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The protective effect of dietary eicosapentaenoic acid against impairment of spatial cognition learning ability in rats infused with amyloid $\beta_{(1-40)}$

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Abstract

Background: Amyloid β (Aβ) peptide (1–40) can cause cognitive impairment.
Experimental design: We investigated whether dietary preadministration of eicosapentaenoic acid (EPA) is conducive to cognition learning ability and whether it protects against the impairment of learning ability in rats infused with Aβ peptide (1–40) into the cerebral ventricle.
Results: Dietary EPA administered to rats for 12 weeks before the infusion of Aβ into the rat brain significantly decreased the number of reference memory errors (RMEs) and working memory errors (WMEs), suggesting that chronic administration of EPA improves cognition learning ability in rats. EPA preadministered to the Aβ-infused rats significantly reduced the increase in the number of RMEs and WMEs, with concurrent proportional increases in the levels of corticohippocampal EPA and docosahexaenoic acid (DHA) and in the DHA/arachidonic acid molar ratio. Decrease in oxidative stress in these tissues was evaluated by determining the reactive oxygen species and lipid peroxide levels. cDNA microarray analysis revealed that altered genes included those that control synaptic signal transduction, cell communication, membrane-related vesicular transport functions, and enzymes and several other proteins.
Conclusion: The present study suggests that EPA, by acting as a precursor for DHA, ameliorates learning deficits associated with Alzheimer's disease and that these effects are modulated by the expression of proteins involved in neuronal plasticity.

Keywords: Alzheimer's disease; Hippocampus; Spatial memory; Fatty acid; Rat; Amyloid β

1. Introduction

Fish oil provides a host of health benefits because of its major polyunsaturated fatty acid (PUFA) components: eicosapentaenoic acid [EPA; C20:5(*n*−3)] and docosahexaenoic acid [DHA; C22:6(*n*−3)]. The beneficial effects of fish oil on brain functions, however, have largely focused on and highlighted only DHA, the elongated/desaturated product of EPA. That is probably due to the fact that DHA, but not EPA, constitutes the major PUFA of brain lipids: DHA alone constitutes >17% of the total fatty acids in the rat brain [1], while EPA, as a precursor for DHA, constitutes only a tiny percentage of the total fatty acids in the brain, thus the extensive studies on the beneficial effects of DHA on cognition learning ability.

Alzheimer's disease (AD) is a primary degenerative disease of the central nervous system, and the histopathological hallmark of AD is the presence of neurofibrillar tangles and amyloid plaques of insoluble amyloid peptide aggregates, which ultimately leads to dementia and behavioral and cognitive impairments [2]. Epidemiological studies show that intake of fish oil is associated with a reduced risk of AD [3,4]. Chronic administration of DHA improves spatial learning ability by increasing the level of DHA in the hippocampus and cerebral cortex of young and aged rats [5,6]. DHA administration also protects against [7] and ameliorates [8] memory deficits in amyloid β (Aβ)-peptide-induced AD model rats. DHA protects against behavior deficits and dendritic pathology in the AD mouse model [9].

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There are few reports on the effects of pure EPA on cognition learning ability. Chronic administration of pure EPA attenuates memory impairment induced by interleukin 1β administration, but does not significantly enhance memory in control rats, although whether their normal laboratory chow diet contained any *n*−3 PUFAs is unclear [10]. *n*−3 PUFAs can induce gene expression with concomitant effects on synaptic transmission [11] and related signal transduction. In a parallel set of experiments, we also investigated whether dietary EPA could induce gene expressions related to its beneficial effects on learning-related ability. EPA is probably taken into brain tissue, since the presence of Δ^4-desaturation enzymes in the brain is still unclear [12]. Nonetheless, ^{14}C-labeled EPA detected in the rat brain 1 h after its oral administration to rats decreases time dependently, while [^{14}C]DHA, a metabolite of EPA, increases time dependently [13]. It is also speculated that in the de novo system, each PUFA that is metabolized after being taken into cerebral endothelial cells and astrocytes (constituent cells of the blood–brain barrier) is released from those cells, and that DHA is taken into neurons as metabolite [14]. Here, we estimated the effects of the chronic administration of pure EPA on spatial learning ability in rats and examined whether EPA can protect against the impairment of learning ability in $A\beta_{(1-40)}$-induced AD model rats.

