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Omega-3 fatty acids prevent LPS-induced passive avoidance learning and memory and CaMKII-α gene expression impairments in hippocampus of rat

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ABSTRACT

Background: Neuroinflammation is considered to be a major factor in several neurodegenerative diseases. Recently, the polyunsaturated fatty acid omega-3 has been shown to have anti-inflammatory effects and might play an effective role in improving memory impairment due to inflammation. In order to test this, we stimulated neuroinflammation in an animal model and induced memory dysfunction as measured by reduced retention of passive avoidance learning (PAL) and altered expression of *CaMKII-α*, a gene known to be crucial for memory formation. We then investigated whether treatment with dietary omega-3 prevents inflammation-induced memory dysfunction in this model.

Methods: Male wistar rats (200–220 g) were fed either a control diet or a diet containing omega-3 (400 mg/kg, po) for 1 month prior. Rats then received injection of either saline or LPS (500 μg/kg, *ip*) and were subjected to the PAL acquisition task. The retention test was performed 24 h later, and animals were sacrificed immediately. Hippocampi were dissected and stored at −80 °C. Finally, TNF-α levels and *CaMKII-α* gene expression were measured by ELISA and qRT-PCR, respectively.

Results: We found that LPS treatment significantly impaired PAL and memory, increased TNF-α levels and impaired *CaMKII-α* gene expression. In control and LPS-injected animals, pre-treatment with omega-3 improved performance on the PAL task and increased *CAMKII-α* gene expression.

Conclusion: Taken together, these data suggest that dietary omega-3 may improve cognitive function and provide a potential therapy for memory impairment due to neuroinflammation.

Introduction

Neuroinflammation and proinflammatory cytokine cascades play an important role in neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease and memory impairments [1–4]. Inflammation of brain involves recruitment of immune cells to the injured areas in the tissue and production of inflammatory signaling molecules such as cytokines and chemokines [5,6]. The primary inflammatory response is produced by activated microglia cells, which perform local phagocytosis and release cytokines such as tumor necrosis factor (TNF-α) [1,7].

TNF-α acts as a key inflammatory cytokine and stimulates secretion of other inflammatory mediators [2]. Lipopolysaccharide (LPS), an endotoxin isolated from bacteria, stimulates proinflammatory cytokine cascades by plasma membrane proteins, such as CD14 and toll-like receptor 4 (TLR4), causing TNF-α and other proinflammatory cytokines to be produced. Systemic injection of LPS induces neuroinflammation, neuronal apoptosis and amyloidogenesis in the cortex and hippocampus [2]. LPS-induced inflammation in animal models has also been demonstrated to cause memory impairment [3].

Hippocampus has a key role in passive avoidance learning (PAL) and memory [8,9]. LTP (long-term potentiation) is a cellular model for learning and memory that has been observed at hippocampus and other brain regions [10,11]. Induction and expression of LTP is dependent upon the alpha isoform of calcium/calmodulin-dependent protein kinase II (CaMKII-α) which is highly expressed in neurons in throughout the brain [12]. CaMKII-α is a serine/threonine protein kinase and is activated by Ca^{2+}/calmodulin. It phosphorylates several synaptic proteins, including AMPA receptors. CaMKII-α regulates targeting

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of AMPA receptors to the post-synapse as well as their endocytosis, both of which are essential for the induction of LTP [13–16].

Previous studies suggest that omega-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid [EPA; C20: 5 (n-3)] and docosahexanoic acid [DHA; C22: 6 (n-3)] have key roles in cell membrane structure and cytokine regulation and may be involved in regulating brain function [17–19]. Chronic administration of DHA improves the spatial memory in both young and aged rats, and PUFA treatment reverses age-related impairments in LTP [18–21]. Furthermore, decreased omega-3 PUFA levels have been shown to correlate with the onset of Alzheimer's disease, suggesting that PUFA levels in the brain may be involved in cognition [18,20,22]. Omega-3 PUFAs may be able to regulate gene expression; previous studies demonstrate that increasing dietary intake of omega-3 PUFA has widespread effects on gene expression [23,24].

