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Effects of eicosapentaenoic acid on synaptic plasticity, fatty acid profile and phosphoinositide 3-kinase signaling in rat hippocampus and differentiated PC12 cells

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Abstract

Placebo-controlled clinical studies suggest that intake of n-3 polyunsaturated fatty acids improves neurological disorders such as Alzheimer's disease, Huntington's disease and schizophrenia. To evaluate the impact of eicosapentaenoic acid (EPA), we orally administered highly purified ethyl EPA (EPA-E) to rats at a dose of 1.0 mg/g per day and measured long-term potentiation of the CA1 hippocampal region, a physiological correlate of synaptic plasticity that is thought to underlie learning and memory. The mean field excitatory postsynaptic potential slope of the EPA-E group was significantly greater than that of the control group in the CA1 region. Gene expression of hippocampal p85α, one of the regulatory subunits of phosphatidylinositol 3-kinase (PI3-kinase), was increased with EPA-E administration. Investigation of fatty acid profiles of neuronal and glia-enriched fractions demonstrated that a single administration of EPA-E significantly increased neuronal and glial EPA content and glial docosahexaenoic acid content, clearly suggesting that EPA was indeed taken up by both neurons and glial cells. In addition, we investigated the direct effects of EPA on the PI3-kinase/Akt pathway in differentiated PC12 cells. Phosphorylated-Akt expression was significantly increased in EPA-treated cells, and nerve growth factor withdrawal-induced increases in cell death and caspase-3 activity were suppressed by EPA treatment. These findings suggest that EPA protects against neurodegeneration by modulating synaptic plasticity and activating the PI3-kinase/Akt pathway, possibly by its own functional effects in neurons and glial cells and by its capacity to increase brain docosahexaenoic acid.

Keywords: Eicosapentaenoic acid; Brain; n-3 fatty acids; Phosphatidylinositol 3-kinase; Long-term potentiation; Neuron

1. Introduction

A number of clinical studies and epidemiological studies have shown that intake of long-chain n-3 polyunsaturated fatty acids (n-3 PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), is associated with reduced risk of neuropsychiatric and neurodegenerative diseases, including schizophrenia, depression, dementia and Alzheimer's disease (AD) [1–5]. Studies of the beneficial effects of n-3 PUFAs on brain function have largely focused on DHA, since it is the predominant structural n-3 PUFA in the brain. Extensive studies have been reported on the beneficial effects of DHA and indicated relationships between its effects on membrane fluidity and the activity of some enzymes, ionic channels and signal transduction [1]. Although the precise mode of action of EPA on neuronal function is unknown, the efficacy of n-3 PUFA treatment, including high proportion of EPA, for neurological disorders has been confirmed in animal and clinical studies [2,6].

EPA is detected as a trace component in the brain and has been recognized as one of the precursor fatty acids of DHA [7]. The n-3 PUFAs cannot be formed *de novo* but can be synthesized from the essential fatty acid α-linolenic acid (ALA). This parent n-3 fatty acid is progressively elongated and desaturated by endoplasmic and peroxisomal enzymes to form EPA and DHA. This conversion occurs primarily in the liver, and the synthesized DHA is transferred to the brain across the blood–brain barrier [8–10]. In an *in vitro* study, it has been shown that the cerebral microvascular endothelium can produce EPA, and that cultured astrocytes actively elongate and desaturate the precursor fatty acids, ALA and EPA, producing DHA [7,11,12]. However, it remains unclear whether EPA can be introduced into neurons and function in them.

Abbreviations: AA, arachidonic acid; Abeta, amyloid-beta peptide; AD, Alzheimer's disease; ALA, α-linolenic acid; APP, beta-amyloid precursor protein; ATP, adenosine 5′-triphosphate; CNS, central nervous system; CREB, cyclic AMP response element-binding protein; DAPI, 4′,6-diamidino-2-phenylindole; DG, dentate gyrus; DHA, docosahexaenoic acid; DMEM, Dulbecco's Modified Eagle's Medium; DPA, docosapentaenoic acid; DPBS, Dulbecco's phosphate-buffered saline; EPA, eicosapentaenoic acid; EPA-E, ethyl EPA; FAMEs, fatty acid methyl esters; fEPSPs, field excitatory postsynaptic potentials; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; LTP, long-term potentiation; NGF, nerve growth factor; n-3 PUFAs, n-3 polyunsaturated fatty acids; PG, prostaglandin; PI3-kinase, phosphatidylinositol 3-kinase; PLA_2, phospholipase A_2; RT-PCR, reverse transcription-polymerase chain reaction; TBS, theta burst conditioning stimulation.

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The n-3 PUFAs have been linked with several neuronal functions preventing neurodegeneration, including effects on inflammation, oxidation, membrane fluidity and phospholipase A_2 (PLA_2) activity [13]. Lynch et al. [14–16] have reported that EPA can improve impairment of synaptic plasticity by suppressing oxidative stress induced by gamma irradiation or aging, and reverse the depression of long-term potentiation (LTP) induced by interleukin-1beta or beta-amyloid. Previous studies on the relationship between EPA and cognitive function have mainly focused on pathological conditions caused by oxidative stress or inflammation. It has been demonstrated that DHA directly modulates the neuronal plasticity related to ionotropic receptors, enzymes, neural signaling factors and growth factors [17–20]. Indeed, chronic administration of DHA improves spatial learning ability by increasing the level of DHA in the hippocampus and the cerebral cortex in young rats [21].

The objectives of this study were to investigate (1) whether administration of EPA to rats influences synaptic plasticity (LTP) and (2) phosphatidylinositol 3-kinase (PI3-kinase), the signaling molecules involved in regulation of LTP; and (3) whether EPA can be introduced into neurons and glial cells of the central nervous system (CNS).

2. Materials and methods

2.1. Animals

Male Wistar rats (Charles River Laboratories Japan, Yokohama, Japan) were housed in stainless steel cages. They were maintained under the following environmental conditions: room temperature, 21–25°C; relative humidity, 40–70%; illumination, 12 h from 7:30 a.m. to 7:30 p.m.

All animal experiments were carried out in accordance with the guidelines for the care and use of laboratory animals of Mochida Pharmaceuticals.

2.2. Experimental design

For fatty acid analysis of plasma and brain, the first set of rats (at 3 weeks of age) was randomly divided into four groups: control I, control II, ethyl EPA (EPA-E) 0.3 mg/g per day and EPA-E 1.0 mg/g per day group (n=10 for each group). Control group I rats were fed a laboratory commercial chow pellet (standard diet, Funabashi Farm, Funabashi, Japan), and rats of the control group II and EPA-E-administered group were fed a laboratory commercial EPA and DHA-deficient diet prepared by deleting fish oil from standard diet (Funabashi Farm). The fatty acid composition of dietary lipids is shown in Table 1. EPA-E (over 97% purity, Mochida Pharmaceuticals) was suspended in 5% gum arabic solution with an ultrasonic cell homogenizer (NS-56S, Microtec, Funabashi) and orally administered by gavage. EPA-E (EPA-E 0.3 mg/g per day and EPA-E 1.0 mg/g per day group) or an equal volume of vehicle (Control group I and Control group II) were administered to rats (starting at 6 weeks of age) daily for 8 weeks.

For fatty acid analysis of neuronal and glia-enriched fractions, the second set of rats that had been fed EPA and DHA-deficient diet (from 8 weeks of age) was used. After rats at 12–13 weeks of age were fasted for 14–16 h, they were administered EPA-E at a single dose of 1.0 mg/g (EPA-E group, n=6) or an equal volume of vehicle (Control group II, n=6).

