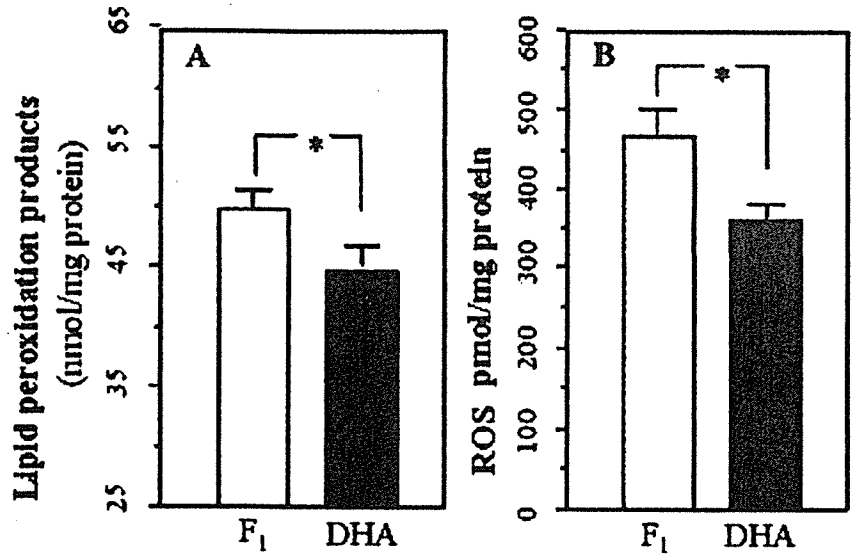

Fig. 3. Effects of DHA administration on synaptic plasma membrane lipid per-oxidation, as measured by thiobarbituric acid reactive substances (A), and on the basal levels of reactive oxygen species (ROS) in the cortex whole homogenate (B). Results are mean ± SE for 10 to 12 rats each with duplicate determinations. See Materials and methods section for details. * $p < 0.05$, unpaired student's t test; □ = F1, control rats; ■ = F1 + DHA, DHA-administered rats.

increased significantly upon DHA administration. Docosapentaenoic acid was present as trace amount, however, it increased significantly in the SPM of DHA rats. The presence of eicosapentaenoic acid in the synaptic membrane is often disputed. Thus, it remains to be clearly known whether it is the retro-conversion product of synaptic DHA or whether it comes from extra-neuronal sources and are misidentified as contaminating n-3 fatty acids. Other fatty acids increased slightly, but not significantly,

Table 2
Effect of dietary administration of DHA on the Liver Fatty acid Profile (μg/mg of protein)

	F_1	F_1 + DHA
Palmitic acid ($C_{16:0}$)	28.0 ± 1.70	33 ± 1.30
Stearic acid ($C_{18:0}$)	19.0 ± 1.15	20.0 ± 0.5
Oleic acid ($C_{18:1,\ n\text{-}9}$)	11.0 ± 1.50	11.0 ± 0.9
Linoleic acid ($C_{18:2,\ n\text{-}6}$)	26.5 ± 2.50	30.0 ± 1.2
Linolenic acid ($C_{18:2,\ n\text{-}6}$)	0.45 ± 0.05	0.50 ± 0.04
Arachidonic acid ($C_{20:4,\ n\text{-}6}$)	25.5 ± 1.00	20.0 ± 0.50*
Eicosapentaenoic acid ($C_{20:5,\ n\text{-}3}$)	0.47 ± 0.04	2.05 ± 2.05*
Docosapentaenoic acid ($C_{22:5,\ n\text{-}3}$)	1.40 ± 0.04	1.97 ± 0.09*
Docosahexaenoic acid ($C_{22:6,\ n\text{-}3}$)	8.25 ± 0.25	16.9 ± 0.40*
n-3 USI	0.55 ± 0.03	0.93 ± 0.01*
USI	1.22 ± 0.009	1.55 ± 0.012*
n-6 USI	0.56 ± 0.01	0.53 ± 0.001
n-3 USI/n-6 USI	1.01 ± 0.08	1.75 ± 0.05*

Results are mean ± SE with n = 10 rats from each group. Unsaturation index (USI) was calculated as [Σ(mole% of each polyunsaturated fatty acid × number of double bonds)]/100. n-3 USI = [3 × ($C_{18:3}$) + 5 × ($C_{20:5}$) + 6 ($C_{22:5}$) + 6 × ($C_{22:6}$)]/100, and n-6 USI = [2 × ($C_{18:2}$) + 4 × ($C_{20:4}$)]/100.