2. Materials and methods

2.1. Animals and diet

The experimental schedule is shown in Fig. 1. Rats were handled and killed in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Shimane Medical University (Shimane, Japan), as compiled from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory of Animal Science. Wistar rats (first generation) (*Jcl*: Wistar; Clea Japan Co., Osaka, Japan) were housed in a room under conditions of controlled temperature (23±2°C), relative humidity (50±10%) and light–dark cycles (light: 0800–2000 h; dark: 2000–0800 h), and were provided fish-oil-deficient pellet diet (F-1; Funabashi Farm, Funabashi, Japan) and water ad libitum. The inbred third-generation male rats (*n*=48; 5weeks old) were divided into two groups: an EPA group (*n*=24) administrated EPA-95E (300 mg/kg/day; Mochida Pharmaceutical Co., Tokyo, Japan) dissolved in 5% gum arabic solution by gavage for 7 weeks initially, and a control group (*n*=21) given 5% gum arabic solution only.

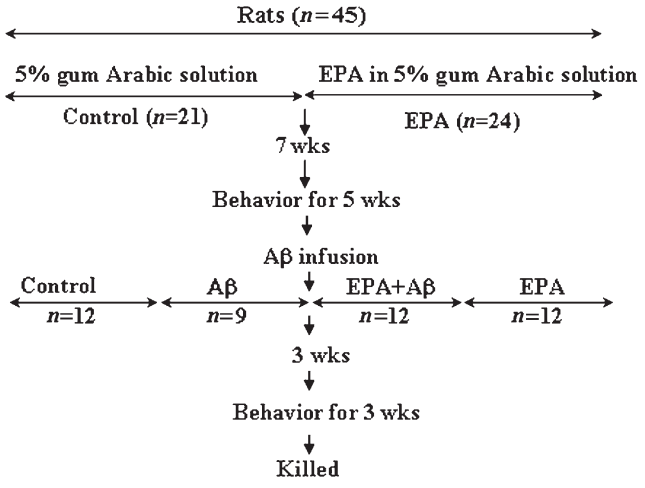

Fig. 1. Schema and schedule of experimental rat groups.

2.2. Preparation of Aβ-infused rats

The surgical techniques for preparing Aβ-infused rats were essentially the same as those described previously [7,8]. Briefly, each rat was anesthetized with sodium pentobarbital (50 mg/kg body weight ip), and its skull was exposed and drilled with two holes (right and left, relative to bregma; 0.8 mm posterior, 1.4 mm lateral) using a stereotaxic frame (Narishige, Tokyo, Japan). A solvent of 35% (vol/vol) acetonitrile plus 0.1% (vol/vol) trifluoroacetic acid (pH 2.0) was used as vehicle for Aβ peptide (1–40) (Peptide Inst., Osaka, Japan). A miniosmotic pump (Alzet 2002; Durect Co., Cupertino, CA, USA) containing either Aβ peptide (1–40) solution (234±13.9 μl) or vehicle alone was quickly implanted into the back of the rats. The outlet of the pump was inserted 3.5 mm into the left ventricle and attached to the skull with screws and dental cement. The infusion rate was 0.56 μl/h, and the total amount infused was approximately 4.9–5.5 nmol/L Aβ. Spontaneous infusion for 2 weeks thus brought about completion of the volume used in the miniosmotic pump.

2.3. Radial maze learning ability

Seven weeks after the start of EPA administration, the learning-related behavior of the rats was assessed by their completing a task in an eight-arm radial maze as previously described [5,8]: four reward pellets were placed randomly on four arms of the maze, and the number of total selections in obtaining the four pellets was counted. Two parameters of memory function were examined: reference memory error (RME), which was determined by the number of entries into unbaited arms, and working memory error (WME), which was estimated by the number of repeated entries into arms that had already been visited within a trial. Lower numbers of RMEs and WMEs implied better spatial learning ability in the rats. Performance was calculated from memory-related behavior. Each rat was given two daily trials, 6 days/week for a total of 3 weeks. After completing the behavior test, each of the two groups of rats was subdivided into two groups (allowing for the number of errors made by each rat in the last six trials of the preliminary behavior test) and infused with either Aβ or the vehicle as follows: a control group was divided into an Aβ-solvent-infused group [control (vehicle) group; *n*=12)] and an Aβ-infused group (Aβ group; *n*=9); an EPA group was divided into a vehicle-

infused EPA group (EPA+vehicle group; n=12) and an Aβ-infused EPA group (EPA+Aβ group; n=12). The four groups of rats were again behaviorally tested 3 weeks after the implantation of the miniosmotic pump to assess the effect of EPA preadministration on the impairment of learning ability in Aβ-infused rats. This testing lasted for a total of 3 weeks. The same protocol used for the preliminary behavior test was followed in the final behavior test, except for the adaptation periods.

2.4. Preparation of sample

After undergoing the behavioral tests for 3 weeks, the rats were anesthetized with sodium pentobarbital (65 mg/kg body weight, ip), blood was drawn for plasma analysis, and the hippocampus and cerebral cortex were separated as described previously [7]. The tissues were stored at −80°C by flash-freezing in liquid N_2 until use.