The third set of rats that have been fed EPA and DHA-deficient diet (from 3 weeks of age) was used for LTP measurement. Rats had been administered EPA-E at a dose of 1.0 mg/g per day (EPA-E group, n=11) or vehicle (Control group II, n=9) from 5 weeks of age for 8 weeks.

The last set of rats fed standard diet (Control group I) or EPA and DHA-deficient diet (control II and EPA-E groups) from 3 weeks of age was used for quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. EPA-E group rats were administered EPA-E at a dose of 1.0 mg/g per day, and Control group I and Control group II rats were administered an equal volume of vehicle from 4 weeks of age for 8 weeks.

2.3. Fatty acid analysis of plasma and brain

At 14 weeks of age, rats were anesthetized with diethyl ether, heparinized blood was collected and the whole brain was removed. The brains were flash-frozen in liquid nitrogen and stored at −80°C until analysis.

Total lipids in plasma (200 μl) were extracted in water and 2:1 (v/v) chloroform/methanol. The mixture was shaken for 10 min, mixed with water and then centrifuged. The organic layer was collected and dried under a nitrogen atmosphere.

To obtain total lipids in rat brain, brains were homogenized in water and 2:1 (v/v) methanol/chloroform and centrifuged, and the supernatant was collected. The supernatant obtained by the above extraction procedure, performed twice, was mixed with water and chloroform and centrifuged. The organic layer obtained was dried under a nitrogen atmosphere.

The total lipids extracts obtained above were hydrolyzed and converted to fatty acid methyl esters (FAMEs), then examined by gas–liquid chromatography (GC-17A, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector. Tricosanoic acid (23:0) in hexane was added to each sample as an internal standard before FAMEs analysis. Peaks were identified by comparison of retention times with external FAME standards from Sigma-Aldrich. Fatty acid profiles are expressed in concentrations (micrograms per milliliter or micrograms per milligram of tissue).

2.4. Fatty acid analysis of neuronal and glia-enriched fractions

Six hours after administration, they were anesthetized with diethyl ether. The brain was perfused with saline, and the whole brain was removed and immediately used for isolation of neuronal and glia-enriched fractions. An isolation technique described previously was used [22].

Total lipid extraction in the neuronal fraction (600 μg) and the glia-enriched fraction (200 μg) was performed using the same method as for total lipid extraction in plasma.

The total lipids extracts obtained were hydrolyzed and converted to FAMEs, then were hydrolyzed and fluorescence labeled with 4% pyridine/ethanol, 25 mmol/L DMEQ-hydrazide/*N*,*N*-dimethylformamide and 1 mol/L *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride/water at 37°C for 10 min.

Fluorescence-labeled FAMEs were analyzed by high-performance liquid chromatography (Waters, Milford, MA, USA) equipped with a fluorescence detector (FP-920, JASCO, Hachioji, Japan). Pentadecanoic acid (15:0, Sigma-Aldrich) was added to each sample as an internal standard before analysis.

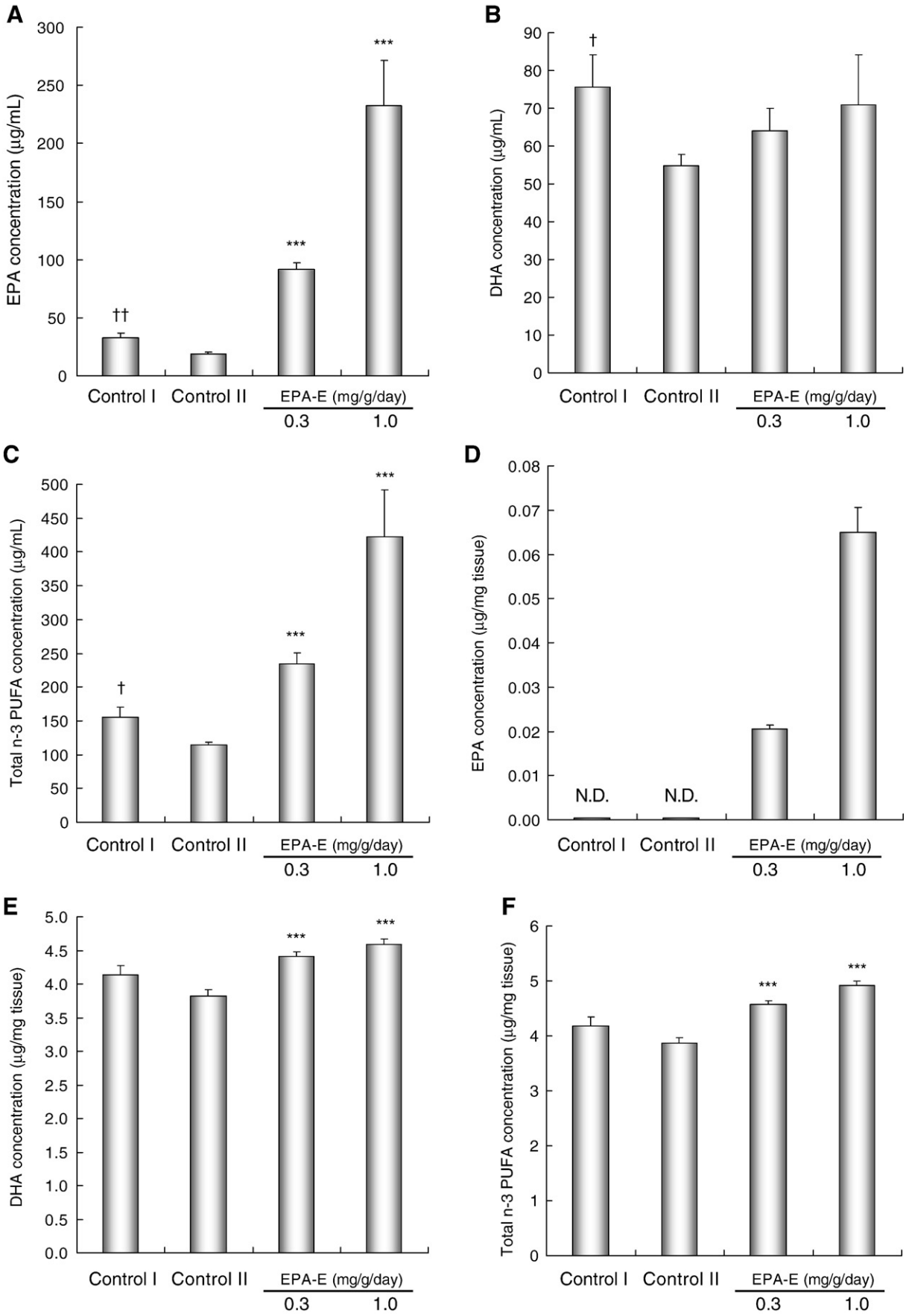
2.5. Immunostaining of neurons and glial cells

Cell suspensions of neurons or glial cells were fixed with 3% paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS) (pH 7.4), spun (Shandon Cytospin 2 Cytocentrifuge, Thermo Fisher Scientific, Waltham, MA, USA) at 600 rpm for 15 min and dried. After washing with DPBS, cells were treated with DPBS containing 10% goat serum (Invitrogen, Carlsbad, CA, USA) and 0.3% Triton X-100 for permeabilization and blocking. They were then incubated with primary antibodies against neuron-specific Class III β-tubulin (mouse monoclonal 1:125, R&D Systems, Minneapolis, MN, USA) or glial fibrillary acidic protein (GFAP, rabbit polyclonal 1:500, Sigma-Aldrich) in 1% goat serum/DPBS. After washing with DPBS, cells were incubated with anti-mouse IgG-FITC (SouthernBiotech, Birmingham, AL, USA) or anti-rabbit IgG-TRITC (Sigma-Aldrich)-conjugated secondary antibody (1:100 or 1:400, respectively) in DPBS. After washing with DPBS, slides were mounted and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). Images were acquired on a fluorescence microscope (IX70, Olympus, Tokyo) using a CCD camera (DP70, Olympus).

