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Food Science and Human Wellness

journal homepage: <https://www.sciopen.com/journal/2097-0765>

Docosahexaenoic acid improves synaptic structure and function in APP/PS1 mice through the TrkB-Erk1/2-CREB pathway

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ARTICLE INFO

Article history:

Received 12 September 2024

Received in revised form 6 December 2024

Accepted 6 January 2025

Keywords:

Docosahexaenoic acid

Alzheimer's disease

Amyloid-beta

Synapse

ABSTRACT

Alzheimer's disease (AD) is a serious disease associated with cognitive impairment, and synaptic loss and amyloid-beta (A β) deposition are closely related to its pathogenesis. Docosahexaenoic acid (DHA) has been reported to alter the cognitive impairment associated with aging and reduce the risk of long-term development of AD. However, the effects of A β on synapses and whether DHA has a protective effect on synapses remain unclear. In this study, APPSwe/PSEN1dE9 transgenic and wild type mice were used as experimental subjects to explore the effect of DHA on synaptic structure and function damaged by A β . Results showed that a large amount of A β was deposited in the brain of AD mice, and DHA intervention decreased the A β deposition ($P < 0.05$). DHA decreased the apoptosis of nerve cells and the oxidative stress level induced by A β ($P < 0.01$). Transmission electron microscopy results showed that DHA improved the synaptic structure and number, and the expression of synaptophysin was significantly upregulated by DHA ($P < 0.01$). Besides, neurotransmitter release was regulated after DHA intervention, including the decreased Glu level and increased γ -aminobutyric acid level, as well as the activity of ATPase was also increased by DHA. Importantly, the phosphorylation levels of tyrosine kinase B (TrkB), phospholipase C- γ -1, calcium/calmodulin-dependent protein kinase II, extracellular regulated protein kinases (ERK1/2), and cAMP-response element binding protein (CREB) were upregulated by DHA ($P < 0.01$). These data indicated that DHA could improve synaptic structure and function through the TrkB-Erk1/2-CREB pathway, thus playing a positive role in the pathological process of AD.

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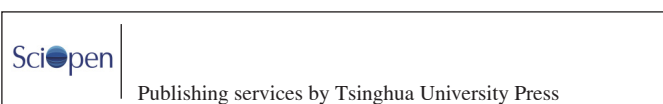
1. Introduction

Alzheimer's disease (AD) is a serious neurodegenerative disease that often occurs in the elderly over the age of 65 and imposes a heavy burden on patients. The main symptoms of AD include impairments in physical, cognitive, and emotional functions, as well as changes in cognitive and sensory functions due to long-term apraxia^[1]. There are many pathogenic

factors of AD, including oxidative stress, amyloid-beta (A β) deposition, synaptic loss, genetics, inflammatory response, neurofibrillary tangles, mitochondrial dysfunction and hormone imbalance^[2]. At present, some drugs for anti-A β , anti-tau, anti-inflammatory, etc. have been developed, but most are still in different stages of preclinical or clinical development^[3]. And the currently available treatments only relieve symptoms and delay disease progression, but do not modify the disease mechanism. Indeed, there is an urgent need to develop more effective treatments for this disease, especially positive interventions that occur early or even before the disease develops.

Studies point out that A β begins to accumulate in the brain 15–20 years before the onset of clinical symptoms in AD^[4]. And the presynaptic accumulation of soluble A β is the most likely cause of synaptic

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Peer review under responsibility of Beijing Academy of Food Sciences.



dysfunction in patients with AD, and mediates loss of dendritic spines and excitatory synapses^[5-6]. Wang et al.^[7] showed that A β promoted the activation of mammalian Ste20-like kinase1 (MST1), thereby mediating synaptic loss and neuronal apoptosis during the development of AD. Synapses are the connection points between the structure and function of neurons, and key parts of neural information transmission. There is evidence that synaptic dysfunction occurs in some brain areas prior to neuron loss, and is the pathological factor most closely related to cognitive decline in AD^[8]. Brain-derived neurotrophic factor (BDNF) and its receptor tyrosine kinase B (TrkB) are one of the most important regulators of synaptic plasticity and neuronal survival in the brain. However, decades of efforts to develop a drug that targets BDNF have failed. Wang et al.^[9] developed a TrkB agonist to enhance synaptic function and cell survival by activating TrkB and its downstream signals, thereby saving spatial cognition in AD mice. Therefore, TrkB and its downstream signaling pathways may be the best targets for “synaptic repair and neuroprotection” strategies, significantly promoting synaptic transmission, plasticity, growth, and enhancing neuronal survival.

As the main component of cell membranes, docosahexaenoic acid (DHA) can readily cross the blood-brain barrier (BBB) to exert its biological effects. DHA is important for cognitive development, learning ability, neuromembrane plasticity, and synaptogenesis, all of which are involved in synaptic transmission and normal brain functions^[10]. Some epidemiological studies have shown that DHA supplementation may alter the cognitive impairment associated with aging and the risk of long-term development of AD^[11-12]. Studies by Zhang et al.^[13] and Wang et al.^[14] showed that DHA alleviates AD symptoms primarily by regulating A β production, oxidative or nitrifying stress, neurotrophic factors, neuroinflammation, and apoptosis. In our previous study, we have demonstrated that DHA intake could reduce neuronal damage and improve cognitive dysfunction by reducing A β production and aggregation in APPSwe/PSEN1dE9 transgenic (APP/PS1) mice^[15]. However, the relationship between A β and synaptic function and structure, as well as the role and mechanism of DHA on synapses, still need to be further explored.

APP/PS1 mice are constructed by crossing APPSwe transgenic mice with PSEN1dE9 transgenic mice, and the gene mutations in presenilin 1 (*PS1*), presenilin 2 (*PS2*), or amyloid precursor protein (*APP*) are responsible for increasing the production of A β ^[16]. Studies have shown a large number of A β plaques begin to accumulate in the brain of APP/PS1 mice at 6–7 months^[17-19]. Therefore, the fundamental aim of this investigation centered on the examination of DHA on synaptic damage induced by A β using APP/PS1 mice and wild-type (WT) mice as the subjects. The deposition of A β protein in the brain was examined. The apoptosis of nerve cells was

detected by TUNEL staining and the oxidative level was measured. Transmission electron microscopy (TEM) was used to observe the ultrastructure of synapses and immunofluorescence was used to detect the expression of synaptophysin (SYP), a presynaptic marker protein. And neurotransmitter levels and the activity of ATPase in mice brain were measured to exam the function of synapses. In addition, the expression of TrkB-extracellular regulated protein kinases (ERK1/2)-cAMP-response element binding protein (CREB) pathway was determined to elucidate the mechanism of DHA's protective effect on synapses. With a view to providing new targets for the prevention and treatment of AD through this study.