2.5. Measurement of fatty acid profile and oxidative status

The brain samples were immediately homogenized on ice in 1.0 ml of ice-cold 0.32 mol/L sucrose buffer (pH 7.4) containing 2 mmol/L EDTA, 0.5 mg/L leupeptin, 0.5 mg/L pepstatin, 0.5 mg/L aprotinin and 0.2 mmol/L phenylmethylsulfonyl fluoride, using a Polytron homogenizer (PCU-2-110; Kinematica GmbH, Steinhofhalde, Switzerland), and the residual tissues were stored at −80 °C by flash-freezing in liquid N_2 until use. The homogenates were immediately subjected to the assays described below or stored at −80 °C after liquid N_2 flash-freezing and bathing until use.

Lipid peroxide (LPO) concentration was assessed by the thiobarbituric-acid-reactive substance assay of Ohkawa et al. [15] as described by Hashimoto et al. [7,8], and its levels were measured in nanomoles of malondialdehyde per milligram of protein. Malondialdehyde levels were calculated relative to a standard preparation of 1,1,3,3-tetraethoxypropane.

The levels of reactive oxygen species (ROS) were determined as described previously [7,8]. Briefly, 50 μl of freshly prepared tissue homogenate was mixed with 4.85 ml of 0.1 mol/L potassium phosphate buffer (pH 7.4) and incubated with 2′,7′-dichlorofluorescin diacetate (Molecular Probes, Eugene, OR, USA) in methanol at a final concentration of 5 μmol/L for 15 min at 37°C. The dye-loaded samples were centrifuged at 12,500×g for 10 min at 4°C. The pellet was mixed on a vortex at 0°C in 5 ml of 0.1 mol/L potassium phosphate buffer (pH 7.4) and incubated for 60 min at 37°C. Fluorescence was measured with a Hitachi 850 spectrofluorometer (Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 488 and 525 nm, respectively. A cuvette holder was maintained at 37°C. ROS was quantified from a dichlorofluorescin standard curve in methanol.

The fatty acid compositions of plasma and brain tissues were determined by gas chromatography as described previously [7].

Protein concentration was estimated by the method of Lowry et al. [16].

2.6. Gene expression analysis

Gene expression analysis was carried out with the GeneChip system (Affymetrix) in accordance with the manufacturer's protocol [17]. Briefly, double-stranded DNA was synthesized from 5 μg of total RNA, and the cDNA obtained was used as a template for in vitro transcription. Fragmented in vitro transcripts were hybridized overnight with Rat Expression Array 230A (Affymetrix), stained, washed and scanned with an Affymetrix GeneArray scanner, where the intensity of the fluorescence for each feature was measured. The expression value (average difference) for each gene was determined by calculating the average of differences in intensity (perfect match intensity minus mismatch intensity) between its probe pairs. The image files obtained were analyzed with the Affymetrix data suite system Microarray Suite 5.0 (MAS 5.0). The expression analysis file created from each sample (chip) was imported into GeneSpring 5.1 (Agilent Technologies, Inc., Palo Alto, CA) for further data characterization. Briefly, a new experiment was generated after importing data from the same organ in which data were normalized by array to the 50th percentile of all measurements on that array. Data filtration based on flags present or marginal in at least one of the samples was first performed, and a corresponding gene list based on those flags was generated. Lists of same-phenotype genes that were either induced or suppressed were created by filtration-on-fold function. Gene Ontology (GO) category analyses were performed using the GeneSpring GO browser, which calculates hypergeometric P-values to measure statistical significance for a specific GO category.

2.7. Statistical analysis

Results are expressed as mean±S.E.M. Behavioral data were analyzed by a two-factor (Group and Block) randomized block factorial analysis of variance (ANOVA), and all other parameters were analyzed for intergroup differences by one-way ANOVA. ANOVA was followed by Bonferroni post hoc comparisons. Correlation was determined by simple regression analysis. The statistical programs used were GB-STAT 6.5.4 (Dynamic Microsystems, Inc., SilverSpring, MD, USA) and StatView 4.01 (MindVision Software; Abacus Concepts, Inc., Berkeley, CA, USA). P<.05 was considered statistically significant.