Table 1
Fatty acid composition of the diets

		Standard diet	EPA and DHA-deficient diet
Composition of the diet (%)			
Water		8.0	8.0
Crude protein		21.3	21.5
Fat		5.1	4.4
Fiber		3.1	2.6
Mineral		5.0	4.9
Carbohydrates		57.5	58.6
Fatty acid composition of dietary lipids (%)			
Myristic acid	C14:0	0.6	0.4
Palmitic acid	C16:0	16.8	17.3
Palmitoleic acid	C16:1n-7	0.5	0.2
Heptadecanoic acid	C17:0	0.4	0.3
Stearic acid	C18:0	4.8	4.7
Oleic acid	C18:1n-9	19.4	19.5
Linoleic acid	C18:2n-6	48.0	51.2
α-Linolenic acid	C18:3n-3	4.1	4.8
Arachidic acid	C20:0	0.3	0.3
Eicosenoic acid	C20:1n-9	1.1	0.5
Eicosapentaenoic acid	C20:5n-3	1.1	ND
Behenic acid	C22:0	0.2	0.3
Docosenoic acid	C22:1	0.5	ND
Docosapentaenoic acid	C22:5n-3	0.3	ND
Docosahexaenoic acid	C22:6n-3	1.0	ND
Lignoceric acid	C24:0	ND	0.2
Not identified		0.9	0.3

ND: Not detected.


A
EPA concentration (μg/mL)
300
250
200
150
100
50
0
††
***
***
Control I
Control II
EPA-E (mg/g/day)
0.3
1.0
B
DHA concentration (μg/mL)
90
80
70
60
50
40
30
20
10
0
†
Control I
Control II
EPA-E (mg/g/day)
0.3
1.0
C
Total n-3 PUFA concentration (μg/mL)
500
450
400
350
300
250
200
150
100
50
0
†
***
***
Control I
Control II
EPA-E (mg/g/day)
0.3
1.0
D
EPA concentration (μg/mg tissue)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
N.D.
N.D.
Control I
Control II
EPA-E (mg/g/day)
0.3
1.0
E
DHA concentration (μg/mg tissue)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
Control I
Control II
EPA-E (mg/g/day)
0.3
1.0
F
Total n-3 PUFA concentration (μg/mg tissue)
6
5
4
3
2
1
0
***
***
Control I
Control II
EPA-E (mg/g/day)
0.3
1.0

2.6. Long-term potentiation measurement

2.6.1. Slice preparation

Rats were sacrificed at 13 weeks of age by decapitation after anesthesia with diethyl ether. The brains were immediately placed in an ice-cold oxygenated preparation solution of the following composition: 124 mM NaCl, 26 mM $NaHCO_3$, 10 mM glucose, 3 mM KCl, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$ and 2 mM $MgSO_4$, for 4–10 min. Then 300-μm slices were prepared using a vibrating tissue slicer (VT1000S, Leica Microsystems, Tokyo), and the hippocampal area was dissected rapidly from the slices. Hippocampal slices were incubated at 27.5°C for 1 h in the preparation solution.

2.6.2. Electrophysiology

The hippocampal slices were gently placed on the center of 0.1% polyethyleneimine-coated multi-electrode dishes in a multichannel extracellular recording system (MED64 system, Alpha MED Sciences, Osaka) [22]. Electrophysiological experiments were performed in an incubator box with perfusion with artificial cerebrospinal fluid of the following composition: 124 mM NaCl, 26 mM $NaHCO_3$, 10 mM glucose, 3 mM KCl, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$ and 1 mM $MgSO_4$, at 32°C. For measurement of dentate gyrus (DG), another important region for LTP, picrotoxin (50 μM) was added to the artificial cerebrospinal fluid. A stimulation electrode was selected either in the stratum radiatum to stimulate Schaffer collateral/commissural fibers projecting to the CA1 region or the perforant path. The recorded field potentials were confirmed to be field excitatory postsynaptic potentials (fEPSPs) by paired-pulse facilitation. In each experiment, after responses were stable, stimulus intensity was adjusted to produce an fEPSP slope of about 30% of the maximum response. Control fEPSPs were recorded for over 10 min at intervals of 60 s before conditioning stimulation. LTP was induced by theta burst conditioning stimulation (TBS) [23], including a 10-burst train of four pulses (100 Hz) with 200-ms intertrain intervals, at the same stimulus intensity as used for test stimulation. After TBS, fEPSPs were recorded every 60 s for 60 min.

2.7. Quantitative real-time RT-PCR analysis

Total RNA was extracted from the hippocampus (at 12 weeks of age) using TRIzol reagent (Invitrogen) and an RNeasy mini kit (QIAGEN GmbH Hilden, Germany) according to the manufacturer's instructions. Total RNA (2 μg) from each sample was converted to cDNA using the Superscript III first-strand synthesis system (Invitrogen), according to the manufacturer's instructions. cDNA (2 μg) was diluted 1:10 or 1:100. Quantitative real-time RT-PCR was performed on a 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) with the following thermal cycler conditions: hold for 20 s at 95°C; 40 cycles at 95°C for 3 s and 60°C for 30 s. The TaqMan probes and primers for p85α, p85β, p55γ and p110β (assay identification numbers Rn00564547_m1, Rn00573376_m1, Rn00573586_m1 and Rn00585107_m1, respectively) were assay-on-demand gene expression products (Applied Biosystems). The rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control (assay identification number Rn99999916_s1, Applied Biosystems). cDNA was mixed with 20× TaqMan gene expression assay mix (Applied Biosystems) and TaqMan Fast Universal PCR master mix (2×) (Applied Biosystems), and total volume was made up to 20 μl with DEPC-treated water. Statistical analysis was not performed since the data were obtained from only three rats for each group.

2.8. Cell culture conditions and fatty acid supplementation

Rat adrenal pheochromocytoma PC12 cells, a cell line widely used as a neurite outgrowth studies mediated by PI3-kinase/Akt pathway, were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated horse serum (Invitrogen), 5% heat-inactivated fetal bovine serum (Moregate Biotech, Bulimba, Australia), penicillin and streptomycin (Invitrogen) in poly-D-lysine-coated flasks (Becton Dickinson, Franklin Lakes, NJ, USA) under a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

For measurement of cell viability and transcriptional activity of cyclic AMP response element-binding protein (CREB), one of the targets for Akt [24], cells were plated on poly-D-lysine-coated 24-well plates (Becton Dickinson) at densities of 1.8×10^4 cells/well and 2.5×10^5 cells/well, respectively. For measurement of caspase-3 activity and immunoblotting, cells were plated on poly-D-lysine-coated six-well plates (Becton Dickinson) at densities of 2×10^6 cells/well and 1×10^6 cells/well, respectively. For direct exposure of cells to EPA (Nu-chek Prep, Elysian, MN, USA), EPA was bound to fatty acid-free bovine serum albumin (Merck, Darmstadt, Germany). EPA stock solutions in 5% bovine serum albumin/DMEM were diluted in DMEM under an argon atmosphere so that the final concentration of bovine serum albumin became 0.5%. To test the effects of EPA supplementation, PC12 cells were maintained in DMEM supplemented with EPA, 0.5% bovine serum albumin, 20 μM α-tocopherol (Sigma-Aldrich) and 100 ng/ml nerve growth factor (NGF, Sigma-Aldrich)-7S for 48 h. Then, to induce cell death, cells were maintained in DMEM for

