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N-3 polyunsaturated fatty acid consumption produces neurobiological effects associated with prevention of depression in rats after the forced swimming test☆

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Abstract

Epidemiological data and clinical trials suggest that n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have preventive and therapeutic effects on depression; however, the underlying mechanism remains elusive. The present study aimed to examine the behavioral effects and antidepressant mechanism of n-3 PUFA using a forced swimming test. Eleven-week-old male Sprague–Dawley rats were fed an American Institute of Nutrition-93M diet containing 0%, 0.5% or 1% EPA and DHA relative to the total energy intake in their diet for 12 weeks (n=8 per group). Total dietary intake, body weight and hippocampus weights were not significantly different among groups. The groups administered 0.5% and 1% EPA+DHA diets had significantly higher levels of n-3 PUFA in their brain phospholipids compared to those in the control group. The immobility time was significantly decreased and the climbing time was significantly increased in the 0.5% and 1% EPA+DHA groups compared with those in the 0% EPA+DHA group. Plasma serotonin concentration and hippocampus c-AMP response element binding protein (CREB) expression were significantly increased in the 0.5% and 1% EPA+DHA groups compared with those in the 0% EPA+DHA group. Conversely, interleukin (IL)-6 expression was significantly reduced in the 0.5% and 1% EPA+DHA groups compared with that in the 0% EPA+DHA group. However, there were no dose-dependent effects of n-3 PUFA and no significant differences in expressions of IL-1β, tumor necrosis factor-α, brain-derived neurotrophic factor or phosphorylated CREB. In conclusion, long-term intake of EPA+DHA induced antidepressant-like effects in rats and overexpression of CREB via decreased IL-6 expression.
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Keywords: BDNF; CREB; Cytokines; Depression; Forced swimming test; n-3 polyunsaturated fatty acids

1. Introduction

A growing number of clinical and epidemiological studies suggest that n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA; 205n3) and docosahexaenoic acid (DHA; 22:6n3) may be effective in the treatment and prevention of depressive disorders [1–4]. The cerebral cell membrane contains high concentrations of DHA, which cannot be synthesized *de novo* in mammalian tissue but must be obtained directly from the diet or through elongation of its shorter-chain, nutritionally essential precursors including α-linolenic acid (ALA; 18:3n3) [5]. The double bonds in the structure of n-3 PUFA result in conformations that prevent dense packing of phospholipids, thereby increasing membrane fluidity [6]. Although the precise mechanism remains unclear, n-3 PUFA-induced suppression of eicosanoids is involved in anti-inflammatory effects, particularly inhibitions of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β [7,8]. Cyclic AMP (cAMP) response element binding protein (CREB) up-regulation via n-3 PUFA-mediated inhibitions of eicosanoids and cytokines may activate the downstream target brain-derived neurotrophic factor (BDNF), which increases neuroplasticity and cell survival [9,10]. Furthermore, DHA supplementation is reported to increase rat brain levels of acetylcholine, norepinephrine, serotonin and dopamine [11–13].

Despite evidence about the interaction between n-3 PUFA and depressive disorders, experimental evaluations remain scarce and have contradictory results. A few previous studies have shown that dietary supplementation with n-3 PUFA reduced immobility in a forced swimming test (FST), widely used as a valid animal model for depression, when administered for 30 days to 15 weeks [11,14–16], although the effect was not observed after only 3 or 10 days [14]. Unfortunately, most previous studies investigated the short-term effect of n-3 PUFA, except the study by DeMar et al. [11], where ALA-deficient and -adequate diets were used; and only one study has evaluated the underlying mechanisms.

Major depression has been shown to be associated with neuronal atrophy in the hippocampus [17], but the underlying mechanisms, particularly changes that occur in the hippocampus after long-term intakes of EPA and DHA, have not been investigated [18]. Thus, the purpose of the present study was to investigate our hypothesis that low, medium and high levels of EPA and DHA consumption for 12 weeks would confer dose-dependent effects on neurobehavioral

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aspects in rats through FST and neurotropic mechanisms, particularly the cAMP cascade of the hippocampus. This is the first study to evaluate the long-term effects of EPA and DHA on antidepressant behavior and their underlying mechanisms.

