

Activation of $\alpha 7$ nAChR by PNU-282987 improves synaptic and cognitive functions through restoring the expression of synaptic-associated proteins and the CaM-CaMKII-CREB signaling pathway

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ABSTRACT

Ligands of nicotinic acetylcholine receptors (nAChRs) are widely considered as potential therapeutic agents. The present study used primary hippocampus cells and APP^{swe}/PSEN1^{dE9} double-transgenic mice models to study the possible therapeutic effect and underlying mechanism of the specific activation of $\alpha 7$ nAChR by PNU-282987 in the pathogenesis of Alzheimer's disease. The results indicated that activation of $\alpha 7$ nAChR attenuated the A β -induced cell apoptosis, decreased the deposition of A β , increased the expression of synaptic-associated proteins, and maintained synaptic morphology. Furthermore, in the APP/PS1_{DT} mice model, activation of $\alpha 7$ nAChR attenuated A β -induced synaptic loss, reduced the deposition of A β in the hippocampus, maintained the integral structure of hippocampus-derived synapse, and activated the calmodulin (CaM)-calmodulin-dependent protein kinase II (CaMKII)-cAMP response element-binding protein signaling pathway by upregulation of its key signaling proteins. In addition, activation of $\alpha 7$ nAChR improved the learning and memory abilities of the APP/PS1_{DT} mice. Collectively, the activation of $\alpha 7$ nAChR by PNU-282987 attenuated the toxic effect of A β *in vivo* and *in vitro*, which including reduced deposition of A β in the hippocampus, maintained synaptic morphology by partially reversing the expression levels of synaptic-associated proteins, activation of the Ca²⁺ signaling pathway, and improvement of the cognitive abilities of APP/PS1_{DT} mice.

INTRODUCTION

Alzheimer's disease (AD) is the most common type of neurodegenerative disorder. The main clinical manifestations of AD patients are progressive memory decline, abnormal brain function and a decline in social adaptability. The pathological features of AD include the formation of extracellular senile plaques (SPs), intraneuronal neurofibrillary tangles aggregating of hyperphosphorylated tau protein and the loss of neurons

[1]. SPs mainly consist of extracellular amyloid β (A β), which contains 38-43 amino acids and plays a key role in the pathogenesis of AD [2]. Deposition of A β in the human brain is considered to be associated with cross-sectional synaptic network dysfunction, progressive brain atrophy, an imbalance in neuronal calcium homeostasis and longitudinal cognitive decline [3]. Therefore, investigating methods to prevent the toxic effects of A β could be one of the promising strategies of treating AD patients.

Nowadays, multiple lines of transgenic mice have been developed to serve as animal models to study the progress of AD. APP^{swe}/PSEN1^{dE9} double-transgenic mice (APP/PS_{DT}) harbor a mutated human amyloid precursor (APP^{swe}, Swedish mutation) and human presenilin (PS DeltaE9) genes. A Swedish mutation of the amyloid precursor protein (APP) gene causes abnormal metabolism of APP and leads to excessive deposition of A β in the brain, which can induce neuronal apoptosis and necrosis [6]. Mutation of the PS1 gene can change the activity of γ -secretase and increase A β_{42} production [7, 8]. Double mutation of APP and PS1 genes in APP/PS1_{DT} mice leads to the accumulation of A β in the brain of these mice, thus causes deficits in learning and memory.

The neuropathological process of AD patients is highly associated with synaptic impairment. Synapses are the junction between two neurons and are the functionally connected parts of neurons, which serve a key role in the transmission of information. The primary function of the synapse is to mediate intercellular communication by releasing neurotransmitters from the presynaptic terminal and to modulate neural plasticity. The synaptic connection is an essential part of neurons that transmits information and is also the basis of learning and memory abilities. Numerous studies have also demonstrated that the decline of cognitive function in patients with AD is closely related to the impairment of synaptic function [15, 16]. Studies on the postmortem brains of individuals with AD, as well as on animal models connected with the disease, indicate that synapses are affected at the earliest stages of the neurodegenerative processes [4]. Loss of synapses in the brain tissues of patients with AD is associated with cognitive impairment [5].

Furthermore, nicotinic acetylcholine receptors (nAChRs) are involved in a variety of vital physiological processes, particularly in memory and cognition. Receptor-ligand binding studies have demonstrated that AD-associated loss of nAChRs is positively correlated with the presence of SPs in the temporal lobe [6]. nAChRs, including hetero- and homo-pentameric subtypes, consist of different combination of α ($\alpha 2$ - $\alpha 10$) and β ($\beta 2$ - $\beta 4$) subunits. In the human brain, $\alpha 4\beta 2$ and $\alpha 7$ are located in cortical, striatal and hippocampal regions, which are involved in memory and cognition, and also the only subtypes of nAChRs that have been implicated in the pathogenesis of AD. A previous study reported that the APP-a7KO model (Tg2576 mice expressing the human amyloid precursor protein sequence with the Swedish mutation crossed with $\alpha 7$ nAChRs null-mutant) have worsened cognitive deficits when $\alpha 7$ nAChR is absent, and a decrease of $\alpha 7$ nAChRs levels are associated with synaptic damage in AD patients [19]. Furthermore, using

specific agonists, which stabilize $\alpha 7$ nAChRs in the open state, has been shown to improve cognitive impairment in different AD models [7].

As activation of $\alpha 7$ nAChRs has a potentially beneficial effect on patients who suffer from cognitive impairments, extensive studies have been performed to study the underlying mechanism of the neural protective effects of $\alpha 7$ nAChRs agonist. It has been reported that nicotine, an unselective $\alpha 7$ nAChRs agonist, protects synapses from morphological destruction and synaptic impairment, which is exerted by A β oligomers. Activation of $\alpha 7$ nAChRs by agonist also prevents both early postsynaptic and late presynaptic impairments induced by A β via the $\alpha 7$ nAChRs/phosphatidylinositol-3 kinase (PI3K) signaling pathway and cross-talk with the Wnt/ β -catenin pathway. Many selective agonists have also been used to activate $\alpha 7$ nAChRs as a potential treatment for patients with AD, including CCMI and PNU-1206596 [8]. Recently, a novel $\alpha 7$ nAChR selective agonist (PNU-282987) has been demonstrated to improve motor ataxia and anxiolytic effect in an AD mouse model [9]. It also has been reported that activation of $\alpha 7$ nAChRs by PNU-282987 can be used to ameliorate cognitive symptoms in schizophrenia and depression [7]. However, the underlying mechanism of PNU-282987 on synaptic changes and its neuroprotective signaling pathway are not fully understood.

In the present study, both primary hippocampus neuron cells and APP/PS1_{DT} mice models were used to investigate the effects of $\alpha 7$ nAChRs activation by PNU-282987 on synaptic morphology, the expression levels of synaptic-associated proteins, cognitive functions and the Ca²⁺ signaling pathway. The present results may provide a theoretical basis for further elucidating the pathogenesis of AD. Furthermore, it may shed light on the functions of $\alpha 7$ nAChRs during the development of AD.

RESULTS

Activation of $\alpha 7$ nAChR attenuates A β -induced apoptosis

To test whether A β oligomers increase the apoptosis rate of neuronal cells, flow cytometry was used to quantify the percentage of apoptotic cells by using the FITC Annexin V Apoptosis Detection Kit. The results demonstrated that the apoptosis rate of A β oligomer and MLA (an antagonist of $\alpha 7$ nAChR)-treated primary neuronal cells was significantly increased compared with the control group. Whereas PNU-282987 attenuated A β -induced apoptosis and MLA amplified A β -induced apoptosis (Figure 1).

Activation of $\alpha 7$ nAChR enhances learning and memory abilities in APP/PS1_DT mice

To examine whether activation of $\alpha 7$ nAChR by PNU-282987 alleviates the cognitive deficits in APP/PS1_DT mice, the present study measured the potential of $\alpha 7$ nAChR to alleviate AD-induced declines in spatial memory and learning ability by using the Morris water maze [10]. The APP/PS1 group demonstrated a longer escape latency compared with the control group. However, the increased escape latency of APP/PS1_DT mice was alleviated by the administration of PNU-282987 (AP group) (Figure 2A). The platform in the water maze was removed from the pool on day 5 of testing, and all groups of mice were allowed to swim for one minute. The time of staying at the platform and the number crossing the platform of PNU-282987-treated mice (AP group) was dramatically increased compared with the APP/PS1_DT mice (APP/PS1 group; Figure 2B and 2C). These results suggested that activation of $\alpha 7$ nAChR improves the spatial learning and memory disorders in APP/PS1_DT mice.

Activation of $\alpha 7$ nAChR reduces the deposition of A β in the hippocampus of APP/PS1_DT mice

A β plays a vital role in the cognitive dysfunction of AD. Thus, the present study observed the expression of A β

by immunohistochemical staining. As presented in Figure 3 (Figure 3A and 3F for 6- and 10-month old mice, respectively), the levels of A β integrated optical density (IOD) were significantly increased in the APP/PS1 group, both in the 6-month-old and 10-month-old APP/PS1_DT mice groups (Figure 3A and 3F). Whereas A β deposition was partially alleviated by PNU-282987 treatment (Figure 3B–3E and 3G–3J). The results indicated that $\alpha 7$ nAChR could reduce the deposition of A β in the hippocampus of APP/PS1_DT mice, thus, alleviate the toxic effect of A β on neuronal cells.

Activation of $\alpha 7$ nAChR promotes the expression of synaptic-associated proteins in A β oligomers treated neurons

To explore whether $\alpha 7$ nAChR could restore synaptic-associated proteins in A β oligomers-treated neurons, reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis were used to quantify the expression of synaptophysin (SYN), postsynaptic density of 95 (PSD95), synaptosomal-associated protein of 25 kD (SNAP25), dynamin 1 (DYN1) and adaptor protein of 180 (AP180) in hippocampal neurons. As presented in Figure 4, compared with the control group, the relative transcription levels of SYN (Figure 4A), PSD95 (Figure 4C), SNAP25 (Figure 4E), DYN1 (Figure 4G),

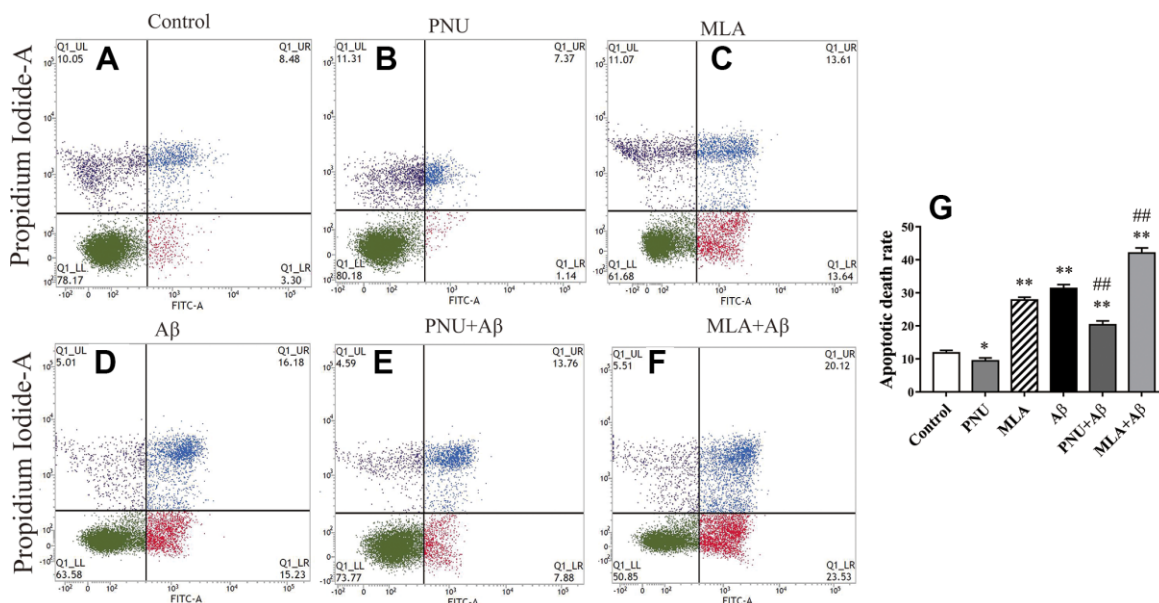


Figure 1. Activation of $\alpha 7$ nAChR attenuates apoptosis induced by A β oligomers. (A) The primary hippocampus cell group (Control), (B) the primary hippocampus cells treated with PNU (PNU), (C) the primary hippocampus cell treated with MLA (MLA), (D) the primary hippocampus cell treated with A β (A β), (E) the primary hippocampus treated with PNU and A β (PNU+A β), (F) the primary hippocampus treated with MLA and A β (MLA+A β) and (G) a histogram presenting the apoptotic death rates of the different groups. (C and D) The apoptosis rate of primary neuronal cells treated with MLA and A β oligomers was significantly increased, (E) while $\alpha 7$ nAChR attenuated apoptosis induced by A β oligomers. Data are presented as the mean \pm standard deviation. *P<0.05, **P<0.01 vs. control group; #P<0.05, ##P<0.01 vs. A β group.

AP180 (Figure 4I) were significantly decreased in A β oligomer-treated neurons. Correspondingly, compared with the control group, the protein levels of SYN (Figure 4B), PSD95 (Figure 4D), SNAP25 (Figure 4F), DYN1 (Figure 4H), AP180 (Figure 4J) were also significantly decreased in A β oligomer-treated neurons. Whereas, the decrease of these protein levels was partially reversed by PNU-282987 treatment. To verify that the neuroprotective effect is $\alpha 7$ nAChRs-dependent, the $\alpha 7$ nAChR-selective competitive antagonist methyllycaconitine (MLA) was used to reduce activation of $\alpha 7$ nAChRs. As presented in Supplementary Figure 4, the protein expression levels of SYN (Supplementary Figure 4A), PSD95 (Supplementary Figure 4B) and DYN1 (Supplementary Figure 4D) in the MLA+A β group (WT neuron cells treated with 100 nmol/l MLA and 0.5 μ mol/l A β for 24 hours) were decreased compared with the A β group (WT neuron cells treated with 0.5 μ mol/l A β for 24 hours). Notably, there were no significant differences in

the protein levels of SNAP25 (Supplementary Figure 4C) and AP180 (Supplementary Figure 4E) compared with the A β group. These results indicate alleviation of synaptic dysfunction *in vitro* by PNU-282987 is $\alpha 7$ nAChR-dependent.