3. Results

3.1. Body weight

The final body weights did not differ among the groups (vehicle group: 393±14; Aβ group: 401±12; EPA+vehicle group: 385±36; EPA+Aβ group: 404±35 g). The brain slices

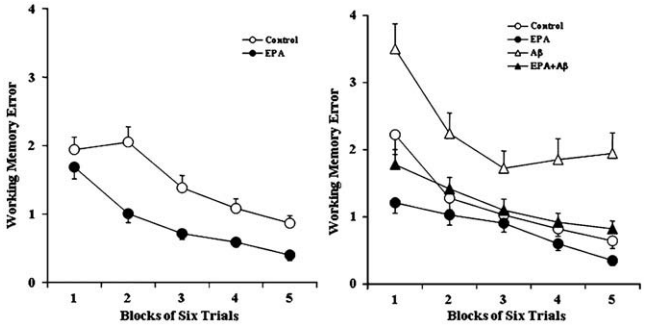

Fig. 2. Effects of the chronic administration of EPA (left) and the infusion of Aβ peptide (1–40) into the rat cerebral ventricle (right) on the number of WMEs evaluated by the radial maze task (see Materials and Methods for details). Left: Control rats (5% gum-arabic-administered rats; n=21) and EPA rats (n=24). After completing the initial behavior test, each of the two groups (control and EPA groups) was subdivided into two groups and infused with either (right) Aβ (Aβ group; n=9) or Aβ solvent (control group; n=12): the EPA group was divided into a vehicle-infused EPA group (EPA group; n=12) and an Aβ-infused EPA group (EPA+Aβ group; n=12). The four groups of rats were again behaviorally tested (with six trials) after the implantation of the miniosmotic pump. Each value represents the number of WMEs, presented as mean±S.E.M. in each block of six trials. The statistical significance of differences between the groups was determined by randomized two-factor (Block and Group) ANOVA followed by Bonferroni post hoc test.

prepared 16–17 days after infusion of the Aβ peptides clearly indicated the deposition of the infused $A\beta_{(1-40)}$ in the corticohippocampal regions (data not shown).

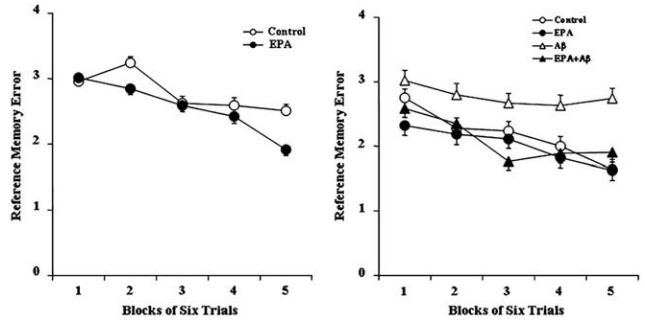

Fig. 3. Effects of the chronic administration of EPA (left) and the infusion of Aβ peptide (1–40) into the rat cerebral ventricle (right) on the number of RMEs evaluated by the radial maze task (see Materials and Methods for details). Left: Control rats (5% gum-arabic-administered rats; n=21) and EPA rats (n=24). After completing the initial behavior test, each of the two groups (control and EPA groups) was subdivided into two groups and infused with either (right) Aβ (Aβ group; n=9) or Aβ solvent (control group; n=12): the EPA group was divided into a vehicle-infused EPA group (EPA group; n=12) and an Aβ-infused EPA group (EPA+Aβ group; n=12). The four groups of rats were again behaviorally tested (with six trials) after the implantation of the miniosmotic pump. Each value represents the number of RMEs, presented as mean±S.E.M. in each block of six trials. The statistical significance of differences between the groups was determined by randomized two-factor (Block and Group) ANOVA followed by followed by Bonferroni post hoc test. Groups without a common letter are significantly different at P<.05. The mole percentages of the unsaturated fatty acids times the number of double bonds in each fatty are presented.

3.2. Effect of EPA on radial maze learning ability

The effect of the chronic administration of EPA on working-memory- and reference-memory-related learning ability is presented as the mean number of WMEs and RMEs for each group, with data averaged over blocks of six trials [Figs. 2 (left) and 3 (left), respectively]. Randomized two-factor (Block and Group) ANOVA revealed a significant main effect of both blocks of trials (P=.0005) and groups (P<.0001), with a significant Block×Group interaction (P=.0018), on the number of WMEs (Fig. 2, left). Similarly, ANOVA revealed a significant main effect of both blocks of trials (P<.0001) and groups (P<.0001), with a significant Block×Group interaction (P=.0484), on the number of RMEs (Fig. 3, left). These results indicate that EPA administration improves working-memory- and reference-memory-related learning ability in young rats.

The effect of EPA preadministered to the vehicle and Aβ-infused groups on working-memory- and reference-memory-related learning ability is presented as the mean number of WMEs and RMEs for each group, with data averaged over six trials [Figs. 2 (right) and 3 (right), respectively]. The number of WMEs was significantly higher in the Aβ group (P=.0011) than in the vehicle group (Fig. 2, right), suggesting learning impairment — a well-known characteristic of AD. The number of WMEs and RMEs was significantly lower in the EPA+Aβ group (WMEs: P<.0001; RMEs: P=.0451) than in the Aβ group [Figs. 2 (right) and 3 (right), respectively], indicating that preadministration of EPA prevents cognitive deficits caused by the infusion of Aβ into the cerebral ventricle of rats.

