Title

n-3 fatty acids effectively improve the reference memory-related learning ability associated with increased brain docosahexaenoic acid-derived docosanoids in aged rats

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Journal

Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, Volume 1851, Issue 2

Published

February 2015

URL

https://doi.org/10.1016/j.bbalip.2014.10.009

n-– 3([-] of all[n - 3] and/or[n - 6] in the profis very long. Please check the correct lenght.) fatty acids effectively improve the reference memory-related learning ability associated with increased brain docosahexaenoic acid-derived docosanoids in aged rats

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Abstract

We investigated whether a highly purified eicosapentaenoic acid (EPA) and a concentrated n-– 3 fatty acid formulation (prescription TAK-085) containing EPA and docosahexaenoic acid (DHA) ethyl ester could improve the learning ability of aged rats and whether this specific outcome ~~has~~ had any relation with the brain levels of EPA-derived eicosanoids and DHA-derived docosanoids. The rats were tested for reference memory errors (RMEs) and working memory errors (WMEs) in an eight-arm radial maze. Fatty acid compositions were analyzed by GC, whereas brain eicosanoid/docosanoids were measured by LC-–ESI-MS–MS-based analysis. The levels of lipid peroxides (LPOs) were measured by thiobarbituric acid reactive substances. ~~Compared with the control rats,~~ The administration of TAK-085 at 300 mg-–kg^{-1} day^{-1} for 17 weeks reduced the number of RMEs in aged rats compared with that in the control rats. Both TAK-085 and EPA administration increased plasma EPA and DHA levels in aged rats, with concurrent increases in DHA and decreases in arachidonic acid in the corticohippocampal brain tissues. TAK-085 administration significantly increased the formation of EPA-derived 5-HETE and DHA-derived 7-, 10-, and 17-HDoHE, PD1, RvD1, and RvD2. ARA-derived PGE_2, PGD_2, and $PGF_{2\alpha}$ significantly decreased in TAK-085-treated rats. DHA-derived mediators demonstrated a significantly negative correlation with the number of RMEs, whereas EPA-derived mediators did not exhibit any relationship. Furthermore, compared with the control rats, the levels of LPO in the plasma, cerebral cortex, and hippocampus were significantly reduced in TAK-085-treated rats. The findings of the present study suggest that long-term EPA + DHA administration may be a possible preventative strategy against age-related cognitive decline.

Abbreviations: ARA, arachidonic acid; BHT, 2,6-di-t-butyl-4-methyl-phenol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; 5-HETE, 5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid; 5-HEPE, 5-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid; 12-HEPE, 12-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid; 15-HEPE, 15-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid; HDoHE, hydroxy docosahexaenoic acid; HPLC, high-performance liquid chromatography; LPO, lipid peroxide; PG, prostaglandin; PD1, protectin D1; PLA_2, phospholipase A_2; PUFAs, polyunsaturated fatty acids; RME, reference memory error; SRM, selected reaction monitoring; WME, working memory error

Keywords: Brain; PUFA-derived mediators; Memory; Aging; EPA and DHA

1 Introduction

Docosahexaenoic acid (DHA, C22:6, n-3) and eicosapentaenoic acid (EPA, C20:5, n-3) are the primary n-3 polyunsaturated fatty acids (PUFAs) in fish oil, and epidemiological studies have revealed that fish oil intake has significant neuroprotective effects in aging [1,2]. Increased fish consumption and DHA + EPA intake are both associated with reduction in cognitive decline [3]. In addition, daily DHA and EPA supplementation has beneficial effects against age-related cognitive decline in otherwise health elderly Japanese individuals with very mild dementia [4]. These findings suggest that increased consumption of n-3 PUFAs is associated with reduced risk of age-related cognitive decline and dementia.

DHA deficiency, as observed with aging and dementia [5], impairs memory and learning and promotes age-related neurodegenerative diseases [6]. Vertebrates do not have adequate metabolic capacity to biosynthesize DHA; thus, vertebrates, including humans, depend on the diet to supply this fatty acid. As expected, dietary DHA ameliorates the learning-related spatial memory of DHA-deficient rats [7–10]. Positively, the administration of a DHA precursor (i.e., EPA) should be expected to cause the neurobehavioral outcome caused by DHA. Dietary EPA has been shown to increase DHA levels in brain tissues of rats, concurrently with the enhancement of spatial cognition [11] and modulation of synaptic plasticity [12]. From these results, it is apparent that EPA and/or DHA ~~could~~ can be used to prevent memory deficit. However, the roles of DHA- or EPA-derived mediators such as docosanoids and eicosanoids, which have strong anti-inflammatory properties [10], on learning memory have remained largely unexplored. In particular, DHA-derived mediator neuroprotectin/protectin D1 (PD1) is an endogenous compound with anti-inflammatory, antioxidant, and antiapoptotic effects [13–15].

Aging is characterized by a low neuroinflammatory and high brain oxidative stress with low levels of DHA and DHA-derived mediators [6]; cognitive decline is also associated with the decreased DHA levels in the brain. Literature reviews suggest improved performance after n-3 PUFA supplementation in aged animals [16,17]. However, the relationship between the learning ability and these n-3 PUFA-derived mediators in the brain has not been studied. We therefore investigated whether the prescription administration of n-3 fatty acids (TAK-085; highly purified and concentrated EPA and DHA ethyl esters) or EPA alone improves cognitive learning ability and, if so, whether the brain levels of PUFA-derived eicosanoids and docosanoids are related to the enhancement of spatial learning-related cognitive functions.