Activation of $\alpha 7$ nAChR increases the expression of synaptic-associated proteins in the hippocampus of APP/PS1_DT mice

The present study subsequently evaluated the expression of synaptic-associated proteins (SYN, PSD95, SNAP25, DYN1 and AP180) at the mRNA and protein level in the hippocampus of APP/PS1_DT mice (6- and 10-months old). As shown in Figure 5, RT-qPCR and western blot analysis revealed that in APP/PS1_DT group, the SYN, PSD95, SNAP25, DYN1 and AP180 mRNA (Figure 5A, 5C, 5E, 5G and 5I) and protein levels (Figure 5B, 5D, 5F, 5H and 5J) in the hippocampus were significantly reduced. Whereas,

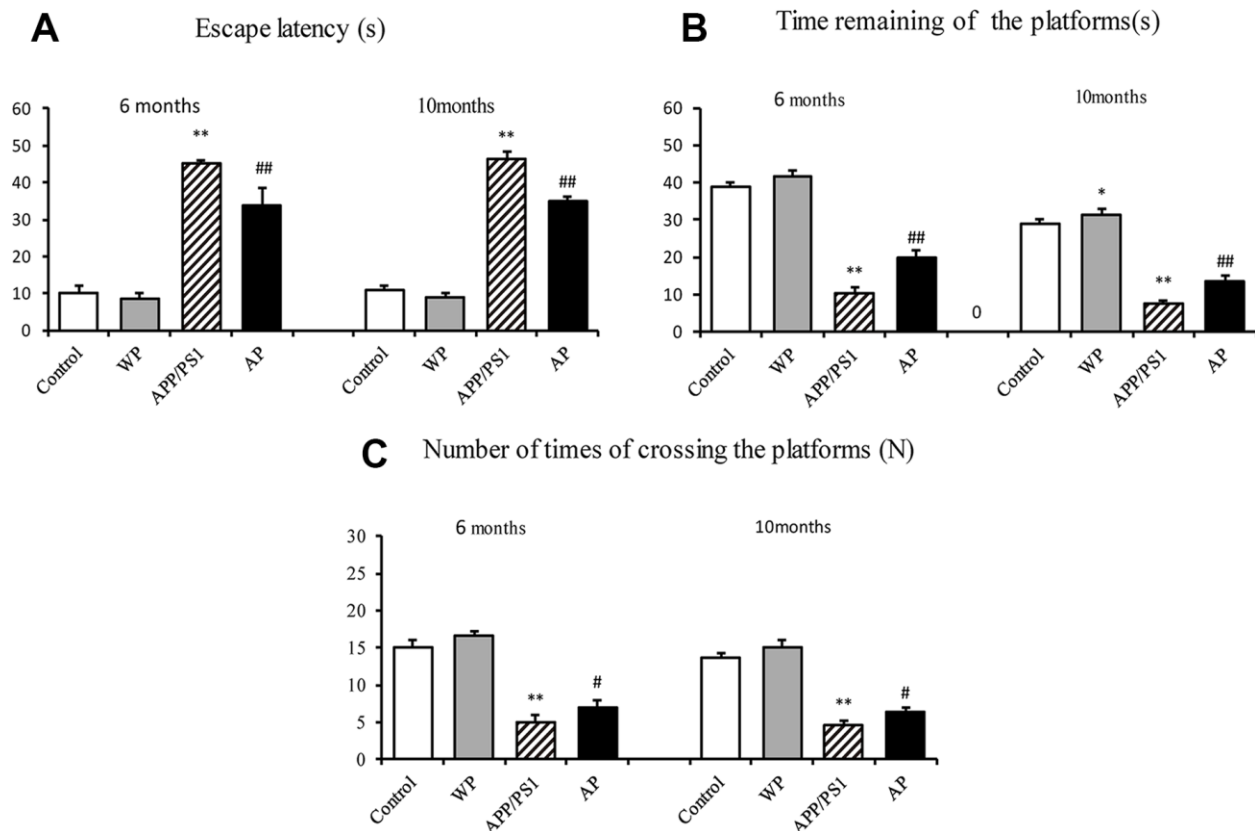


Figure 2. Activation of $\alpha 7$ nAChR alleviates cognitive deficits in APP/PS1_DT mice. (A) Escape latency of mice from different groups. (B) Times of staying in the platform (C) The numbers of crossing the platform. The abscissa indicate the wild-type C57 mice injected with saline (control), wild-type C57 mice injected with PNU-282987 (WP), APP/PS1_DT mice injected with saline (APP/PS1) and APP/PS1_DT mice injected with PNU-282987 (AP). The Morris water maze demonstrated that both spatial learning, and spatial memory abilities were declined in 6- and 10-months-old APP/PS1_DT mice, while $\alpha 7$ nAChR alleviated cognitive deficits in APP/PS1_DT mice. These results indicate that the expression level of $\alpha 7$ nAChR is associated with improved cognitive function in the APP/PS1_DT mice. Data are presented as the mean \pm standard deviation. *P<0.05, **P<0.01 vs. control group; #P<0.05, ##P<0.01 vs. APP/PS1 group. s, seconds; N, times.

following PNU-282987 treatment, the expression levels of SYN, PSD95, SNAP25, DYN1 and AP180 were significantly increased compared with the APP/PS1_DT group. These data indicated that $\alpha 7$ nAChR partially reverses the loss of synaptic-associated proteins.

Expression of SYN in primary hippocampus neurons detected by immunofluorescence

Evidence has shown that the levels of PSD95 and SYN are reduced in AD transgenic mice models [11, 12] and

the brains of patients with AD [13]. SYN and PSD95 are markers of the pre- and post-synapse, respectively. Furthermore, both *in vivo* and *in vitro* experiments have shown that A β monomer can lead to synaptic plasticity damage and synaptic loss. The A β oligomers can cause synaptic dysfunction [14]. The present study used immunofluorescence to investigate whether $\alpha 7$ nAChR could restore SYN expression in A β oligomers-treated neurons. As shown in Figure 6, the expression level of SYN was significantly decreased in the A β oligomer-treated group, and this decreasing trend was partially

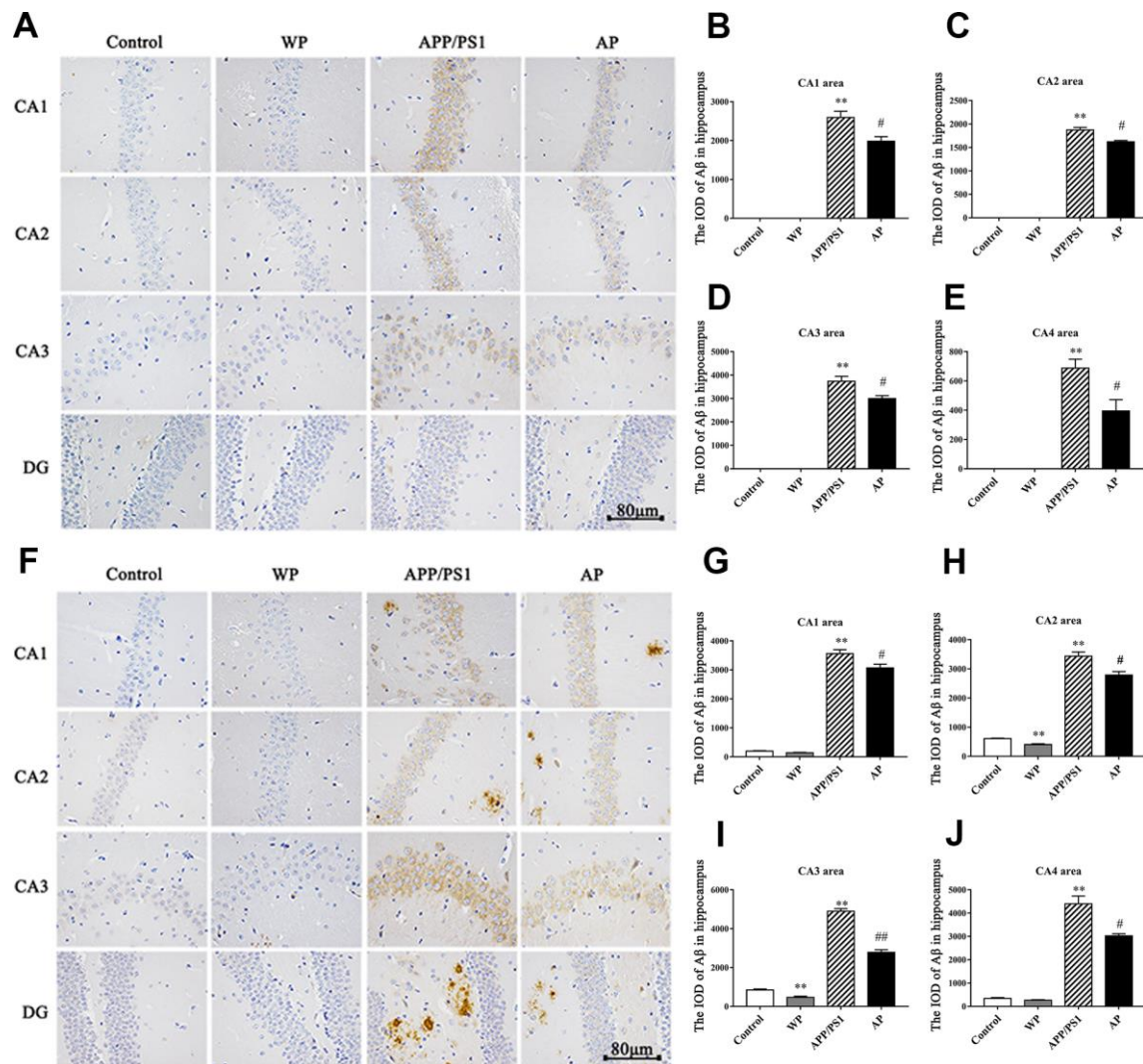


Figure 3. Activation of $\alpha 7$ nAChR reduces the deposition of A β in the hippocampus of the APP/PS1_DT mice. The deposition of A β in the hippocampus of APP/PS1_DT mice (A) 6 months old and (F) 10 months old. The IOD of A β in the hippocampus area of 6 months old mice in the (B) CA1 area, (C) CA2 area, (D) CA3 area, (E) CA4 area, and 10 months old mice in the (G) CA1 area, (H) CA2 area, (I) CA3 area and (J) CA4 area. Control, WT C57 mice injected with saline; WP, WT mice injected with PNU; APP/PS1, APP/PS1_DT mice injected with saline; AP, APP/PS1_DT mice injected with PNU. CA1, CA2, CA3 and DG indicate the CA1 area, CA2 area, CA3 area and DG area of the hippocampus, respectively. Compared with the control group, A β deposition in the hippocampus of APP/PS1_DT mice was increased significantly, and this trend was partially reversed by PNU treatment (AP group). The results demonstrated that $\alpha 7$ nAChR partially reduced the deposition of A β in the hippocampus of the APP/PS1_DT mice. Data are presented as the mean \pm standard deviation. *P<0.05, **P<0.01 vs. control group; #P<0.05, ##P<0.01 vs. APP/PS1 group.

reversed by PNU-282987 treatment (Figure 6E and 6F). This result indicates that $\alpha 7$ nAChR could attenuate synaptic loss *in vitro*.

Expression of SYN in a mice model detected by immunofluorescence staining

To investigate whether $\alpha 7$ nAChR could restore SYN expression in APP/PS1_DT mice, immunofluorescence was used to determine the

expression of SYN in the hippocampus of the DG area. As presented in Figure 7, the expression level of SYN was significantly decreased in the hippocampus DG area of APP/PS1_DT mice (in both 6- and 10-months-old mice). The A β distribution and SYN expression were negatively correlated, and the reduction of SYN could be partially reversed by PNU-282987 treatment (Figure 7G and 7H). These results suggested that $\alpha 7$ nAChR partially attenuates synaptic loss in the hippocampus of APP/PS1_DT mice.