Table 1
Major fatty acid composition of plasma, cerebral cortex and hippocampus in control, EPA, Aβ and EPA+Aβ rats

	Control	EPA	Aβ	EPA+Aβ
Plasma (%)				
EPA	0.36±0.01^{c}	4.53±0.29^{a}	0.48±0.04^{c}	3.80±0.21^{b}
AA	26.7±0.86^{a}	21.5±0.68^{b}	25.2±0.46^{a}	22.0±1.17^{b}
DHA	2.47±0.06^{b}	3.53±0.09^{a}	2.46±0.07^{b}	3.50±0.14^{a}
DHA/AA	0.09±0.00^{b}	0.17±0.01^{a}	0.10±0.00^{b}	0.16±0.01^{a}
USI	185±2.35^{b}	199±1.64^{a}	184±1.34^{b}	198±2.69^{a}
Cortex (%)				
AA	12.0±0.23^{a,c}	11.1±0.11^{b}	12.4±0.41^{a}	11.4±0.18^{b,c}
EPA	0.10±0.00^{b}	0.13±0.01^{a}	0.09±0.00^{b}	0.13±0.01^{a}
DHA	16.8±0.29^{b,c}	18.1±0.17^{a}	16.1±0.44^{b}	17.6±0.29^{a,c}
DHA/AA	1.41±0.05^{b}	1.63±0.03^{a}	1.32±0.08^{b}	1.56±0.05^{a}
USI	167±1.15^{b}	173±0.86^{a}	164±1.48^{b}	171±1.20^{a}
Hippocampus (%)				
AA	13.4±0.21^{a}	12.5±0.17^{b}	13.3±0.11^{a}	12.7±0.16^{b}
EPA	0.10±0.00^{b}	0.14±0.01^{a}	0.10±0.00^{b}	0.14±0.00^{a}
DHA	14.5±0.12^{c}	15.6±0.16^{a}	15.0±0.13^{b}	15.8±0.11^{a}
DHA/AA	1.09±0.01^{c}	1.25±0.02^{a}	1.13±0.01^{b}	1.25±0.02^{a}
USI	161±0.91^{c}	166±0.97^{a}	163±0.65^{b}	167±0.69^{a}

Values are expressed as mean±S.E.M. and as mole percent of the total fatty acids (n=9–12). Means in a row with superscripts without a common letter differ (P<.05). USI (unsaturation index) was calculated as a function of the sum of the mole percentages of unsaturated fatty acids times the number of double bonds in each fatty acid.

Table 2
Correlation between the mole percentages of plasma EPA and corticohippocampal EPA, DHA and USI

Plasma EPA (y)	x					
	Cortex			Hippocampus		
	EPA	DHA	USI	EPA	DHA	USI
	0.81 (P<.05)	0.57 (P<.05)	0.62 (P<.05)	0.80 (P<.05)	0.67 (P<.05)	0.60 (P<.05)

Results are evaluated with simple regression analysis. P values are expressed inside the parentheses.

3.3. Fatty acid profiles of plasma and brain

The major fatty acid composition in the rat plasma, cortex and hippocampus is shown in Table 1. In the plasma, the proportion of EPA was significantly higher — and that of arachidonic acid [AA; 20:4(n−6)] was significantly lower (P<.05) — in both EPA and EPA+Aβ rats than in the vehicle and Aβ rats, respectively. The proportion of DHA was higher in both EPA and EPA+Aβ rats than in the vehicle rats. EPA administration brought about a significant increase in the plasma DHA/AA molar ratio and USI value in both EPA and EPA+Aβ rats.

Chronic administration of EPA significantly enhanced the EPA proportion in both the cortex and the hippocampus of the EPA and EPA+Aβ rats. In the hippocampus, the proportion of DHA increased, whereas that of AA decreased significantly, effecting a significant increase in the DHA/AA ratio in both the hippocampus and the cortex. EPA administration brought about a significant increase in the corticohippocampal USI values in both these groups of rats.

Highly significant positive correlations were observed between the percent compositions of plasma EPA and EPA, DHA or USI values of both the cortex and the hippocampus (Table 2), indicating that dietary administration of EPA accumulates EPA and DHA in brain tissues.