2 Materials and methods

2.1 Animals and treatment

Aged male Wistar rats (70 weeks old), the offspring of rats maintained for two generations on a standard F1 pellet containing no fish products (Funabashi Farm, Funabashi, Japan), were used in the study. After acclimatization, the rats were randomly divided into three groups: control rats ($n = 8$), TAK-085-treated rats ($n = 8$), and EPA-treated rats ($n = 8$). The TAK-085 group was orally fed TAK-085 (Pronova BioPharma ASA, Oslo, Norway; containing 467 mg/g EPA, 365 mg/g DHA, and 3.8 mg/g α-tocopherol); the EPA group was orally fed EPA (Nisshin Pharma Inc., Tokyo, Japan; containing 980 mg/g EPA and 1.9 mg/g α-tocopherol) emulsified in 5% gum Arabic solution at 300 mg/kg BW/day; and the control group was orally fed a similar volume of 4.8 mg/g α-tocopherol in 5% gum Arabic. The rats were cared for and killed in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. The oral administration of TAK-085 and EPA emulsion and/or gum Arabic solution was continued for 17 weeks.

2.2 Eight-arm radial maze task

Ten weeks after the start of TAK-085/EPA administration, the learning-related behavior of the rats was assessed by an eight-arm radial maze, as described previously [7,9]. Each rat was tested by two daily trials for 6 days/week for a total of 5 weeks. The trial consisted of baiting only four arms (consistently the same arm for any one rat) with reward pellets and placing the rat in the center of the platform facing a randomly selected arm. Two parameters of memory function were examined: (i) reference memory error (RME), determined by the number of entries into the unbaited arms, and (ii) working memory error (WME), determined by the number of repeated entries into arms that had already been visited during the trial.

2.3 Sample preparation

After completing the behavioral studies, the rats were anesthetized with sodium pentobarbital (65 mg/kg BW, i.p.), blood was collected, and the cerebral cortex and hippocampus were separated as described previously [8]. The tissues were stored at − 80 °C by flash-freezing in liquid N_2 until use or immediately homogenized in ice-cold phosphate buffer (pH 7.4) containing 0.005% 2,6-di-t-butyl-4-methyl-phenol (BHT).

2.4 Sample preparation for analysis of fatty acid-derived metabolites

Brain homogenates were adjusted to 67% methanol and were centrifuged at 5000 × *g* for 10 min at 4 °C to remove precipitated proteins. The supernatant fractions were adjusted to 10% (v/v) methanol. Internal standards (5 ng of PGE_2-*d4*, PGD_2-*d4*, $PGF_{2\alpha}$-*d4*, 5-HETE-*d8*, and ARA-*d8*) were added to each sample. Samples were acidified to pH 4.0 with 0.1 M HCL and immediately applied to preconditioned solid-phase extraction cartridges (Sep-Pak C18; Waters, Milford, MA, USA) to extract the fatty acid metabolites. Finally, fatty acid metabolites were eluted with 10 mL methyl formate.

2.5 LC–ESI-MS–MS-based analysis

Fatty acid metabolites in the cerebral cortex were measured as described previously, with a slight modification [18,19]. High-performance liquid chromatography (HPLC) was combined with ESI–MS using a TSQ quantum mass spectrometer (Thermo Fisher Scientific K.K., Tokyo, Japan). HPLC was performed using a Luna 3u C18(2) 100 Å LC column (100 × 2.0 mm; Phenomenex, Torrance, CA, USA) at 30 °C. MS–MS analyses were conducted in the negative ion mode, and fatty acid metabolites were detected and quantified by selected reaction monitoring (SRM). Peaks were selected, and their areas were calculated using the Xcalibur 2.1 software (Thermo Fisher Scientific K.K.).

2.6 Measurement of LPO and fatty acid profiles

LPO concentrations were assessed as described previously [8], against 1,1,3,3-tetraethoxypropane as the standard. The fatty acid composition of plasma and brain tissues was determined by gas chromatography, as described previously [8]. Protein concentrations were estimated using the method of Lowry et al. [20].

2.7 Statistical analysis

The results are expressed as mean ± SEM. Behavioral data were analyzed using two-way (group and block) repeated measures ANOVA and all other parameters were analyzed for intergroup differences by one-way ANOVA. ANOVA was followed by Fisher's PLSD for post hoc comparisons. Correlations were determined by simple regression analysis. The statistical programs used were GB-STAT™ 6.5.4 (Dynamic Microsystems) and Stat-View® 4.01 (MindVision Software, Abacus Concepts). Differences with P values < 0.05 were considered significant.

3 Results

3.1 Body weight

Final body weights did not differ among the three groups (control group, 379 ± 7 g; TAK-085, 374 ± 7 g; EPA, 365 ± 6 g).