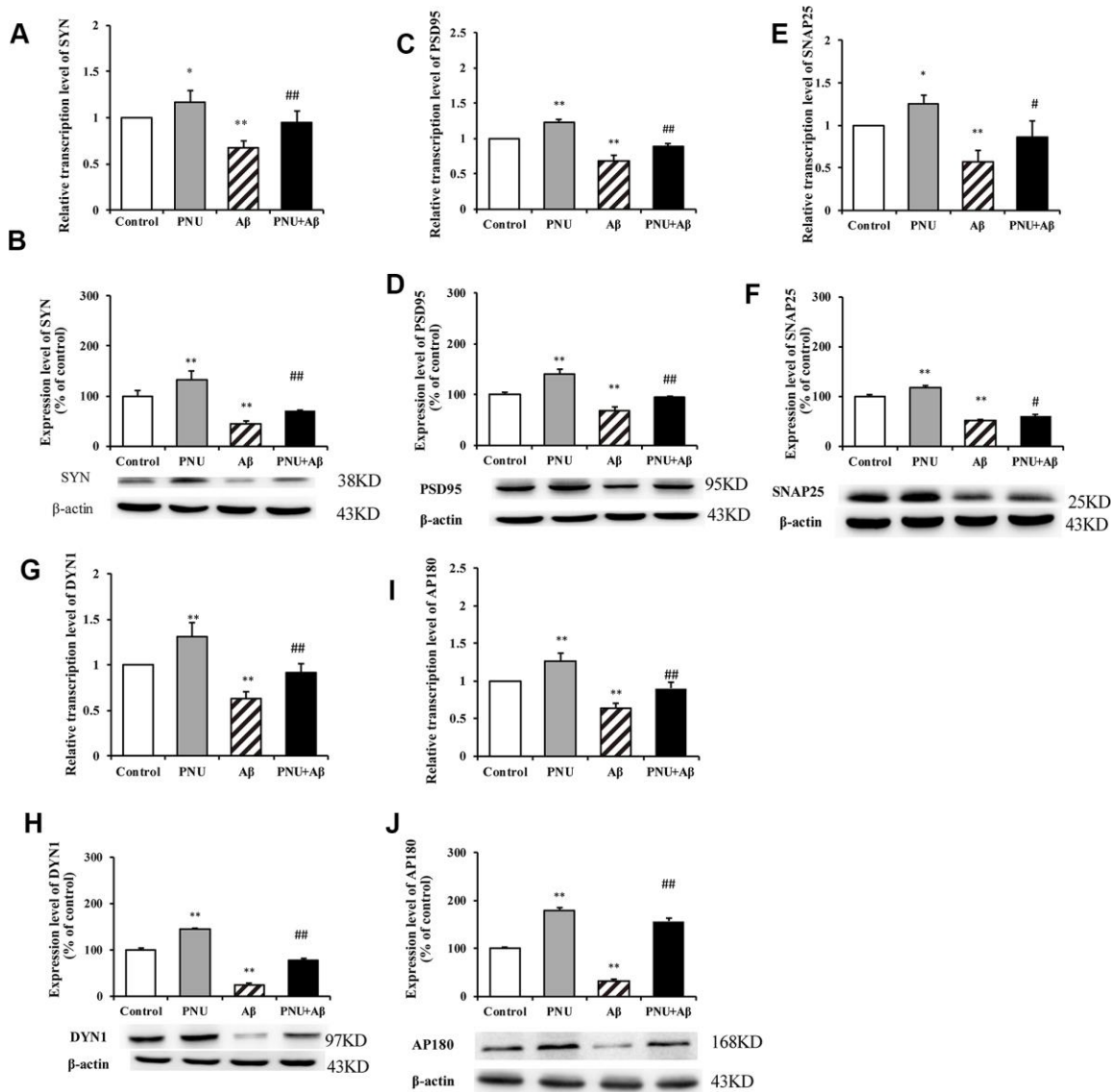


Figure 4. Activation of $\alpha 7$ nAChR promotes the expression of synaptic-associated proteins in A β oligomer-treated neurons.

The x-axis labels are the neurons isolated from the WT rat (control), the WT neuron cells treated with PNU (PNU), the WT neuron cells treated with A β (A β) and the WT neuron cells treated with PNU and A β (PNU+A β). The y-axis indicates the relative level of mRNA or protein (% of control). Detection of SYN (A) mRNA and (B) protein; PSD95 (C) mRNA and (D) protein; SNAP25 (E) mRNA and (F) protein; DYN1 (G) mRNA and (H) protein; AP180 (I) mRNA and (J) protein. The relative level in each group was measured by RT-qPCR and western blot analysis, and β -actin was used as an internal control. The results demonstrated that the protein expression levels of SYN, PSD95, SNAP25, DYN1 and AP180 were significantly decreased in A β oligomer-treated neurons, and this decrease was partially reversed by PNU treatment. Data are presented as the mean \pm standard deviation. *P<0.05, **P<0.01 vs. control group; #P<0.05, ##P<0.01 vs. A β .

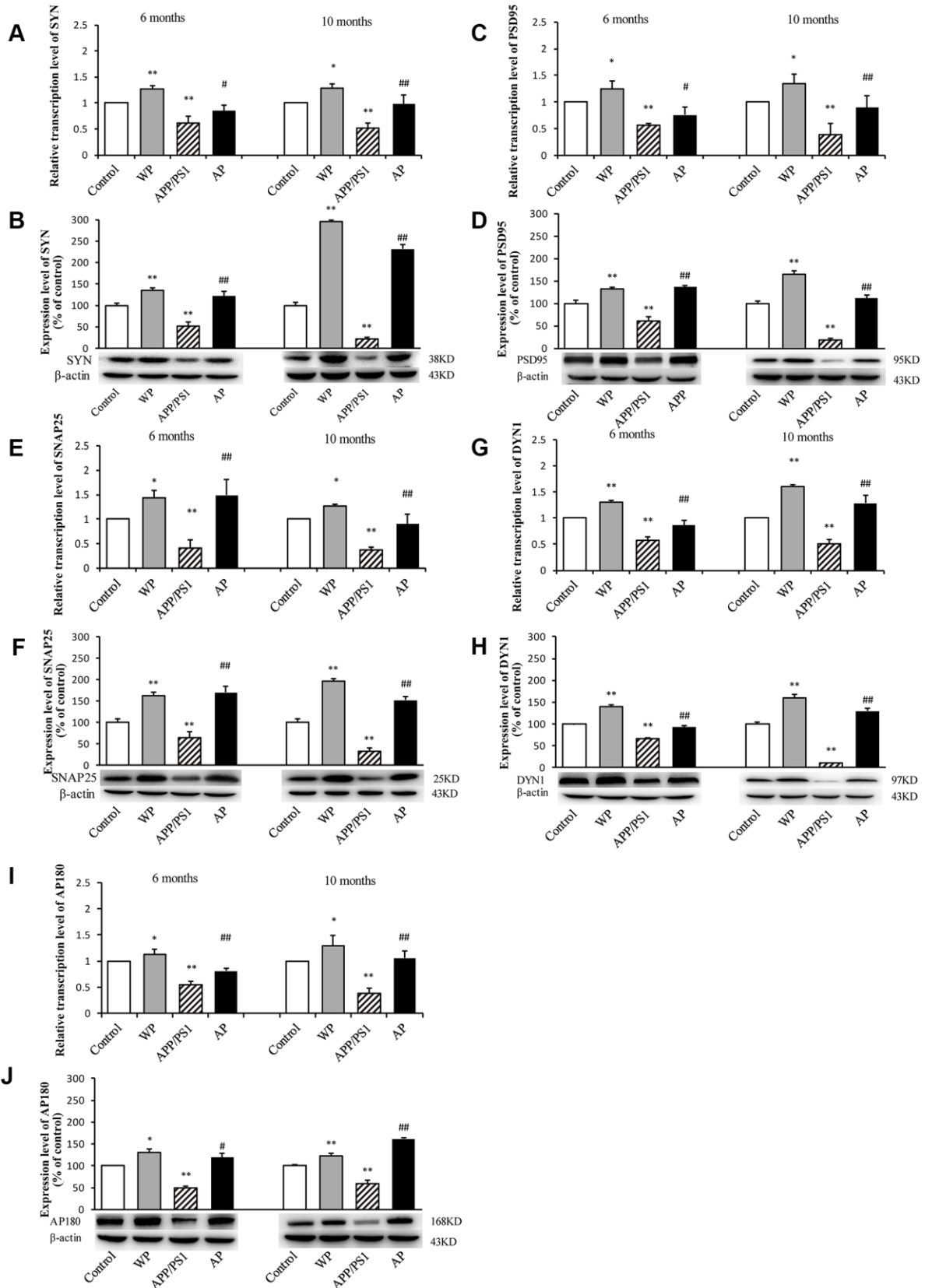


Figure 5. Activation of $\alpha 7$ nAChR increases the expression of synaptic-associated proteins in the hippocampus of APP/PS1_{DT} mice. The x-axes are the WT mice (control), the WT mice treated with PNU (WP), the APP/PS1_{DT} mice (APP/PS1) and the APP/PS1_{DT} mice treated with PNU (AP). The y-axes are the relative level of mRNA or protein (% of control group). Detection of SYN (A)

mRNA and (B) protein; PSD95 (C) mRNA and (D) protein; SNAP25 (E) mRNA and (F) protein; DYN1 (G) mRNA and (H) protein; AP180 (I) mRNA and (J) protein by RT-qPCR and western blot analysis. Protein expression levels were detected by western blot analysis (β -actin was used as an internal control). RT-qPCR and western blot analysis demonstrated that the expression levels of SYN, PSD95, SNAP25, DYN1 and AP180 in the hippocampus of APP/PS1_DT mice were significantly decreased compared with the control group, and this decreasing trend was partially reversed by PNU treatment. Data are presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. APP/PS1 group.

Expression of PSD95 in the mice model detected by immunohistochemistry

The PSD95 is closely related to synaptic fitness, and a decrease in its expression indicates synaptic malfunction [15]. The PSD95 interacts with a variety of post-synaptic membrane proteins, participates in the formation of synaptic connections and participates in post-synaptic signal transduction. Immunohistochemical staining was used to measure the expression of PSD95. The expression levels of PSD95 were significantly decreased in the 6-month-old APP/PS1_DT group (Figure 8A–8E), and the expression level of PSD95 was significantly reduced in the hippocampus of 10-month-old APP/PS1_DT

mice (Figure 8F–8J). As expected, the expression level of PSD95 was partially reversed by PNU-282987 treatment (Figure 8B–8E and 8G–8J).

Synapse morphology observed by transmission electron microscopy (TEM)

Synapses are the basic structure for the connection between neurons. In the central nervous system, the number of synapses directly affects the efficiency of information transmission. Synaptic transmission efficiency is correlated with synaptic gap width. Additionally, postsynaptic density and curvature regulates synaptic plasticity [16]. The image of TEM revealed that compared with the control group, the number of

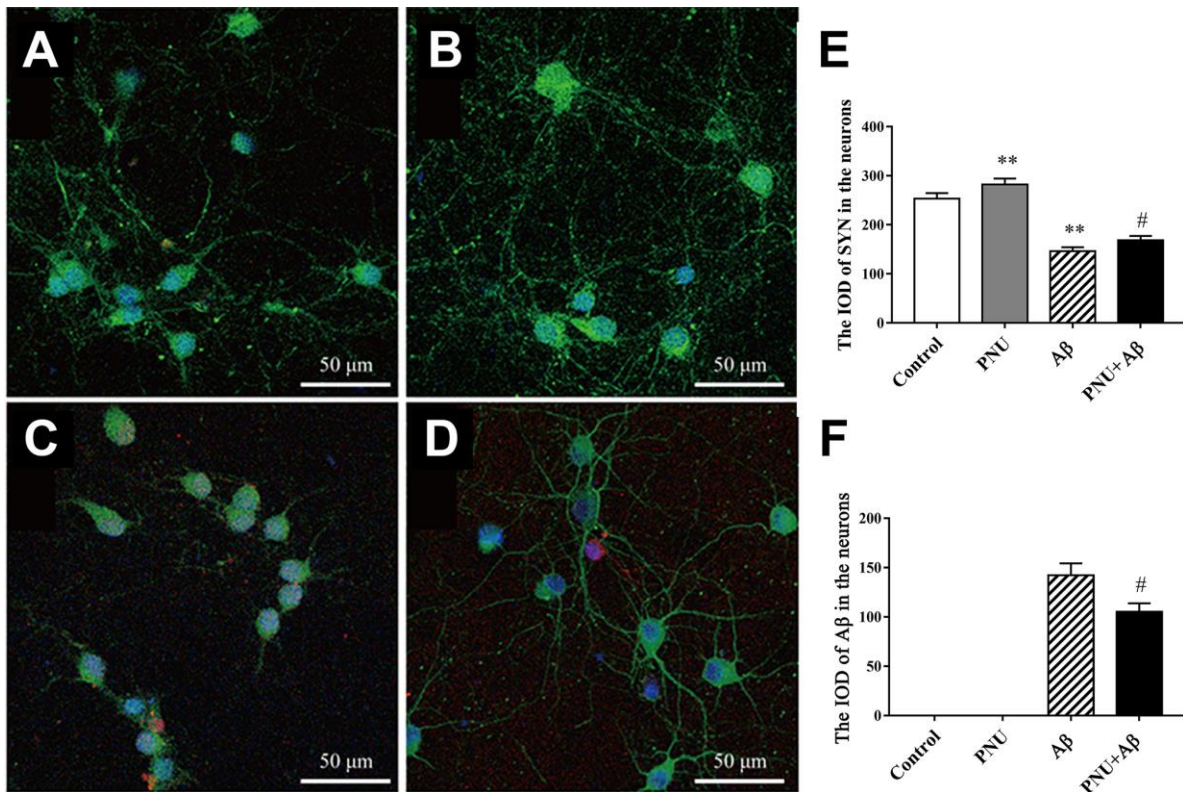


Figure 6. Activation of $\alpha 7$ nAChR promotes the expression of SYN in A β oligomer-treated neurons. (A) Hippocampus cells isolated from WT mice (control), (B) control treated with PNU (PNU), (C) control treated with A β (A β) and (D) control treated with PNU and A β (PNU+A β). (E) The IOD of SYN in the hippocampus neurons. (F) The IOD of A β in the hippocampus neurons. SYN, A β oligomers and the nuclei were labeled green, red and blue, respectively. Magnification, x200. Compared with the (A) control group, the expression level of SYN (green) was significantly decreased in the (C) A β oligomer group, (D) and this decreasing was partially reversed by PNU treatment. Data are presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. A β group.

synapses and synaptic vesicles was significantly decreased, and activation of $\alpha 7$ nAChR increased the number of synapses in the hippocampus of APP/PS1_DT mice (Figure 9).

The hippocampal neurons of the 6-month and 10-month-old APP/PS1_DT mice underwent the following morphological changes: i) The number of synapses in both 6- and 10-months-old mice was decreased (Figure 9C and 9G); ii) synaptic gap became wider (Figure 9D and 9H); iii) the post-synaptic dense substance in the hippocampus of APP/PS1 group became thinner (vs. the control group) and the post-synaptic dense substance in the AP groups (either 6-month-old or 10-month-old) was thicker than in the APP/PS1 group (Figure 9E and 9I); and iv) the curvature of the synaptic interface became smaller in 10-month-old mice (Figure 9F and 9J). Furthermore, there were no significant differences in synaptic gap width and synaptic interface curvature between the APP/PS1 and AP groups (Figure 9D, 9F, 9H and 9J). These results indicated that $\alpha 7$ nAChR is beneficial to maintain the integrity of the synaptic

structure and increase the number of synapses, thus could improve synaptic functions.