In the present study, we investigate the effect of neuroinflammation on memory formation *in vivo*, and whether dietary consumption of omega-3 PUFA may have neuroprotective effects. We found that, as predicted, induction of brain inflammation by LPS injection produced significant memory impairment, as measured by the passive avoidance learning (PAL) task. LPS injection also impaired *CaMKII-α* gene expression. Consumption of a diet high in omega-3 prevented both LPS-induced memory impairment and changes in *CaMKII-α* gene expression. We conclude that a diet rich in omega-3 PUFA may protect against cognitive decline related to neuroinflammation.

Material and methods

Animals

Adult male wistar rats weighing 200–220 g were housed under standard conditions, according to the NIH Guide for the Care and Use of Laboratory Animals.

Material

Lipopolysaccharide (LPS L3775) and omega-3 were, respectively, purchased from Sigma, USA, and Zahravi Co, Iran.

Passive avoidance learning (PAL) test (step-through test)

Passive avoidance apparatus

The step-through passive avoidance apparatus consisted of two compartments: one illuminated cubicle (20 cm × 20 cm × 30 cm) made of transparent plastic and another dark cubicle of the same size made of dark opaque plastic. One rectangular opening (6 cm × 8 cm) was located between the two compartments and could be closed by an opaque guillotine door. The floor of both compartments was made of stainless steel rods (3 mm diameter) spaced 1 cm apart. In addition, the floor of the dark chamber could be electrified using a shock generator [25,26].

Acquisition training

All experimental groups were given two trials to be habituated to the apparatus. For the first of these sessions, an individual rat was placed in the illuminated compartment; 5 s later, guillotine door was raised and rat was allowed to enter the dark compartment. Immediately after, the guillotine door was closed and after 30 s, rat was returned to its home cage. The second habituation trial (with the same intervals as the first) was performed after 30 min. The step-through latency to the dark compartment (STL) was defined as the time it took the animal to fully enter the dark compartment and place all four paws in that chamber. After the animal had spontaneously entered the dark compartment, the guillotine door was lowered and mild electrical shock (0.2 mA, 1.5 s) was delivered to the grid floor. After 30 s, the rat was returned to its home cage. The procedure was repeated until the rat remained in the light compartment for 120 consecutive seconds. The number of trials required to reach this level of acquisition was recorded.

Retrieval test

The retention test was performed 24 h later, after the PAL acquisition trial described above. The rat was placed in the illuminated compartment as in the acquisition trial, facing away from the door. After 5 s, the guillotine door was raised and the rat was allowed to enter the dark compartment. To assess memory retention, the step-through latency (STL) and the time spent in the dark compartment (TDC) were recorded for up to 300 s. If the rat did not enter the dark compartment within 300 s, the retention test was terminated and a ceiling score of 300 s was assigned.

Experimental protocol

The animals were divided into four equal groups ($n = 8$), and received saline, LPS, omega-3, and combination of omega-3 and LPS. LPS was dissolved in saline, and injected intraperitoneally (*ip*) (500 μg/kg), 4 h before retrieval test [6]. This dose was used for induction of moderate inflammation [27]. Also, it has been reported that this dose is in the range which does not affect motor activity [28].

Animals received omega-3 (400 mg/kg/day) by oral gavage for 30 days until they were sacrificed [29]. To control the similarity of injections among the four groups, all groups received 1 month saline or omega-3 by gavage and one dose saline or LPS by *ip* injection. The control group received saline by oral gavage (400 mg/kg) for 30 days followed by pre-test *ip* injection of saline (500 μg/kg) and was tested after 4 h. The LPS-treated group received saline by oral gavage (400 mg/kg) for 30 days followed by a pre-test *ip* injection of LPS (500 μg/kg) and was tested after 4 h. The omega-3 group received omega-3 by oral gavage (400 mg/kg) for 30 days followed by pre-test *ip* injection of saline (500 μg/kg) and was tested after 4 h. The last group (omega-3 + LPS) received omega-3 by gavage (400 mg/kg) for 30 days followed by pre-test *ip* injection of LPS (500 μg/kg) and was tested after 4 h. The interval between the last gavage and *ip* injection was 30 min.