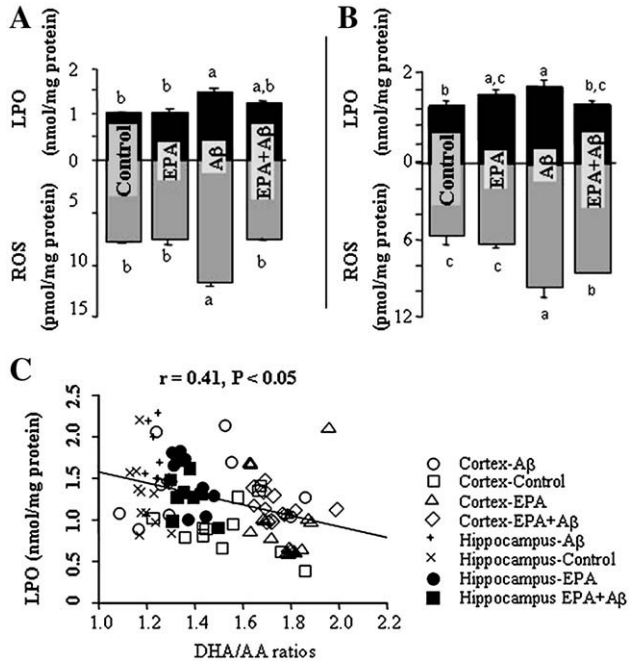

Fig. 4. The effects of EPA on the LPO (upper; A and B) and ROS (lower; A and B) levels of the cortex (A) and hippocampus (B) of the Aβ rats. The correlation between corticohippocampal DHA/AA molar ratio and LPO levels (C) is presented.

Table 3
Effects of chronic administration of EPA on hippocampal gene expression

Up-regulated genes	
1. Signal transduction	
Cystatin C	2.80
$GABA_B$ receptor1	2.04
mGluR8	1.97
Pyruvate dehydrogenase kinase 2	1.84
Regulator of G-protein signaling 4	1.79
2. Cell communication	
Syntaxin 1a	1.79
PLD1	1.65
Suppressor of K^+ transport defect 3	1.73
3. Nucleic acid binding	
Forkhead box E1 (thyroid transcription factor 2)	2.38
Basic helix–loop–helix domain containing class B2	2.02
v-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein G	1.72
4. Microtubular dynamics	
VAPs B and C	1.67
5. Enzyme	
VCP	2.33
PLD1	1.65
Protein arginine *N*-methyltransferase 3-like 3	1.60
Pyridoxine 5-phosphate oxidase	1.59
Down-regulated genes	
1. Signal transduction	
Insulin-like growth factor binding protein 2	2.09
ATP-binding cassette, subfamily G (WHITE), member 5 (sterolin 1)	1.69
GABA-α receptor γ3 subunit	1.63
Hyperpolarization-activated cyclic nucleotide-gated K^+ channel 2	1.61
2. Cell communication	
TTR	3.10
Integrin α1	2.66
Aquaporin 1	1.70
Solute carrier family 9, member 1	1.63
Transferrin receptor	1.61
3. Nucleic acid binding	
Telomeric repeat binding factor 2	2.17
ETS domain transcription factor Pet-1	1.80
Myogenin	1.73
Gonadotropin-inducible ovarian transcription factor 2	1.56
4. Membrane component	
Ectonucleotide pyrophosphatase/phosphodiesterase 2	2.01
5. Enzyme	
Coagulation factor 5	2.83
Glycerol kinase	2.00
Triadin 1	1.82
Metalloprotease/disintegrin	1.78
HP33	1.77

Results are the average of triplicate determination, expressed as the ratio of EPA-administered rats to control rats.

The levels of both LPO and ROS were significantly higher in the cerebral cortex of the Aβ rats (Fig. 4A) than in the cerebral cortex of the vehicle controls and EPA rats. In the cortex of the EPA+Aβ rats, both LPO and ROS levels decreased, but decreased significantly only in the latter. In the hippocampus of the Aβ rats, the level of LPO was significantly higher than that in vehicle rats, but not significantly higher than that in EPA rats (Fig. 4B). The LPO levels were significantly lower in EPA+Aβ rats than in Aβ rats. The ROS level in the hippocampus was also significantly high in Aβ rats and significantly low in EPA+Aβ rats. Finally, the change in oxidative stress in these two tissues demonstrated a significant negative correlation between LPO levels and corticohippocampal DHA/AA ratios (Fig. 4C).

3.4. Effects of EPA administration on hippocampal gene expression

EPA administration triggered a substantial change in the expression of both up-regulated and down-regulated genes (Table 3). The up-regulated genes strongly affected by EPA were signal transduction proteins (cystatin C and $GABA_B$ receptor 1), cell communication protein (syntaxin 1a), nucleic acid binding protein (thyroid transcription factor 2) and microtubular dynamics [vesicle-associated membrane proteins (VAPs) B and C]. The down-regulated genes strongly affected by EPA were signal transduction protein (ATP-binding cassette protein), cell communication proteins [transthyretin (TTR) and integrin α1], nucleic acid binding protein (telomeric repeat binding factor 2) and membrane-associated ectonucleotidase.