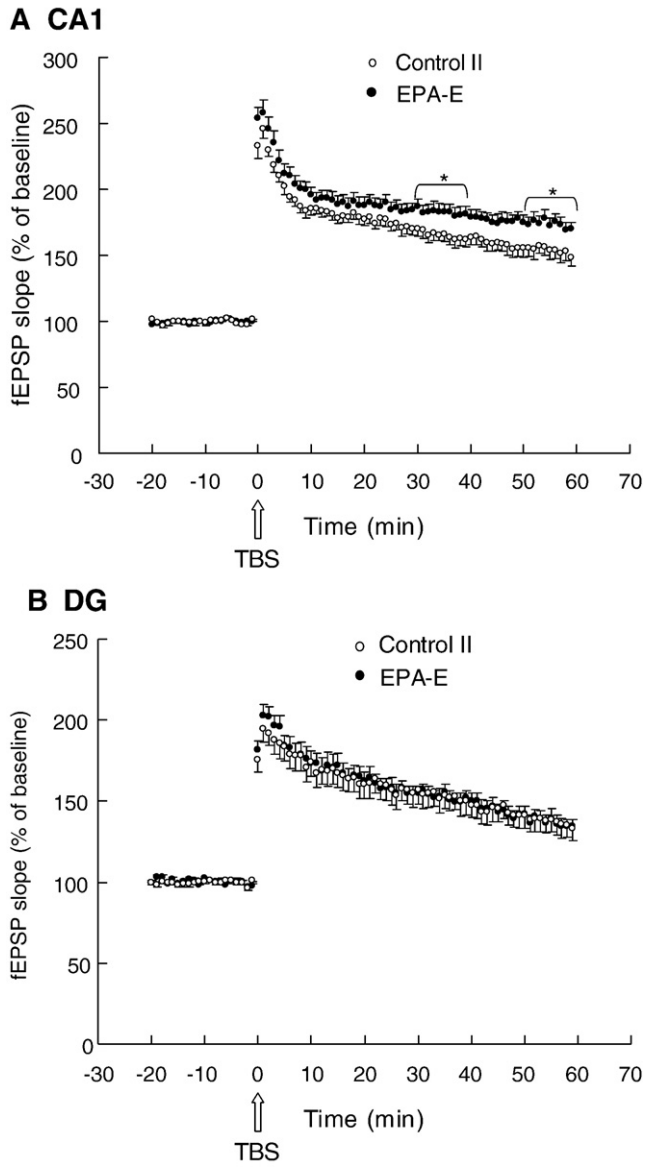


Fig. 2. LTP in *ex vivo* hippocampal slices from rats administered EPA-E for 8 weeks. The EPA and DHA-deficient diet-fed rats were administered vehicle (Control group II) or EPA-E at a dose of 1.0 mg/g per day (EPA-E group) daily for 8 weeks. LTP measured in the CA1 region (A) and DG (B) is shown. Values are the mean percentage change in fEPSP slope (n=9 or 12 slices) relative to the slope during the 10-min period preceding TBS. *Depicts results significantly different from Control group II: *P<.05 (Student's t test).

Fig. 1. Effects of chronic EPA-E administration on fatty acid profiles of total lipids in rat plasma and brain. Control group I rats were fed standard diet; and Control group II, EPA-E 0.3 mg/g per day and EPA-E 1.0 mg/g per day group rats were fed an EPA and DHA-deficient diet (n=10 for each group). EPA-E at a dose of 0.3 or 1.0 mg/g per day was administered to rats daily for 8 weeks, and the Control group I and Control group II rats were administered an equal volume of vehicle only. EPA (A and D), DHA (B and E) and total n-3 PUFA (C and F) concentrations in rat plasma (A–C) and brain (D–F) are shown. †Depicts results significantly different from Control group II: †P<.05, ††P<.01 (Student's t test or Aspin–Welch t test). *Depicts results significantly different from Control group II: ***P<.001 (Dunnett test or Steel test). Since EPA concentration was not detected in brains of the Control group I and Control group II rats (D), statistical analysis was not performed. ND: Not detected.

another 48 h (cell viability) or 6 h (caspase-3 activity measurement). Controls were treated similarly, but with EPA omitted.

2.9. Cell viability and caspase-3 activity measurement

Cell viability was monitored using an assay kit (CellTiter-Glo luminescent cell viability assay, Promega, Madison, WI, USA). This kit is based on luminometric quantitation of adenosine 5′-triphosphate (ATP) content as a marker of metabolically active cells. Procedures were performed according to the manufacturer's instructions. To calculate the number of cell deaths, the luminescence signal obtained before cell death induction was subtracted from each luminescence signal measured after cell death induction (48 h later). Proportion of cell death was expressed as percentage decrease in ATP content during deprivation of NGF and serum (for 48 h).

Caspase-3 activity was measured using a colorimetric assay kit (CaspACE Assay System, Fluorometric, Promega) according to the manufacturer's protocol.

2.10. Immunoblotting

After PC12 cells had been supplemented with EPA for 48 h, they were washed with cold DPBS and were suspended in 100 μl of cell lysis buffer that contained 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2 EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 μg/ml leupeptin (Cell Signaling Technology, Danvers, MA, USA) supplemented with protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 2 mM PMSF (Santa Cruz) and 50 mM sodium fluoride (Sigma-Aldrich), followed by centrifugation at 10,000 rpm for 10 min. The protein extract (14.4 μg of protein) was electrophoresed, transferred from the gel to a polyvinylidene difluoride membrane and blocked in 5% (v/v) membrane blocking agent (GE Healthcare, Buckinghamshire, UK) in Tris-buffered saline (pH 7.5) supplemented with 0.1% Tween 20. Akt and Akt phosphorylated at Ser-473 were immunoblotted with anti-Akt antibody and anti-phosphorylated-Akt antibody (Cell Signaling Technology), respectively. Enhanced chemiluminescence (ECL plus Western Blotting Detection System, GE Healthcare) of HRP-labeled antirabbit IgG antibody (Cell Signaling Technology) was visualized with an image analyzer (Typhoon 9410, GE Healthcare). Quantitative densitometric analyses were performed on digitized images of immunoblots using software (ImageQuant TL v2003.02, GE Healthcare).

2.11. CREB transcriptional activity

Ten minutes after the PC12 cells were plated on 24-well plates, transient transfection was carried out using lipofectamine. Cells were co-transfected with pCMV-CREB plasmid DNA (CREB dominant-negative vector set, Becton Dickinson), which constitutively expresses the human wild-type CREB protein, and pCRE-Luc plasmid DNA (Mercury pathway profiling luciferase system 1, Becton Dickinson), a reporter plasmid encoding the firefly luciferase gene driven by a specific *cis*-acting DNA enhancer element responsive to CREB. Seven hours after transfection, the medium was changed. The cells were then incubated for an additional 24 h with the indicated concentration of EPA and 100 ng/ml NGF. Luciferase assays were performed with an assay kit (Steady-Glo luciferase assay system, Promega) according to the manufacturer's protocol.

2.12. Statistical analysis

Values are the mean±S.E. All results were tested for homogeneity of variance using the *F* test (between two groups) or Bartlett's test (among three groups or more). For evaluation of differences between two groups, a Student's *t* test was performed if the variance was homogeneous, and an Aspin–Welch *t* test if heterogeneous. For evaluation of differences among three groups or more, a Dunnett test was performed if the variance was homogeneous, and a Steel test if heterogeneous. To assess the significance of differences in LTP findings, comparisons were made between the average slope for 30–40 or 50–60 min after TBS. SAS statistical programs were used (ver 9.1.3, SAS Institute Japan, Tokyo). Differences with $P<.05$ were considered significant.