Activation of $\alpha 7$ nAChR activated the CaM-CaMKII-CREB signaling pathway in $A\beta$ oligomer-treated neurons

The calcium disorder theory suggests that the concentration of calcium ions determines whether neuronal cells can produce excitatory and complete excitatory signals. The accumulation of $A\beta$ in AD patients causes intracellular calcium (Ca^{2+}) ion disorder; thus, damages synaptic plasticity [17]. Ca^{2+} can activate calpain kinase, which damages the cellular structure and leads to decreased cognitive ability in patients [18]. Therefore, the CaM-CaMKII-CREB signaling pathway, which regulates Ca^{2+} concentration in neuronal cells, plays an important role in synaptic degeneration and memory loss in AD, and it is also a potential therapeutic target for treating AD [19]. The present study investigated whether PNU-282987 activated the CaM-CaMKII-CREB signaling pathway in hippocampal

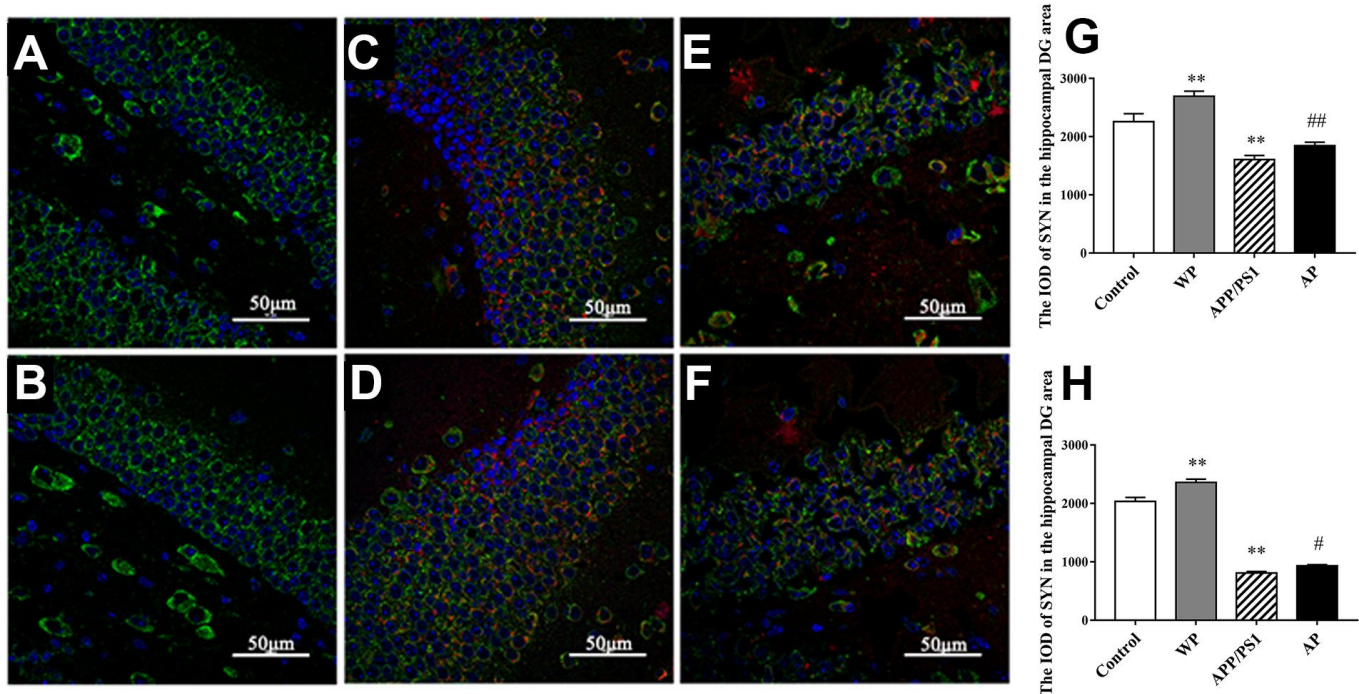


Figure 7. Activation of $\alpha 7$ nAChR attenuates the expression of SYN in the hippocampus of APP/PS1_DT mice. (A) Hippocampus cells isolated from WT mice (Control); (B) Controls treated with PNU (WP); (C) 6-month-old APP/PS1_DT mice; (D) 6-month-old APP/PS1_DT mice treated with PNU; (E) 10-month-old APP/PS1_DT mice and (F) 10-month-old APP/PS1 mice treated with PNU. (G) The IOD of SYN in the hippocampus DG area, which was collected from 6-month-old mice. (H) The IOD of SYN in the hippocampus DG area, which was collected from 10-month-old mice. SYN, $A\beta$ and nucleus are labeled in green, red and blue, respectively. Magnification, x200. Compared with the (A) control group, the expression level of SYN was significantly decreased in the hippocampus DG area of (C) 6- and (E) 10-month-old APP/PS1_DT mice. (D and F) The $A\beta$ distribution and SYN expression were negatively correlated, and the decrease of SYN could be partially reversed by PNU treatment. Data are presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. APP/PS1 group.

neurons. As shown in Figure 10, the relative mRNA and protein levels of CaM (Figure 10A and 10B) were increased; however, the mRNA expression levels of CaMKII α (Figure 10C) and CREB (Figure 10E) were decreased in the A β oligomer-treated neurons. The protein

level of CaMKII α (Figure 10D) remained unchanged, while the protein levels of CREB (Figure 10F), phosphorylated (p)-CaMKII α (Figure 10G), p-CREB (Figure 10H), and the ratio of phosphorylated to non-phosphorylated proteins, p-CaMKII α /CaMKII α

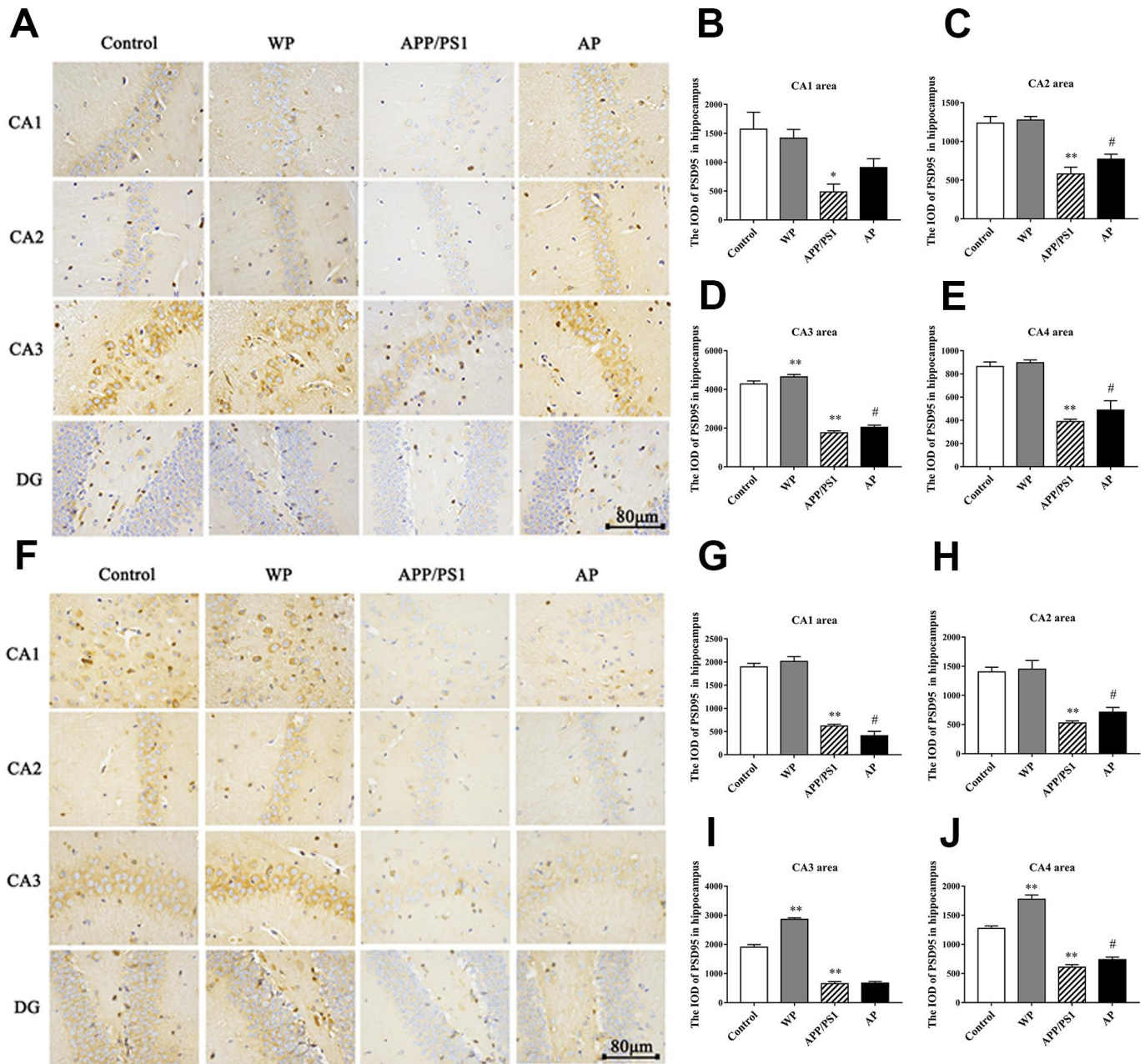


Figure 8. Activation of $\alpha 7$ nAChRs increases the expression of PSD95 protein in the hippocampus of APP/PS1_{DT} mice. The expression of PSD95 protein in the hippocampus of the APP/PS1_{DT} mice at (A) 6-months and (F) 10-months old. The IOD of PSD95 in hippocampus (B) CA1 area, (C) CA2 area, (D) CA3 area and (E) CA4 area of 6-month-old APP/PS1_{DT} mice. The IOD of PSD95 in hippocampus (G) CA1 area, (H) CA2 area, (I) CA3 area and (J) CA4 area of 10-month-old APP/PS1_{DT} mice. Control, WT C57 mice injected with saline; WP, WT mice injected with PNU; APP/PS1, APP/PS1_{DT} mice injected with saline; and AP, APP/PS1_{DT} mice injected with PNU. Compared with the control group, the expression of PSD95 protein in the hippocampus of the APP/PS1_{DT} mice group was significantly decreased and was partially reversed by PNU. This result suggested that activation of $\alpha 7$ nAChR partially reversed the expression level of PSD95 protein in the hippocampus of APP/PS1_{DT} mice. Data are presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. APP/PS1 group.

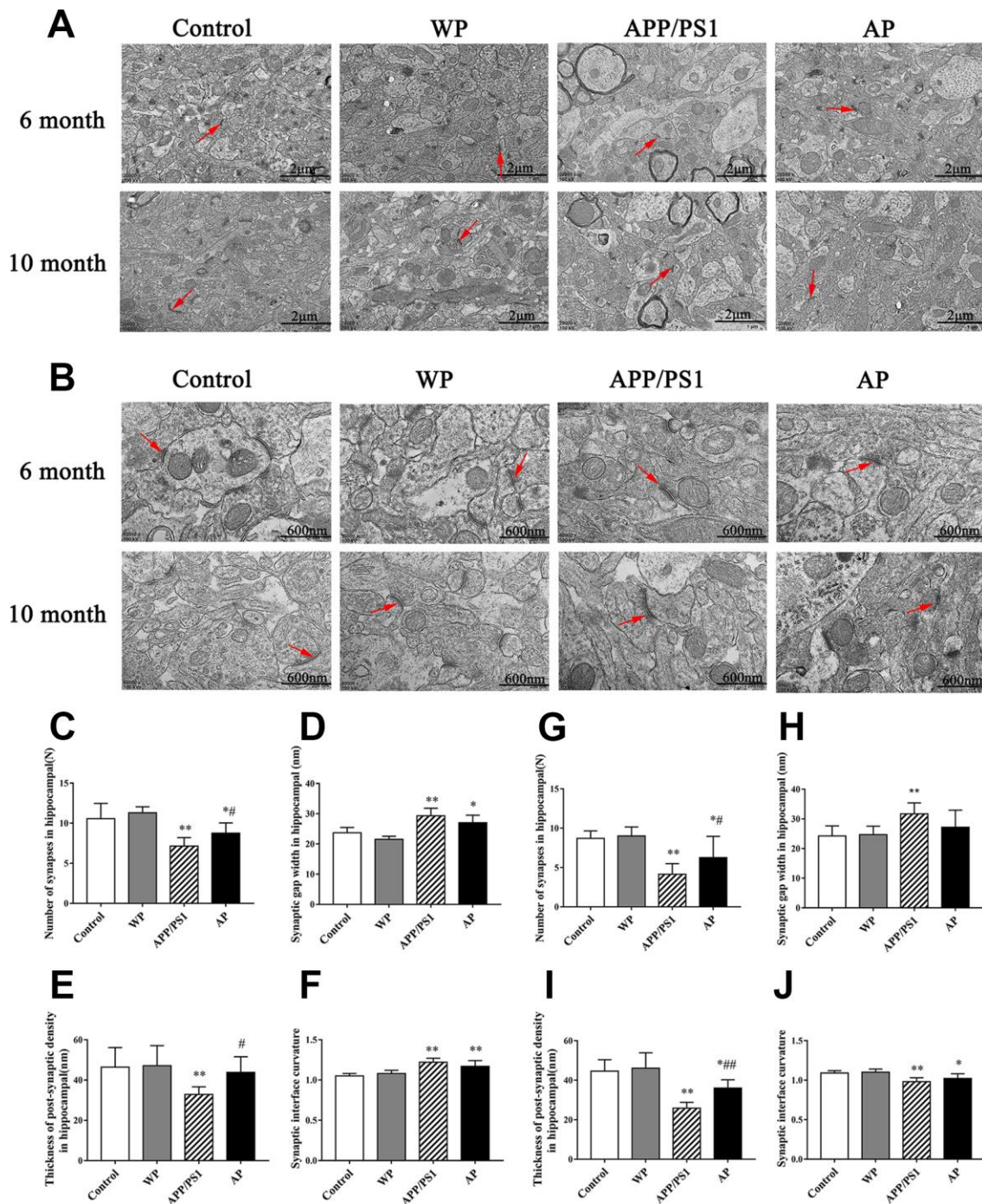


Figure 9. Activation of $\alpha 7$ nAChR increases the number of synapses and maintains the structural integrity of synapses in the hippocampus of APP/PS1_{DT} mice. The synapses were visualized using transmission electron microscopy. Control, the WT mice; WP, the WT treated with PNU; APP/PS1, the APP/PS1_{DT} mice; and AP, the APP/PS1_{DT} mice treated with PNU. Magnification, (A) x20 and (B) x50. The number and characteristics of synapses in (C–F) 6- and (G–J) 10-months-old mice are presented in the figure. Arrows indicate synapses. The results demonstrated that synapses underwent the following changes: (C and G) The number of synapses in both 6- and 10-month-old mice was decreased; (D and H) the synaptic gap became wider; (E and I) the post-synaptic dense substance in the hippocampus of the APP/PS1 group became thinner (vs. the control group) and the post-synaptic dense substance in the AP groups (either 6- or 10-month-old) were thicker than the APP/PS1 group; and (F and J) the curvature of the synaptic interface became smaller in 10-month-old mice. (D, H, F and J) Moreover, there were no significant differences in synaptic gap width and synaptic interface curvature between the APP/PS1 and AP groups. These results indicate that $\alpha 7$ nAChR is beneficial to maintain the integrity of the synaptic structure and increase the number of synapses, thus could improve synaptic functions. Data are presented as the mean \pm standard deviation. *P<0.05, **P<0.01 vs. control group; #P<0.05, ##P<0.01 vs. APP/PS1 group.