Tissue preparation

Immediately after the completion of behavioral test, the animals were decapitated. The rats were first anesthetized with halothane and then sacrificed using a rodent guillotine. The hippocampi were quickly isolated in ice and were transferred to liquid nitrogen where it was kept at −80 °C for further processing. After this step, right or left hippocampus of each animal randomly prepared for analysis by ELISA or for analysis of mRNA expression.

ELISA quantification of TNF-α

Hippocampus samples with different treatments were homogenized in ice-cold lysis buffer (Tris–HCl 50 mM, NaCl 150 mM, Triton X-100 0.1%, EDTA 1 mM, SDS 0.1%, sodium deoxycholate 0.25% and protease inhibitor cocktail 1%). In order to obtain the total protein extract, homogenates were centrifuged at 12,000 rpm for 20 min at 4 °C and supernatants were collected for determining TNF-α concentration [27].

TNF-α levels were evaluated in rat hippocampus by a sandwich-type enzyme-linked immunoassay according to the manufacturer's instructions (R & D Systems, UK). Briefly, hippocampus homogenates were incubated in duplicate in a 96-well

microplate which had been coated with an anti TNF-α monoclonal antibody. The completed reaction was read by a spectrophotometer (Awareness Technology Inc., USA) at an absorbance of 450 nm.

RNA isolation and reverse transcription

Total hippocampal RNA was extracted using RNX plus kit (Cinnagen, Tehran, Iran) according to the protocol described by the manufacturer. The purity of the isolated total RNA was analyzed by absorption at 260 and 280 nm with Eppendorf spectrophotometer (Eppendorf, Hamburg, Germany). The integrity of the total RNA was evaluated with TAE agarose gel electrophoresis, and the samples were treated with the DNAaseI, RNAase-free kit (Fermentase, Vilinius, Lithuania). The first standard complementary DNA (cDNA) was synthesized from 1 μg total RNA in a reaction mixture containing random hexamer primer using RevertAid™ first standard cDNA synthesis kit (Fermentase) and stored at −20 °C.

RT-PCR amplification

Real-time polymerase reaction (PCR) was performed in a Chromo4 Detection System (BioRad, Hercules, CA, USA). All PCR quantification procedures were performed in duplicate. Reaction progress was evaluated by alternations of florescence intensity of SYBR premix Ex TagTM II (Takara, Tokyo, Japan) dye attached to the double-standard DNA. Primer sequences were designed using Beacon Designer 7.5 and oligo6 software to amplify 145-bp amplification for CaMKII-α, 146-bp for β-actin (Table 1). A control cDNA dilution series was made for each gene to access a standard curve. Each reaction was evaluated regarding the melting point analysis to confirm single amplified products. The amplification was performed in a total volume of 25 μl containing of 10 ng of cDNA, gene-specific primers and SYBR Green Master Mix (Takara, Tokyo, Japan). In each experiment, the β-actin housekeeping gene was amplified as a reference standard and used to normalize all values. Reactions were performed in duplicate and PCR parameters were 1 cycle at 95 °C for 30 s, 45 cycles at 94 °C for 5 s, 57 °C for 20 s and 72 °C for 15 s. The specificity of the PCR products was confirmed using single peak of dissociation curves and using agarose gel electrophoresis. Relative gene expression data were quantified by the $2^{-\Delta\Delta CT}$ method, where CT is the threshold cycle.

Statistical analysis

All results are shown as means ± SEM. Data were analyzed by one-way analysis (ANOVA) followed by Tukey's multiple comparison tests. In all statistical comparisons, a probability level of 0.05 or less was considered significant.

Results

Treatments do not alter PAL acquisition

Step-through latency (STL) in the first acquisition trial of passive avoidance learning (PAL) was determined for control rats and the three experimental groups (LPS, omega-3, LPS + omega-3; Fig. 1). One-way ANOVA revealed no significant difference in the STL between the four different groups of rats in the first acquisition trial ($F(3,28) = 1.26$, $p = 0.3$). This result shows that in the absence of electrical shock, the exploratory behavior of the four groups of rats did not differ.

