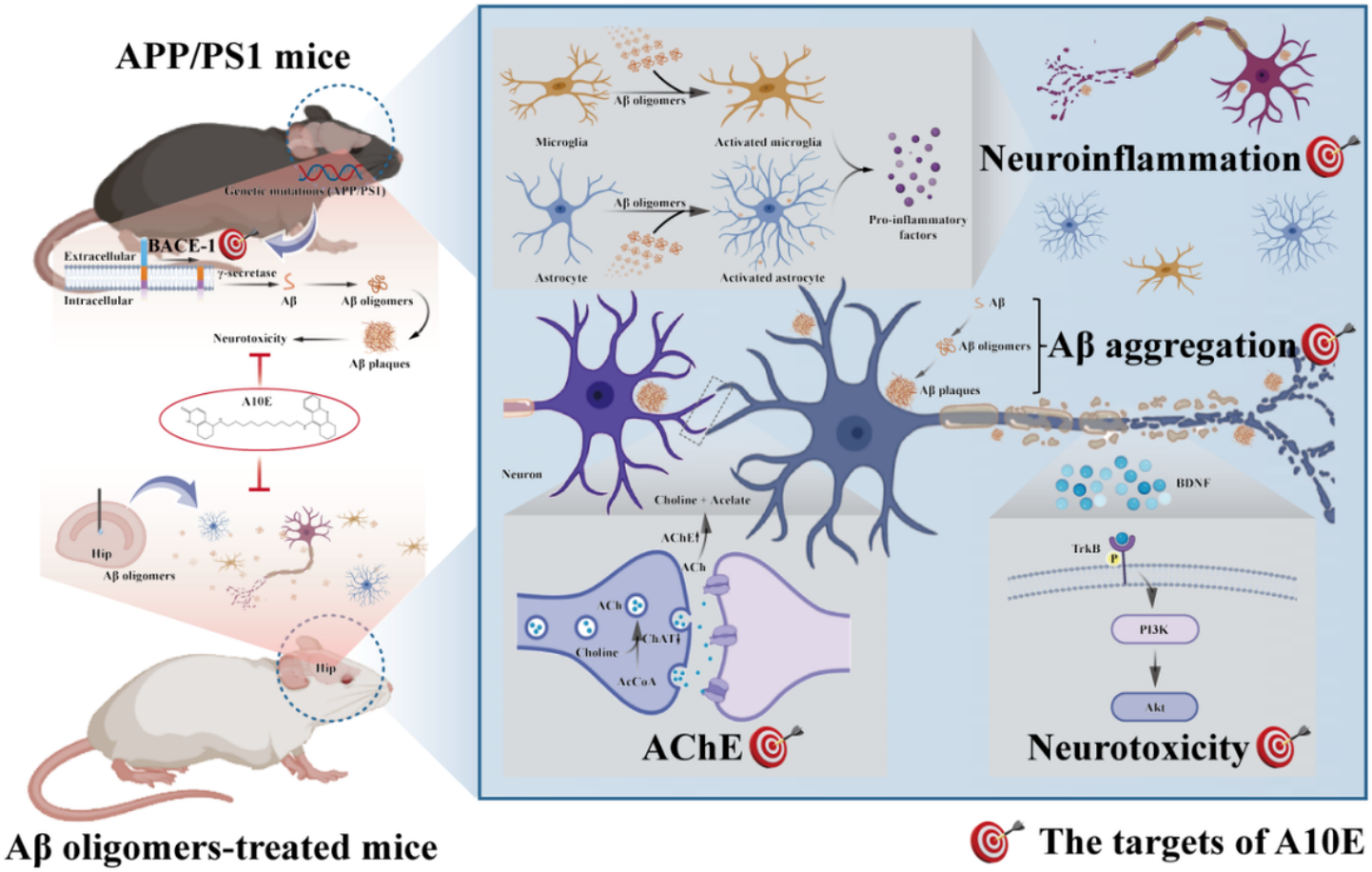

Figure 8

The schematic diagram of multi-target anti-AD mechanisms of A10E. APP/PS1 mice and Aβ oligomers-treated mice, were applied in this study. A10E, as a MTDL, might manipulate multiple targets of AD including Aβ aggregation, AChE, the BDNF/TrkB pathway, BACE-1, and neuroinflammation, indicating that A10E might be developed as a promising anti-AD lead.