3.2 Effect of TAK-085 and EPA administration on radial maze learning ability

The effects of long-term administration of TAK-085 and EPA alone on reference and working memory-related learning abilities are presented in Fig. 1A and B, respectively, as the mean number of RMEs and WMEs for each group, with data averaged in blocks of 6 trials. Detailed results are shown in Supplemental Table 1. Two-way (block and group) repeated measures ANOVA revealed a significant main effect of both groups and blocks of trials, with a significant group × block interaction in relation to the number of RMEs (Fig. 1A). With respect to the number of WMEs, two-way (block and group) repeated measures ANOVA revealed a significant main effect of both groups and blocks of trials, without a significant group × block interaction (Fig. 1B).

Post hoc analyses of RMEs and WMEs in each block revealed that the number of RMEs but not WMEs was significantly lower in TAK-085-treated rats and EPA-treated rats (than in the controls) in blocks 7 to 10 and in blocks 7 to 9, respectively (Fig. 1A). Interestingly, the number of RMEs was significantly lower in TAK-085-treated rats than in EPA-treated rats in block 10 (Fig. 1A).

Subtest analyses of RMEs and WMEs revealed that the number of RMEs but not WMEs was significantly lower in the TAK-085-treated rats than in the controls (Fig. 1). However, subtest analysis revealed that there was no significant difference in the number of RMEs and WMEs between the EPA-treated rats and the control rats (Fig. 1). Subtest analysis also revealed that there was no significant difference in the number of RMEs and WMEs between the TAK-085- and EPA-treated rats (Fig. 1). These results finally suggest that long-term administration of TAK-085 but not EPA alone improved reference memory-related learning ability but not working memory-related learning ability in aged rats.

3.3 Lipid profiles of plasma and brain

The plasma fatty acid profiles of the rats are shown in Table 1. The plasma levels of EPA, DHA, and docosapentaenoic acid [DPA, C22:5(n−3)] were significantly higher in the EPA- and TAK-085-treated rats than in the control rats, whereas the plasma level of ARA was significantly lower in the EPA-treated rats than in the control rats. The plasma levels of EPA and DPA were significantly higher in the EPA-treated rats than in the TAK-085-treated rats, and those of DHA were higher in the TAK-085-treated rats than in the EPA-treated rats. The plasma levels of oleic acid were significantly lower in the EPA- and TAK-085-treated rats than in the control rats, and those of stearic acid were significantly lower in the TAK-085-treated rats than in the control rats. EPA and TAK-085 administration significantly decreased the plasma n−6/n−3 molar ratio and increased the plasma DHA/AA molar ratio and the unsaturated index (USI). However, their administration did not affect the plasma levels of palmitic acid and linoleic acid.

Table 1 Plasma fatty acid profiles.

	Control group (n = 9)	EPA group (n = 10)	TAK-085 group (n = 9)
Palmitic acid $_{C16:0}$	23.35 ± 0.37	22.33 ± 0.43	23.11 ± 0.25

Stearic acid$_{C18:0}$	16.49 ± 0.52^{a}	15.09 ± 0.43^{a,b}	14.17 ± 0.25^{b}
Oleic acid$_{C18:1(n-9)}$	15.34 ± 0.66^{a}	12.18 ± 0.55^{b}	12.34 ± 0.64^{b}
Linoleic acid$_{C18:2(n-6)}$	18.06 ± 0.65	18.57 ± 0.43	19.40 ± 0.53
Arachidonic acid$_{C20:4(n-6)}$	22.07 ± 1.15^{a}	17.93 ± 0.92^{b}	18.96 ± 0.78^{a,b}
Eicosapentaenoic acid$_{C20:5(n-3)}$	0.82 ± 0.03^{c}	7.99 ± 0.37^{a}	5.13 ± 0.25^{b}
Docosapentaenoic acid$_{C22:5(n-3)}$	0.37 ± 0.03^{c}	1.49 ± 0.06^{a}	1.01 ± 0.04^{b}
Docosahexaenoic acid$_{C22:6(n-3)}$	1.54 ± 0.06^{c}	2.74 ± 0.11^{b}	4.21 ± 0.16^{a}
n-6/n-3	13.69 ± 0.44^{a}	2.97 ± 0.12^{b}	3.67 ± 0.15^{b}
C22:6(n-3)/C20:4(n-6)	0.069 ± 0.003^{b}	0.154 ± 0.005^{a}	0.223 ± 0.012^{a}
Unsaturation index (USI)	156.3 ± 3.39^{b}	186.0 ± 2.78^{a}	184.2 ± 2.82^{a}

The fatty acid values are expressed as mol%; results are means ± SE. Values without a common alphabet in the same ~~column~~ ~~(row)~~ ("column" is "row") are significantly different at $P < 0.05$.

The fatty acid profiles in the rat cerebral cortex are shown in Table 2. The DHA, DPA, and linoleic acid levels in the cerebral cortex were significantly higher in the EPA- and TAK-085-treated rats than in the control rats, whereas the ARA levels were significantly lower in the EPA- and TAK-085-treated rats than in the control rats. The linoleic acid and ARA levels did not differ between the EPA- and TAK-085-treated rats. The cerebral cortex DPA levels were significantly higher in the EPA-treated rats than in the TAK-085-treated rats, and the DHA levels were higher in the TAK-085-treated rats than in the EPA-treated rats. EPA and TAK-085 administration significantly decreased the cerebral cortex n-6/n-3 molar ratio and increased the DHA/AA molar ratio; however, their administration did not affect the cerebral cortex levels of palmitic acid, stearic acid, oleic acid, and EPA.

Table 2 Fatty acid profiles of cerebral cortex.