(Figure 10I) and p-CREB/CREB (Figure 10J) were decreased in the A β oligomer-treated neurons group. Furthermore, the abundance of all proteins (except CaMKII α) was largely restored following PNU-282987 treatment and the protein level of CaM (Figure 10B) was decreased.

Activation of $\alpha 7$ nAChR activates the CaM-CaMKII-CREB signaling pathway in the hippocampus of APP/PS1_DT mice

The present study further investigated whether $\alpha 7$ nAChR can activate the CaM-CaMKII-CREB signaling

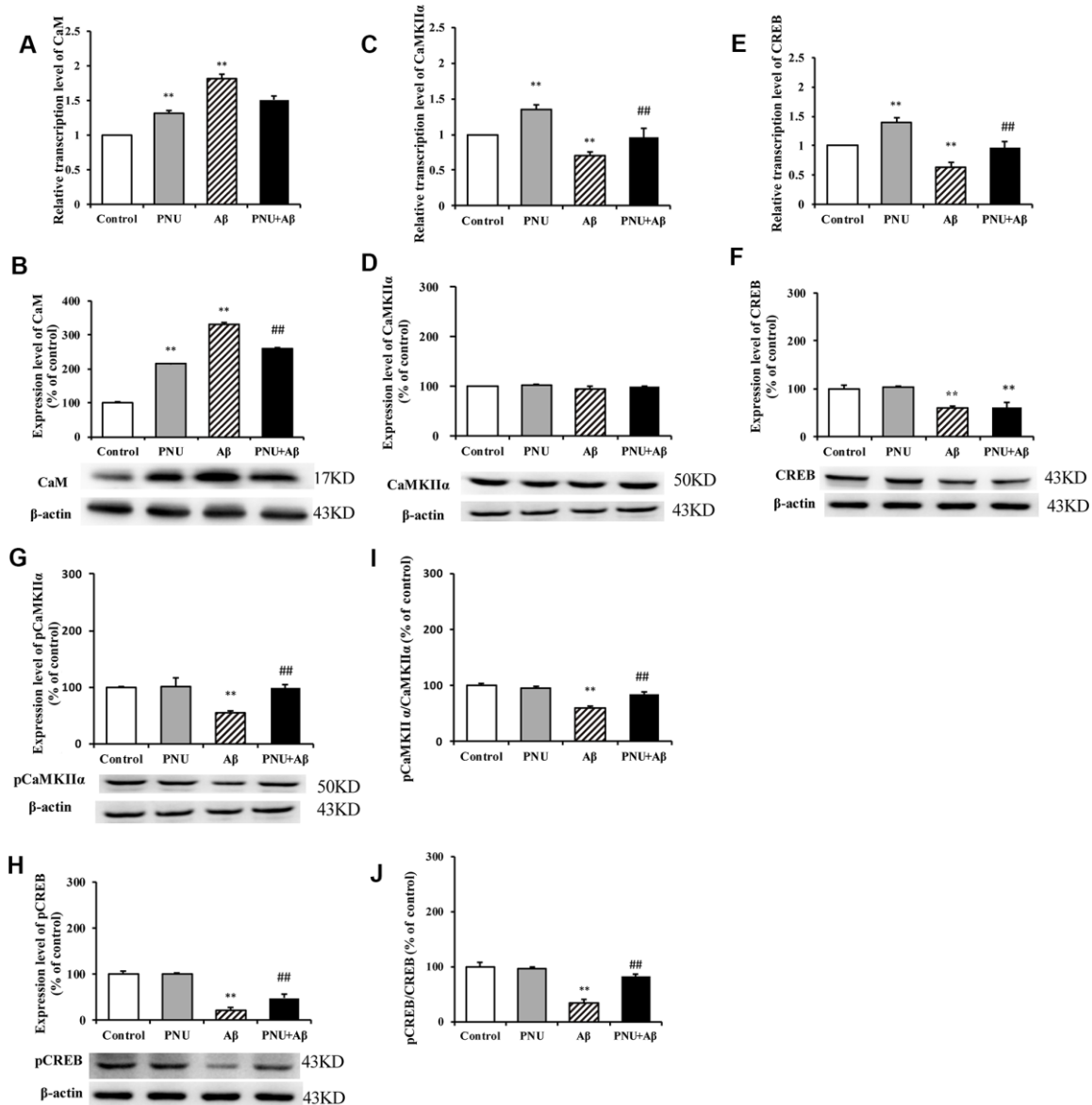


Figure 10. Activation of $\alpha 7$ nAChR activates the CaM-CaMKII-CREB signaling pathway in A β oligomer-treated neurons. The x-axis labels are Control, hippocampus cells from the WT rat; PNU, WT hippocampus cells treated with PNU; A β , the WT hippocampus cells treated with A β ; and PNU+A β , the WT hippocampus cells treated with PNU and A β . The y-axis indicates the relative mRNA or protein levels as a percentage of the control. mRNA and protein expression levels in each group were measured by RT-qPCR and western blot analysis, respectively. Detection of the levels of CaM (A) mRNA and (B) protein, CaMKII α (C) mRNA and (D) protein, CREB (E) mRNA and (F) protein, (G) p-CaMKII α protein and (H) p-CREB protein, and the (I) p-CaMKII α /CaMKII α and (J) p-CREB/CREB ratios. The results demonstrated that the transcription of CaM and the protein level of CaM were significantly increased in the A β group, while the expression level of $\alpha 7$ nAChR was decreased. The transcription level of CaMKII α was significantly decreased, and the expression levels of p-CaMKII α , CREB and p-CREB, and p-CaMKII α /CaMKII α and p-CREB/CREB ratios were significantly decreased in the A β group. All these protein levels were largely restored following activation of $\alpha 7$ nAChR by PNU treatment. Data are presented as the mean \pm standard deviation. *P<0.05, **P<0.01 vs. Control group; #P<0.05, ##P<0.01 vs. A β .

pathway in APP/PS1_DT mice. As shown in Figure 11, compared with the control group, the transcription levels of CaM (Figure 11A), CaMKII α (Figure 11C) and CREB (Figure 11E) were significantly decreased in the hippocampus of APP/PS1_DT mice. The protein expression levels of CaM (Figure 11B), CaMKII α (Figure 11D), CREB (Figure 11F), p-CaMKII α (Figure 11G) and p-CREB (Figure 11H) were also significantly reduced. Moreover, the p-CaMKII α /CaMKII α (Figure 11I) and p-CREB/CREB (Figure 11J) ratios were significantly reduced. The expression levels of CaM, CaMKII and CREB proteins in the hippocampus of APP/PS1_DT mice were significantly increased after PNU treatment (except CREB and CaMKII α /CaMKII α in 6-month mice in the AP group). These results suggested that $\alpha 7$ nAChR activates the CaM-CaMKII-CREB signaling pathway.

Activation of $\alpha 7$ nAChR activates the calcium signaling pathway in KN93 treated neurons

To examine whether $\alpha 7$ nAChR activates the CaM-CaMKII-CREB signaling pathway, the present study measured the CaMKII α protein level after adding KN93, which is a Ca²⁺/calmodulin-dependent protein kinase pathway inhibitor. As shown in Figure 12, compared with the control group, the levels of CaMKII α were decreased in the A β and KN93 groups. Whereas, the expression level of CaMKII α protein was significantly increased after PNU treatment. These results demonstrated that PNU282987 can activate the KN93-inhibited CaM-CaMKII-CREB signaling pathway. This result indicated that the neuroprotective effects of $\alpha 7$ nAChR is CaM-CaMKII-CREB signaling pathway dependent.

DISCUSSION

AD is an intractable progressive neurodegenerative disease characterized by cognitive decline and dementia [20]. With the increase in the average life expectancy worldwide, the incidence of AD has also increased significantly [21]. Over the past few years, the potential therapeutic role of $\alpha 7$ nAChRs has been extensively studied [9, 22–24]. Using an agonist, such as PNU-282987, to modulate the activity of $\alpha 7$ nAChRs is a promising strategy to alleviate cognitive impairment [25]. However, the underlying mechanism of PNU-282987 on cognitive performance, synaptic change and its neuroprotective signaling pathway remains to be fully elucidated [26]. Thus, the aim of the present study was to evaluate the possible therapeutic role and underlying mechanism of PNU-282987 in synapse dysfunction and the neuropathological process of AD using both primary hippocampus cells and APP/PS1_DT mice models.

Following treatment with the $\alpha 7$ nAChR agonist PNU-282987, a specific $\alpha 7$ nAChR agonist with an efficacy of 100%, the deposition of A β was reduced, the brain weights were increased, and the learning and memory abilities were also improved in APP/PS1_DT mice both 6- and 10-months old. The expression levels of synaptic-associated proteins, including SYN, PSD95, SNAP25, DYN1 and AP180, were partially restored compare with the A β treated group. This result was in line with a study by Kroker et al, which found that an $\alpha 7$ nAChR agonist (SSR180711) was able to rescue impaired synaptic plasticity induced by A β infusion in hippocampal CA1 slices [27]. Additionally, transmission electron microscopy demonstrated that activation of $\alpha 7$ nAChR increased the number of synapses in hippocampal neurons of APP/PS1_DT mice, and the synaptic morphology was partially recovered, which could contribute to improving synaptic function and cognitive abilities. Immunohistochemistry and immunofluorescence experiments confirmed that the deposition of A β is negatively correlated with the expression of SYN and PSD95 proteins; therefore, the increase of SYN and PSD95 could decrease A β deposited in neural cells. Notably, the present study also found that the expression level of calcium signaling pathway proteins, including CaMKII, CREB, p-CaMKII and p-CREB, in PNU-282987 treated hippocampal neurons or the hippocampus of APP/PS1_DT mice was increased compared with in A β -treated groups. In summary, it has been demonstrated that activation of $\alpha 7$ nAChR by PNU-282987 could reduce the deposition of A β , alleviate its toxic effects on neural cells, reduce synaptic damage and activate the calcium signaling pathway. To the best of our knowledge, this is the first report of PNU-282987 decreasing the deposition of A β .

Several $\alpha 7$ nAChR agonists, such as GTS-21 [28], Encenicline [29] and Nelonicline [30], have also been considered as therapeutic drugs for AD. However, Encenicline use was halted in a clinic trial by the US Forum in 2016 due to its severe gastrointestinal response during the Phase III clinical phase. A peer-reviewed paper on the results of the monotherapy trial of Nelonicline revealed no significant improvement with any of the ABT-126 doses tested [28, 30]. As a novel agonist of nAChRs, GTS-21 stimulates the release of dopamine in the rat striatum and also enhances the learning ability of monkeys; however, it binds to human $\alpha 4\beta 2$ nAChR 100 times stronger than that of $\alpha 7$ nAChR [28]. Compared with the aforementioned agonists, the main advantage of PNU282987 is that it binds to $\alpha 7$ nAChR specifically and activates $\alpha 7$ nAChR at an efficacy of 100%, thus it has been considered a promising candidate for improving the cognitive function of AD patients.