PAL task performance was impaired by neuroinflammation but improved by dietary omega-3

STL was recorded in the retention test 24 h after training (Fig. 2A). One-way ANOVA revealed a significant difference in the STL among the different groups ($F(3,28) = 33.03$, $p < 0.001$). Post hoc analysis by Tukey's test showed that STL of LPS-treated group was significantly shorter than the latency observed in the control group ($p < 0.001$), suggesting that LPS-induced neuroinflammation impaired memory. Interestingly, STL of omega-3-treated and omega-3 + LPS-treated animals were significantly longer ($p < 0.001$) than the control group. The STL of the LPS-treated rats was also significantly shorter than the omega-3 + LPS-treated group ($p < 0.001$).

Neuroinflammation also affected the length of time spent in the dark compartment (TDC), which is an additional measure of memory retention of the shocks received during learning acquisition (Fig. 2B). There was also a significant difference in TDC among the experimental groups ($F(3,28) = 45.74$, $p < 0.001$). TDC of the LPS-treated group was significantly longer than the control group ($p < 0.01$). However, the TDC of omega-3 ($p < 0.001$) and omega-3 + LPS ($p = 0.001$)-treated animals were significantly shorter than control groups. Animals pre-treated with omega-3 before LPS treatment also displayed significantly shorter TDC than rats which received LPS treatment only ($p < 0.001$).

Stimulation of TNF-α by neuroinflammation was prevented by consumption of omega-3

Systemic injection of LPS was used in the present study to induce an acute neuroinflammatory response in the brain. To confirm LPS-induced neuroinflammation, 4 h after LPS injection, levels of the proinflammatory cytokine TNF-α were measured in the hippocampus using ELISA (Fig. 3). One-way ANOVA revealed a

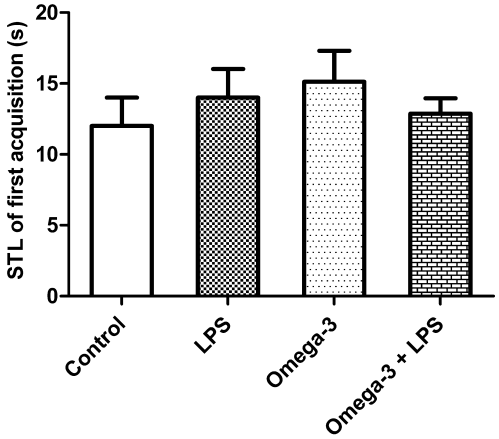

Fig. 1. There was no significant difference in the step-through latency (STL) between the four different groups in the first acquisition trial. This indicates that in the absence of electrical shock, the exploratory behavior of the four groups of rats did not differ. Each value represent mean ± SEM, $n = 8$.

Table 1
Primer sequences for endogenous control and *CaMKII-α* genes.

Name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Primer length	Prod. length (bp)
CaMKII-α	CAGGAATCCTCTGAGAGCACC	CGGTTCAAAGGCTGTCATTCC	21	145
B-actin	GGAGAAGATTTGGCACCACAC	GGATGGCTACGTACATGGCTG	21	146