3. Results

3.1. Effects of chronic EPA-E administration on fatty acid profiles of plasma and brain

To determine the effects of repeated administration of EPA-E on fatty acid profiles of total lipids in rat plasma and brain, we treated the rats with EPA-E for 8 weeks.

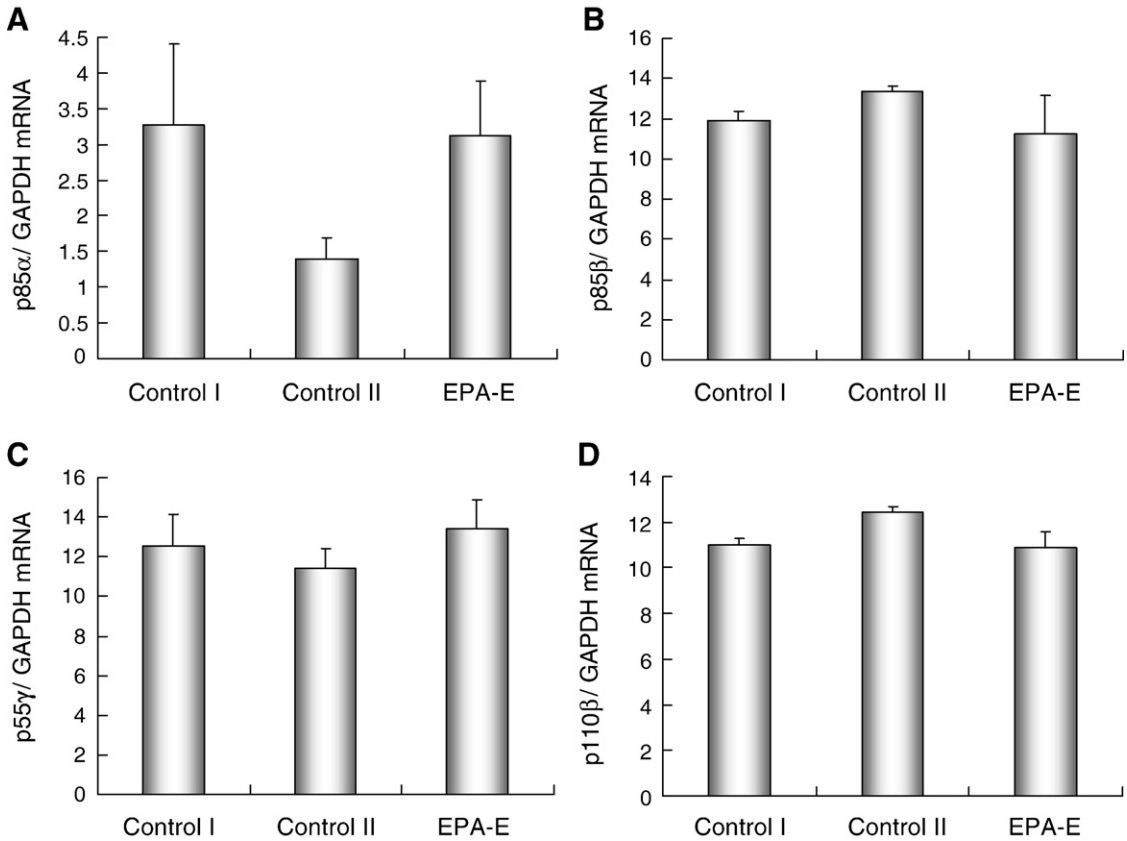


Fig. 3. Quantitative real-time RT-PCR measurements of hippocampal PI3-kinase mRNA in rats administered EPA-E for 8 weeks. Control group I rats were fed standard diet, and control II and EPA-E group rats were fed an EPA and DHA-deficient diet. EPA-E group rats were administered EPA-E at a dose of 1.0 mg/g per day, and Control group I and Control group II rats were administered vehicle daily for 8 weeks ($n=3$ for each group). mRNA levels of regulatory subunits p85α (A), p85β (B) and p55γ (C), and catalytic subunit p110β (D) are shown. Results have been normalized to GAPDH.

The plasma concentrations of EPA, DHA and total n-3 PUFAs decreased with fish oil depletion (Fig. 1A–C, respectively). EPA-E supplementation for 8 weeks clearly increased the plasma levels of EPA and total n-3 PUFAs significantly above those in Control group II.

The effects of fish oil depletion and chronic administration of EPA-E on brain total lipid profiles are shown in Fig. 1D–F. Although EPA concentration was not detected in brains of Control group I and Control group II rats, chronic administration of EPA-E increased EPA content (Fig. 1D). The concentrations of DHA and total n-3 PUFAs in the brain also significantly increased (Fig. 1E and F, respectively).

3.2. Effects of chronic EPA-E administration on hippocampal LTP

To investigate the effects of repeated EPA-E administration on the CNS, we measured synaptic plasticity (LTP) in *ex vivo* hippocampal slices from rats administered EPA-E for 8 weeks.

The effects of EPA-E administration for 8 weeks on LTP in the hippocampal CA1 region and DG are shown in Fig. 2A and B, respectively. In the CA1 region, EPA-E significantly increased mean fEPSP slope (% of baseline) in the 30 to 40 min period following TBS. EPA-E also significantly increased mean fEPSP slope (% of baseline) in the 50 to 60 min period following TBS (EPA-E group, 173.90±5.65%, n=12 slices from 11 rats; Control group II, 154.27±6.86%, n=9 slices from nine rats). On the other hand, there were no significant differences in mean fEPSP slope in the DG region.

3.3. Effects of chronic EPA-E administration on hippocampal PI3-kinase mRNA

It has been reported that DHA, a metabolite of EPA, prevented reduction of PI3-kinase regulatory subunit p85α expression and attenuated behavioral deficits in a mouse model of AD [25]. We therefore examined the effects of EPA-E administration for 8 weeks on PI3-kinase mRNA in the hippocampus of rats using quantitative real-time RT-PCR.

EPA-E administration led to a 2.2-fold increase in hippocampal p85α mRNA compared to that in Control group II (Fig. 3A). There were no differences in levels of regulatory subunits p85β, p55γ or catalytic subunit p110β mRNA between the EPA-E group and Control group II (Fig. 3B–D, respectively).

3.4. Effects of EPA-E single administration on fatty acid profiles of neuronal and glia-enriched fractions

To determine whether exogenously administered EPA-E can increase neuronal and glial EPA, or increases only DHA, we isolated neuronal and glia-enriched fractions from rats after a single EPA-E administration using the discontinuous sucrose density gradient centrifugation technique and examined their fatty acid profiles.

Representative photographs of the neuronal and glia-enriched fractions obtained are shown in Fig. 4A and B, respectively. Although the glia-enriched fraction was less pure, containing some neurite debris, the cells in the neuronal fraction were not immunoreactive to the astrocyte marker GFAP, suggesting that there was no contamination by astrocytes in the neuronal fraction. We examined the purity of the neuronal fraction by counting the cells labeled with a neuron-specific Class III β-tubulin antibody within randomly selected fields and confirmed that it was over 96.6% (data not shown).

The effects of single EPA-E administration on lipid profiles of neuronal and glia-enriched fractions of rat brain are shown in Table 2. A single administration of EPA-E significantly increased only EPA concentration, and other fatty acids including DHA were not changed in the neuronal fraction. On the other hand, concentrations of EPA, DHA and other fatty acids were significantly changed in the glia-enriched fraction.