2. Methods and materials

2.1. Animals and diets

An animal protocol approved by the Institutional Animal Care and Use Committee of Hanyang University was used for all animal experiments (HY-IACUC-10-016). Ten-week-old male Sprague–Dawley rats (Jung Ang Lab. Animal Inc., Seoul, Korea) were housed in individual ventilated cages in an air-conditioned room maintained at 22°C±2°C with a 12-h light–dark cycle. After a 1-week acclimatization phase, 24 rats were randomly divided into three isoenergetic diet groups (n=8 each) in which the diets were formulated based on a purified American Institute of Nutrition (AIN)-93M diet with 0%, 0.5% or 1% EPA+DHA relative to the total energy intake. The diets were provided to all animals for 12 weeks. The 0% EPA+DHA diet contained 40 g of grape seed oil per kg diet, the 0.5% EPA+DHA diet contained 20.77 g of grape seed oil and 19.23 g of fish oil (30% pure EPA+DHA; Carlson fish oil, Carlson lab, Norway) per kg diet, and the 1% EPA+DHA diet contained 1.54 g of grape seed oil and 38.46 g of fish oil per kg diet. The original AIN-93M diet included soybean oil, which contains n-3 PUFA; in our study, soybean oil was replaced with grape seed oil. Rats were provided food pellets and fresh tap water *ad libitum*. Table 1 shows the fatty acid compositions of the three diets.

Food intake was measured daily, and body weight was measured weekly. At the end of the experiment, rats were fasted overnight and euthanized the next day via exsanguination under anesthesia with an intraperitoneal injection of tiletamine (25 mg/kg), zolazepam (25 mg/kg) and xylazine (10 mg/kg). Blood was collected in EDTA and SST tubes via heart puncture and centrifuged at 3000 rpm for 15 min (HA 1000-3, Hanil Sciences Industrial Co. Ltd., Incheon, Korea) to obtain plasma and serum, respectively. The organs were harvested, rinsed with physiological saline and weighed. Blood and tissue samples were stored at −80°C until further analysis.

2.2. Forced swimming test

The detailed procedures of FST have been described elsewhere [19]. Briefly, each rat was placed individually into a vertical plexiglas cylinder (40 cm in height and 20 cm in diameter) containing 25°C water for a 15-min pretest. At the end of this pretest phase, the rat was removed from the water, dried with a towel and placed in a cage. Twenty-four hours later, the rat was exposed to the same experimental conditions outlined above for a 5-min FST. Immobility, swimming and climbing times were recorded by two trained observers who were blind to the dietary treatment.

2.3. Fatty acid compositions of brain and erythrocytes

Brain tissue (100 mg) was mixed with 5 ml of chloroform:methanol:distilled water at a ratio of 2:2:1 (vol:vol:vol). Tissue phospholipids were separated using thin-layer chromatography (silica gel G, Analtech, Newark, DE, USA) and re-extracted in hexane:ether:acetic acid, 40:10:1 (vol:vol:vol). Tissue phospholipids or erythrocytes were methylated through the addition of boron trifluoride methanol benzene (B1252; Sigma-Aldrich, St. Louis, MO, USA) and were heated at 100°C for 10 min. Fatty acid methyl esters were analyzed using gas chromatography (Shimadzu 2010AF; Shimadzu Scientific Instrument, Tokyo, Japan) with a 100-m SP2560 capillary column (Supelco, Bellefonte, PA, USA) [20]. Fatty acids were identified by comparison with known standards (GLC-727; Nu-Check Prep, Elysian, MN, USA). The quality control sample was composed of pooled erythrocytes, and the coefficient of variation was 4.6%.

Table 1
Fatty acid compositions of the experimental diets

%	0% EPA+DHA [a]	0.5% EPA+DHA	1% EPA+DHA
SAT	14.24	19.21	25.27
MUFA	30.87	31.12	30.96
n-6 PUFA	53.12	34.78	11.90
n-3 PUFA	0.71	14.23	31.39
18:3n3	0.67	0.69	0.80
20:5n3	0.03	8.14	18.20
22:5n3	0.00	0.94	2.11
22:6n3	0.00	4.47	10.29

SAT, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.
[a] % EPA+DHA relative to the total energy intake in the diet.