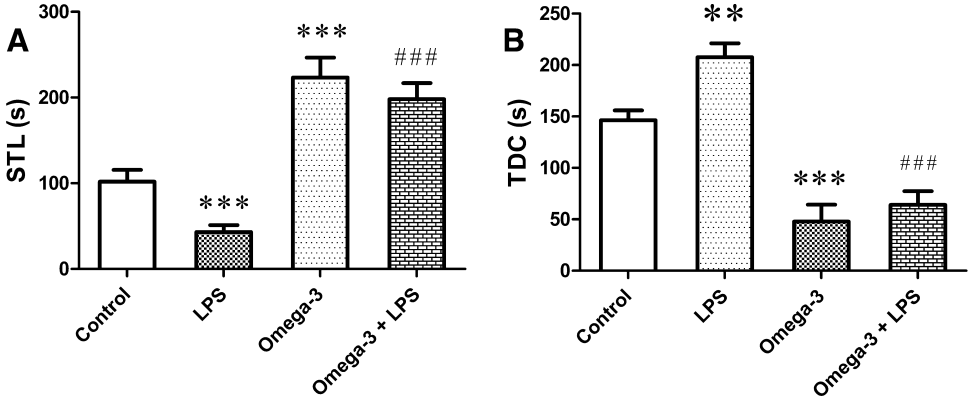

Fig. 2. Performance on memory retention task was impaired by LPS treatment and enhanced by omega-3 consumption. Retention of PAL acquisition was assessed by determining the STL 24 h after task acquisition. (A) STL was significantly ($p < 0.001$) longer in rats injected with LPS but shorter ($p < 0.001$) in animals that received dietary omega-3. On the other hand, rats that received omega-3 before LPS injection displayed significantly ($p < 0.001$) longer STL regardless of whether they received LPS injection. (B) LPS-treated animals also displayed a longer time ($p < 0.01$) spent in the dark compartment (TDC), which is an additional measure of impairment in memory retention. Conversely, rats that received dietary omega-3 displayed significantly ($p < 0.001$) shorter TDC, indicating improved retention of the PAL task. Also rats that received omega-3 before LPS displayed significantly ($p < 0.001$) shorter TDC regardless of whether they received LPS injection ($p < 0.001$). Each value represents mean ± SEM, $n = 8$, $^{***}p < 0.001$ and $^{**}p < 0.01$ *vs.* control group and $^{###}p < 0.001$ *vs.* LPS group.

significant difference in the TNF-α levels among the different groups ($F(3,28) = 62.19$, $p < 0.001$). As expected, a single *ip* injection of LPS significantly increased TNF-α levels with respect to the control group ($p < 0.001$), whereas dietary omega-3 treatment had no effect ($p = 0.07$). However, pre-treatment with omega-3 fatty acids significantly prevented the LPS-induced changes in TNF-α ($p < 0.001$). These data suggest that the protective effect of omega-3 on LPS-induced memory impairment may be due to reduction of inflammatory cytokines such as TNF-α.

Hippocampal CaMKII-α expression was decreased by LPS treatment and increased by omega-3 consumption

CAMKII-α plays a crucial role in memory formation. Since we observed memory impairment on the behavioral task, we next determined whether neuroinflammation and omega-3 treatments can affect CAMKII-α expression. Hippocampal CaMKII-α mRNA was quantified by RT-PCR for all four groups (Fig. 4). One-way ANOVA revealed a significant difference in the CaMKII-α mRNA expression ($F(3,28) = 121.03$, $p < 0.001$) among the different groups. LPS treatment significantly decreased CaMKII-α mRNA expression with respect to control group ($p < 0.05$), whereas in omega-3-treated rats, CaMKII-α mRNA expression was significantly increased ($p < 0.001$). Pre-treatment with dietary omega-3 significantly increased CaMKII-α mRNA levels in LPS-treated rats compared to rats receiving LPS only ($p < 0.001$). Consequently, CaMKII-α mRNA expression in omega-3 + LPS was indisguishable from rats receiving omega-3 only ($p = 0.76$). LPS-induced memory impairment may therefore be due to reduction in CaMKII-α expression; dietary intake of omega-3 reverses this effect and improves memory formation.

Discussion

Neuroinflammation, which may occur after traumatic brain injury, is known to be associated with memory impairment. Accumulating research suggests that persistent inflammation may increase cytokine release, alter protein expression, and ultimately disrupt memory networks causing dementia. Suppression of inflammation may therefore improve cognitive function. The polyunsaturated fatty acid omega-3 has been demonstrated to