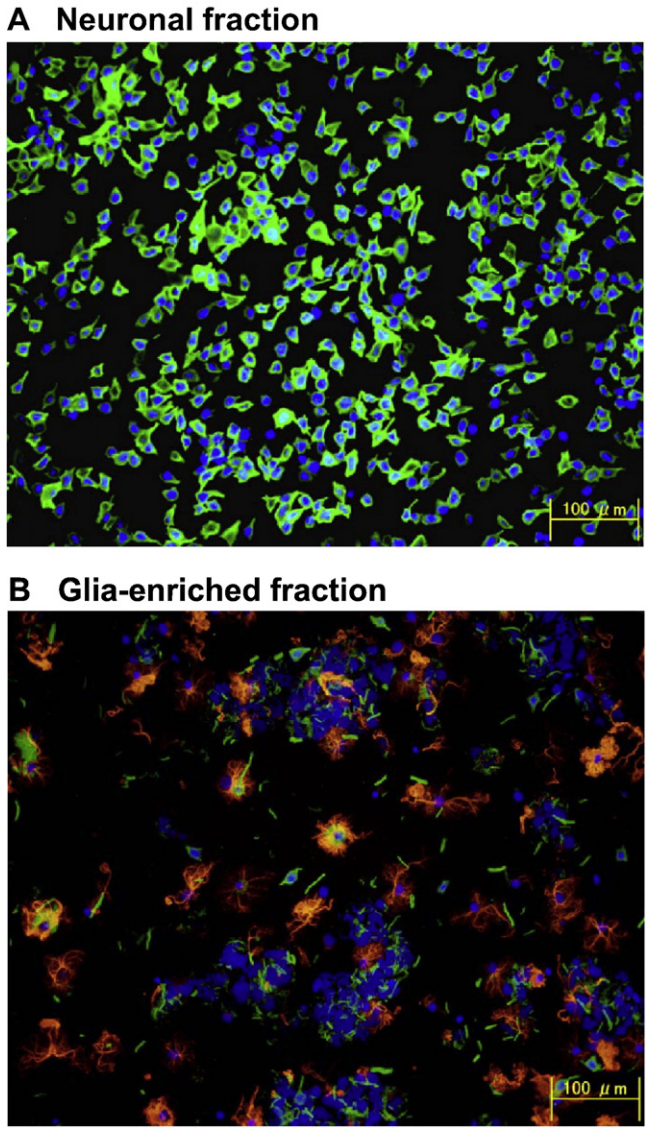


Fig. 4. Photographs of neuronal and glia-enriched fractions from rat brain. The neuronal fraction was separated from debris by discontinuous sucrose density gradient centrifugation. Cells were stained with antibodies to neuron-specific Class III β-tubulin (green) and GFAP (red), together with the nuclear marker DAPI (blue). Representative photographs of the neuronal fraction (A) and glia-enriched fraction (B) are shown.

3.5. Effects of EPA on serum and NGF withdrawal-induced cell death and caspase-3 activity

To investigate whether EPA has direct effects of its own on the PI3-kinase/Akt pathway, and not as a DHA precursor, we examined the effects of EPA using differentiated PC12 cells, which have been used to study NGF-induced cell survival mediated by PI3-kinase/Akt pathway.

Incubation of differentiated PC12 cells under serum-free and NGF-free conditions for 48 h induced cell death. Preincubation of cells with EPA for 48 h before serum and NGF deprivation dose-dependently decreased the cell death induced by deprivation

(Fig. 5A). In addition, EPA did not affect cell viability up to the highest concentration (50 μM) under standard conditions (in the presence of NGF) (data not shown).

Similar results were obtained for the activity of caspase-3, a member of the cysteine protease family that has been shown to mediate apoptosis in mammalian cells. Fig. 5B shows the increase in caspase-3 activity induced by serum and NGF deprivation, while preincubation of cells with EPA for 48 h before serum and NGF deprivation decreased caspase-3 activity induced by deprivation.

3.6. Effect of EPA supplementation on Akt phosphorylation

To further investigate the direct effects of EPA on the PI3-kinase/Akt pathway, we measured the expression of phosphorylated-Akt in PC12 cells treated with EPA and NGF for 48 h.

Western blotting analysis revealed that the level of expression of Akt phosphorylated at Ser-473, an active form of Akt, was significantly increased in EPA-treated differentiated PC12 cells compared with the control. In contrast, no difference was observed in total Akt expression between EPA-treated cells and the control (Fig. 6A and B).

3.7. Effects of EPA on CREB transcriptional activity

Transient co-transfection with a pCRE-Luc plasmid harboring the luciferase reporter gene under the control of a CRE-binding sequence and pCMV-CREB into PC12 cells followed by treatment with EPA for 24 h resulted in a dose-dependent increase in luciferase activity (Fig. 7).

4. Discussion

The present study clearly demonstrated that oral administration of EPA affected hippocampal synaptic plasticity in the rat. This effect appeared to reflect the pharmacological activity of DHA, which is known to affect synaptic plasticity, because the circulating EPA was efficiently converted to DHA in the brain. It has been reported that ALA is one of the precursors of DHA, and that it can be converted to DHA in astrocytes, although its bioavailability in human brain is low, and less than 1% of ALA is converted to DHA in humans [7,26–28]. EPA is metabolized and increases the content of newly formed DHA in the phospholipids of cells from human neuroblastoma and retinoblastoma cell lines, although the membrane DHA content resulting from EPA supplementation does not match the value resulting from direct supplementation with DHA [29–31]. Langelier et al. [29] reported that EPA down-regulates overaccumulation of DHA by suppressing the expression of Δ6-desaturase, one of the key enzymes in the synthesis of DHA from docosapentaenoic acid (DPA), in SH-SY5Y neuroblastoma cells. Our findings indicated that oral administration of highly purified EPA-E supplied DHA to the rat brain under conditions of sufficient ALA supplementation. EPA could thus be a rich source of DHA without suppressing the activity of Δ6-desaturase in rats.

Importantly, EPA was immediately taken up by astrocytes through the blood–brain barrier and converted to DHA before plasma DHA level increased. EPA, but not DHA, can be transformed into anti-inflammatory prostaglandin (PG) and thromboxane of 3-class by cyclooxygenase and into leukotriene of 5-class by 5-lipoxygenase.