Table 2
Dietary intake, body weight and various organ weights [a]

	0% EPA+DHA [b]	0.5% EPA+DHA	1% EPA+DHA
Dietary intake (g/day)	19.43±0.44	19.88±0.57	19.96±0.56
Initial body weight (g)	371.88±4.09	371.00±3.58	371.50±3.66
Final body weight (g)	538.63±10.74	553.88±13.34	558.00±20.21
Liver weight (g)	13.10±0.39	13.38±0.48	14.21±0.68
Kidney weight (g)	2.58±0.06	2.56±0.06	2.69±0.11
Frontal cortex weight (g)	0.13±0.01	0.11±0.01	0.10±0.01
Hippocampus weight (g)	0.23±0.01	0.21±0.01	0.23±0.01

[a] Data are mean±S.E.M. values and were tested using one-way analysis of variance.
[b] % EPA+DHA relative to the total energy intake in the diet.

2.4. Plasma serotonin level

Plasma was centrifuged at 4500g for 10 min at 4°C to obtain platelet-free plasma. The level of 5-hydroxytryptamine was determined using a commercial enzyme-linked immunosorbent assay kit (IBL, Hamburg, Germany). Serotonin in samples and controls was acylated with acetic anhydride in acetone, and then samples, controls and standards were added to 96-well microplates coated with goat anti-rabbit IgG. Biotinylated serotonin and rabbit antiserum to serotonin were added to each well and incubated overnight at 4°C. *para*-Nitrophenyl-phosphate in a diethanolamine solution was used as a substrate following the application of alkaline phosphatase-conjugated goat antibiotin antibody. Samples were analyzed at 405 nm on a microplate reader (ELx 800 UV, BIO-TEK Instruments, Inc., Winooski, VT, USA).

2.5. Western blot analysis

Hippocampus tissue was homogenized in ice-cold radioimmunoprecipitation buffer. The tissue homogenates were centrifuged at 10,000g for 10 min, and then the supernatants were collected. Protein concentration in the supernatant was determined using Bio-Rad protein reagent (BioRad, Richmond, CA, USA) with bovine serum albumin as the standard. Equal amounts of protein (50 μg) were separated on 10% polyacrylamide gels, transferred to polyvinylidene fluoride membranes, blocked for 1 h at room temperature with 5% skim milk in TBST and then incubated with anti-CREB (1:1000, Cell Signaling, New England Biolabs, Beverly, MA, USA), phosphorylated CREB (pCREB; 1:1000, Cell Signaling, New England Biolabs, Beverly, MA, USA), TNF-α (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), IL-1β (1:800, Santa Cruz Biotechnology, Santa Cruz, CA, USA), IL-6 (1:800, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or BDNF (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) with 5% skim milk in TBST overnight at 4°C. After several washes with TBST, membranes were incubated with horseradish-peroxidase-conjugated secondary antibody (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was either anti-rabbit IgG or anti-goat IgG, for 1 h at room temperature. Immunoreactive bands were visualized with enhanced chemiluminescence and were captured on X-ray film. For quantification of the relative amounts of CREB, pCREB, TNF-α, IL-1β, IL-6 and BDNF, the blots were scanned and the images were converted to TIFF files and quantified using NIH Image software (ImageJ version 1.37 v). The relative amounts of protein on the blots were obtained by normalizing the values to 0% EPA+DHA (control) values.

2.6. Statistical analyses

Data were analyzed using the SPSS-PC+ statistical software package for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA). All data are expressed as mean±standard error of the mean (S.E.M.). Differences between the control group and each test group were analyzed using one-way analysis of variance, followed by Duncan's multiple-range test. Differences with P<.05 were considered to be statistically significant.

3. Results

Dietary intake; initial and final body weights; and weights of liver, kidney, frontal cortex and hippocampus did not significantly differ among the 0%, 0.5% and 1% EPA+DHA groups (Table 2). Table 3 shows

Table 3
Behavioral responses and plasma serotonin levels *

	0% EPA+DHA †	0.5% EPA+DHA	1% EPA+DHA
Immobility (s)	20.71±3.87 [a]	9.27±3.37 [b]	5.67±1.73 [b]
Swimming (s)	273.50±4.62	280.54±3.99	281.67±2.10
Climbing (s)	5.79±1.38 [a]	10.20±0.72 [b]	12.66±2.00 [b]
Serotonin (ng/ml)	22.11±2.63 [a]	117.24±25.75 [b]	76.71±12.64 [b]

* Data are mean±S.E.M. values. Values with different superscripts a,b within a row are significantly different at P<.05 according to Duncan's multiple range test.
† % EPA+DHA relative to the total energy intake in the diet.