4. Discussion

The present study provides evidence that not only the oral administration DHA but also the oral administration of EPA protects against memory impairment in the AD model rats infused with amyloid peptide. The protective effect was accompanied by corticohippocampal increases in EPA and DHA, DHA/AA molar ratio and USI values. The 10- to 12-fold increase in the proportion of plasma EPA in the EPA and EPA+Aβ rats (Table 1), as compared with that in the controls, clearly suggests effective intestinal absorption of this PUFA after oral administration. Moreover, the correlation between plasma EPA and corticohippocampal DHA or the DHA/AA ratio was highly positive, suggesting that plasma EPA effectively deposits DHA in brain tissues after crossing the blood–brain barrier.

We speculate that EPA increases the DHA (and DHA/AA ratio) of the corticohippocampal tissues and, in doing so, exerts beneficial effects on memory formation/protection in EPA or EPA+Aβ rats. The basis of the speculation is that the level of radiolabeled EPA ([^{14}C]EPA) detected in the rat brain 1 h after its oral administration to rats decreases time dependently with a concomitant increase in the levels of [^{14}C]docosapentaenoic acid and [^{14}C]DHA, metabolites of EPA [13]. It is also inferred that each PUFA is metabolized after being taken into cerebral endothelial cells and astrocytes (constituent cells of the blood–brain barrier), that it is released from both cells and that DHA is taken into neurons as metabolite. This speculation is consistent with the fact that DHA is taken into neurons from the extracellular medium after its release from glial cells or the capillary endothelium [14]. Indeed, increased DHA and EPA levels in the plasma raise the fatty acid unsaturation index in rat caudal arteries [18,19]. Moreover, lysophosphatidylcholine may be a transporter of DHA in the blood–brain barrier [20]. Remaining to be explored, however, is whether the transformation of [^{14}C]DHA from [^{14}C]EPA occurs in the liver and is then transported to the brain, or whether the conversion occurs solely and/or partly in brain tissues. Regardless of where EPA is transformed into DHA, it is evident from our study that EPA was converted into DHA and exerted its effects on memory functions.

The brain has an intrinsic capacity to retain its DHA; however, if DHA is depleted, huge differences in brain functions occur [21]. Thus, a change in brain DHA level might be related to behavioral impairments [22]. We reported that a small increase (in mol%) in DHA content contributed significantly to limiting memory deficits in DHA-deficient rats [8]. Thus, a small but highly significant increase in corticohippocampal DHA composition (8–9 mol%) in the EPA/EPA+Aβ rats after EPA administration is consistent with our previous reports [8]. EPA-mediated actions directly or indirectly could involve effects on antioxidative status, amyloid processing, apoptosis, expression of a host of proteins, membrane lipid (disorder) fluidity and exocytosis. An increased DHA/AA ratio is associated with increased memory-related learning ability in young [5], aged normal [6] and AD model rats [7,8], with a concurrent decrease in brain LPO levels. Consistent with our previous investigations [7,8], the DHA/AA ratios correlated negatively with the repression of lipid peroxidation (Fig. 4C). The mechanism by which this correlation affects memory enhancement and amyloid burden is not clear yet. Free-radical theory of AD pathology involves amyloid-induced oxidative stress [23]. Increasing levels of DHA in the cortex of aged rats significantly increase antioxidative enzymes, including catalase, glutathione peroxidase and reduced glutathione [24]. We have hypothesized that the DHA/AA ratio acts as an indicator of antioxidants indirectly by inhibiting the level of AA in the neuronal plasma membrane [7]. An increase in the DHA/AA ratio thus, at least partially, protects the corticohippocampal regions from oxidative insult and provides protection against the impairment of memory in Aβ-infused rats. DHA inhibits the accretion of Aβ peptide (1–40) in detergent insoluble neuronal membrane domains of the cerebral cortex [25] and of Aβ-induced apoptosis-like neuronal cell death [7]. Thus, the finding of EPA-administration-induced protection against memory

impairment, with concurrent DHA accretion in the brain, is in line with our studies [7,8] and those of others [10,26].