*$p < 0.05$ vs. F_1 control. n-3 USI = USI calculated using only n-6 polyunsaturated fatty acids.

Table 3
Effect of dietary administration of DHA on the synaptosomal plasma membrane fatty acid profile and cholesterol-phospholipid composition in rat cerebral cortex

	Control rats (n = 10)	DHA-fed rats (n = 10)
Fatty acid (nmol/mg protein)		
Palmitic acid ($C_{16:0}$)	16.9 ± 0.65	20.4 ± 0.95
Stearic acid ($C_{18:0}$)	61.2 ± 2.30	69.1 ± 2.40
Oleic acid ($C_{18:1, n-9}$)	8.10 ± 0.60	10.3 ± 0.70[a]
Linoleic acid ($C_{18:2, n-6}$)	1.10 ± 0.08	1.60 ± 0.90
Arachidonic acid ($C_{20:4, n-6}$)	15.1 ± 0.70	12.3 ± 0.90[a]
Eicosapentaenoic acid ($C_{20:5, n-3}$)	1.05 ± 0.03	0.95 ± 0.06
Docosapentaenoic acid ($C_{22:5, n-3}$)	0.45 ± 0.02	0.60 ± 0.05[a]
Docosahexaenoic acid ($C_{22:6, n-3}$)	42.0 ± 1.25	48.8 ± 1.50[a]
$C_{22:6, n-3}/C_{20:4, n-6}$	2.85 ± 0.15	4.20 ± 0.35[a]
Cholesterol-Phospholipid (nmol/mg protein)		
Cholesterol	40.7 ± 3.85	34.5 ± 3.20
Phospholipid	100 ± 6.40	120 ± 6.35[a]
Cholesterol-Phospholipid	0.41 ± 0.25	0.29 ± 0.02[a]

Results are mean ± SE.
[a] $p < 0.05$, significantly different with control rats.

as a result of the dietary administration of DHA. The total cholesterol content of the cerebral cortex SPM was not altered by DHA administration, while the total phospholipid content of the SPM increased significantly in the DHA-fed rats, leading to a significant decrease in the cholesterol/phospholipid molar ratio.

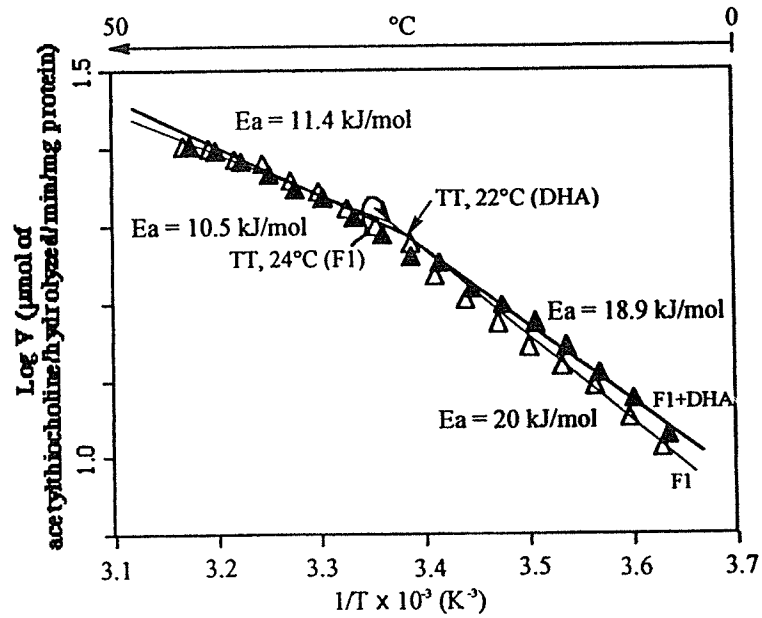

Fig. 4. Arrhenius plots showing the effects of DHA administration on the temperature-dependence of synaptic plasma membrane-bound AChE activity of control (△) and DHA-fed (▲) rats.