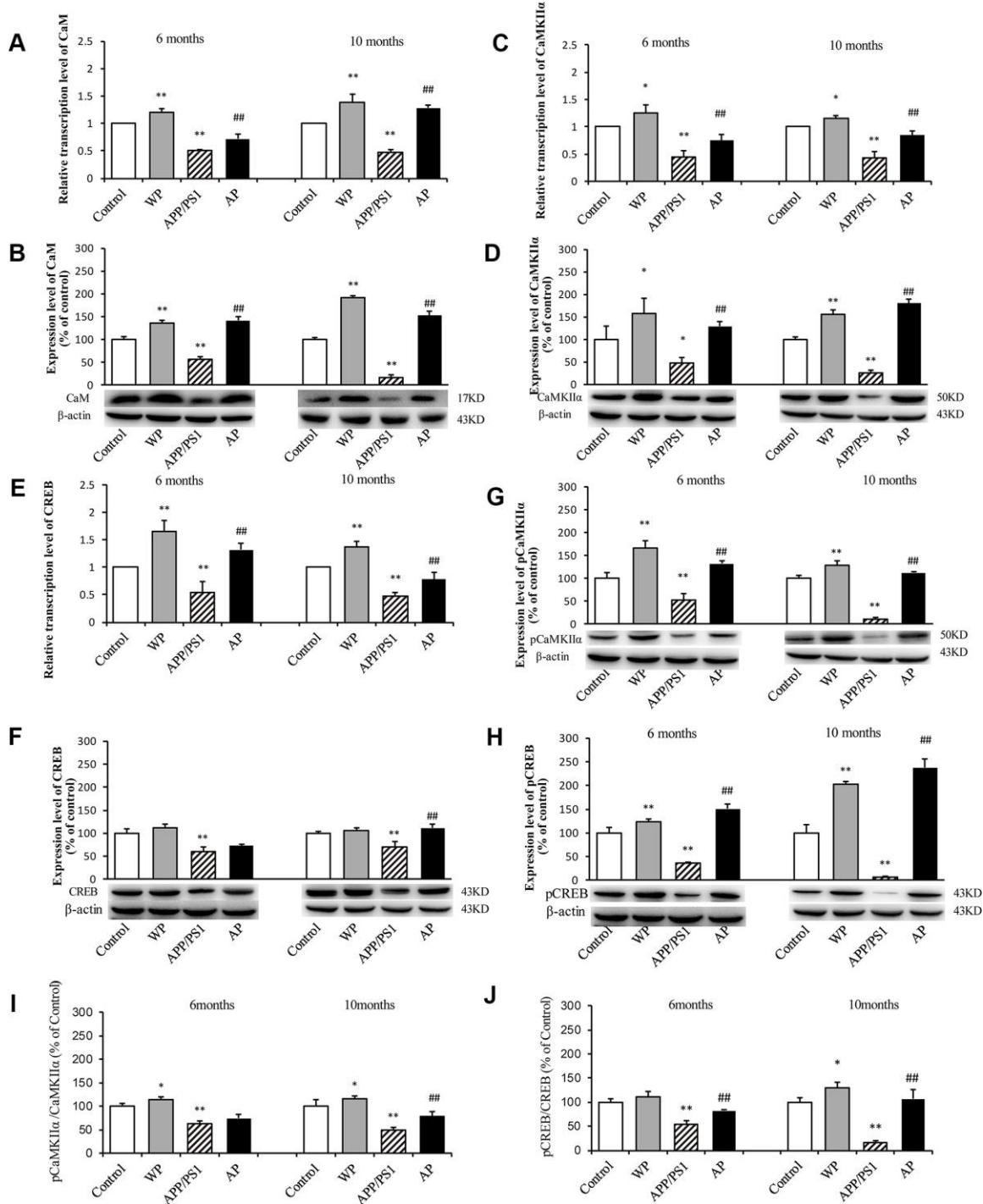


Figure 11. Activation of $\alpha 7$ nAChR activates the CaM-CaMKII-CREB signaling pathway in the hippocampus of APP/PS1_{DT} mice. The x-axis indicates the WT mice (control); WT mice treated with PNU (WP); APP/PS1_{DT} mice (APP/PS1); and APP/PS1_{DT} mice treated with PNU (AP). The y-axes indicate the relative mRNA and protein levels as a percentage of the control. Compared with the control group, the transcription levels of CaM (A), CaMKII α (C) and CREB (E) were significantly decreased in the hippocampus of APP/PS1_{DT} mice. The protein expression levels of CaM (B), CaMKII α (D), CREB (F), p-CaMKII α (G) and p-CREB (H) were also significantly reduced. Moreover, the p-CaMKII α /CaMKII α (I) and p-CREB/CREB (J) ratios were significantly reduced. The protein expression levels of CaM, CaMKII and CREB in the hippocampus of APP/PS1_{DT} mice were significantly increased after PNU treatment (except CREB and CaMKII α /CaMKII α in 6-month mice in the AP group). Data are presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. APP/PS1 group.

A pioneer study has shown that antagonists of $\alpha 7$ nAChR impair the performance of spatial memory in rodents [31]; therefore, numerous agonists of $\alpha 7$ nAChR have been tested for treating cognitive degeneration, and positive effects have been reported [24, 32, 33]. Nicotine activation of neurotransmitter release from presynaptic nAChRs elicits both long-term and short-term potentiating effects on synaptic plasticity [34] and enhances synaptic transmission [35]. Evidence has shown that 5 mg/kg PNU-282987 decreases motor activity in mice and appears to reverse stress effects [26, 36]. Another study has demonstrated that PNU-282987 can be used to improve cognitive symptoms in depression and schizophrenia [7]. The selective activation of $\alpha 7$ nAChRs by agonist has been reported to improve a variety of cognitive functions, including the attention of rodents and non-human primates, spatial learning, working memory and episodic memory [37, 38]. Additionally, activation of $\alpha 7$ nAChRs is considered to promote or prevent the damaging effects of A β on synaptic transmission [39]. Despite the ambiguity of the interaction of A β $\alpha 7$ nAChRs, preclinical evidence suggests that activation of $\alpha 7$ nAChRs restores behavioral defects associated with A β accumulation in animal models [9, 40]. In recent years

it has been found that stimulating $\alpha 7$ nAChRs can promote not only neuronal excitation [41] but also attenuate A β -induced neuronal apoptosis [42]. Therefore, this improves spatial learning and memory abilities of AD patients [40]. The present study provides understanding of how $\alpha 7$ nAChRs exert a neuroprotective effect on an AD model, which may be by reducing the deposition of A β , restoring the expression of synaptic proteins and maintaining the synapse fitness, including the number and morphology.

Changes in Ca²⁺ homeostasis in AD patients are associated with synaptic dysfunction and neuronal decline. In a primary hippocampus cell model, the present study found that the expression levels of CaM-CaMKII-CREB signaling pathway-associated proteins were significantly reduced in neurons treated with A β . Activation of $\alpha 7$ nAChRs by PNU-282587 induced Ca²⁺ influx, which leads to intracellular Ca²⁺ overload and increases the expression of CaM. These results were consistent with a study by Haass et al [43]. In the hippocampus of APP/PS1_DT mice, the CaM-CaMKII-CREB signaling pathway proteins demonstrated a decreasing trend, and this trend became more significant as the age of the mice increased. A previous study has

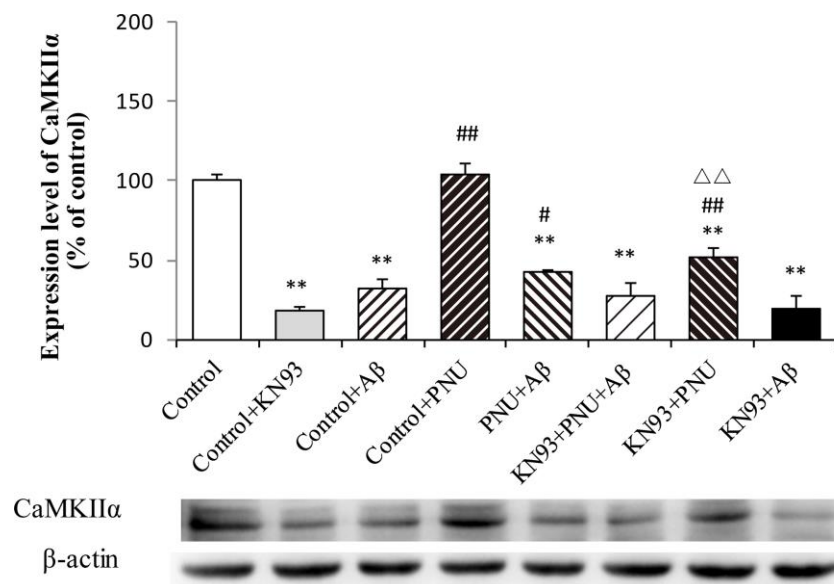


Figure 12. Activation of $\alpha 7$ nAChR activates the calcium signaling pathway in A β oligomer and KN93-treated neurons. Protein levels in each group were determined by western blot analysis and β -actin was used as an internal control. The x-axis indicates the Control, hippocampus cells of the WT rat; Control + KN93, the WT hippocampus cells treated with CaMKII inhibitor (KN93); Control + A β , the WT hippocampus cells treated with A β ; Control + PNU, the WT hippocampus cells treated with PNU; PNU + A β , the WT hippocampus cells treated with PNU and A β ; KN93+PNU+A β , the WT hippocampus cells treated with KN93, PNU and A β ; KN93+PNU, the WT hippocampus cells treated with KN93 and PNU; KN93+A β , the control group treated with KN93 and A β . The Y-axis indicates the protein expression levels as a percentage of the control. The results demonstrated that the protein expression of CaMKII α was significantly decreased in KN93 or A β oligomer-treated neurons, whereas expression of CaMKII α was increased in the PNU groups (PNU+A β , KN93+PNU+A β and KN93+PNU). This result indicated that $\alpha 7$ nAChR activated the CaM-CaMKII-CREB signaling pathway. Data are presented as the mean \pm standard deviation. *P<0.05, **P<0.01 vs. control; #P<0.05, ###P<0.01 vs. Control+A β , $\Delta\Delta$ P<0.01 vs. Control+KN93.

shown that activation of presynaptic $\alpha 7$ nAChRs by nicotine activates CaMKII, which triggers the long-term enhancement of glutamate release [44]. Activation of $\alpha 7$ nAChR by PNU-282987 increased Ca^{2+} influx and CaM expression level. As a result, the level of the Ca^{2+} /CaM complex was increased. This may further activate CaMKII and the downstream CaM-CaMKII-CREB signaling pathway, thereby exerting neuroprotective effects on neural cells and significantly improving learning and memory abilities in APP/PS1_DT mice (Figure 13). In primary hippocampus neurons treated with the $\alpha 7$ nAChR antagonist MLA, certain CaM-CaMKII-CREB signaling pathway-associated protein expression levels were reduced, demonstrating to some extent the effect of PNU282987-specific agonistic $\alpha 7$ nAChR on this signaling pathway. However, changes in the expression of CaM protein were detected to be consistent with changes in agonistic $\alpha 7$ nAChR treatments, which may be related to cell processing *in vitro*.

The present study explored the possible therapeutic role and underlying mechanism of PNU-282987 in synapse dysfunction and the neuropathological process of AD, by using both primary hippocampus cells and APP/PS1_DT mice models. To the best of our knowledge, the present study provides comprehensive and in-depth understanding on the neuroprotective effect of $\alpha 7$ nAChRs and the underlying mechanisms. However, there are a number of limitations of the present study, such as the following: i) the animal and cell models may fail to fully mimic the pathogenesis of AD in human; ii) not all synapse-related and Ca^{2+} signaling-associated proteins were tested in the current study; iii) further studies should be designed to fully elucidate the neural protective effect of $\alpha 7$ nAChRs against the toxicity of $\text{A}\beta$; and iv) we didn't monitor the status of the $\alpha 7$ nAChRs (activation or desensitization) in our experiment, however, the results suggested that low probability of desensitization and predominant activation may contribute the effect of PNU-282987 on $\alpha 7$ nAChRs.

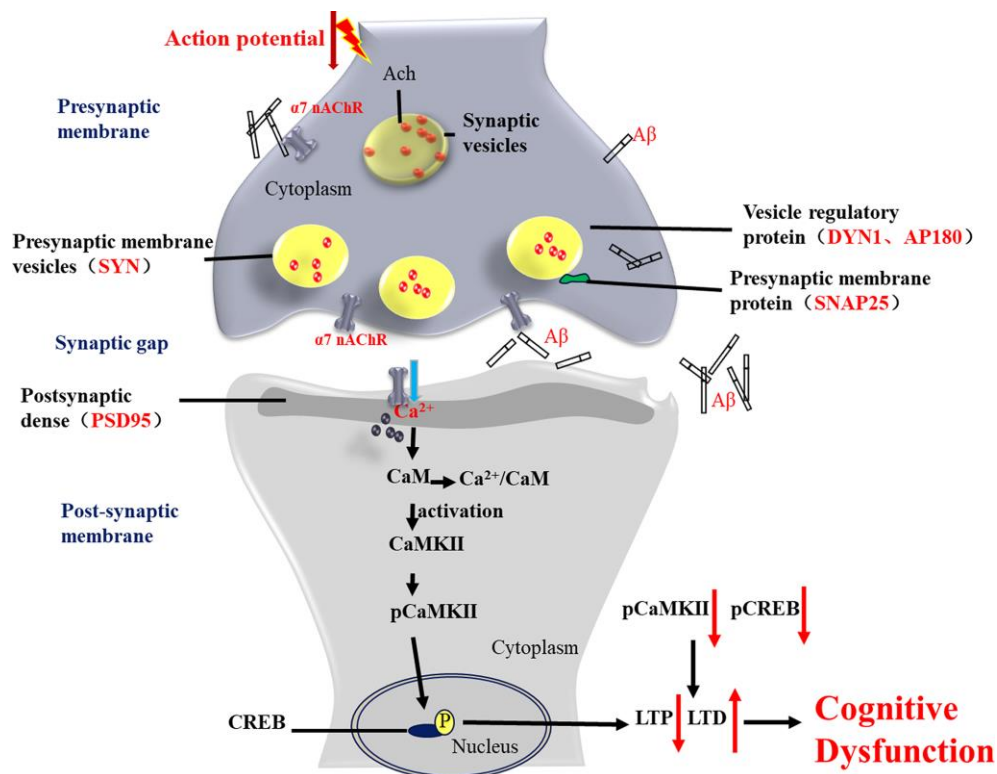


Figure 13. Activation of $\alpha 7$ nAChR by PNU-282987 improves synaptic and cognitive functions through restoring the expression of synaptic-associated proteins and the CaM-CaMKII-CREB signaling pathway. Synapses are the basic structure for the connection between neurons, and the function of a synapse is largely associated with synaptic-associated proteins. The present study found that activation of $\alpha 7$ nAChRs by PNU-282987 could largely restore the expression level of synaptic-associated proteins, including SYN, PSD95, SNAP25, DYN1 and AP180, which are downregulated by deposition of $\text{A}\beta$. $\alpha 7$ nAChRs belong to the ligand-gated ion channel coupled receptors, and form an ion channel in the center, which mainly regulates the flow of Ca^{2+} plasma inside and outside the cell. As a second cytoplasmic messenger, Ca^{2+} can flow into the cells through $\alpha 7$ nAChRs and bind to CaM to form an active CaM complex. The CaM complex can bind to CaMKII, which changes the CaMKII spatial conformation and phosphorylates CaMKII protein. Phosphorylated CaMKII enters the nucleus to catalyze CREB phosphorylation. CREB is located downstream of the $\alpha 7$ nAChR signal transduction pathway and acts as a transcription factor that regulates the growth and development of neurons and the formation of long-term memory.