2. Materials and methods

2.1 Materials

DHA (22:6 *n*-3, non-esterified fatty acid, purity > 99%) and xanthan gum were purchased from Sigma (St. Louis, MO, USA). Primary antibodies of phospho-TrkB (Tyr817), phospho-ERK1 (Thr202/Tyr204)/ERK2 (Thr185/Tyr187), phospho-CREB (Ser133), SYP, A β ₁₋₄₂, β -actin, horseradish peroxidase-conjugated secondary antibodies and FITC-labeled goat anti-rabbit immunoglobulin G (IgG) were purchased from Beyotime (Jiangsu, China). Primary antibodies of phospholipase C-gamma-1 (PLC γ 1) (phospho-Tyr783) and calcium/calmodulin-dependent protein kinase II (CaMKII) (phospho-Thr286) were purchased from EnoGene (Nanjing, China). Glutamine (Glu) and aminobutyric acid (GABA) were purchased from Solarbio (Beijing, China). All other required chemicals were of the purest grade available for analysis.

2.2 Animals and experimental design

A total of 30 female APP/PS1 mice of 5 months old (in C57BL/6J background) and 20 female littermates WT C57BL/6J mice of 5 months old were provided by the Experimental Animal Center of China Academy of Chinese Medical Sciences, Beijing, China. This study was approved by the local ethics committee (approval number: SCXK(JING)2019-0010) and the procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. After 1 week of adaptive feeding, the mice were randomly divided into 5 groups with 10 mice in each group by double-blind method. The feeding environment temperature was (22.0 $\pm$ 0.5) °C, the humidity was (50 $\pm$ 5)%, the light-dark cycle was 12 h:12 h. The feeding duration lasted for 60 days to ensure A β plaque deposition in the brains of APP/PS1 mice. Grouping details are shown in Table 1.

Table 1
Group details.

Group	Mice (number)	Treatment	Duration (day)
WT(Veh)	C57BL/6J (10)	0.9% Saline (0.01 L/kg)	60
APP(Veh)	APP/PS1 (10)	0.9% Saline (0.01 L/kg)	60
APP(Xanthan)	APP/PS1 (10)	0.1% Xanthan gum solution (0.01 L/kg)	60
APP(DHA)	APP/PS1 (10)	DHA (400 mg/kg)	60
WT(DHA)	C57BL/6J (10)	DHA (400 mg/kg)	60

Note: DHA was diluted in 0.1% xanthan gum solution. The APP(Xanthan) group was used to observe the effect of xanthan gum on the protective function of DHA.

2.3 TUNEL staining

After the feeding process, the mice were killed by cervical dislocation after being fasted 12 h. The brain tissues were rapidly removed, washed with 0.9% cold saline, weighted. Half of the brain tissue from 3 of these mice were removed and placed in formaldehyde solution for subsequent sectioning. The remaining brain tissues were placed in liquid nitrogen for subsequent determination. The hippocampus tissue was separated from the brain to obtain paraffin wax tissue sections. The sections were deparaffinized with dimethylbenzene. Then DNase-free proteinase K was added on sections at 37 °C for 30 min and the sections were washed by PBS for 3 times. TUNEL reaction mixture (mixture of terminal deoxynucleotidyl transferase (TdT) and Cy3-labeled dUTP) was added on the sections and incubated at 37 °C in the dark for 60 min. The sections were washed by PBS three times, mounted with Dapi-Fluoromount-G and observed under a fluorescence microscope (OLYMPUS, FSX100, Japan).

2.4 Determination of oxidative stress level

Brain tissue (100 mg) was mixed with 1 mL extract solution and homogenized on ice. After centrifugation at $8\,000 \times g$ at 4 °C for 10 min, the supernatant was collected and the content of malondialdehyde (MDA) and activity of superoxide dismutase (SOD) were determined according to the instructions of SOD kit and MDA kit (Solarbio, China).

2.5 Immunofluorescent staining

Tissue sections were deparaffinized, antigen repaired, and blocked in bovine serum albumin blocking solution at 37 °C for 30 min. Next, 100 µL of A β primary antibody (1:100 diluted in PBS) was added to the tissue sections and incubated overnight at 4 °C. After washing with PBS for 3 times, the brain tissues were incubated with fluorescein FITC-labeled goat anti-rabbit IgG antibody (1:500 diluted in PBS) at 37 °C away from light for 1 h. Thereafter, the sections were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma, USA) and washed with PBS. Images were observed and recorded using laser scanning confocal microscopy (Leica, Germany).

2.6 Transmission electron microscope analysis

The hippocampal tissues of mice were removed, washed with normal saline and placed in 2.5% precooled glutaraldehyde fixed solution. The fixed solution was removed and the osmic acid solution was added to fix the sample for 1–2 h. After washed by PBS (0.1 mol/L, pH 7.0), the fixed tissues were dehydrated with ethanol at a gradient concentration for 15 min. The samples were treated with acetone for 20 min, the mixture of embedding agent and acetone (1:1, *V/V*) for 1 h, the mixture of embedding agent and acetone (3:1, *V/V*) for 3 h, and pure embedding agent overnight, respectively. After heated overnight at 70 °C, the tissue embedding blocks were cut into ultrathin sections, which were stained with lead citrate solution and uranium dioxide acetate solution for 5–10 min respectively. After drying, the sections were observed under TEM (FEI Tecnai F20).

2.7 Immunohistochemistry staining

The paraffin sections were deparaffinized, antigen repaired and blocked in BSA blocking solution at 37 °C for 30 min. After that, SYP primary antibody (1:100 diluted by PBS) was added to the tissues in 100 µL per piece, and incubated overnight at 4 °C. After being washed 3 times with PBS, the brain tissues were incubated with HRP-labeled goat anti-rabbit IgG antibody (1:500 diluted by PBS) at 37 °C for 30 min. Thereafter, DAB color development reagent was added to the sections for 5–10 min. After staining with hematoxylin, the sections were dehydrated, sealed and observed under a microscope (Olympus BH22, Japan).

2.8 Determination of neurotransmitters

Glu and GABA standards (5.0 mg) were weighed and dissolved in 50% methanol solution to 25, 50, 100, 125, 150, 200 µg/mL of standard series. Brain tissue (100 mg) was homogenized with 900 mL methanol solution/water (1:1, *V/V*) on the ice. The supernatant was collected after centrifugation at 3 000 r/min for 30 min. The supernatant (200 µL) or standard solution was mixed with 200 µL of 0.5 mol/L NaHCO₃ and 100 µL of 0.5% 2,4-dinitrofluorobenzene (DNFB), and the mixture reacted at 65 °C for 55 min away from light. The sample solution (20 µL) was injected into high performance liquid chromatography (HPLC). An LC-10AT series of HPLC system (Shimadzu, Chengdu, China) was used. The LC separation was performed on a Shim Pack CLC ODS C₁₈ column (250 mm $\times$ 4.6 mm, 5.0 µm) and ultraviolet detector. The total flow rate was 1.0 mL/min. The column temperature was at 37 °C. Mobile phase: acetonitrile (A); sodium acetate (B); water (C). The eluent gradient was: 0 min, A:B:C = 6:88:6 (*V/V*); 0–5 min, A:B:C = 17.5:6.5:17.5; 5–13 min, A:B:C = 30:40:30; 13–25 min, A:B:C = 6:88:6, followed by washing and reconditioning the column.