Effect of dietary DHA administration on the temperature dependence of AChE activity

The Arrhenius plot showed that the break point for the AChE activity from DHA-fed rats was 22 °C and that of the F1 rats was 24 °C (Fig. 4). The activation energy (Ea) of the enzyme below and above the transition temperatures (TT) were not altered significantly between the two rat groups (Ea (kJ/mol) below the TT: F1 rats, 20.0 and F1 + DHA, 18.9; above the TT: F1, 10.5 and F1 + DHA, 11.5).

Correlation of SPM fatty acids, LPO and membrane order

Pyrene-determined membrane lateral mobility of annular and global regions both correlated positively with the DHA/AA molar ratio of the cerebral cortex SPM (annular mobility, $r = 0.59$, $p < 0.005$; average or global mobility, $r = 0.45$, $p < 0.05$). DPH polarization of the SPM correlated negatively with both DHA content ($r = -0.53$, $p < 0.05$) and the DHA/AA molar ratio ($r = -0.58$, $p < 0.02$). Synaptosomal membrane LPO correlated positively with DPH-polarization ($r = 0.44$, $p < 0.05$) and negatively with pyrene-determined lateral mobility, of both the annular ($r = -0.61$, $p < 0.005$) and global ($r = -0.48$, $p < 0.05$) regions. The DHA/AA molar ratio in the SPM correlated negatively with the level of LPO ($r = -0.39$, $p < 0.05$).

Discussion

The present experiment revealed that DHA administration increases the disorder of the annular and hydrophobic core regions of the cerebral cortex SPM and also reduces the levels of LPO and ROS, while having no effect on SPM-bound AChE activity. Increased DHA content of the cerebral cortex SPM failed to alter the order of the water-lipid interfacial surface region of the bilayer leaflet, as indicated by the absence of change in TMA-DPH fluorescence polarization. This TMA-DPH fluoroprobe result is consistent with our previous demonstration in endothelial cell plasma membranes (Hashimoto et al., 1999b), but it appears, however, to conflict with the results of Yorek et al. (1989), who reported an increase in surface membrane disorder in DHA-enriched retinoblastoma (Y-76) cells. This discrepancy may be based on the differences in cell types, the extent of membrane perturbations and/or the sensitivity of the TMA-DPH probe to the small increases (by 16% only) in DHA content in the SPM of DHA-fed rats observed in our experiment as opposed to higher increases (1400%) in DHA content in the Y-76. AChE remains attached to the surface of the SPM (Fernandez, 1996), and since the surface membrane order of the SPM was not altered, DHA enrichment thus may not affect the activity of the surface-bound AChE.

Mitchell and Litman (1998) reported that polyunsaturated fatty acids particularly DHA confers a greater degree of disorder at the midplane of the bilayer than do monounsaturated and/or saturated fatty acids or even polyunsaturated n-6 arachidonic acid. Conceivably, therefore, DHA administration caused an increase in disorder of the DPH-probed mid-acyl chain hydrophobic regions of the SPM, but the SPM-bound AChE activity was not, however, affected by the increase in hydrophobic core lipid disorder. The extent to which AChE is sensitive to alterations in the hydrophobic lipid core is thus uncertain (Nemat-Gorgani and Meisami, 1979). This finding contrasts with the increased activity of synaptosomal AChE found in rats fed high versus low 18:2/18:3 diets (Foot et al., 1983). Our results also contrast with those of several other studies, where aqueous (Tanii et al., 1995), organic (Edelfors and

Ravn-Jonsen, 1992; Engelke et al., 1992) or anesthetic compounds (Ondrias et al., 1983; Sidek et al., 1984; Mazzanti et al., 1986) were shown to alter AChE activity with concomitant changes in DPH-determined membrane disorder of the SPM. Although it is difficult to account for this discrepancy, it may be considered that these organic molecules were encountered by the acyl chains of the phospholipid bilayer acyl chain as an extrinsic factor, whereas DHA after in vivo administration is intrinsically esterified to the phospholipid glycerol backbone. Following this line of reasoning, the extent of membrane perturbation, regarding the effects on membrane free volume and net effects on activation energy conferred by the organic molecules (viz. alcohol, toluene and anesthetics) and by dietary DHA enrichment would obviously be different and their respective effects on AChE would also be different. This inconsistency led us to measure the mobility of annular lipid region, which remains intimately contacted with the membrane-bound proteins. The random distribution of DPH, TMA-DPH and pyrene provide average values for all microregions of the bilayers even if the bilayers have microregions of different disorder. The changes in the membrane order of the annular region, rather than those of the non-annular region, would thus have a more intense effect, if there is an effect at all, on the properties of the membrane-bound proteins.