Table 2
Fatty acid concentrations in neuronal and glia-enriched fractions

Fatty acid		Fatty acid concentration in plasma (μg/ml)		Fatty acid concentration in glia-enriched fraction (μg/g protein)		Fatty acid concentration in neuronal fraction (μg/g protein)	
		Control II	EPA-E	Control II	EPA-E	Control II	EPA-E
Saturates							
Lauric acid	C12:0	1.9±0.1	3.0±0.8	73±5	ND	33±2	37±1
Myristic acid	C14:0	7.9±0.6	7.0±0.9	90±17	49±9	90±3	97±5
Palmitic acid	C16:0	452.3±32.7	412.5±41.4	38335±479	41813±1221*	21739±554	22215±751
Stearic acid	C18:0	275.4±12.5	264.0±18.2	28873±352	31151±888*	19112±535	19520±515
Arachidic acid	C20:0	1.8±0.2	1.6±0.2	678±30	722±33	112±9	110±5
Behenic acid	C22:0	4.4±0.2	3.2±0.2**	514±28	519±23	39±2	43±4
Lignoceric acid	C24:0	10.8±0.5	8.5±0.5**	560±34	570±22	33±2	35±3
Total saturates		754.6±39.9	699.9±60.6	69123±609	74824±2139	41158±1088	42057±1271
Monounsaturates							
Myristoleic acid	C14:1n-5	0.5±0.1	0.5±0.1	92±8	48±5***	29±4	30±3
Palmitoleic acid	C16:1n-7	21.6±2.1	28.1±6.4	633±16	720±13**	600±26	585±27
Vaccenic acid	C18:1n-7	38.7±2.6	35.8±5.0	7803±110	8697±246**	4092±174	4183±170
Oleic acid	C18:1n-9	179.8±18.7	190.5±37.0	40688±518	44832±1464*	13605±541	13778±595
Eicosenoic acid	C20:1n-9	1.0±0.1	1.2±0.2	842±41	883±41	513±22	521±14
Erucic acid	C22:1n-9	5.2±1.5	5.5±1.4	855±109	954±102	194±52	166±55
Nervonic acid	C24:1n-9	12.0±0.7	8.4±0.5**	726±34	734±39	36±2	36±2
Total monounsaturates		257.1±22.9	269.1±49.5	51639±634	56869±1753*	19070±791	19271±836
PUFAs							
n-6 PUFAs							
Linoleic acid	C18:2n-6	421.8±38.5	416.4±68.4	2443±115	2805±131	1086±35	1115±47
γ-Linolenic acid	C18:3n-6	8.9±0.8	17.1±4.4	ND	ND	ND	ND
Eicosadienoic acid	C20:2n-6	2.5±0.2	2.6±0.5	191±9	220±7*	152±6	151±6
Dihomo-γ-linolenic acid	C20:3n-6	5.9±0.8	6.1±0.9	608±19	677±24*	329±14	322±12
Arachidonic acid	C20:4n-6	682.8±38.4	576.3±36.4	24485±119	27324±670**	18157±797	18298±786
Docosatetraenoic acid	C22:4n-6	10.4±0.8	9.9±1.8	3603±68	3991±100**	3243±132	3363±135
Total n-6 PUFAs		1132.3±64.6	1028.5±109.0	31329±299	35017±850**	22966±977	23250±970
n-3 PUFAs							
α-Linolenic acid	C18:3n-3	13.0±1.9	14.9±3.7	16±2	26±2*	9±0	10±1
Eicosapentaenoic acid	C20:5n-3	18.4±2.2	561.6±220.6	57±2	232±22***	14±0	44±5**
Docosapentaenoic acid	C22:5n-3	ND	ND	ND	ND	ND	ND
Docosahexaenoic acid	C22:6n-3	62.6±3.4	46.9±7.6	15471±98	17096±408**	13689±718	13791±713
Total n-3 PUFAs		93.9±7.0	623.3±231.2	15544±100	17354±423**	13711±719	13845±717
Total PUFAs		1226.2±70.7	1651.8±336.1	46873±382	52372±1198**	36677±1694	37095±1681
Total fatty acids		2237.9±131.3	2620.8±443.7	167635±1357	184065±5020*	96904±3537	98422±3717

The EPA and DHA-deficient diet-fed rats were administered EPA-E at a single dose of 1 mg/g (EPA-E group, $n=6$) or an equal volume of vehicle (Control group II, $n=6$). *Depicts results significantly different from the Control group II: *$P<.05$, **$P<.01$, ***$P<.001$ (Student's t test or Aspin–Welch t test), ND = not deleted.

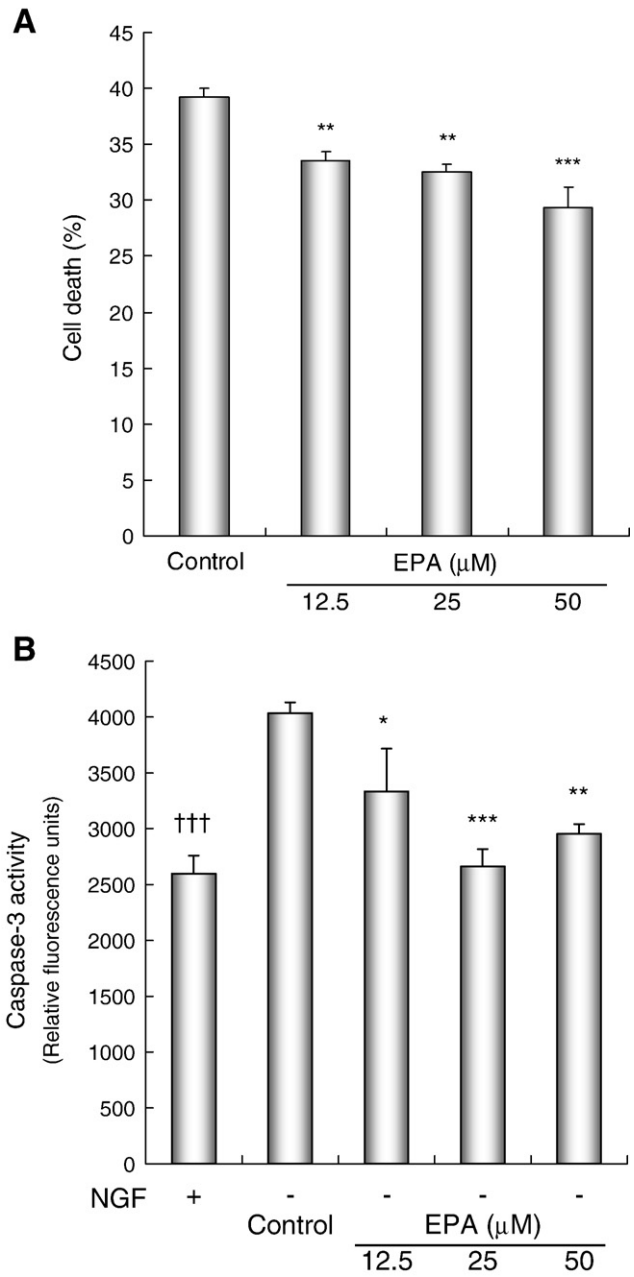


Fig. 5. Effects of EPA on serum and NGF withdrawal-induced cell death and caspase-3 activity in differentiated PC12 cells. (A) Cells were cultured for 48 h in medium including EPA and then exposed to serum-free and NGF-free DMEM medium for 48 h. Cell death was significantly decreased in EPA-treated cells compared with control (Dunnett test, $^{**}P<.01$, $^{***}P<.001$, $n=6$). (B) PC12 cells were first incubated with EPA for 48 h and then exposed to serum-free and NGF-free DMEM medium for up to 6 h. At 0 or 6 h after the start of serum and NGF deprivation, caspase-3 activity was measured. Serum and NGF withdrawal-induced increase in caspase-3 activity was suppressed by EPA treatment. †Depicts results significantly different from control cells cultured in medium without EPA prior to serum and NGF withdrawal: $^{†††}P<.001$ (Student's t test). *Depicts results significantly different from the control: $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$ (Dunnett test, $n=4$).

Therefore, highly inflammatory condition can be attenuated by inhibiting arachidonic acid (AA) metabolism competitively, resulting in reduced production of pro-inflammatory PG and thromboxane of 2-class and leukotriene of 4-class [32,33]. PGE_2 stimulates the production of beta-amyloid precursor protein (APP) and the activation of gamma-secretase and then generates amyloid-beta peptides (Abeta) 40 and 42, respectively [34]. Transgenic mice expressing the mutant type of APP, which lack either PGE_2 receptor EP2 or EP4 receptor, exhibit lower levels of Abeta 40 and 42, and oxidative injury to the brain [34,35]. PGE_2 receptor expressed in microglia is associated with