2.9 Determination of ATPase activity

Brain tissue (100 mg) was homogenized with 900 µL of 0.9% saline on the ice and centrifuged at 2 500 r/min for 10 min. The supernatant was collected and protein content was determined by Coomassie Brilliant Blue reagent. Na⁺,K⁺-ATPase, Ca²⁺,Mg²⁺-ATPase and T-ATPase were determined by using ATPase kits (Nanjing Jiancheng Bioengineering, China).

2.10 Western blot analysis

Brain tissue (100 mg) was crushed and homogenized with 900 mL Tris (pH 7.8) on the ice and the supernatant was collected after centrifugation at 12 000 r/min for 5 min. Coomassie brilliant blue method was used to determine protein content. Polyacrylamide gel electrophoresis (12% separation gel and 5% stacking gel) was conducted to separate the protein sample. And the sample was transferred to nitrocellulose (NC) membrane on the ice for 2 h. After that, the NC membrane was blocked with 5% skimmed milk for 1.5 h at room temperature. Then the membranes were incubated with primary antibody (1:1 000 diluted by PBS) at 4 °C overnight and HRP-conjugated secondary antibody (1:1 000 diluted by PBS) at room temperature for 1.5 h. After being washed by PBS and adding

the fluorescence enhancement solution, the membranes were observed under an image-quant Las4000 (GE, USA).

2.11 Statistical analysis

The results were shown as mean $\pm$ SE, and each experiment was repeated at least 3 times. SPSS (version 18.0) software was used for statistical analysis to determine the significance of the difference. Pixel intensity detection of protein expression or phosphorylation was performed by ImageJ software to test its variability and statistical significance. $P < 0.05$ were considered statistically significant.

3. Result

3.1 Effect of DHA on A β plaque deposition in AD and WT mice

A β plaque deposition is the main pathological feature of AD, and the amyloid cascade hypothesis is also the most studied theory of AD occurrence and development^[20]. A β deposition leads to neuronal

degradation and death, as well as synaptic dysfunction, and eventually manifests as cognitive impairment^[21]. Immunofluorescence staining was performed to measure the deposition of A β in the hippocampi of AD and WT mice. The results were showed in Fig. 1, there was no significant A β protein plaque deposition in WT(Veh) mice. But there was a significant increase in the fluorescence intensity and A β plaque deposition in hippocampi from APP(Veh) mice compared with those from WT mice ($P < 0.01$) (Figs. 1A and B). However, the immunofluorescence intensity was significantly reduced in the hippocampal from the APP(DHA) group stained with the A β antibody, and the number of protein deposits had reduced compared with APP(Veh) group ($P < 0.05$). The immunofluorescence intensity in WT(DHA) mice was not significantly different from WT(Veh) mice ($P > 0.05$). Besides, the expression of A β was measured and the results were showed in Fig. 1C. The expression of A β in APP(Veh) mice was increased compared with that in WT(Veh) mice. Moreover, the expression of A β in APP(DHA) mice was remarkably downregulated by 10.7% compared with that in APP(Veh) mice ($P < 0.01$), which further corroborated our immunofluorescence results.

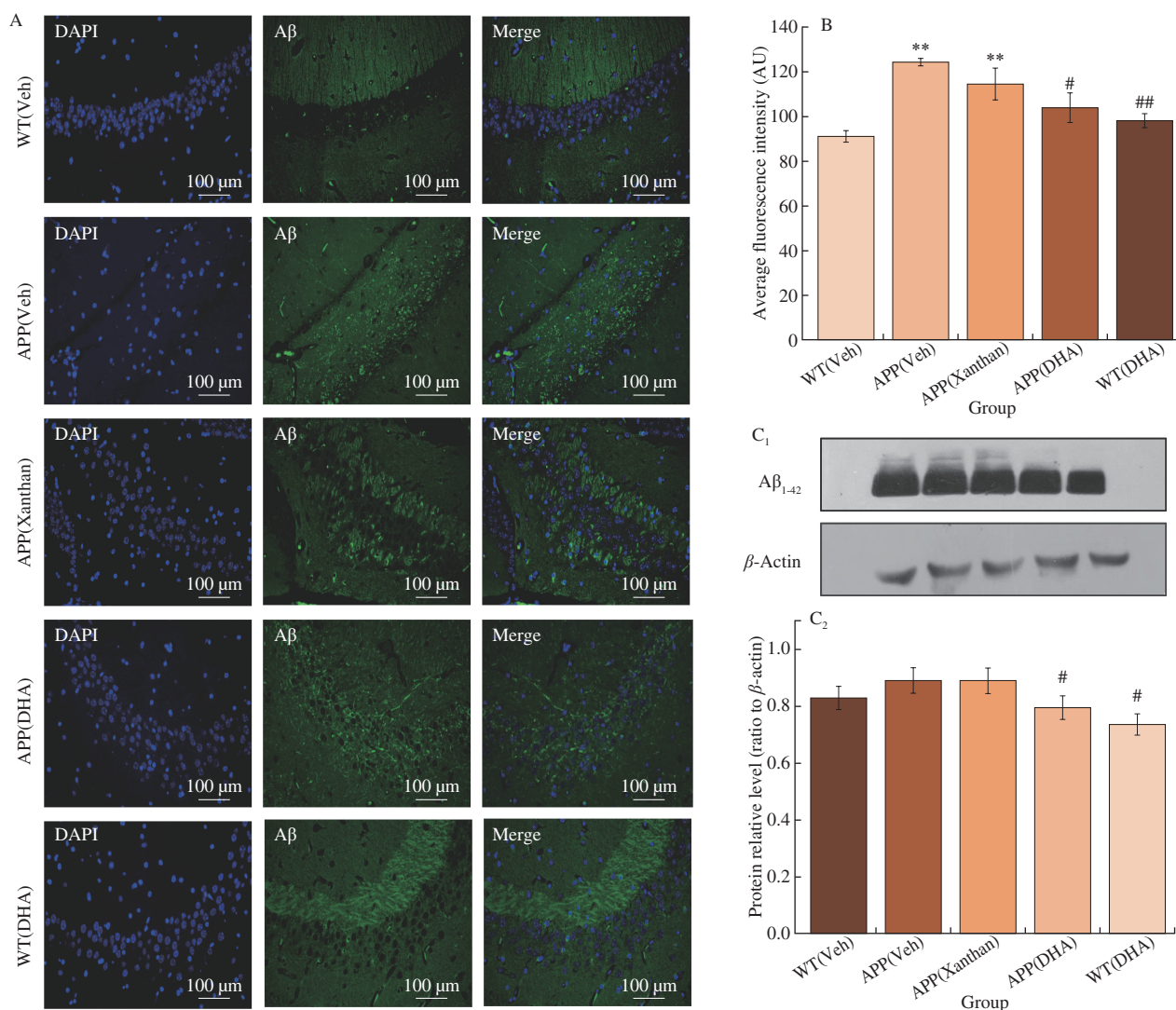


Fig. 1 Effect of DHA on A β protein deposition in the hippocampus of mice. (A) Immunofluorescence with anti-A β_{1-42} (green) and DAPI (blue). Scale bar: 100 μ m. (B) Average fluorescence intensity. (C) Expression of A β_{1-42} protein by Western blot. ** $P < 0.01$ vs. WT(Veh). # $P < 0.05$, ## $P < 0.01$ vs. APP(Veh). Values are expressed as mean $\pm$ SE, $n = 3$.