	Control group (n = ~~=~~9)	EPA group (n = ~~=~~10)	TAK-085 group (n = ~~=~~9)
Palmitic acid$_{C16:0}$	25.24 ± 0.29	25.48 ± 0.32	25.18 ± 0.27
Stearic acid$_{C18:0}$	25.57 ± 0.27	25.53 ± 0.18	25.33 ± 0.23
Oleic acid$_{C18:1(n-9)}$	19.21 ± 0.48	18.37 ± 0.43	18.94 ± 0.42
Linoleic acid$_{C18:2(n-6)}$	0.72 ± 0.03^{b}	0.90 ± 0.03^{a}	0.89 ± 0.02^{a}
Arachidonic acid$_{C20:4(n-6)}$	12.30 ± 0.33^{b}	11.27 ± 0.30^{a}	10.75 ± 0.16^{a}
Eicosapentaenoic acid$_{C20:5(n-3)}$	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Docosapentaenoic acid$_{C22:5(n-3)}$	0.10 ± 0.01^{c}	0.34 ± 0.01^{a}	0.22 ± 0.01^{b}
Docosahexaenoic acid$_{C22:6(n-3)}$	15.39 ± 0.20^{c}	16.80 ± 0.38^{b}	17.33 ± 0.27^{a}
n-6/n-3	0.84 ± 0.03^{a}	0.71 ± 0.24^{b}	0.66 ± 0.13^{b}
C22:6(n-3)/C20:4(n-6)	1.26 ± 0.04^{b}	1.50 ± 0.05^{a}	1.61 ± 0.03^{a}
Unsaturation index (USI)	163.8 ± 1.16	168.8 ± 2.04	169.8 ± 1.52

The fatty acid values are expressed as mol%; results are means ± SE. Values without a common alphabet in the same ~~column~~ ("column" is "row".) are significantly different at $P < 0.05$.

The fatty acid profiles of the rat hippocampus are shown in Table 3. The DHA levels in the hippocampus were significantly higher in the TAK-treated rats than in the EPA-treated and control rats, and the DPA and linoleic acid levels were significantly higher in the EPA- and TAK-085-treated rats than in the control rats. The ARA levels were significantly decreased in the EPA- and TAK-085- treated rats than in the control arts, whereas the ARA levels did not differ between the EPA- and TAK-085-treated rats.

The hippocampal stearic acid levels were significantly lower in the EPA-treated rats than in the TAK-085-treated and control rats, and the oleic acid levels were significantly higher in the EPA-treated rats than in the TAK-085-treated and control rats. EPA and TAK-085 administration significantly decreased the hippocampal n-6/n-3 molar ratio and increased the DHA/AA molar ratio; however, their administration did not affect the hippocampal levels of palmitic acid and EPA.

Table 3 Fatty acid profiles of hippocampus.

	Control group (n = 9)	EPA group (n = 10)	TAK-085 group (n = 9)
Palmitic acid$_{C16:0}$	25.12 ± 0.09	24.68 ± 0.24	24.88 ± 0.12
Stearic acid$_{C18:0}$	26.01 ± 0.10^{a}	25.28 ± 0.10^{b}	25.78 ± 0.13^{a}
Oleic acid$_{C18:1(n-9)}$	19.50 ± 0.24^{b}	20.85 ± 0.45^{a}	19.66 ± 0.19^{b}
Linoleic acid$_{C18:2(n-6)}$	0.54 ± 0.01^{b}	0.69 ± 0.02^{a}	0.65 ± 0.01^{a}
Arachidonic acid$_{C20:4(n-6)}$	13.81 ± 0.31^{a}	12.35 ± 0.21^{b}	12.95 ± 0.15^{b}
Eicosapentaenoic acid$_{C20:5(n-3)}$	0.06 ± 0.01	0.08 ± 0.01	0.07 ± 0.01
Docosapentaenoic acid$_{C22:5(n-3)}$	0.10 ± 0.01^{c}	0.34 ± 0.01^{a}	0.21 ± 0.01^{b}
Docosahexaenoic acid$_{C22:6(n-3)}$	13.17 ± 0.11^{b}	13.49 ± 0.23^{b}	14.16 ± 0.19^{a}
n-6/n-3	1.08 ± 0.02^{a}	0.94 ± 0.01^{b}	0.94 ± 0.02^{b}
C22:6(n-3)/C20:4(n-6)	0.96 ± 0.02^{b}	1.09 ± 0.02^{a}	1.10 ± 0.02^{a}
Unsaturation index (USI)	156.7 ± 1.23	156.1 ± 1.4	160.2 ± 0.9

The fatty acid values are expressed as mol%; results are means ± SE. Values without a common alphabet in the same column ("column" is "row".) are significantly different at $P < 0.05$.

3.4 LPO levels in plasma, cerebral cortex, and hippocampus

The LPO levels in the plasma, cerebral cortex, and hippocampus are shown in Table 4. Compared with the control rats, the LPO levels were significantly lower in the cerebral cortex and hippocampus of TAK-085-treated rats, whereas only the LPO level in the hippocampus was significantly lower in the EPA-treated rats. However, their administration did not affect plasma levels of LPO (Table 4).

Table 4 Lipid peroxide levels of plasma, cerebral cortex and hippocampus.