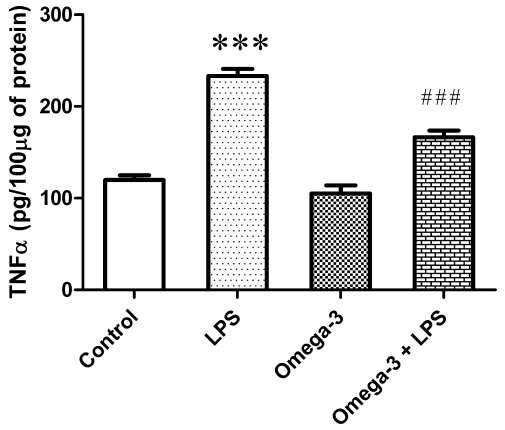

Fig. 3. LPS treatment increases TNF-α concentrations in rat hippocampus. Hippocampal tissue samples were collected after behavioral experiment and TNF-α levels were determined by ELISA. TNF-α concentrations were increased following LPS injection, and this change was prevented by pre-treatment with omega-3 dietary supplement. Each value represents mean ± SEM, $n = 8$, $^{***}p < 0.001$ *vs.* control group and $^{###}p < 0.001$ *vs.* LPS group.

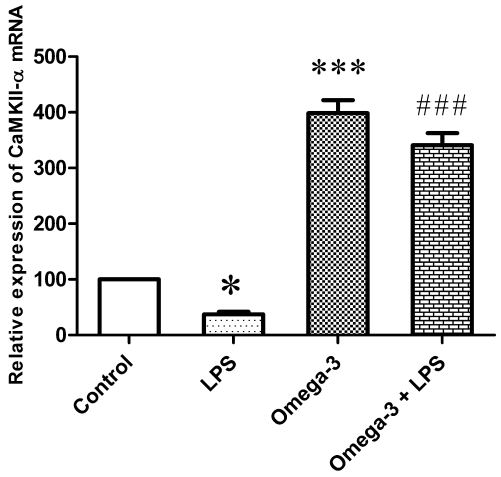

Fig. 4. CaMKII-α mRNA levels are decreased by LPS treatment and enhanced by omega-3. RT-PCR analysis of hippocampal CaMKII-α expression was determined for each of the four treatment groups. CaMKII-α mRNA levels were significantly reduced following LPS injection, but increased by omega-3 treatment. Each value represents mean ± SEM, $n = 8$, $^{*}p < 0.05$ and $^{***}p < 0.001$ *vs.* control group and $^{###}p < 0.001$ *vs.* LPS group.

have anti-inflammatory effects, and recent studies suggest that dietary omega-3 consumption may promote memory retention and protect against cognitive decline. However, the mechanism by which omega-3 might protect against inflammation-induced memory loss is not known.

In order to investigate the mechanism further, we tested whether omega-3 treatment could protect against inflammation-induced memory loss using a rat model of memory acquisition. We found that stimulation of neuroinflammation by LPS injection significantly impaired retention of the passive avoidance learning (PAL) task. Pre-treatment with 1 month of dietary omega-3 significantly improved PAL performance in both unstimulated and LPS-stimulated rats. Interestingly, these results suggest that omega-3 may improve memory even in the absence of neuroinflammation.

In the present study, we used acute intraperitoneal injection of LPS (500 μg/kg), given 4 h before memory testing, and showed a significant impairment on PAL retention. These data support the previous studies indicating that a single dose of LPS is sufficient to induce memory impairment [6,30,31]. We hypothesized that the LPS-induced changes in memory function may be due to increased brain inflammation, since previous studies indicate peripheral administration of LPS induces neuroinflammatory reactions [2,30]. To determine whether LPS injection could stimulate inflammation in our rat model, we quantified hippocampal concentrations of TNF-α, a pro-inflammatory cytokine and found it to be significantly increased. Our finding agrees with a previous study which showed, in mice, systemic injection of LPS induces TNF-α production in serum and central nervous system within 1 h [32]. TNF-α has been shown to be upregulated following brain trauma and can activate other cytokines to propagate the inflammatory response [2]. In addition to activating this proinflammatory cascade, TNF-α levels may remain increased in the brain for up to 1 week. Serum levels, however, return to baseline within 1 day [2]. Changes in TNF-α levels are due to activation of microglia and astrocytes [33–36] and are related to changes in cell morphology and expression of new proteins including cyclooxygenase-2 (COX-2), iNOS and other proinflammatory cytokines [4,37,38]. It has been reported that LPS activates nuclear factor kappa B (NF-κB) which primarily regulates expression of inflammatory proteins [39]. However, the relationship between LPS-induced changes in TNF-α levels and memory impairment is not fully understood.