3.2 Effect of DHA on neuronal apoptosis and cognitive function in AD and WT mice

Before the mice were sacrificed, a water maze test was performed to determine the effects of DHA on cognitive function of the mice. And in our previously published paper, we have demonstrated that AD mice showed obvious learning and memory impairment, and DHA intervention could alleviate the cognitive dysfunction^[15]. The underlying cause of memory impairment in AD is believed to be the loss of neurons and synapses in the cerebral cortex^[22]. Therefore, a TUNEL assay was used to evaluate the apoptosis of neuronal cells in APP/PS1 and WT mice and the results are shown in Fig. 2. The number of TUNEL-stained cells in the brain of APP(Veh) mice was increased compared with that of WT(Veh) group. After DHA treatment, the number of TUNEL-staining cells was significantly decreased, indicating that neuronal apoptosis was reverted. The number of TUNEL-stained cells of WT(DHA) group was not significantly different from that of WT(Veh) group, suggesting that DHA intervention would not affect WT mice. As the result of

APP(Xanthan) group was not significantly different from that of APP(Veh), it was not shown in Fig. 2.

3.3 Effect of DHA on oxidative stress in AD and WT mice

The increase of oxidative stress level in the brain of AD patients is a key factor that causes the increased susceptibility of neurons to excitotoxicity and apoptosis^[23]. Therefore, the content of MDA and the activity of SOD were measured and the results are shown in Fig. 3. The MDA level of mice in the APP(Veh) and APP(Xanthan) groups increased significantly compared with mice in the WT(Veh) group ($P < 0.01$). DHA treatment decreased the MDA level by 22.0% compared with the APP(Veh) group ($P < 0.01$) (Fig. 3A). As shown in Fig. 3B, the SOD activity of mice in the APP(Veh) and APP(Xanthan) groups decreased by 33.5% and 32.1%, respectively, compared to WT(Veh) ($P < 0.01$). However, in the mice gavaged with DHA, the SOD activity increased significantly when compared to the mice in APP(Veh) ($P < 0.01$).

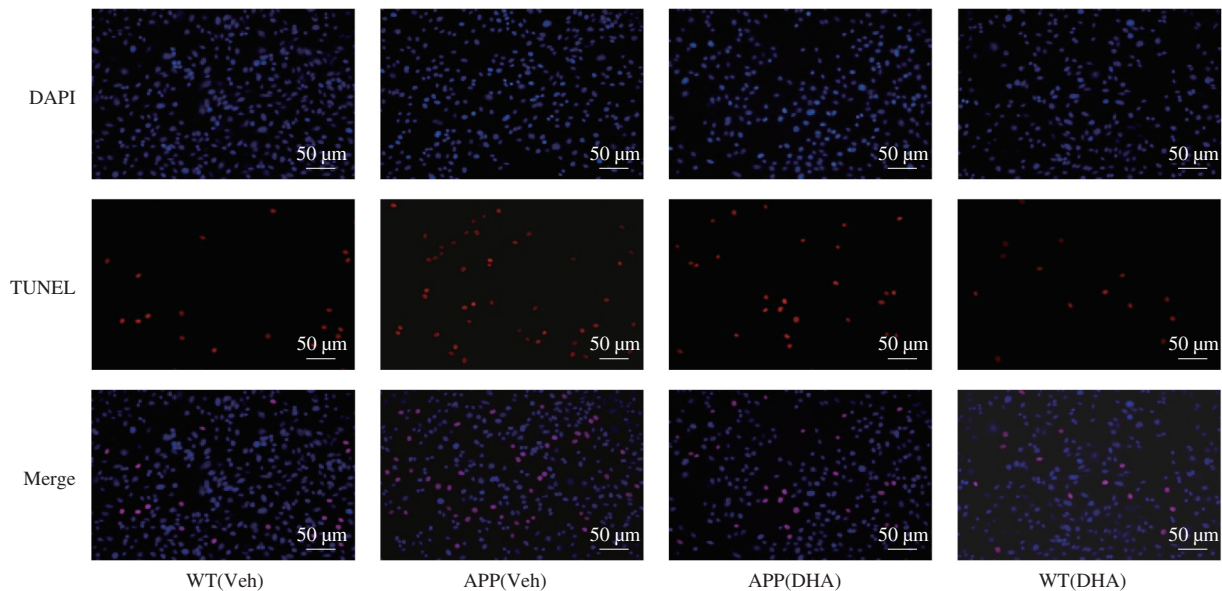


Fig. 2 Effect of DHA on neuronal apoptosis in brain of APP/PS1 and WT mice. The brain tissue was stained with TUNEL and DAPI. Scale bar = 50 μ m.

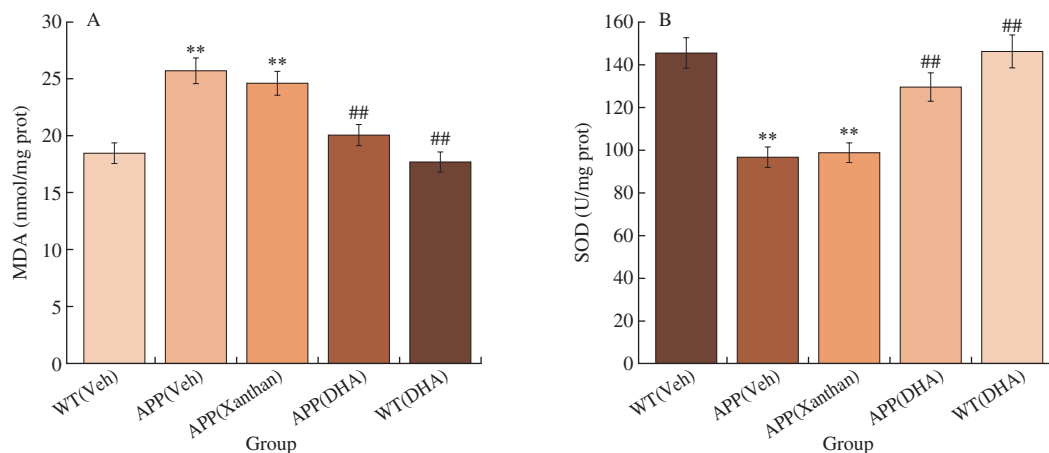


Fig. 3 Effect of DHA on (A) MDA level, (B) SOD activity in brain of APP/PS1 and WT mice. Values are expressed as mean $\pm$ SE, $n = 6$. ** $P < 0.01$ vs. WT(Veh); ## $P < 0.01$ vs. APP(Veh).