	Control group (n = 9)	EPA group (n = 10)	TAK-085 group (n = 9)
Plasma (nmol/mL)	16.65 ± 1.22	13.52 ± 0.79	15.34 ± 1.20
Cerebral cortex (nmol/mg protein)	0.28 ± 0.04^{a}	0.20 ± 0.02^{a,b}	0.16 ± 0.01^{b}
Hippocampus (nmol/mg protein)	0.34 ± 0.02^{a}	0.27 ± 0.01^{b}	0.28 ± 0.01^{b}

The levels of lipid peroxides (LPO) were measured as thiobarbituric acid reactive substances. Results are means ± SE. Values without a common alphabet in the same column ("column" is "row".) are significantly different at $P < 0.05$.

3.5 Levels of eicosanoids and docosanoids in the cerebral cortex of aged rats

The levels of ARA-, EPA-, and DHA-derived mediators in the cerebral cortex are shown in Fig. 2. TAK-085 administration significantly increased the formation of EPA-derived 5-HETE and DHA-derived 7-, 10-, and 17-HDoHE, PD1, RvD1, and RvD2. On the other hand, compared with the control rats, the endogenous formation of ARA-derived PGE_2, PGD_2, and $PGF_{2\alpha}$ was significantly decreased in the TAK-085-treated rats. Compared with the control rats, the formation of ARA-derived 5-, 12-, and 15-HETE, EPA-derived 5-, 12-, and 15-HEPE, and DHA-derived PD1 and RvD2 was significantly increased in the EPA-treated rats. However, the formation of DHA-derived PD1, RvD1, and RvD2 was significantly lower in the EPA-treated rats than in the TAK-085-treated rats.

The formation of ARA-derived PGD_2 and 5- and 12-HETE in the EPA-treated rats was significantly higher than that in the TAK-085-treated rats.

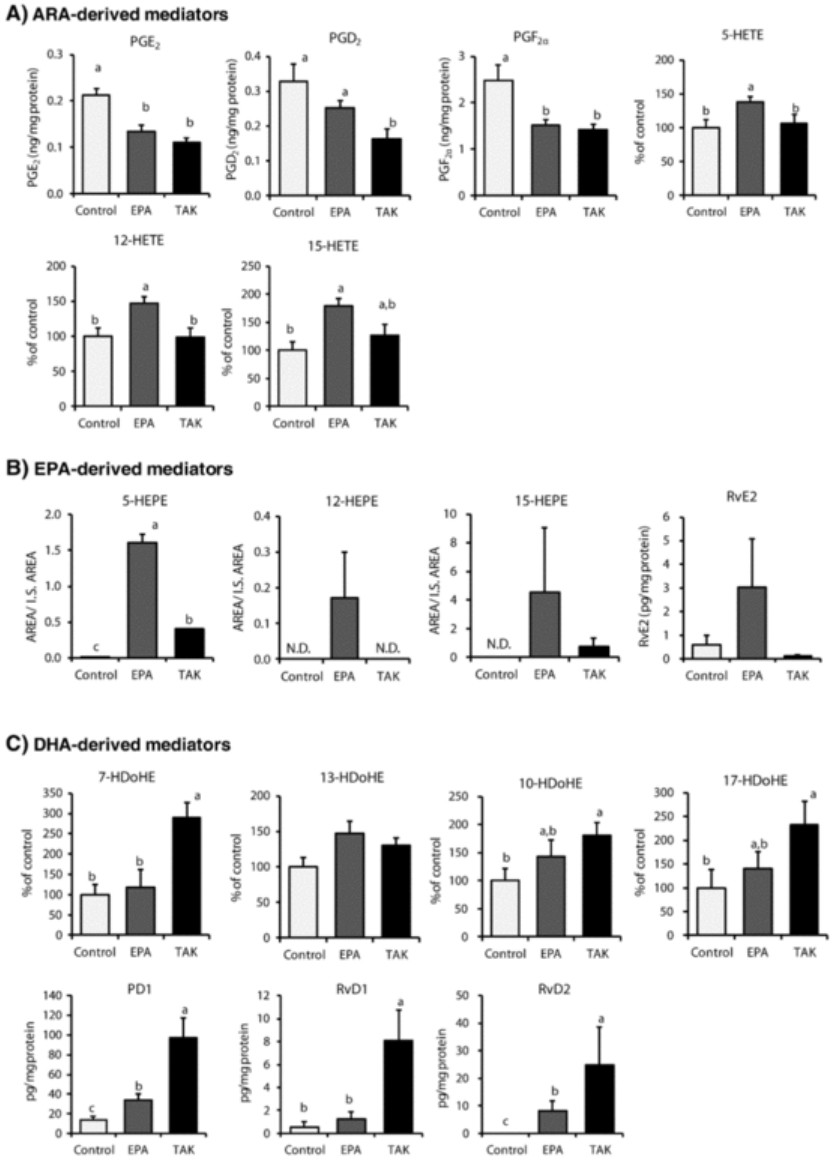

Fig. 2 Effect of TAK-085 and EPA administration on the eicosanoids and docosanoids in the rat brain cortex.