The increase in the annular lipid mobility in the DHA-fed rats did not affect the activity of AChE. It can be speculated that the interaction of DHAs of phospholipids in the SPM and AChE did not reach the extent that might lead to a change in the AChE activity.

Cholesterol decreases the membrane free volume by intimate interaction with the saturated acyl chains (Mitchell and Litman, 1998). A decrease in the cholesterol/phospholipid molar ratio causes an increase in plasma membrane order (North and Fleischer, 1983; Hashimoto et al., 1999a,b; Hossain et al., 1999a). This ratio was also significantly lower in the more disordered synaptic membranes of the DHA-fed rats. The similarity in AChE activity levels in the SPM of the control and DHA rats, despite differences in their cholesterol/phospholipid molar ratio, goes against the assumption that the modulation of rat AChE activity reflects changes in the membrane sterol components rather than changes in its acyl composition (Hrboticky et al., 1989). In addition, an increase in membrane lipid peroxidation has been found to reduce membrane order in the isolated synaptosomes (Aksentsev et al., 1995; Urano et al., 1997), platelets (Hossain et al., 1999b) and endothelial cells (Hashimoto et al., 1999a,b). In the present study, DPH polarization correlated positively and pyrene-determined annular/global lateral mobility correlated negatively with LPO. This suggests that the enhancement of annular lipid and/or global mobility in the SPM of DHA-fed rats resulted from the combined effects of the reduced cholesterol/phospholipid molar ratio and lipid peroxidation, and the increased synaptosomal DHA and DHA/AA molar ratio. These results also indicate that DHA in vivo did not play an oxidative role, in which case SPM disorder would have been reduced rather than increased.

The activity of AChE was not affected by DHA administration. An increased membrane disorder allows the enzyme more conformational freedom, thus decreasing the activation energy. To evaluate this, the enzyme AChE activity was measured at different temperatures and the energy of activation was calculated. In this study, Arrhenius plots of synaptosomal AChE activity were discontinuous, with a 2 °C lower transition temperature in the DHA rats than that in the control rats. Activation energies of AChE of both above and below the transition temperatures were not altered significantly between the SPMs of control and DHA-fed rats. Though a lower transition temperature suggests a more disordered or fluid microenvironment for the AChE in the synaptosomal membrane, such environment, however, could not affect the activity of the enzyme. Thus the behavior of AChE is not consistent with the defined changes in the fatty acid composition of the SPM of DHA vs.

control rats. This may suggest that acyl chain structure of the lipid fatty does not affect the temperature dependence of the AChE activity so long as the lipids that surround and/or hold the enzyme are in the liquid-crystalline state as seen above the transition temperature. The activity of AChE in the DHA-fed rats tended to increase, though not significantly, below the transition temperatures and this may relate to increased membrane free volume and hence smaller activation energy conferred by the DHA acyl chain. Or, in other words, the catalytic unit of the AChE does not interact substantially with the lipid acyl chain regions that undergo phase transition, and is to be classified as extrinsic protein. This behavior of AChE is also consistent with the fact that the activity of this enzyme was not altered even though the SPM annular lipid mobility increased significantly. Our result on the effect of n-3 DHA on the temperature-dependence of AChE is consistent with that of the Horboticky et al. (1989).