An alternative mechanism of EPA-induced amelioration of memory may be as follows: we have previously reported that DHA increases the expression of the Fos protein, the immediate early gene *c-fos* (which acts as a transcription factor and as a functional marker of neuronal activity) of the rat CA1 hippocampus [27]. *n*−3 PUFAs induce the expression of a host of genes that control synaptic plasticity and underlying signal transduction mechanism(s) [11]. Using microarray analysis, we also examined whether the administration of EPA induces the expression of genes, and whether their function is correlated with EPA-induced memory protection in the AD model rats. The gene chip data are correlative and appear to be the only data from EPA-versus-control rats in the present experimental scenario. However, if such data had been obtained also from Aβ and EPA+Aβ rats, then gene expression would have been conferred to the neuroprotective actions of EPA in the EPA+Aβ rats. Consistent with this possibility, we found that EPA up-regulated 16 genes and down-regulated 25 genes (Table 3). The γ-aminobutyric acid (GABA) receptor 1 ($GABA_B$) and the metabotropic glutamate receptor (mGluR) 1 were expressed twice, as compared with those of control rats. The $GABA_B$ receptor interacts with mGluR1-mediated excitatory transmission [28], suggesting that EPA administration might contribute to a mechanism of regulatory synaptic plasticity. Several other genes that participate in cell communication were also overexpressed. For example, the expression of syntaxin 1a and VAPs B and C increased concurrently (Table 3). Syntaxin 1a forms a complex with VAP [29] in order to release neurotransmitters and regulates their transporter [30]. These activities further support the implication of the *n*−3 PUFA-induced increase in neurotransmitter release and synaptic plasticity. In addition, the expression of the *cystatin C* gene, a lysosomal cysteine protease inhibitor [31], increases upon EPA administration. Lysosomal proteases (including cathepsins B and D) are up-regulated in the AD brain [32], and cystatin C relates to neurogenesis in neural stem cells [33]. In this regard, we have recently shown that DHA significantly enhances neurogenesis both in vivo and in vitro [34]. Phospholipase D1 (PLD1) regulates both the release of secretory vesicles from the trans-Golgi network (TGN) [35] and exocytosis [36]. Amyloid proteolysis and subsequent trafficking from the TGN to the cell surface are impaired in AD. Up-regulation of PLD1 in AD rescues impaired APP trafficking from TGN to the membrane surface [37]. In our study, expression of the valosin-containing protein (VCP) increased concomitantly in EPA-fed rats. VCP senses and pulls abnormal protein accumulation in the cell, and pulls from the endoplasmic reticulum and nucleus [38]. DHA-induced clearance of Aβ from neuronal membranes is suggested to be mediated by exocytosis — a process that may be facilitated by the DHA-induced increase in membrane fluidity [24,39,40]. Consistently, EPA-induced memory augmentation is attributed to neurotransmitter (noradrenaline) release [10], as facilitated by an increase in membrane disorder (fluidity) [41]. A DHA-induced increase in memory-related performance is accompanied by increased levels of acetylcholine [42]. Therefore, all these effects are speculated to be in concert with the EPA-induced increase in memory protection of the EPA+Aβ rats. Our microarray data also suggest that EPA suppressed the genes involved in signal transduction, cell communication and/or membrane-bound components to a remarkable extent (Table 3). The contributions of EPA to the repression of these genes have remained largely unrecognized. For example, TTR is a thyroid hormone transporter that is secreted by the liver in plasma and by choroid epithelial plexus in cerebrospinal fluid. The TTR gene was suppressed in our feeding paradigm. To date, the effects of *n*−3 PUFAs on the expression of TTR are inconsistent and conflicting. Short-term administration of *n*−3 fatty acids from fish oil for 1 month increases TTR expression in 2-year-old rats [43]. In contrast, TTR gene expression is unchanged in mice fed a high-DHA diet [44]. Our result is, however, consistent with the report of Tanabe et al. [27] wherein the rats were fed *n*−3 PUFA containing 8% fish oil diet. In our study, the young rats were fed 99% purified EPA at a dose of 300 mg/kg body weight for 12 weeks. The discrepancies may thus relate to the age of the rats, the feeding duration, the fatty acid composition of the supplemented oil and the chow diet itself. Also, the debate on whether TTR is expressed in the hippocampus and/or whether the level found in brain tissues is just an effect of contamination from the plexus epithelium must be resolved [45]. Integrin α1 expression decreased consistently on EPA feeding. Inhibition of this cell-communicating protein by echistatin or antibodies protects against Aβ-induced degeneration of neurons in vitro [46]. Because the gene chip data are unsubstantiated, with no validation at either the mRNA level (by RT-PCR) or the protein level, the correlation of EPA-induced memory amelioration with other altered genes has remained without further predication. The induction of gene expression thus needs to be confirmed by real-time RT-PCR.

To summarize, EPA protects against Aβ-peptide-induced memory deficit in AD model rats after its transformation into DHA. This is accompanied by the accumulation of DHA and/or an increase in the DHA/AA ratio in the corticohippocampal tissues, with a corresponding decrease in oxidative stress and an increase in the expression of synaptic-plasticity-related proteins. Nonetheless, further studies are needed for additional data on EPA.

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