In summary, the present study used an $\alpha 7$ nAChRs specific agonist PNU-282987 to active $\alpha 7$ nAChRs in both *in vitro* and *in vivo* models, and then analyzed its role in synapse morphology and functionality, the Ca^{2+} signaling pathway and learning-memory abilities. The results indicated that activation of $\alpha 7$ nAChRs could reduce $\text{A}\beta$ deposition in the hippocampus and protect neuron cells against $\text{A}\beta$ toxicity. The protective mechanism of $\alpha 7$ nAChRs was proposed as follows: i) a decrease of the deposition of $\text{A}\beta$; ii) an increases expression of synaptic-associated proteins; and iii) maintenance of Ca^{2+} homeostasis by activation of the CaM-CaMKII-CREB signaling pathway. The present study may provide understanding on the underlying pathogenesis of AD.

MATERIALS AND METHODS

Reagents and antibodies

The following antibodies were used in the present study: Rabbit anti-SYN polyclonal antibody (cat. no. ab32594), rabbit anti-AP180 polyclonal antibody (cat. no. ab33898) and rabbit anti-p-CaMKII α (Thr286) polyclonal antibody (cat. no. Ab5683) were purchased from Abcam (Cambridge, MA, USA). Rabbit anti-p-CREB (Ser133) monoclonal antibody (cat. no. 9198), horseradish peroxidase (HRP)-labeled anti-mouse secondary antibody (cat. no. 7076) and HRP-labeled anti-rabbit secondary antibody (cat. no. 7074) were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). Rabbit anti-PSD95 polyclonal antibody (cat. no. GTX133091), rabbit anti-SNAP25 polyclonal antibody (cat. no. GTX113839), rabbit anti-DYN1 polyclonal antibody (cat. no. GTX110379), rabbit anti-CREB monoclonal antibody (cat. no. GTX61140) and rabbit anti- β -actin polyclonal antibody (cat. no. GTX109639) were purchased from GeneTex, Inc. (Irvine, CA, USA). Mouse anti-CaM monoclonal antibody (cat. no. sc-137079) and mouse anti-CaMKII α monoclonal antibody (cat. no. sc-13141) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). GFAP (cat. no. ab7260) and NeuN3018822 were from Merck KGaA (Darmstadt, Germany).

Other reagents used in the present study were as follows: B-27 Additive, GlutaMAX Additive, NuPAGE LDS Sample Buffer (4X), NuPAGE Sample Reducing Agent (10X), NuPAGE Novex 4-12% Bis-Tris Precast, MES SDS Electrophoresis, Alexa FluorTM488 goat anti-rabbit IgG antibody and Cy3[®] goat anti-mouse IgG were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Hibernate-E Medium, DMEM high glucose medium and Neurobasal-A medium were purchased from Thermo Fisher Scientific, Inc. The ABC immunohistochemistry kit was purchased from Vectastain, USA. The primers for amplification of SYN,

PSD-95, SNAP25, AP180, DYN1, CaM, CaMKII, CREB and β -actin mRNA were synthesized by Shanghai Genecore Bio Technologies, Shanghai, China. $\text{A}\beta_{1-42}$, PNU-282987, HFIP (1,1,1,3,3,3-hexafluoro-2-propanol), DMSO and the other chemicals used were purchased from Sigma-Aldrich; Merck KGaA. MLA was purchased from Tocris Bioscience (Bristol, UK).

$\text{A}\beta$ oligomers preparation

$\text{A}\beta$ was prepared according to a previously reported method [45]. Synthetic human $\text{A}\beta_{1-42}$ was suspended in pre-cooled HFIP at a final concentration of 1 mM and then incubated at room temperature for 60 min, followed by incubation on ice for 10 min. Aliquots of $\text{A}\beta$ solution were transferred into non-siliconized micro-centrifuge tubes, HFIP was allowed to evaporate overnight in a hood at room temperature, and the tubes were then stored at -80°C . Prior to treatment of the cells, the $\text{A}\beta_{1-42}$ was dissolved in DMSO to obtain a final concentration of 5 mmol/l. For the preparation of oligomers, this solution was diluted with modified DMEM and then incubated for 24 h or at 4°C . $\text{A}\beta$ oligomers were identified by western blot analysis.

Primary neuron culture and treatment

Sprague-Dawley rats (within 24 hours of birth) were provided by the experimental animal center of Guizhou Medical University. The entire brain was immersed in pre-cooled D-Hank's balanced salt solution. The hippocampus was isolated, and the meninges and blood vessels were removed. Subsequently, it was cut into pieces with eye scissors and digested with 0.25% trypsin at 37°C for 15 min. The trypsin solution was then discarded and DMEM containing 10% FBS was added to terminate digestion. Following two washes with Hank's buffered saline solution, the digestion was resuspended in 2 ml Neurobasal/B27 complete medium (Neurobasal A medium with 2% B27, 1% GlutaMAX supplement, 100 U/ml penicillin and 100 mg/ml streptomycin) and broken apart with Calibre Pasteur pipettes. Following removal of the supernatant, neurobasal medium supplemented with 2% B27 was added and gently pipetted 20 times. The supernatant cell suspension was gently pipetted, the cells were counted by trypan blue staining and cells were seeded on PLL-coated 6-well plates at a density of $\sim 1 \times 10^6$ cells/ml. The cells were then incubated in an incubator at 37°C with 5% CO_2 and a saturated humidity, and half of the medium was replaced every 3 days. The immunofluorescence staining of glial fibrillary acidic protein (GFAP) and Hexaribonucleotide Binding Protein-3 (NeuN) was used to identify hippocampal neurons [46].

The primary neurons isolated were determined by double immunofluorescence staining with mouse anti-NeuN

antibody (diluted 1:200), anti-mouse IgG labelled with CY-3 (red), rabbit anti-GFAP antibody (diluted 1:300) and anti-rabbit IgG labelled with FITC (green). After 10 days of incubation, the medium was replaced with neurobasal medium without the B27, and the neurons then were treated with A β oligomers (0.5 μ mol/l) and/or PNU-282987 (10 μ mol/l) and/or KN-93 (1 μ mol/l) for 24 h. Cells were then harvested for subsequent experiments.

Reproduction and identification of APP / PS1 double transgenic mice

Male and female APP/PS1_{DT} mice (B6. Cg-Tg) (weight, 20-30 g) were purchased from Shanghai Southern Model Biology Co., Ltd. (animal license no. SCXK 2014-0002). The genotypes of these mice were further confirmed by PCR to confirm the presence of the APP_{swe}/PS1_{E9} gene mutations. Verification primers designed for APP and PS1 are listed in Supplementary Table 1. The expected lengths of the PCR products were 400 and 600 bp, respectively. APP/PS1 double transgenic mice were denoted APP/PS1_{DT} mice thereafter.

Different drugs/operations were applied to APP/PS1_{DT} mice to set up the following groups: i) WP group, wild-type C57 mice treated with PNU-282987; ii) AP group, APP/PS1_{DT} mice treated with PNU that were given daily intraperitoneal injections of 1 mg/kg PNU-282987 at the age of 6- and 10-months old for 5 days [26]; iii) APP/PS1 group, APP/PS1_{DT} mice injected intraperitoneally with the same amount of normal saline for 5 days; and iv) control group, wild type C57 mice injected intraperitoneally with the same amount of normal saline for 5 days. All operations on mice were approved by the Experimental Animal Ethics Committee of Guizhou Medical University (approval no. 1702153).

Hippocampal tissue preparation

After opening the head skeleton and careful removal of adjacent, non-neural tissue, isolated hippocampal tissue was collected into a container and cooled on ice. Subsequently, 1 mm³ hippocampus was transferred into 4% glutaraldehyde solution for transmission electron microscope analysis. Brains were transferred into 4% paraformaldehyde in PBS (pH 7.4) for histological analysis, into ice-cold protein extraction buffer for western blot analysis or into TRIzol reagent for RT-qPCR analysis.

Flow cytometry

Primary neuron cells were treated with A β , PNU-282987 (A β oligomers, 0.5 μ mol/l; PNU282987, 10 μ mol/l; KN93, 1 μ mol/l) and MLA (100nmol/l) for 24 h. The cells were then washed three times with PBS and digested with trypsin for 3 mins. Digested cells were collected by

centrifugation and suspended in binding buffer (BD Biosciences; FITC Annexin V Apoptosis Detection Kit I; cat. no. 556547) to a concentration of 1x10⁶ cells/ml. Annexin V-FITC dye and PI solutions were added to the cells and mixed gently, and then incubated for 15 min at 2-8°C in the dark. The treated cells were then immediately analyzed by flow cytometry.

Morris water maze test

The Morris Water Maze test is widely used to study spatial learning and memory [47]. Mice in the different groups were subjected to four trials a day for total of 4 days, with a 5-7 min rest interval between trials. The movement of the mice was monitored with video track software (View Point, DNS-2). In each trial, the duration of a mouse to escape a platform was determined (escape latency). The mouse was allowed to sit on the platform for 5 sec after successfully locating it. Mice that failed to find the platform within 60 sec were manually guided to the platform, then allowed to settle on it for 20 sec, and the escape latency was recorded as 60 sec. The test was performed for 4 consecutive days. On the fifth day, the central platform was removed and the number of mice passing through the platform in 60 sec was recorded. Furthermore, the mice were subjected to a 60 sec probe trial, in which they were allowed to search for the location of the platform, and the time spent in each quadrant of the maze was recorded (quadrant search time). All these behavioral tests were conducted in a quiet environment with subdued lighting.

Synapse morphology changes observed by transmission electron microscope

Brain tissue was fixed by 4% glutaraldehyde solution for >2 h and washed with 0.1 M PBS three times. Then samples were fixed by 1% osmium tetroxide for 2 h. Subsequently, the samples were dehydrated by gradient ethanol and acetone. Finally, the specimens were impregnated, embedded and polymerized by epoxy resin. Slices of 0.5 μ m and 60 nm were prepared, and then doubled dyed with uranium acetate and lead citrate. A H-7500 electron microscope (HITACHI) was used to observe the changes of the synapse morphology in hippocampal neurons. The number of synapses, the width of the synaptic cleft, the thickness of synapse and the curvature of the synaptic interface were analyzed using an image analysis system (Image-pro plus 6.0).

Analysis of the expression of synaptic-associated proteins and CaM-CaMKII-CREB signaling pathway proteins by RT-qPCR

The expression levels of synaptic-associated proteins (SYN, PSD95, SNAP25, DYN1 and AP180) and CaM-

CaMKII-CREB signaling pathway proteins were determined by RT-qPCR, as described previously [48]. In brief, 1 µg total RNA extracted from the primary hippocampal cells was used as the template to reverse transcribe RNA to cDNA. RT-qPCR was performed in a 96-well with the ABI Step One Plus System and analyzed with SDS 1.4 (Applied Biosystems, Step One Plus Real-Time PCR system). These reactions were carried out with the universal TaqMan 2xPCR SYBR green mastermix in a 25-µl volume, which contained primers for analyzing the expression of synaptic-associated proteins. The relative transcript levels were calculated by the $2^{-\Delta\Delta C_q}$ method [49].

Analysis of the protein levels of synaptic-associated proteins and CaM-CaMKII-CREB signaling pathway proteins by western blot analysis

The levels of the synaptic-associated proteins (SYN, PSD95, SNAP25, DYN1 and AP180) and CaM-CaMKII-CREB signaling proteins in tissues/cell lysates were quantified by western blot analysis as described previously [26]. In brief, total protein concentration was measured with the Coomassie brilliant blue protein assay. The proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Following blocking with 5% fat-free milk for 2 h, the membranes were incubated with primary antibodies against synaptic-associated proteins and CaM-CaMKII-CREB signaling proteins. The membranes were then washed and incubated with secondary antibody conjugated with HRP for 60 min. Finally, the proteins were visualized with the ECL chemiluminescence detection system (Syngene, GeneGnome XRQ NPC). Subsequently, the antibodies were washed off the membranes and the membranes were incubated with antibodies against β -actin (1:5,000) for 120 min and then with HRP-conjugated anti-mouse IgG for 60 min. The expression of the target proteins was calculated relative to the expression level of β -actin, which used as an internal control.

A β 1-42 and PSD95 expression analysis by microscopy and immunohistochemistry

Brain tissues were fixed by 4% paraformaldehyde in PBS for 24 h. In brief, 4% PFA-fixed and paraffin-embedded brain tissues were cut into 4-µm slices. The slices were then deparaffinized, rehydrated and incubated for 30 min in 3% H₂O₂. Subsequently, slices were unmasked using citrate buffer and then blocked with 5% normal goat serum for 30 min. Followed by incubation overnight with A β ₁₋₄₂ (1:100) and PSD95 (1:100) primary antibodies in a moist chamber at 4°C. For the detection of monoclonal antibodies, peroxidase-conjugated anti-mouse ABC kit (Vector, vectastain

ABC KIT) was used. Negative control slides were incubated non-immune serum to replace the primary antibodies. The analysis was performed under a Nikon microscope with NIS-Elements AR software (Nikon, Model Eclipse Ci-E).

Immunofluorescence detection of the relationship between the expression of SYN and A β 1-42 in brain tissue

The right side of the brain was taken (including the hippocampus), fixed with 4% paraformaldehyde for >24 h, dehydrated, paraffin embedded and sectioned. The section was then dewaxed, citrate repaired, blocked with sheep serum working solution, and incubated with the corresponding primary antibody at 4°C overnight. After the primary antibody was discarded, the diluted secondary antibody (Cy3 labeled anti-mouse IgG and 488-labeled anti-rabbit IgG) was added dropwise, and the plate was sealed with DAPI after 1 h incubation at room temperature. The images were observed and collected using a fluorescence inverted microscope, and at least two fields of views were randomly selected for each group to be photographed 200 times. The background light of each photo was kept consistent. Image-Pro Plus 6.0 software was used to analyze the green and red fluorescence of each photo to obtain the integrated optical density (IOD) of each photo.