A DHA-induced increase in memory related-performance is accompanied by increased levels of acetylcholine (Minami et al., 1997) in the cerebrum, and if DHA could cause an increase in the AChE activity, this increase in the acetylcholine level could not occur. This is because acetylcholine is hydrolysed by AChE, causing its level to decrease. In general, acetylcholine level is decreased and choline levels are increased in memory impairment such as in Alzheimer's disease due to cholinergic dysfunctions including increased AChE activity (Sáez-Valero et al., 2002), decreased choline acyltransferase activity (Nabeshima and Nitta, 1994; Nitta et al., 1994, 1997), and impaired acetylcholine release (Itoh et al., 1996). Furthermore, DHA-induced increases in membrane disorder and antioxidative effects could be extrapolated to at least a partial relationship with increased vesicular neurotransmitter (acetylcholine) release, because an increased proportion of DHA relative to AA, reduced synaptosomal lipid peroxidation and a reduced cholesterol/phospholipid ratio (which all occurred in the present investigation) have been demonstrated to facilitate the increased release of acetylcholine (Urano et al., 1997). Thus the lack of the effects of DHA on the AChE activity may relate with its ability to increase the brain acetylcholine level (Minami et al., 1997) with concurrent increases of cognitive functions after DHA administration (Hashimoto et al., 2002).

Conclusion

Dietary administration of DHA significantly increased the synaptic plasma membrane annular lipid mobility without a concurrent effect on acetylcholinesterase activity. The present observations may have significant physiological implications and may be of importance in understanding the physical and biochemical effects of DHA in connection with the physiological impact of DHA on acetylcholinesterase.

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Chronic Administration of Docosahexaenoic Acid Ameliorates the Impairment of Spatial Cognition Learning Ability in Amyloid β–Infused Rats

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ABSTRACT We investigated whether administration of docosahexaenoic acid (DHA), a major (n-3) fatty acid of the brain, ameliorates the impairment of learning ability in an animal model of Alzheimer's disease (AD), rats infused with amyloid-β (Aβ) peptide (1–40) into the cerebral ventricle. Inbred 3rd generation male rats (20 wk old) fed a fish oil–deficient diet were randomly divided into 4 groups: a vehicle group, an Aβ peptide-infused group (Aβ group), a DHA group, and an Aβ + DHA group. A mini-osmotic pump filled with Aβ peptide or vehicle was implanted in the rats, and they were tested for learning ability–related reference and working memory in an 8-arm radial maze. The rats were then orally fed DHA dissolved in 5% gum Arabic solution at 300 mg/(kg · d) (DHA and Aβ + DHA groups) or vehicle alone (vehicle and Aβ groups) and tested again for learning ability. DHA administered for 12 wk significantly reduced the increase in the number of reference and working memory errors in the Aβ-infused rats, and increased both the cortico-hippocampal level of DHA and the molar ratio of DHA/arachidonic acid, suggesting an amelioration of the impaired spatial cognition learning ability. Furthermore, DHA suppressed the increases in the levels of lipid peroxide and reactive oxygen species in the cerebral cortex and the hippocampus of Aβ-infused rats, suggesting that DHA increases antioxidative defenses. DHA is thus a possible therapeutic agent for ameliorating learning deficiencies due to Alzheimer's disease. J. Nutr. 135: 549–555, 2005.

KEY WORDS: • *docosahexaenoic acid* • *therapeutic agent* • *spatial working memory* • *antioxidative defense* • *Alzheimer's disease*

Docosahexaenoic acid [DHA; 22:6(n-3)],[2] one of the main structural lipids in the mammalian brain, is essential for normal neurological development and for vision (1). Deficiency in this fatty acid is associated with a loss of discriminative learning ability (2,3); thus intake of DHA may restore lost learning ability. Consistent with these findings, we demonstrated that chronic administration of DHA enhances long-term memory in both young (4) and old (5) rats. Treatment with DHA improves the neurological condition in Zellweger's syndrome, a peroxisomal disorder that produces serious mental retardation (6). More interestingly, the DHA level in the hippocampus was found to be very low (7) in patients with Alzheimer's disease (AD), compared with that in brain samples from age-matched human controls. AD is characterized by the formation of neurofibrillary tangles and neuritic plaque of amyloid peptides such as amyloid-β (Aβ) peptide (1–40), as well as by neuronal and memory loss. We reported recently that preadministration of DHA protects against the impairment of learning ability in an animal model of AD, rats infused with Aβ peptide (1–40) into the cerebral ventricle (8).