3.4 Effect of DHA on the synaptic structure in AD and WT mice

Synapses are the fundamental components of nerve cells and form the necessary connections in the neural network of cognition. Synaptic dysfunction and loss caused by A β oligomers are strongly associated with the cognitive decline experienced by AD patients^[24]. In order to intuitively observe the effects of DHA on the ultrastructure and number of synapses in mice brain, TEM was used in this study and the results are shown in Fig. 4. The representative TEM images illustrated that the number of synapses was highly abundant in WT(Veh) group, and abundant synaptic vesicles were observed in the presynaptic region, while mitochondria and endoplasmic reticulum were observed in the postsynaptic region, and no structural abnormalities were found. In contrast, the number of synapses evidently reduced in APP(Veh) and APP(Xanthan) groups ($P < 0.01$), with an inadequate number of presynaptic vesicles, uneven nucleoplasm, and cavitation of the cytoplasm. When compared to APP(Veh) group, the number of synapses in the brain of APP(DHA) mice was significantly increased ($P < 0.01$), the synaptic structure was relatively complete, and a few cytoplasm were condensed (Fig. 4B). In addition, the thickness of postsynaptic dense (PSD) in the brain of APP(Veh) and APP(Xanthan) groups decreased markedly compared with that in WT(Veh) group ($P < 0.01$).

DHA treatment increased the thickness of PSD by 97.8% compared with APP(Veh) group ($P < 0.01$) (Fig. 4C). And compared with WT(Veh) group, the width of synaptic gap in the mice brain of APP(Veh) and APP(Xanthan) groups increased by 42% and 40%, respectively, but it significantly decreased after DHA treatment ($P < 0.01$) (Fig. 4D).

3.5 Effect of DHA on SYP expression in brain of in AD and WT mice

SYP is a specific marker of synaptic vesicles, and its content indirectly reflects the number and density of synapses in the brain. Thus, immunohistochemistry was conducted in this study to detect SYP expression in the mice brain of various groups, and the results are shown in Fig. 5. In the hippocampus of APP(Veh) and APP(Xanthan) groups mice, the positive SYP expression was markedly decreased compared to in WT(Veh) group. Whereas, the positive SYP expression in the brain of DHA-treated mice was significantly increased when compared to that in APP(Veh) group mice, indicating that DHA increases the density and distribution of synapses. In addition, the expression of SYP in the cortex showed similar results with that in the hippocampus, where the expression of SYP decreased in APP/PS1 mice, but increased after DHA treatment.

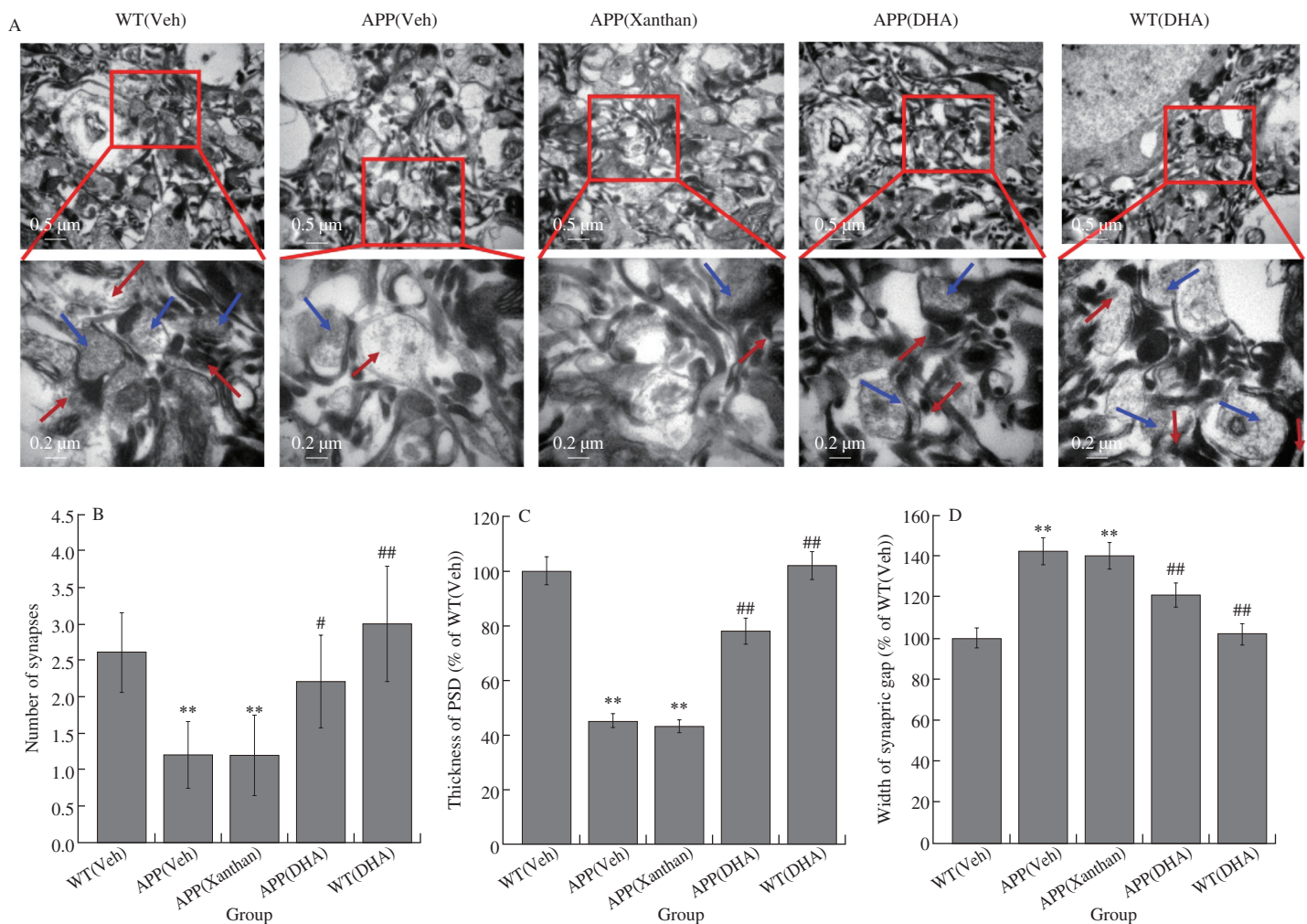


Fig. 4 Effect of DHA on the synaptic structure in APP/PS1 and WT mice. (A) Representative TEM images of synapses in APP/PS1 and WT mice. Scale bar = 0.5 and 0.2 μ m. Blue arrow: presynaptic terminals; red arrow: post-synaptic spines. (B) Number of synapses in APP/PS1 and WT mice. (C) Thickness of PSD in APP/PS1 and WT mice. (D) Width of synaptic gap in APP/PS1 and WT mice. Values are expressed as mean $\pm$ SE, $n = 3$. ** $P < 0.01$ vs. WT(Veh); * $P < 0.05$, ## $P < 0.01$ vs. APP(Veh).

3.6 Effect of DHA on the neurotransmitters level in AD and WT mice

Neurotransmitters are specific chemicals that act as "messengers" in synaptic transmission. Changes in synaptic structure and number inevitably lead to changes in neurotransmitter release and the results of neurotransmitter release are shown in Fig. 6. Glu level in the brain of APP(Veh) mice was significantly increased compared with WT(Veh) group ($P < 0.01$), while DHA treatment group had a significant decrease in Glu ($P < 0.01$) (Fig. 6A). Compared with WT(Veh) group, GABA level showed a significant decrease in the APP(Veh) and APP(Xanthan) groups ($P < 0.01$). DHA treatment significantly increased GABA level by 37.0% and 35.8% compared with APP(Veh) and APP(Xanthan) groups, respectively ($P < 0.01$) (Fig. 6B).