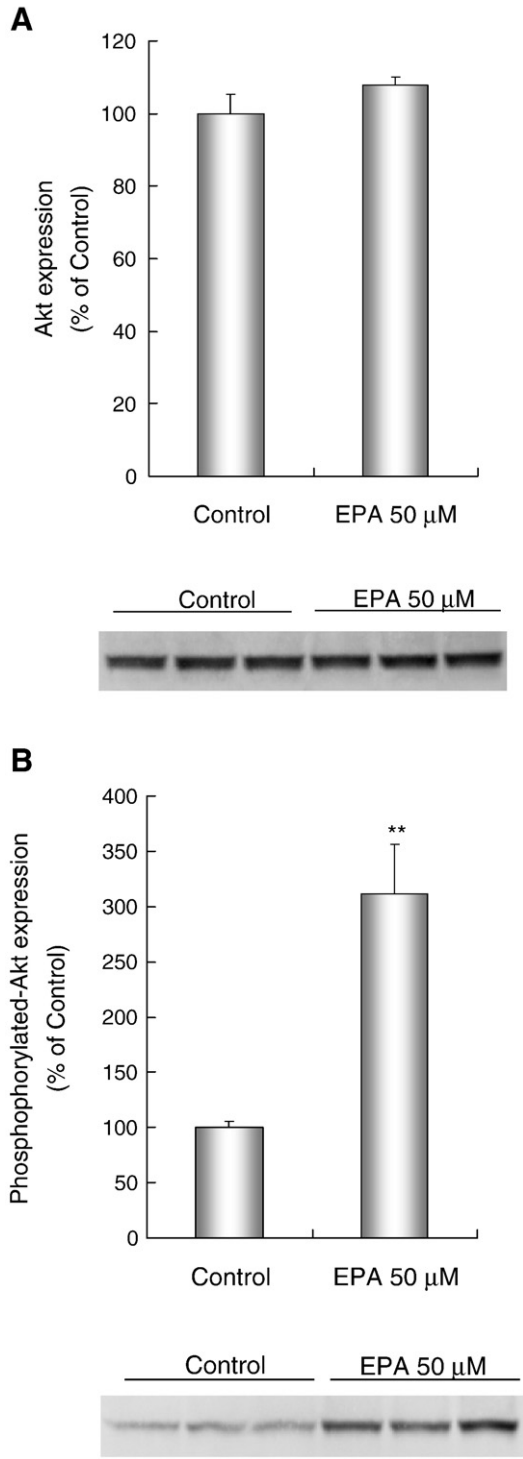


Fig. 6. Effect of EPA on Akt phosphorylation in differentiated PC12 cells. Cells were cultured for 48 h in medium including EPA and then collected and analyzed by Western blotting. No difference in total Akt expression (A) was observed. Expression of Akt phosphorylated at Ser-473 (B) was significantly increased in EPA-treated cells compared with control: $^{**}P<.01$ (Dunnett test, $n=3$). The intensities of bands were analyzed by densitometry and expressed as percentages with respect to control.

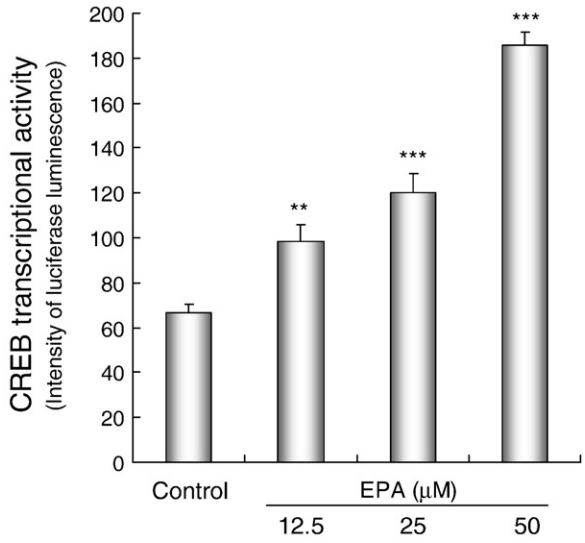


Fig. 7. Effects of EPA on CREB transcriptional activity in PC12 cells. PC12 cells were co-transfected with pCMV-CREB and pCRE-Luc, and the effects of EPA (for 24 h) were tested on firefly luciferase. Significantly different from control: $^{**}P<.01$, $^{***}P<.001$ (Dunnett test, $n=4$).

clearance of Abeta and alpha-synuclein [36,37]. The PGE_2 concentration of cerebrospinal fluid is elevated in AD patients [38]. In schizophrenic patients, the plasma level of PGE_2 is also elevated and correlated with symptoms [39,40]. Peet et al. [4] suggested that EPA supplementation is preferable to DHA supplementation, which performed no better than placebo in alleviating the symptoms in schizophrenic individuals. EPA was transferred to astrocytes from brain endothelial cells and might exhibit additive anti-inflammatory effects compared with DHA in the CNS.

Decreased brain level of DHA is associated with impairment of memory-related learning ability in normal nonaged rats, aged rats and AD model rats, and chronic DHA administration reverses this impairment [21,41,42]. In this study, administration of EPA-E actually modulated synaptic plasticity (LTP) with accompanying increase in DHA level. However, a conflicting finding has been reported which showed that LTP was not affected in nonaged rats administered EPA, unlike the improvement of impaired LTP in aged rats [15]. Gamoh et al. [21] reported that chronic DHA administration improves reference memory-related learning ability in nonaged rats. The previously cited experiment performed by Martin et al. [15] was using standard laboratory chow, while fish oil-deficient chow, the same as in this study, was used in the after-mentioned study performed by Gamoh et al. [21]. These findings thus suggest the importance of n-3 PUFA supply even when a sufficient ALA is supplied in the diet. Further studies are required to clarify the interrelationships among different fatty acids and to develop effective treatment with n-3 PUFAs. However, chronic EPA administration modulated neuronal plasticity by increasing DHA content and probably by exerting several DHA-related biological pathways such as modulation of synaptic plasma membrane fluidity [15]; increase in anti-oxidative enzymes [43]; activation of PI3-kinase [25]; enhancement of pre- and post-synaptic proteins, including synapsin-1, post-synaptic density 95 and syntaxin-3 [44]; and regulation of *N*-methyl-D-aspartate receptors [45].

An issue remaining to be explored, however, is whether the effects of oral administration of EPA are mediated solely by its metabolite DHA or whether EPA exerts effects, at least in part, on synaptic plasticity prior to its transformation to DPA and DHA. Investigation of the fatty acid profiles of neuronal and glia-enriched fractions demonstrated that a single administration of EPA significantly increased neuronal EPA content, clearly suggesting that EPA, but not its metabolite DHA, was indeed taken into neurons. The precise neuronal function of EPA in the brain is not clear, whereas DHA has been reported to promote neuronal differentiation, prevent apoptosis and activate neuronal gene expression [1]. It was reported that DHA activates Akt by promoting phosphatidylserine accumulation in neuronal membranes and translocation of Akt to plasma membrane in mouse neuroblastoma cells, but that DPA does not [46,47]. Here, we confirmed that EPA enhanced cell survival as effectively as DHA in differentiated PC12 cells by facilitating activation of Akt (data not shown). Akt translocation might thus be one of the targets of EPA. On the other hand, it is also possible that EPA directly increases PI3-kinase activity by promoting p85α expression in neurons. Calon et al. [25] reported that DHA treatment preserved the loss of p85α expression and behavioral deficits caused by reduction of dietary n-3 PUFAs in a mouse model of AD, and we have confirmed the effect of ethyl-ester DHA on hippocampal p85α mRNA, which exhibited only a 1.6-fold increase compared to that in the EPA and DHA-deficient diet group (data not shown), whereas EPA-E administration led to 2.2-fold increase in hippocampal p85α mRNA. It has also been reported by Maher et al. [48] that EPA treatment prevented age-related impairment in PI3-kinase activity in cortical tissue. Although further studies are needed to confirm the direct contribution of EPA to neuronal function, these findings suggest that EPA itself might directly exert positive effects in the same fashion as DHA affects neuronal function, although it is less abundant than DHA and AA.

In conclusion, our findings suggest a potential neuroprotective property of EPA in the modulation of synaptic plasticity and activation of the PI3-kinase pathway possibly by its direct effects on neurons and glial cells and by its capacity to increase brain DHA. EPA may thus be of use in preventing or treating neurological disorders.

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