Epidemiologic studies show a relation between sources of dietary fish oil, especially DHA, and AD. Intake of DHA has been associated with reduced risk of AD (9). DHA oil supplementation was shown to improve intellectual function in the elderly (10). We therefore hypothesized that chronic administration of DHA may ameliorate the impairment of learning ability in Aβ-infused rats.

MATERIALS AND METHODS

Animals and diet. Wistar rats (generation 1, G1) (Jcl: Wistar; Clea Japan) were housed in a room under controlled temperature (23 ± 2°C), relative humidity (50 ± 10%), and light-dark cycles (light: 0800 to 2000 h; dark: 2000 to 0800 h). Rats consumed a fish oil–deficient diet (F-1®; Funabashi Farm) (Table 1) and water ad libitum. The inbred 3rd generation male rats [n = 38; 20 wk old; 376.3 ± 3.3 g body weight (BW)], fed the same F-1 diet, were randomly divided into 4 groups: a vehicle group (n = 9), an Aβ peptide-infused group (Aβ group) (n = 10), a DHA group (n = 9) and an Aβ + DHA group (n = 10). The rats were handled and killed in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Shimane Medical University, compiled from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science.

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[2] Abbreviations used: AA, arachidonic acid; Aβ, amyloid-β; AD, Alzheimer's disease; BW, body weight; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; LTP, long-term potentiation; PLSD, protected least significant difference; RME, reference memory error; ROS, reactive oxygen species; USI, unsaturation index; WME, working memory error.

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TABLE 1

Ingredients and fatty acid composition of the F1 diet

	F1[1]	Fatty acid	F1[2]
	g/kg		*g/kg*
Corn grain	191	Myristic acid (14:0)	0.9 ± 0.9
Wheat bran	218	Palmitic acid (16:0)	156 ± 5.3
Wheat flour	358	Palmitoleic acid [17:1(n-7)]	ND
Soybean meal	80	Stearic acid (18:0)	54.1 ± 0.9
Casein	40	Oleic acid [18:1(n-9)]	211 ± 1.7
Dry skim milk	38	Linoleic acid [18:2(n-6)]	524 ± 8
Soybean oil	15	Linolenic acid [18:2(n-3)]	44.9 ± 1.3
Mineral mixture[3]	10	Arachidic acid (20:0)	1.4 ± 0.9
Vitamin mixture[4]	10	Eicosenoic acid [20:1(n-9)]	3.3 ± 1.3
Amino acid mixture[5]	10	Arachidonic acid [20:4(n-6)]	ND
DL-Methionine[6]	1	Eicosapentaenoic acid [20:5(n-3)]	0.6 ± 0.6
Calcium carbonate[6]	9	Docosapentaenoic acid [22:5(n-3)]	ND
		Docosahexaenoic acid [22:6(n-3)]	ND
		Lignoceric acid (24:0)	1.1 ± 0.7

1 The F1 standard diet, containing no fish products, comprised (g/100 g): protein, 21.3; fat 5.1; fiber 1; carbohydrate, 5; nonnitrogen, 57.5; and total energy, 17.7 J/g and was purchased from Funabashi Farm, Chiba, Japan.

2 Values are means ± SEM, n = 4; ND, not detected.

3 Mineral mixture (g/kg) as formulated by Takeda Kagaku Shiryo, Tokyo, Japan: $MnSO_4$, 15.7; $FeSO_4$, 23.8; $CoSO_4$, 0.7; $CuSO_4$, 1.0; Ca $(IO_3)_2$, 0.5; $MgCO_3$, 3.0; NaCl, 300.0; $CaCO_3$, 655.3.

4 Vitamin mixture (g/kg) as formulated by Takeda Kagaku Shiryo: retinal, 300 mg/kg; vitamin D oil, 5 mg/kg; *dl*-α-tocopherol acetate, 5.0; menadione, 1.0; thiamine nitrate, 0.7; riboflavin, 0.8; pyridoxine hydrochloride, 1.0; nicotinamide, 4.0; calcium pantothenate, 1.7; choline chloride, 65.0; cyanocobalamin, 0.5; biotin, 0.015; saccharin sodium, 8.5; mil S-Na_2 [natural flavor (g/100 g): carbohydrate 8; protein 16; lactate 52; fat, 18.5], 100.0; glucose 90.0.