Statistical analysis

All experiments were conducted three times and data are presented as the mean \pm standard deviation (SD). The groups were compared using a two-tail Student's t-test and one-way ANOVA using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Abbreviations

AD: Alzheimer's disease; A β : β -amyloid peptide; APP/PS1_DT: amyloid precursor protein/Presenilin 1 double-transgenic mice; AP180: adaptor protein of 180 kDa; BCA: background-corrected absorbance; CaM: Calmodulin; CaMKII: calmodulin-binding protein kinase II; CREB: cAMP responsive element-binding protein; CA1: Cornu ammonis 1; DG: dentate gyrus; DEPC: diethylpyrocarbonate; DYN1: dynamin 1; DAPI: 4,6-diamidino-2-phenylindole; DMSO: dimethyl sulfoxide; ECL: enhanced chemiluminescence; GFAP: Glial fibrillary acidic protein; HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol; IOD: integral optical density; LTP: long-term potentiation; mRNA: messenger ribonucleic acid; MLA: methyllycaconitine; NFTs: neurofibrillary tangles; nAChR: neuronal nicotinic acetylcholine receptor; IOD:

integral optical density; PBS; phosphate buffered solution; PSD95: post synaptic density of 95 kDa; PMSF: phenylmethanesulfonyl fluoride; RT-qPCR, reverse transcription quantitative-polymerase chain reaction; SNAP-25: synaptosomal-associated protein of 25; SYN: synaptophysin; SP: senile plaques; TEM: transmission electron microscopy.

AUTHOR CONTRIBUTIONS

QXL, WH and ZZG conceived and designed the research. XLW, YXD, YMG, YTD and FW performed the experiments. WH, XLQ and XLW wrote the manuscript. All authors have read and approved the final manuscript.

CONFLICTS OF INTEREST

All authors declare that they have no competing interests. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIALS

Supplementary Methods

Identification of APP/PS1_DT mice

To determine whether desired gene mutations occurred at APP^{swe}/PS1^{dE9} loci in the mouse model. Diagnostic PCR was used to identify the offspring of mice (primers were listed in Supplementary Table 1). Offspring produced both 400-bp and 600-bp amplicons were desired double-transgenic mutants for APP and PS1 genes. These mice denoted APP/PS1_DT were further used (Supplementary Figure 1). Previous research has found that the expression of $\alpha 7$ nAChR gene was not affected by APP^{swe}/PS1^{dE9} gene mutations in APP/PS1_DT mice [1].

Preparation of A β oligomers

A β protein has several different forms monomers, oligomers and fibrous bodies. It is widely recognized that soluble A β oligomers in the early stage of A β aggregation are the most neurotoxic, and play a key role in the pathogenesis of AD. To prepare A β oligomer from a commercially available monomer, we used synthetic human A β ₁₋₄₂ in 1, 1, 1, 3, 3, 3- hexafluoro-2-propanol (HFIP). Western blot analysis showed that the

prepared A β oligomers were mix of A β monomers (4KD, 18%), dimers (8KD, 2%), trimers (12 KD, 74%) and tetramers (16KD, 6%) (Supplementary Figure 2).

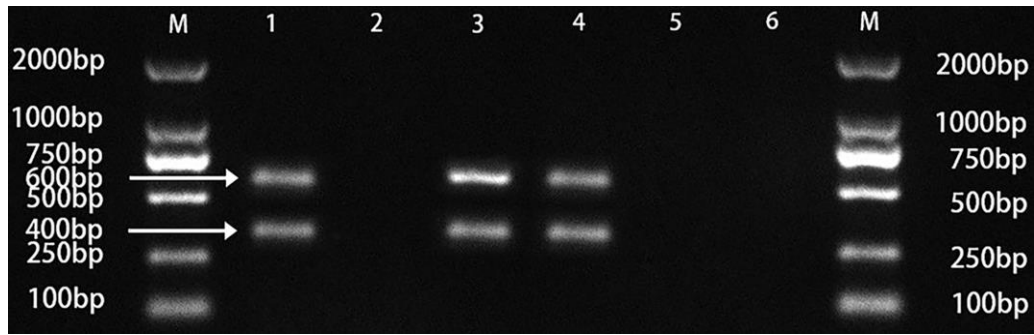
The purity of hippocampal neurons in primary bred rats

The in vitro isolated primary hippocampal neurons (NeuN) is an important model for studying the development and morphological function of neurons. We used immunofluorescence to identify the purity of hippocampal neurons (NeuN and GFAP antibody). The result showed that 81% of neurons cells were NeuN positive and GFAP positive (Supplementary Figure 3). This result indicated that the high purity in vitro neurons cell model was obtained.

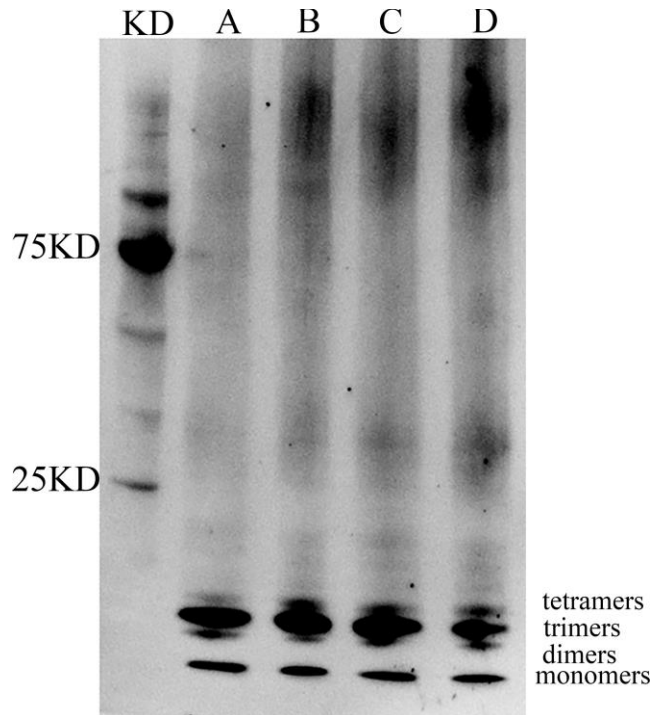
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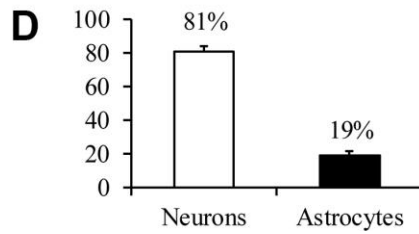
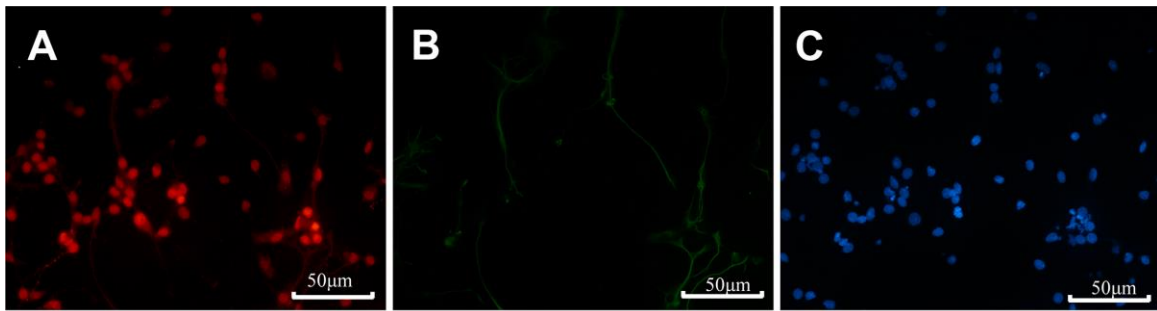
Supplementary Figures



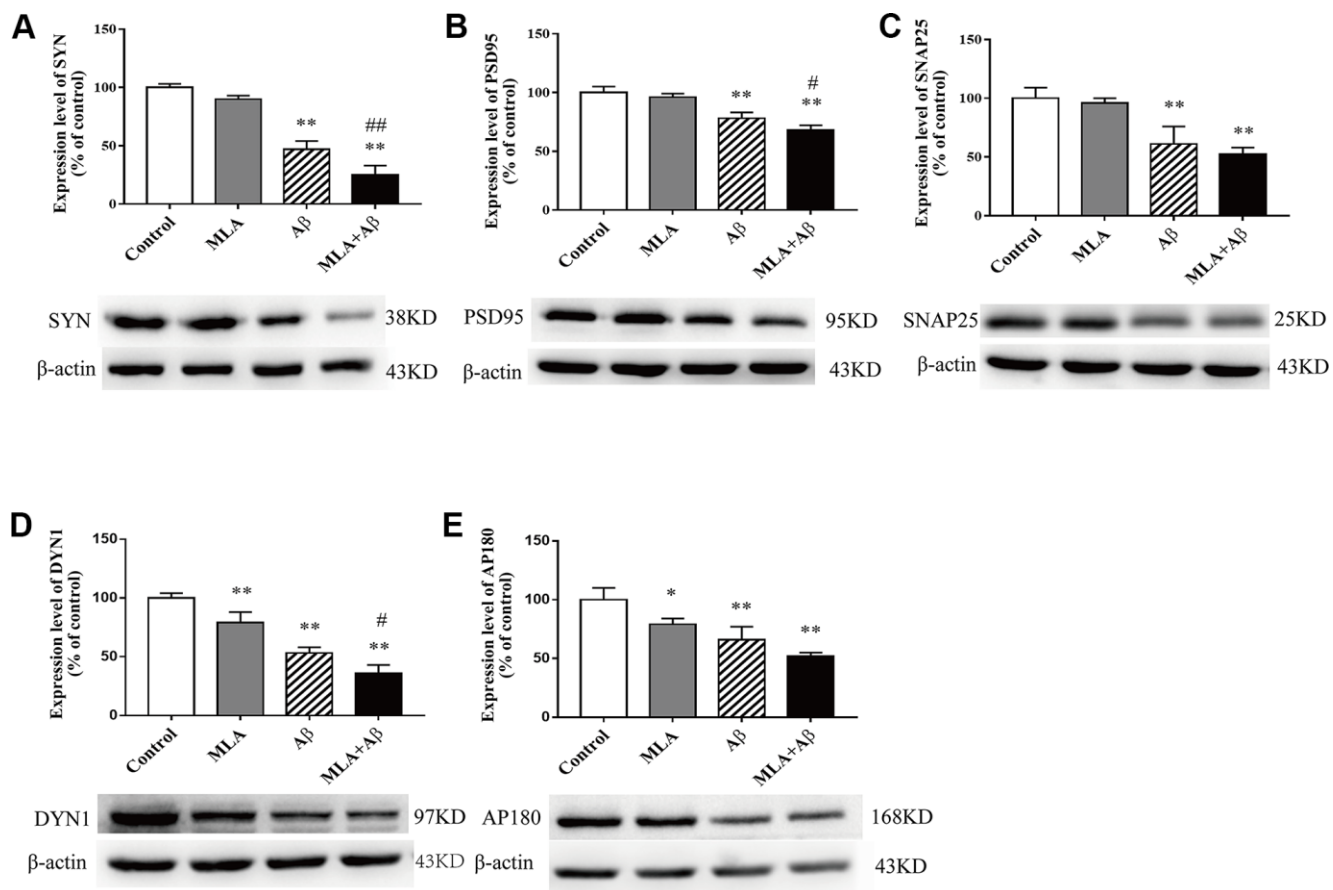
Supplementary Figure 1. Identification of APP/PS1_{DT} mice. Genomic DNA of all offspring mice were extracted from its tail and were used as PCR templates. Primers flank APP and PS1 loci were used to identify APP/PS1_{DT} mice form 1st generation bred offspring. Lane 1 – 6, PCRs using different genomic DNA as template, double-transgenic mice produce two distinct bands of 400 bp and 600 bp, while Control mice produces no band. Lane 1, 3, 4 were APP/PS1_{DT} mice, and lane 2, 5, and 6 were wild-type mice (note that only APP/PS1_{DT} mice will be subjected to further experiments). Lane M, DL2000 DNA marker (Takara, China).



Supplementary Figure 2. Identify Aβ oligomers by western blot analysis. The first lane is protein weight marker, and lanes A, B, C, and D were Aβ samples. The monomer, dimer, trimer, and tetramer of Aβ were indicated at the right bottom. The results showed that we successfully produce a mixture of Aβ monomer (4KD, 18%), dimers (8KD, 2%), trimers (12 KD, 74%), tetramers (16KD, 6%).



Supplementary Figure 3. Purity of primary hippocampal neurons in the APP/PS1 mice. Neurons (A, red); astrocytes (B, green); nucleus of neuron cells (C, blue) and the composition of neuron and astrocyte (D). As determined by immunofluorescent, the primary neurons isolated from 1st generation bred mice were approximately 81% of purity. Double staining of neurons and astrocytes were performed with mouse anti-NeuN and anti-mouse IgG labeled with CY-3 (neurons, red), and with rabbit anti-GFAP and anti-rabbit IgG labeled with FITC (astrocytes, green). The result indicated that the high purity of in vitro neurons cell model was obtained.



Supplementary Figure 4. Antiagitation of $\alpha 7$ nAChR decreases the expression of synaptic-associated proteins in A β -treated neurons. The X-axis labels are the neurons isolated from the WT rat (Control); the WT neuron cell treated with MLA (MLA); the WT neuron cell treated with A β (A β); and the WT neuron cell treated with MLA and A β (MLA+A β). The Y-axis indicates relative level of proteins (% of control). Detection of SYN protein (A); PSD95 protein (B); SNAP25 protein (C); DYN1 protein (D); AP180 protein (E); expression level in each group were measured western blot analysis (β -actin was used as an internal control). The results showed that the expression of SYN, PSD95, SNAP25, DYN1 and AP180 protein was significantly decrease in A β oligomers treated neurons, and this decreasing was enhanced by MLA treatment. Data are presented as the means \pm SD. $P < 0.05$, ** $P < 0.01$ vs. Control group; # $P < 0.05$, ## $P < 0.01$ vs. A β .

Supplementary Table

Supplementary Table 1. Primers sequences used for identification of APP/PS1_DT mice.

Gene	Primer sequence (5'-3')
APP	Upstream: GACTGACCACTCGACCAGGTTCTG Downstream: CTTGTAAGTTGGATTCTCATATCCG
PS1	Upstream: AATAGAGAACGGCAGGAGCA Downstream: GCCATGAGGGCACTAATCAT