Data are presented as the mean ± SEM. Bars without a common alphabet are significantly different at $P < 0.05$. Data were analyzed by one-way ANOVA followed by Fisher's PLSD post hoc for multiple comparisons. Control, control rats (n = 8); EPA, EPA-treated rats (n = 8); TAK, TAK-085-treated rats (n = 8); 7-HDoHE, 7-hydroxy docosahexaenoic acid; 13-HDoHE, 13-hydroxy docosahexaenoic acid; 10-HDoHE, 10-hydroxy docosahexaenoic acid; 17-HDoHE, 17-hydroxy docosahexaenoic acid; 5-HETE, 5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid; 5-HEPE, 5-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid; 12-HEPE, 12-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid; 15-HEPE, 15-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid; PD1, protectin D1; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; RvD1, resolvin D1; RvD2, resolvin D2; RvE2, resolvin E2.

3.6 Correlation between cognitive function and LPO levels and the formation of eicosanoids and docosanoids in the cerebral cortex

Regression analyses revealed significant positive correlations between the number of RMEs in the final block of the radial maze task and the formation of the ARA-derived PGD_2 and negative correlations between the number of RMEs and the formation of the DHA-derived 7- and 17-HDoHE, PD1, and RvD2 in the cerebral cortex of aged rats (Table 5). The formation of ARA-derived $PGF_{2\alpha}$ and the number of RMEs tended to be positively correlated ($P = 0.055$; Table 5).

Table 5 Correlation coefficients between learning ability and LPO levels in the cerebral cortex and the formation of the ARA- and DHA-derived mediators in the cerebral cortex of aged rats.

	ARA-derived mediators			DHA-derived mediators				
	PGE_2	PGD_2	$PGF_{2\alpha}$	7-HDoHE	17-HDoHE	PD1	RvD1	RvD2
Reference memory error								
Coefficiency (r)	NS	0.505	0.396	−0.474	−0.485	−0.605	−0.228	−0.499
P value		0.012	0.055	0.019	0.016	0.002	NS	0.013
TBARS								
Coefficiency (r)	0.558	NS	NS	−0.385	NS	−0.407	−0.364	NS
P value	0.005			0.063		0.048	0.08	

The number of RMEs in block 10 shown in Fig. 1 was used as an indicator of learning ability. Data were analyzed by simple regression analysis. TBARS, thiobarbituric acid reactive substances. Abbreviations of mediators are the same as those used in Fig. 2. Differences with *P* values < 0.05 were considered significant. NS, not significant, $P \geq 0.10$.

A significant positive correlation was observed between cerebral cortex LPO and the formation of ARA-derived PGE_2 (Table 5). On the other hand, there was a significant negative correlation between LPO and the formation of the DHA-derived PD1 and a negative correlation between DHA-derived 7-HDoHE and RvD1 (Table 5).

4 Discussion

This ~~is the~~ report ~~to demonstrate~~demonstrates the relationship between reference memory-related learning ability and the n−3 PUFA-derived mediators in the cerebral cortex of aged rats. The brain membranes of aged rats contain ~~lesser~~less DHA than those of young rats [21]. The age-related spatial cognitive decline may be prevented by the administration of dietary supplements of DHA in aged rats [16,17]. With regard to the controls, TAK-085 (containing 467 mg/g EPA and 365 mg/g DHA) had a more noticeable influence on reference memory-related learning ability than EPA alone (Fig. 1).

A previous human interventional study showed that EPA administration does not affect plasma levels of DHA [22]. However, previous studies involving rats have reported that EPA administration increases plasma levels of DHA [23,24], indicating that dietary EPA administration upregulates rat plasma levels of DHA. EPA infusion increases plasma levels of DHA but does not affect levels of DHA in the liver, heart, lung, kidney, and spleen of rats within 7 days [23]. In the present study, EPA comprised only a small amount of total PUFAs in the brain in comparison with DHA (Tables 2 and 3). The EPA level in the cortex and hippocampus of aged rats was not affected by the administration of EPA and TAK-085, whereas the DHA level in the cortex and/or hippocampus of EPA- and TAK-085-treated rats increased. Thus, an increase in DHA but not EPA in the corticohippocampal tissues may be attributed to the molecular composition of corticohippocampal neurons and the resultant spatial cognition in aged rats. Long-term administration of EPA ameliorated the learning ability of young rats, concurrent with the increase in the corticohippocampal DHA level [11]. Our results are consistent with the fact that ^{14}C-labeled EPA levels in the rat brain decrease after its oral administration, whereas those of [^{14}C]DHA, a metabolite of EPA, increase in a time-dependent manner [25]. Moreover, it has been reported that the low levels of EPA in the brain are maintained by multiple redundant pathways, including β-oxidation, decreased incorporation from the plasma unesterified fatty acid pool, elongation/desaturation to n−3 docosapentaenoic acid, and lower recycling within brain phospholipids [26]. These results indicate that neuronally available EPA is continuously being converted to DHA. Despite the increase in the DHA level in the brain of EPA-treated rats, aged rats have not demonstrated improvements in spatial memory (Fig. 1B). This discrepancy may result from the fact that we used aged rats instead of young rats.