5 Amino acid mixture (g/kg) as formulated by Takeda Kagaku Shiryo: DL-methionine, 300.0; L-lysine hydrochloride, 300.0; defatted rice bran, 400.0.

6 Wako Pure Chemicals (Osaka, Japan).

Preparation of Aβ-infused rats. The surgical techniques for preparing Aβ-infused rats were essentially the same as those described (8). Briefly, each rat was anesthetized lightly with sodium pentobarbital (50 mg/kg BW, i.p.); the skull was then exposed and 2 holes (right and left, relative to the bregma; 0.8 mm posterior, 1.4 mm lateral) were drilled according to the atlas of Paxinos and Watson (11) using a stereotaxic frame (Narishige). Then, 0.5 μg $AlCl_3$ (in 5 μL, intracerebroventricularly, 1 μL/min) was injected through a cannula 3.5 mm into the right ventricle, with a Hamilton syringe. Although the cause of AD is Aβ (1–42), we used Aβ (1–40) because of its better solubility. Moreover, because a small amount of $AlCl_3$ facilitated aggregation of Aβ peptide in vitro, and because the method has limited reproducibility without $AlCl_3$, we used $AlCl_3$ before implanting the osmotic pump for continuous infusion of Aβ. This procedure greatly improved the reproducibility and reliability of producing this animal model of AD, rats with impaired memory. A mini-osmotic pump (Alzet 2002, Durect), containing either Aβ peptide (1–40) solution or vehicle alone was quickly implanted in the back of the rat. The outlet of the pump was inserted 3.5 mm into the left ventricle and attached to the skull with screws and dental cement.

Radial maze-learning ability and DHA administration. The rats were tested for learning ability 4 wk after the implantation of the mini-osmotic pump to verify the memory impairment. Learning-related behavior was assessed using an 8-arm radial maze (Toyo Sangyo) as described (4,5). Briefly, the rats were trained to acquire a reward (food-pellet) at the end of each of 4 arms of an 8-arm radial maze. The performance involved 2 parameters of memory function, i.e., reference memory error (RME), entry into unbaited arms; and working memory error (WME), repeated entry into arms that had already been visited and obtaining the rewards within a trial. Each rat was given 2 daily trials, 6 d/wk for a total of 2.5 wk. The DHA and Aβ + DHA groups were then orally fed DHA-95E [300 mg/(kg · d), an ethyl-ester all-*cis*-4,7,10,13,16,19-docosahexaenoate with a purity of over 95%; Harima Chemicals] gently emulsified in a 5% gum Arabic solution in ice-cold water; the vehicle and Aβ groups were fed an equal volume of vehicle only.

Seven weeks after starting the administration of DHA, the rats were tested again for learning ability using an 8-arm radial maze for a total of 5 wk, to assess the effect of DHA on the impairment of learning ability.

Measurement of fatty acid profiles and oxidative status. 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 (8). The tissues were stored at −80°C by flash-freezing in liquid N_2 until use or immediately homogenized in 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). The homogenates were immediately subjected to the assays described below or stored at −80°C after liquid N_2 flash and bath until use.

Lipid peroxide concentration was assessed by the TBARS assay, as described (8,12). TBARS levels are expressed as nanomoles of malondialdehyde/mg 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 (8,12). Briefly, 50 μL freshly prepared tissue homogenate was mixed with 4.85 mL of 100 mmol/L potassium phosphate buffer (pH 7.4) and incubated with 2′,7′-dichlorofluorescin diacetate 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 100 mmol/L phosphate buffer (pH 7.4) and incubated again for 60 min at 37°C. Fluorescence was measured with a Hitachi 850 spectrofluorometer (Tokyo, Japan) at wavelengths of 488 nm for excitation and 525 nm for emission. The cuvette holder was maintained at 37°C. ROS were quantified from a dichlorofluorescin standard curve in methanol.

Fatty acid composition was determined by the one-step analysis of Lepage and Roy (13) by GC as described (12,14). Protein concentration was estimated by the method of Lowry et al. (15).