3.7 Effect of DHA on ATPase activity in AD and WT mice

Studies have shown that Na^+, K^+ -ATPase and $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase regulates the excitability of neurons by maintaining ion transport and neurotransmitter intake in neurons. In astrocytes, Na^+, K^+ -ATPase regulate central nervous network integration by adjusting the Glu intake and calcium release^[25]. Therefore, the ATPase activities were measured in this study and the results are shown in Fig. 7. The activity of Na^+, K^+ -ATPase and $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase of mice in APP(Veh) and APP(Xanthan) groups significantly decreased compared to that of WT(Veh) mice ($P < 0.01$) (Figs. 7A and B). After DHA treatment, the activities of Na^+, K^+ -ATPase and $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase were increased by 36.7% and 41.4%, respectively ($P < 0.01$). In addition, compared with WT(Veh) group, the mice in APP(Veh) and APP(Xanthan) groups showed decreased T-ATPase activity, which significantly increased after DHA treatment ($P < 0.01$) (Fig. 7C).

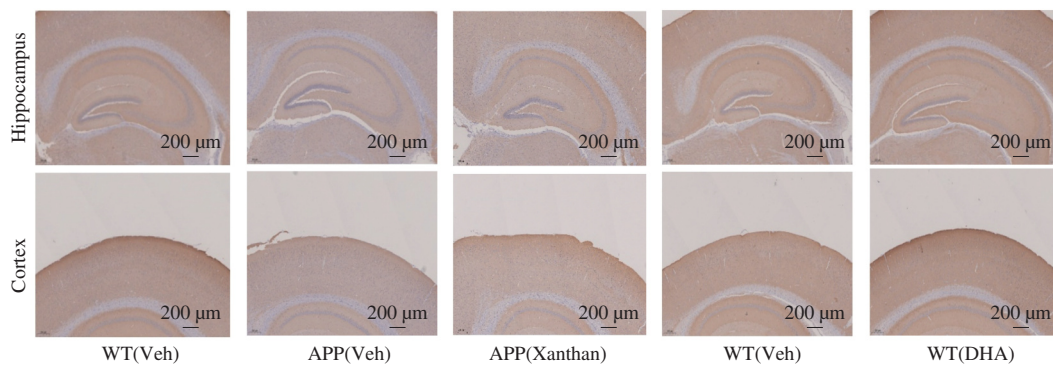


Fig. 5 Effect of DHA on the expression of SYP in hippocampus and cortex in APP/PS1 and WT mice. Scale bar = 200 μm .

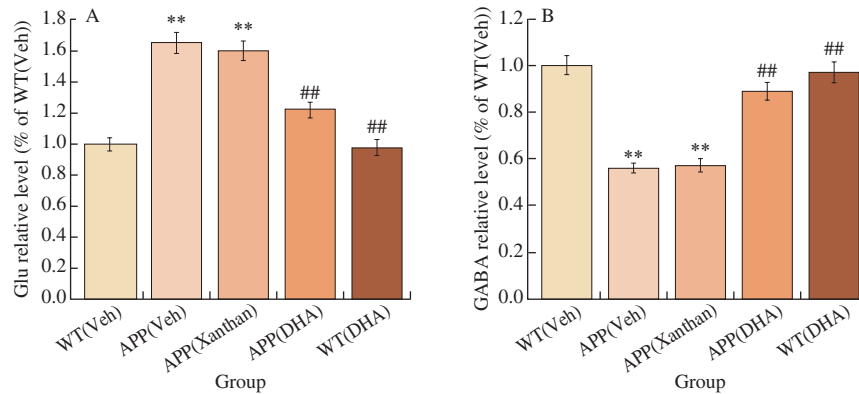


Fig. 6 Effect of DHA on neurotransmitter release. (A) Glu level; (B) GABA level in APP/PS1 and WT mice. Values are expressed as mean $\pm$ SE, $n = 3$. ** $P < 0.01$ vs. WT(Veh). ## $P < 0.01$ vs. APP(Veh).

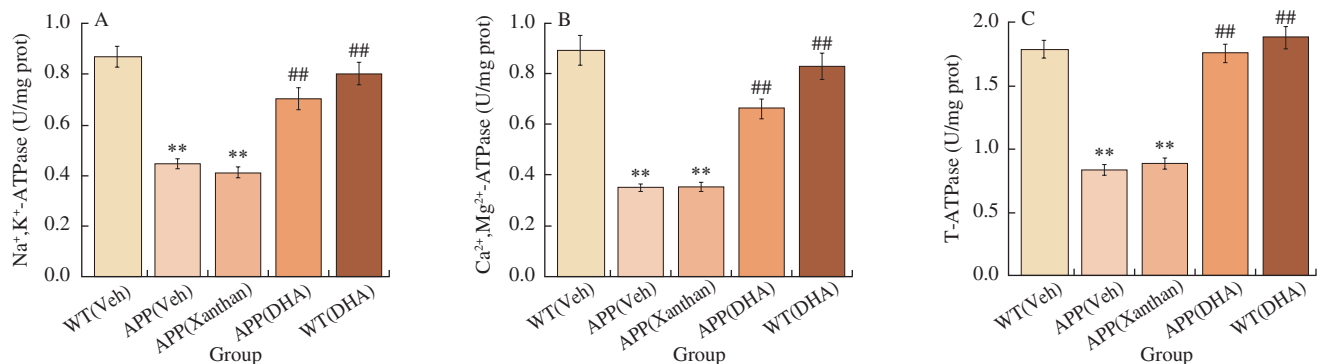


Fig. 7 Effect of DHA on ATPase activity in brain of APP/PS1 and WT mice. (A) Na^+, K^+ -ATPase activity; (B) $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase activity; (C) T-ATPase activity. Values are expressed as mean $\pm$ SE, $n = 6$. ** $P < 0.01$ vs. WT(Veh); ## $P < 0.01$ vs. APP(Veh).

3.8 Effect of DHA on the expression of TrkB-Erk1/2-CREB pathway in AD and WT mice

TrkB, the cell surface specific receptor of BDNF, can activate intracellular signaling pathways, including MAPK/ERK, PI3K/AKT and PLC γ , to transmit signals to the nucleus, activate CREB and regulate protein synthesis, thereby stimulating dendritic growth and regulating transmitter release^[26-27]. We have demonstrated that DHA protected PC12 cells from oxidative damage through the TrkB-Erk1/2-CREB pathway^[28]. In this study, the expression levels of p-TrkB, p-PLC γ 1, p-CaMKII, p-Erk1/2, and p-CREB were determined to reveal the effects of DHA on the cell signaling pathways related to the synaptic function. As shown in

Fig. 8A, the expression levels of p-TrkB and p-PLC γ 1 were significantly downregulated in the APP(Veh) and APP(Xanthan) groups than in the WT(Veh) ($P < 0.01$), whereas their expression was upregulated after DHA administration ($P < 0.01$). In addition, compared with WT(Veh) group, the phosphorylation of CaMKII, Erk1/2, and CREB decreased by 32.0%, 70.1%, and 32.7%, respectively, in the brain of APP(Veh) mice ($P < 0.01$). Whereas DHA treatment significantly upregulated the expression levels of p-CaMKII, p-Erk1/2, and p-CREB, which was shown in Figs. 8B, C and D ($P < 0.01$). These data suggested that the improvement of synaptic structure and function by DHA may be related to the up-regulation of TrkB-Erk1/2-CREB signaling pathway.