In order to determine how neuroinflammation affects mechanisms of memory formation, we investigated the effect of LPS on expression of the calcium-activated enzyme CaMKII-α. Interestingly, in the present study, we found that LPS treatment significantly decreases hippocampal *CaMKII-α* gene expression, CaMKII-α has a key role in memory formation [40] and is involved in activity-dependent changes in LTP and synaptic plasticity [41]. In a mouse model, homozygous knockout of CaMKII-α blocked hippocampal LTP and learning [42]. CaMKII-α also has a key role in the later phases of memory such as retrieval [43–46]. Since the hippocampus plays a critical role in passive avoidance learning and memory [8,47], we hypothesize that, in the present study, the LPS-induced hippocampal deficiency in *CaMKII-α* expression may be involved in the observed PAL impairment. By disrupting CaMKII-α mRNA levels, LPS treatment may severely decrease synthesis of new dendritic CaMKII-α protein, alter LTP induction, and finally disrupt PAL retention.

There is growing evidence that omega-3 fatty acids, DHA and EPA, because of their roles in cell membrane structure and cytokine regulation, have a role in brain function, learning and memory, as well as neuroplasticity [17,20,29,48–51]. Furthermore, activation of NF-κB and expression of inflammatory genes such as iNOS, COX2 and IL-1α are inhibited by omega-3 treatment [52,53]. Therefore, we hypothesized that dietary omega-3 PUFA may prevent deleterious effects of LPS on memory impairment.

Our behavioral studies showed that 1 month pre-treatment with omega-3 significantly decreased the number of trials to acquisition in the PAL task, which demonstrates improvements in acquisition (data not shown). During the retention test, omega-3 increased STL and decreased TDC regardless of LPS treatment, suggesting it improves memory formation. The results of this study are in line with previous reports that omega-3 dietary consumption improves learning and memory in both young and aged animals, enhances LTP, upregulates adult neurogenesis in hippocampus and reduces cognitive deficits during Alzheimer's disease and aging [17,49–51]. Furthermore, previous results indicate that water maze learning and memory improved in rats with cerebral ischemia who received dietary fish oil, which contains high levels of omega-3. These animals also had improved LTP maintenance and reduced oxidative damage and less hippocampal neuronal apoptosis [54].

In the present study, we demonstrated a significant enhancement of hippocampal *CaMKII-α* gene expression following omega-3 treatment. These data suggest that the improved PAL performance we observed in omega-3-treated animals is likely due to increased levels of CaMKII-α. The exact mechanism of omega-3 on upregulation of *CaMKII-α* gene expression is not fully clear. Previous studies indicate that *CaMKII-α* expression may be regulated by retinoid X receptors (RXRs) and nuclear RA receptors (RARs) that, when activated, form heterodimers and regulate transcription [20,55,56]. Interestingly, omega-3 fatty acids are endogenous ligands of RXRs and RARs [20]. During aging, expression of these nuclear receptors significantly decrease and can be reversed by omega-3 dietary supplementation [20]. Thus, it is possible that omega-3 could modulate expression of the *CaMKII-α* gene by binding to these receptors.

Here, we show that dietary omega-3 consumption prevented the neuroinflammation-induced TNF-α increase, loss of *CaMKII-α* gene expression and memory impairment. Our results suggest that inflammation may cause cognitive dysfunction by decreasing *CaMKII-α* gene expression. Furthermore, omega-3 consumption prevents these detrimental effects as well as improved baseline memory function. Taken together, our data suggest that reducing neuroinflammation may be crucial for prevention of dementia and that omega-3 may provide a useful dietary therapeutic intervention.

Conflict of interest

None of the authors claim any conflict of interest.

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