Table 4
The fatty acid compositions of brain phospholipids *

Fatty acids (%)	0% EPA+DHA†	0.5% EPA+DHA	1% EPA+DHA
14:0	0.67±0.03	0.70±0.02	0.73±0.03
16:0	21.78±0.13	22.20±0.38	22.55±0.30
16:1n7	0.14±0.00[a]	0.18±0.01[b]	0.22±0.01[c]
18:0	20.87±0.21	21.12±0.26	20.56±0.22
18:1n9	22.14±0.21	22.26±0.17	22.06±0.33
18:2n6	0.46±0.01[a]	0.42±0.03[a]	0.24±0.02[b]
18:3n3	0.02±0.01[a]	0.05±0.01[b]	0.05±0.01[b]
20:4n6	11.47±0.08[a]	10.19±0.25[b]	9.36±0.09[c]
20:5n3	0.00±0.00[a]	0.07±0.02[b]	0.25±0.02[c]
22:4n6	3.94±0.06[a]	2.87±0.15[b]	2.33±0.05[c]
22:5n6	1.43±0.11[a]	0.16±0.01[b]	0.09±0.01[c]
22:5n3	0.19±0.05[a]	0.67±0.07[b]	0.93±0.05[c]
22:6n3	15.41±0.19[a]	17.07±0.31[b]	18.77±0.25[c]
Total n-3 PUFA	15.60±0.16[a]	17.82±0.28[b]	19.96±0.27[c]
Total n-6 PUFA	17.16±0.15[a]	14.13±0.37[b]	12.51±0.18[c]

* Data are mean±S.E.M. values. Values with different superscripts a,b,c within a row are significantly different at $P<.05$ according to Duncan's multiple range test.

† % EPA+DHA relative to the total energy intake in the diet.

the effects of the n-3 PUFA diet on behavioral activities during FST. The immobility time of rats on both the 0.5% and 1% DPA+DHA diets was significantly shorter than that of rats on the 0% EPA+DHA diet, while the climbing time was significantly longer in both the 0.5% and 1% EPA+DHA groups. However, swimming time did not significantly differ among groups. Thus, there was no dose-dependent effect of EPA+DHA on behavioral response. Plasma serotonin concentration was also significantly higher in both the 0.5% and 1% EPA+DHA groups compared to that in the 0% EPA+DHA group (Table 3).

Table 4 shows that the diets had a significant effect on the fatty acid composition of brain phospholipid. The levels of n-3 PUFA, ALA, EPA, docosapentaenoic acid (22:n3) and DHA were the highest in the 1% EPA+DHA group, followed by those in the 0.5% EPA+DHA group and finally those in the 0% EPA+DHA group. In contrast, the levels of n-6 PUFA, 18:2n6, 20:4n6, 22:4n6 and 22:5n6 were highest in the 0% EPA+DHA group, followed by those in the 0.5% EPA+DHA group and then those in the 1% EPA group. Monounsaturated fatty acid 16:1n7 levels in the brain were also significantly increased with increasing levels of EPA+DHA in the diet. There were no significant differences in other fatty acids among the groups.

In the hippocampus of rats fed 0.5% and 1% EPA+DHA, the expression of IL-6 was significantly reduced and the expression of CREB was significantly increased compared with those in the 0% EPA+DHA group (Fig. 1). However, the expressions of IL-1β, TNF-α, pCREB and BDNF did not significantly differ among the groups.

4. Discussion

As hypothesized, long-term intake of EPA+DHA induced antidepressant-like effects in rats during FST and caused some changes in the hippocampus cAMP cascade, leading to overexpression of CREB via decreased IL-6 expression. This is consistent with recent studies that revealed that dietary supplementation with ALA reduced immobility and climbing during FST when administered for 30 days [14], 6 weeks [15,16] and 15 weeks [11]. Several studies of patients with depression have reported reduced n-3 PUFA in plasma [21,22] or erythrocyte membranes [3,4]. Moreover, significant benefits of n-3 PUFA with antidepressant medications were demonstrated in three double-blind, placebo-controlled trials [2,23,24]. Thus, we observed that consumption of n-3 PUFA, particularly EPA+DHA, has an effect on the development of antidepressant-like behaviors in rats according to the FST. Furthermore, our study results were consistent with those of previous studies demonstrating that the antidepressant-like effects of n-3 PUFA were apparent only with long-term dietary enrichment and not after shorter regimens because short-term