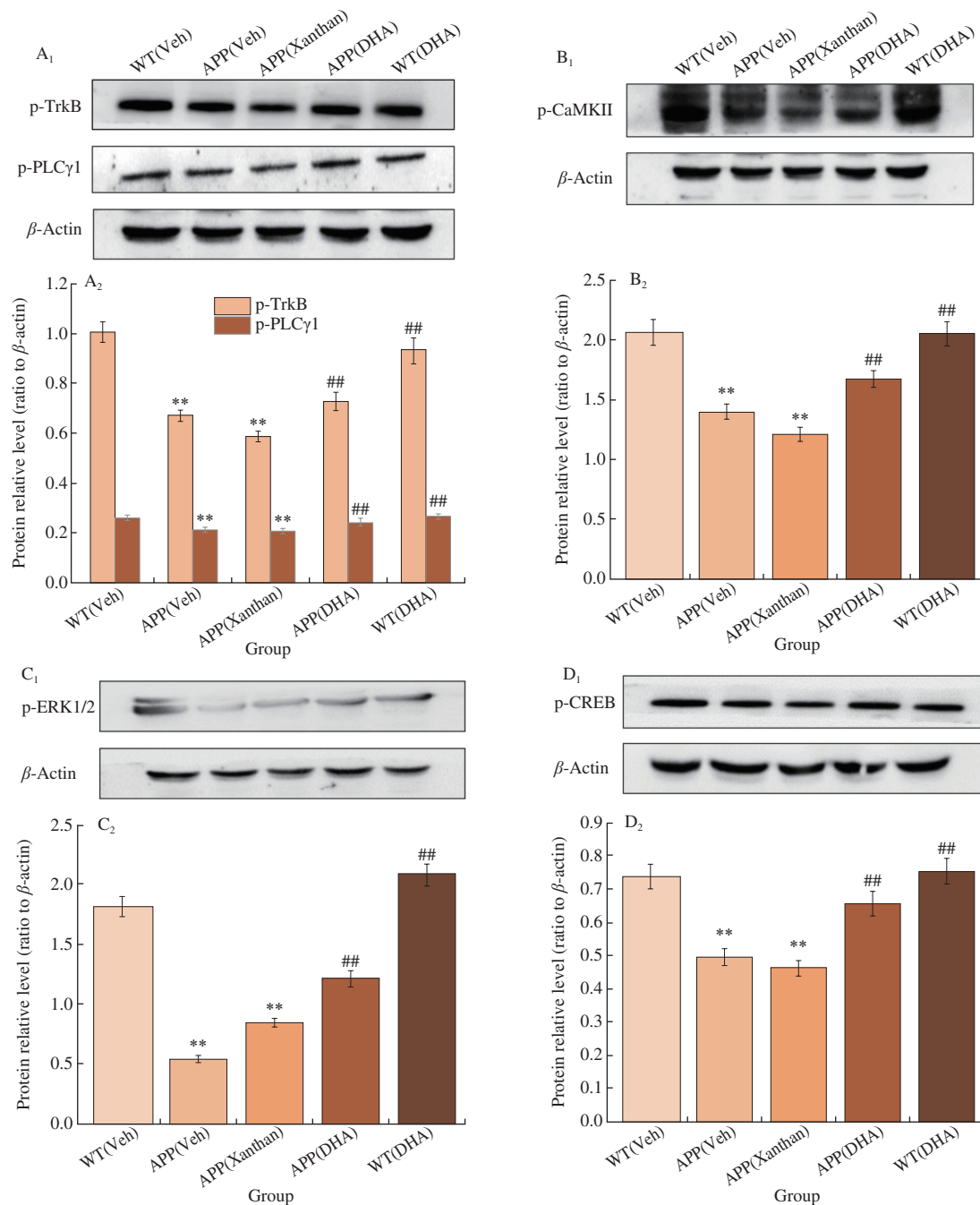


Fig. 8 Effect of DHA on the expression of (A) p-TrkB and p-PLC γ 1, (B) p-CaMKII, (C) p-Erk1/2, and (D) p-CREB in APP/PS1 and WT mice. β -Actin was used as an equal loading control. Values are expressed as mean $\pm$ SE, $n = 3$. ** $P < 0.01$ vs. WT(Veh); ## $P < 0.01$ vs. APP(Veh).

4. Discussion

The molecular pathophysiology of AD is quite complex. Antarctic krill (*Euphausia suprema*) oil (AKO), which is abundant in *n*-3 polyunsaturated fatty acid, such as DHA, could alleviate AD by participating in synaptic plasticity and anti-neuroinflammation^[29]. Besides, AKO may also provide a potential strategy to prevent AD by inhibiting alkaline phosphatase (ALPL) and synergistically regulating BBB function with Nobiletin and Theanine^[30]. In our previous study, we have demonstrated that DHA could reduce A β plaque formation and improve cognitive function of AD mice. However, whether this neuroprotective effect is related to changes in synaptic function needs further investigation. Thus, in this study, the effect of DHA on the structure and function of synapses were evaluated using APP/PS1 and WT mice. Our results suggested that DHA improved the function and structure of synapses and the effect of DHA on synapses is related to the TrkB-Erk1/2-CREB pathway.

APP/PS1 mice are relatively mature AD research models, and the dramatic acceleration of A β deposition in APP/PS1 mice brain owes to the increase in A β ₄₂ production mediated by mutations in PSEN1^[31]. In this study, A large amount of A β deposition was observed in the brain of AD mice, indicating that the model was reliable, while DHA intervention significantly reduced the A β level in the brain of AD mice. As an important pathological feature of AD, A β deposition stimulates the oxidative stress of AD through the production of a large number of reactive oxygen species (ROS)^[32-33]. Wen et al.^[34] showed that DHA inverted the decrease of SOD level and the increased of MDA level in the brain of A β injected rats, which was consistent with our results. In addition, A β can activate microglia and induce the production of pro-inflammatory cytokines, which promote synaptic loss, neuronal death, and ultimately cognitive dysfunction^[35]. We confirmed that there was a large number of neuronal apoptosis in AD mice by TUNEL staining, and DHA intervention alleviated the neuronal apoptosis caused by A β .