DHA, a major component of synaptic membranes, influences the membrane microenvironment and modulates neurotransmitter uptake and release, thereby playing a role in memory and neuroprotection [5]. ARA is preferentially released from membrane phospholipids by the action of endogenous phospholipase A_2 (PLA_2), and ARA-derived mediators participate in signal transduction events. Similarly, free DHA released from synaptic membranes by PLA_2 is transformed into the bioactive docosanoids via enzymatic oxidation ~~[23]~~[27]. The DHA-derived docosanoids set in motion endogenous signaling to sustain homeostatic synaptic and integrity, and docosanoids such as PD1 act as a protective sentinel of neural cell homeostasis [6,~~23~~27]. EPA- and DHA-derived mediators, resolvins and neuroprotectins, are also produced in the brain to counteract and resolve neuroinflammation, which is associated with both neurobiological injury and neurological disease.

However, there are no reports on the role of the DHA-derived mediators in spatial cognitive learning ability. In this experiment, TAK-085 administration significantly increased the endogenous formation of DHA-derived 7-, 10-, and 17-HDoHE, PD1, RvD1, and RvD2, while it significantly decreased the formation of ARA-derived PGE_2, PGD_2, and $PGF_{2\alpha}$ in the cerebral cortex of aged rats (Fig. 2). This was associated with the negative correlation between the number of RMEs in the final block of the radial maze task and the formation of DHA-derived 7- and 17-HDoHE, PD1, and RvD2 and the positive correlation between the number of RMEs in the final block of the radial maze task and the formation of ARA-derived PGD_2 in the cerebral cortex of aged rats (Table 5). Docosanoids regulate the synthesis of eicosanoids derived from ARA ~~[24]~~[28]. These results suggest a possibility of the protective effect of TAK-085 against memory-related learning ability decline in aged rats through DHA and DHA-derived docosanoids such as PD1.

It has been reported that DHA reduces oxidative stress ~~[25,26]~~ [29,30]and protects against oxidative stress-induced cognitive impairment [8,9]. Dietary DHA has been found to improve traumatic brain injury-induced water maze memory deterioration and oxidative stress ~~[27]~~[31]. These findings corroborate our speculation that the TAK-085-induced improvement in memory-related learning ability decline in aged rats may be achieved, at least partially, through the inhibitory effect of DHA on oxidative stress. TAK-085 supplementation reduced the cerebral cortex LPO levels in the aged rats (Table 4). In addition, DHA-derived mediators, PD1 and RvD1, play a role in neuroprotection and memory [6]. Indeed, in the present study, PD1 levels in the cerebral cortex were increased by TAK-085 administration (Table 2), negatively correlating with cerebral cortex LPO levels (Table 5). It is thus conceivable that the potential antioxidant action of DHA and PD1 in the TAK-085-treated rats occurs through mechanisms that maintain synaptic plasticity and increase memory ability. In other words, TAK-085 counteracts the age-related increase in oxidative stress, with subsequent DHA- and PD1-mediated effects on synaptic plasticity and cognition.

Moreover, long-term administration of EPA has a neuroprotective effect on the modulation of rat hippocampal synaptic plasticity by its capacity to increase brain DHA levels and its direct effects on neurons and glial cells [11,12]. Thus, it is suggested that TAK-085 is more effective than DHA or EPA alone in preventing metabolic syndrome- and/or age-related cognitive decline. Finally, n-3 PUFA-induced improvements in memory and learning are thought to be underpinned by various factors, including antioxidative effects, stimulation of hippocampal neurogenesis, and modulation of neuronal signaling pathways (Fig. 3). Regardless of the mechanism, the present study demonstrated that EPA + DHA administration, which enabled the brain cortex to contain more DHA-derived mediators, exhibited a higher beneficial effect on reference memory with the progression of learning trials in aged rats.

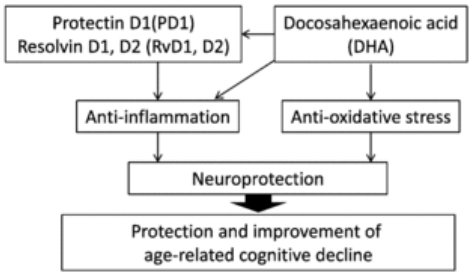

Fig. 3 Neuroprotection and improvement in age-related cognitive decline by DHA and DHA-derived mediators docosanoids and eicosanoids.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbalip.2014.10.009.

~~5 Uncited references~~

~~[28,29,30,31]~~

~~Acknowledgements~~Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education Culture, Sports, Science and Technology, Japan (23500955, M.H.) and a research grant from Takeda Pharmaceutical Company.

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E-Extra

The effects of long-term administration of TAK-085 and EPA alone on reference and working memory-related learning abilities are presented in Fig. 1A and B, respectively, as the mean number of RMEs and WMEs for each group, with data averaged in blocks of 6 trials. Detailed results are shown in Supplemental Table 1. Two-way (block and group) repeated measures ANOVA revealed a significant main effect of both groups and blocks of trials, with a significant group × block interaction in relation to the number of RMEs (Fig. 1A). With respect to the number of WMEs, two-way (block and group) repeated measures ANOVA revealed a significant main effect of both groups and blocks of trials, without a significant group × block interaction (Fig. 1B).