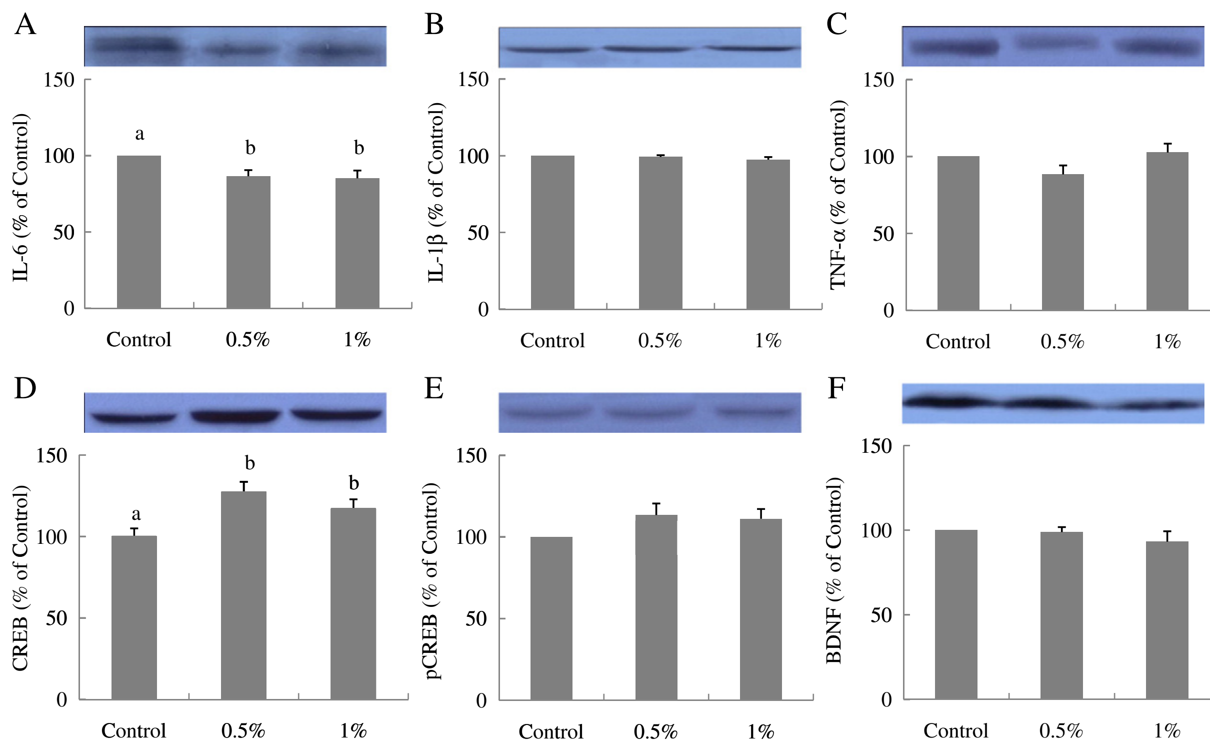

Fig. 1. The expressions of IL-6 (a), IL-1β (b), TNF-α (c), CREB (d), pCREB (e) and BDNF (f) were measured using Western blot. Representative Western blot images for IL-6, IL-1β, TNF-α, CREB, pCREB and BDNF from frontal cortices of subjects in the control (0% EPA+DHA) group, 0.5% EPA+DHA group and 1% EPA+DHA group. Data were compared using one-way analysis of variance and mean±S.E.M. for $n=8$ independent samples, Values with different superscripts within a row are significantly different at $P<.05$ according to Duncan's multiple range test.

administration of n-3 PUFA failed to produce antidepressant-like effects in the FST [14,25]. The reason why a delay of several weeks was necessary to produce an antidepressant-like effect remains undetermined. However, it may be that n-3 PUFA supplementation slowly modifies the cellular membrane phospholipid concentrations that finally lead to the modification of neurotransmission in the serotonergic system [26].