However, could the reduction in A β further affect synapses that directly affect learning and memory? Extracellular A β accumulates around postsynaptic and presynaptic spines and the oligomer A β induces excitotoxicity at the presynaptic terminal, allowing more Ca²⁺ to flow directly into the synapse by increasing Ca²⁺ inflow and forming membrane pores^[36]. A study has suggested that the synaptic dysfunction caused by A β oligomer, as an early event, may be an important cause of memory impairment in patients with AD^[37]. Our results showed that synaptic structure was disrupted in the brain of APP(Veh) group mice, in which a large number of A β produced and aggregated. DHA treatment increased the number of synapses and thickness of PSD, but decreased the width of synaptic gap, indicating synaptic structure was improved. In addition, during the pathogenesis of AD, decreased levels of synaptic related proteins lead to destruction of synaptic plasticity and hippocampal dependent cognitive dysfunction. PSD is the most abundant scaffold protein in the postsynaptic membrane, which is involved in regulating the number of synapses during development and promoting synaptic formation^[38]. SYP is a calcium-binding glycoprotein that is closely related to the structure and function of synapses. It is specifically distributed in the presynaptic membrane and is involved in the recycling of synaptic vesicles and the release of neurotransmitters^[39]. The results of IHC suggested that SYP expression was downregulated in APP/PS1 mice, which further demonstrated that neurotoxicity of A β

resulted in decreased expression of presynaptic SYP and postsynaptic PSD in the hippocampus, and led to the destruction of synaptic plasticity. Our results showed DHA increased the expression of SYP and consistent with previous study.

Since synapses are the specialized junctions between neurons, and play a crucial role in neuronal communication, for which we subsequently measured neurotransmitters release and the activities of ATPase. Accurate and appropriate neurotransmitter release is the key factor to maintain normal physiological system of nervous system and avoid neurotoxicity. In the brain of patients with AD, excessive Glu has an excitatory toxic effect through excessive activation of NMDA receptor and leads to learning and memory disorders, and GABA plays an important protective role in this process^[40]. Importantly, Na⁺,K⁺-ATPase and Ca²⁺,Mg²⁺-ATPase are vital enzymes for cell integrity and homeostasis, influencing neurotransmitter signal. And Ca²⁺ are universal secondary messengers that are also involved in synaptic transmission, learning, and formation and consolidation of memory^[41]. Our previous study showed that DHA treatment reduced Glu level and increased GABA level in H₂O₂-induced PC12 cells. The results of this study suggest a similar conclusion, that DHA regulated neurotransmitter release, increased the activities of ATPase, and reduced mitochondrial metabolic imbalance in the mice brain induced by A β , thus improving the cognitive function of APP/PS1 mice.

The above results suggested that the structure and function of synapses were damaged in APP/PS1 mice, but significantly improved after DHA treatment, then we investigated the mechanism of DHA's protective effect on synapses. TrkB is a specific receptor of BDNF, the activation of TrkB receptors lead to memory formation and storage, as well as enhanced neuroplasticity, neurogenesis, neurite growth, and neuron survival. At the same time, activation of the signaling cascade downstream of TrkB can promote the transcription of genes for neuronal survival, neurogenesis, and neuroplasticity^[42]. Ogawa et al.^[43] found that adolescent nasal obstruction led to decreased learning and memory ability in mice accompanied by decreased expression of TrkB protein in hippocampus, and memory formation requires activation of TrkB in hippocampus CA3 and CA1 regions^[44]. Additionally, TrkB regulated the formation and maintenance of long-term potentiation (LTP) by activating the phosphatidylinositol 3-kinase (PI3K)-Akt pathway and the PLC-Ca²⁺ pathway^[45]. Ca²⁺-regulated Erk1/2, protein kinase A (PKA), and CaMK are activated and transferred into the nucleus to regulate CREB phosphorylation, which further regulates the synthesis of cytoskeleton proteins and synapsis^[46]. Previous studies showed that tetramethylpyrazine (TMP) ameliorated cognitive ability and memory impairments, and upregulated the expression of synapse-related proteins. And the underlying mechanism was that TMP could activate the TrkB/Erk/CREB signaling pathway^[47]. Importantly, A β inhibits PKA and thus down-regulates CREB phosphorylation^[48]. The oligomer A β also interfered with Ras-ERK and PI3K-AKT pathways, which aggravated neurotoxicity. Therefore, we focused on the changes in this pathway in the improvement of synaptic function by DHA. Our results suggested that DHA could protect synapses from A β -induced damage through TrkB-PLC γ 1-CaMKII-Erk1/2-CREB pathway.

5. Conclusion

In this study, the protective effect of DHA on the synaptic structure and function, and related mechanism were investigated

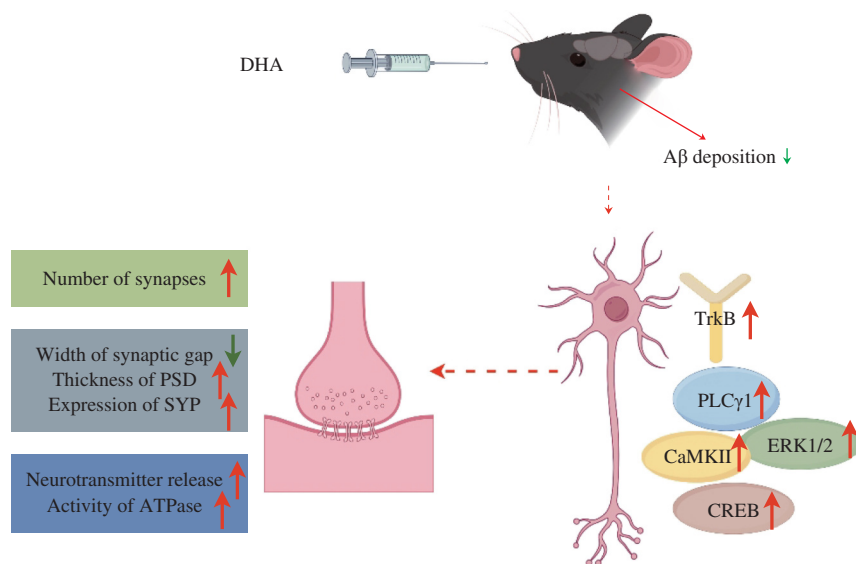


Fig. 9 Schematic diagram of the mechanism of DHA in improving synaptic structure and function in mice.

using APP/PS1 and WT mice as subjects. The results demonstrated that DHA intake reduced neuronal apoptosis and decreased oxidative level induced by Aβ deposition in the brain of AD mice. Importantly, Aβ can promote synaptic loss, while DHA intervention could improve the synapses number and synaptic structure. Besides, DHA increased the expression of SYP, regulated the release of neurotransmitters, and increased the activities of ATPase. And DHA decreased the deposition of Aβ and improved the structure and function of synapses by upregulating the TrkB-Erk1/2-CREB pathway, thereby alleviating cognitive dysfunction in AD. A speculated schematic diagram of DHA improving the synaptic function of mice is depicted in Fig. 9. These results provide a theoretical basis for DHA to improve synaptic function in mice, and provide a new idea for DHA as an adjunct in the treatment of senile cognitive disorders.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgments

This study was supported by Natural Science Foundation of Henan Province of China (242300421536); College Students' Innovation and Entrepreneurship Training Program of Xinxiang Medicine University (202410472024).

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