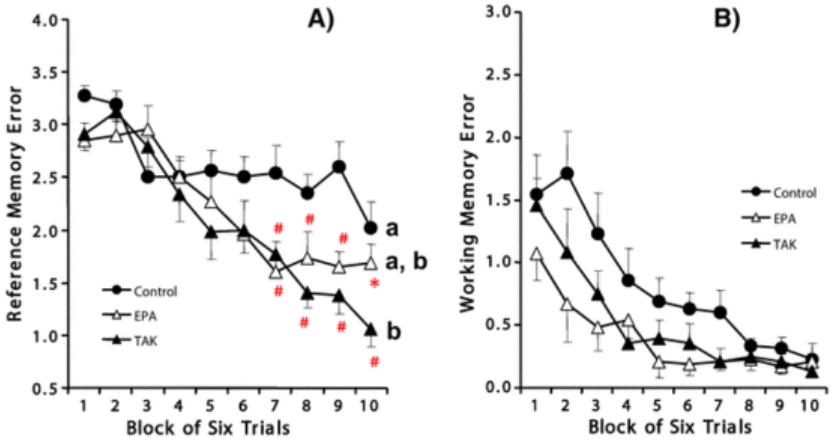


Fig. 1 Effect of TAK-085 and EPA administration on the reference (A) and working (B) memory-related learning ability of the aged rats in the radial maze task. (●) Control rats (n = 8); (▲) TAK-085-treated rats (n = 8); (△) EPA-treated rats (n = 8). Each value represents the number of RMEs and WMEs as the mean ± SEM in each block of six trials. Groups without a common letter of the alphabet for the main effects of groups are significantly different at $P < 0.05$. Details of the statistical descriptions including subtest analysis are given as: Two-way (block and group) repeated measures of ANOVA revealed a significant main effect of groups ($F_{2, 23} = 9.4158$, $P = 0.0012$) and blocks of trials ($F_{9, 216} = 18.7976$, $P < 0.001$), with a significant group × block interaction ($F_{18, 216} = 1.8225$, $P = 0.0252$) in relation to the number of RMEs (Fig. 1A). As for WMEs (Fig. 1B), two-way (block and group) repeated measures ANOVA revealed a significant main effect of both groups ($F_{2, 23} = 4.5612$, $P = 0.0226$) and blocks of trials ($F_{9, 216} = 17.5001$, $P < 0.001$), without a significant group × block interaction ($F_{18, 216} = 1.0151$, $P = 0.4447$).

Two-way ANOVA of RMEs and WMEs was also conducted between controls and TAK-085-treated or EPA-treated rats. Analysis of the RME scores of TAK-085-treated rats vs. control rats revealed a significant group × block interaction ($F_{9, 126} = 2.70$; $P = 0.0065$). This finding indicated that TAK-085 had a significant influence on the RME scores with the progression of the sequence of blocks (from the start to the end of the trials). Analyses of the WME scores did not detect a significant effect on the interaction of groups vs. blocks ($F_{9, 126} = 0.649$; $P = 0.754$), suggesting that TAK-085 did not significantly affect the WME scores until the end of the trials. Subanalysis of RME or WME scores in rats treated with EPA alone vs. control rats showed that TAK-085 did not have a significant effect on the group × block interactions (RMEs: $F_{9, 126} = 1.89$, $P = 0.060$; WMEs: $F_{9, 126} = 1.46$, $P = 0.169$).

Post hoc tests (Fisher's LSD) were performed to determine group differences in each block. #: Significant difference from the control group ($P < 0.05$) and *: significant difference between the EPA and TAK-085 groups ($P < 0.05$).

These results finally suggest that long-term administration of TAK-085 and not EPA alone improved reference memory-related learning ability but not working memory-related learning ability in aged rats.

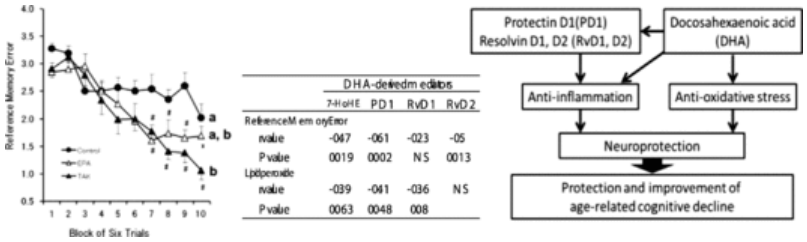
E-component

The following are the Supplementary data related to this article.

Multimedia Component 1

Supplemental Table 1 Mean values and 95% of confidence intervals of reference memory errors at each value in each group.

Graphical abstract



Highlights

- EPA + DHA administration improves learning memory in aged rats.
- EPA + DHA administration increased DHA-derived docosanoids in the cerebral cortex.
- Administration of EPA alone ~~administration~~ increased DHA levels in both plasma and the cortex.
- DHA, but not EPA, -derived mediators were negatively correlated with number of RMEs.
- EPA + DHA administration may be a strategy for protecting against cognitive decline.

Queries and Answers

Query:

Please check the data presentation in Table 1, and correct if necessary.

Answer: OK

Query:

Please check the data presentation in Table 3, and correct if necessary.

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Please confirm that given names and surnames have been identified correctly.

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This sentence has been slightly modified for clarity. Please check that the meaning is still correct, and amend if necessary.

Answer: OK, he meaning is correct.

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Answer: OK, This meaning is correct.

Query:

Uncited references: This section comprises references that occur in the reference list but not in the body of the text. Please position each reference in the text or, alternatively, delete it. Thank you.

Answer: Line 356 and 359, [23] is [27]. Line 375; [24] is [28]. Line 378; [25] and [26] are [29] and [30], respectively. Line 381 [27] is [31].

Query:

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