One of the biological mechanisms that could explain this result is the regulation of neurotransmitters and signal transduction by n-3 PUFA. The change in fatty acid concentration in the brain, induced by dietary intake of n-3 PUFA, may alter serotonergic and dopaminergic neurotransmissions and then lead to an increase in the serotonin receptor level (5-HT_2) [27]. The up-regulation of the 5-HT receptor is thought to play a role in the pathophysiology of depression [28]. High cerebrospinal fluid concentration of 5-hydroxyindoleacetic acid, a metabolite of serotonin and an indicator of brain serotonin turnover [29], has been shown to be positively associated with high plasma concentration of n-3 PUFA in healthy subjects [30], and lower serotonergic activity has been well established in the pathophysiology of depression [31].

In the present study, serotonin concentration was significantly higher in the 0.5% and 1% EPA+DHA groups than in the 0% EPA+DHA group, although there was no dose-dependent effect of n-3 PUFA. Additionally, we found that rats fed the 0.5% and 1% EPA+DHA diets had significantly lower IL-6 levels than did those receiving 0% EPA+DHA, suggesting that decreased IL-6 expression may be related to increased serotonin concentration. The proinflammatory cytokine IL-6 has been shown to be both directly and indirectly associated with depression [9]. Interestingly, antidepressant medications that inhibit serotonin reuptake can also inhibit the releases of IL-6, IL-1β and TNF-α [32].

Eicosapentaenoic acid may act as an inhibitor of phospholipase A_2 to reduce the secretions of eicosanoids and proinflammatory cytokines that might be associated with the reversal of loss of interest, fatigue, loss of energy, poor appetite and inability to concentrate often seen in patients with depression [32,33]. Although DHA is a major structural component of phospholipid in the neuronal cell membrane [15,25,34], EPA rather than DHA appears to be the effective agent in clinical studies [2,23]. This contradiction may suggest that EPA has other important physiological functions, including roles as a precursor for eicosanoids and a modulator of cytokines [9].

In addition to elevated proinflammatory cytokine levels, patients with major depression are more likely to have high levels of plasma prostaglandin E2 [35,36]. Prostaglandin E2 and cytokines may up-regulate phosphodiesterase E4, which increases the expression of CREB, which requires phosphorylation in order to transcribe CREB-regulated genes including BDNF [37].

Venna et al. [16] previously reported that 6 weeks of ALA deprivation in mice significantly decreased BDNF expression. In addition, Rao et al. [10] observed that ALA deprivation in rats for 15 weeks significantly reduced BDNF expression, CREB transcription factor activity and p38 mitogen-activated protein kinase (MAPK) activity. This suggests that decreased basal p38 MAPK activity may be responsible for the decreased phosphorylation of CREB, which could be responsible for the decreased CREB DHA-binding activity and ultimately for the reduced transcription and translation of BDNF.

However, we found that CREB but not BDNF or pCREB expression was significantly increased in the 0.5% and 1% EPA+DHA groups compared with that in the 0% EPA+DHA group. This inconsistency between the present study and other studies [10,16] may be due to the different types of n-3 PUFA (EPA+DHA vs. ALA) or to the differences in the amounts of n-3 PUFA in the diets (control and n-3 PUFA supplementation vs. control and n-3 PUFA deprivation). Deprivation of n-3 PUFA may have a deleterious impact on depression rather than the favorable effect n-3 PUFA supplementation appears to give. In addition, the Rao et al. study [10] failed to result in changes in the fatty acid composition of the brain after deprivation of n-3 PUFA, and Venna et al. [16] did not measure the fatty acid composition. We, however, we found a significant increase in brain n-3 PUFA levels.

Unfortunately, there were a few limitations of the study: (a) there was no active control group, e.g., rats with antidepressant treatment; (b) fatty acid composition was examined in the whole brain, not specifically in the hippocampus; (c) levels of kinases related to the cAMP cascade were not measured; (d) the differences observed in this study could be partly due to decreased n-6 PUFA and (e) a dose-dependency of effect could not be detected partly due to the small sample size.

In conclusion, our results demonstrated that n-3 PUFA EPA and DHA supplementations had a beneficial effect on preventing the development of depression-like behaviors and may exert their therapeutic efficacy through CREB overexpression in this rat model. However, we found that there was no dose-dependent effect of EPA and DHA consumption. This is the first study that not only determined antidepressant-like effects of long-term intake of EPA and DHA in a behavioral study, but also characterized their mechanisms of action. Future studies should examine the roles of n-3 PUFA in the MAP pathway, BDNF expression, neurogenesis and synaptogenesis in other brain diseases.

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