Statistical analysis. Results are expressed as means ± SEM. Behavioral data were analyzed by a 2-factor (group and block) randomized block factorial ANOVA, and all other parameters were analyzed for intergroup differences by 1-way ANOVA. ANOVA was followed by Fisher's PLSD for post-hoc comparisons. Correlation was determined by simple regression analysis. The statistical programs used were GB-STAT™ 6.5.4 (Dynamic Microsystems), and StatView® 4.01 (MindVision Software, Abacus Concepts). Differences with $P < 0.05$ were considered significant.

RESULTS

Body weight. Body weights did not differ among the groups after the administration of DHA for 12 wk (vehicle: 494 ± 8 g; DHA: 481 ± 6 g; Aβ: 484 ± 9 g; Aβ + DHA: 490 ± 10 g).

Effect of DHA administration on radial-maze learning ability. The effects of Aβ peptide (1–40) infused into the rat cerebral ventricle and that of DHA administered to vehicle and Aβ-infused rats for 12 wk on reference and working memory-related learning ability are shown in **Figures 1** and **2**, respectively. The score is expressed as the mean number of RMEs and WMEs for each group, with data averaged over blocks of 6 trials. The *left panels* in both figures indicate the effect of the infused Aβ peptide (1–40). Randomized 2-factor (block and group) ANOVA to analyze the effect of the infused Aβ revealed significant main effects of both blocks of trials (P

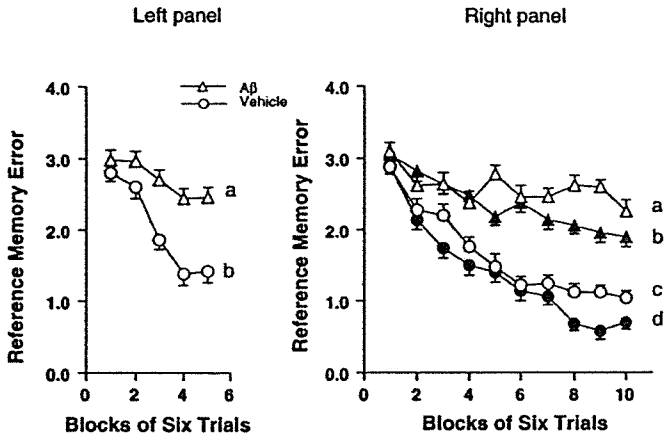

FIGURE 1 Effect of the infusion of amyloid β (Aβ) peptide (1–40) into the rat cerebral ventricle (*left panel*) and the effect of docosahexaenoic acid (DHA) administered to the Aβ-infused rats (*right panel*) on reference memory–related learning ability in the radial maze task. Each value represents the number of RMEs as the mean ± SEM in each block of 6 trials. Groups without a common letter for the main effects of groups are significantly different at $P < 0.05$. *Left panel*: vehicle rats ($n = 19$), Aβ rats ($n = 19$). The significance of differences between the 2 groups was determined by randomized 2-factor (block and group) ANOVA followed by Fisher's PLSD test; main effects of blocks of trials and groups were both significant ($P < 0.0001$), with a significant block × group interaction ($P < 0.0001$) on the number of RMEs. *Right panel*: Aβ rats ($n = 9$), Aβ + DHA rats ($n = 10$), vehicle rats ($n = 9$), DHA ($n = 10$). The significance of differences among the 4 groups was determined by randomized 2-factor (block and group) ANOVA followed by Fisher's PLSD test; main effects of blocks of trials and groups were both significant ($P < 0.0001$), with a significant block × group interaction ($P < 0.0001$) on the number of RME. Details of the subtest analysis between 2 groups of main effects of blocks of trials and groups, and between 2 groups of block × group interaction are shown in Table 2.

< 0.0001) and groups ($P < 0.0001$) with a significant block × group interaction ($P < 0.0001$) on the number of RMEs (Fig. 1, *left panel*). Similarly, a significant main effect of blocks of trials ($P < 0.0001$) and groups ($P < 0.0001$) with a significant block × group interaction ($P = 0.0174$) was observed on the number of WMEs (Fig. 2, *left panel*). These results indicate that Aβ peptide (1–40) infused into the rat cerebral ventricle impaired reference and working memory in the rats, suggesting learning impairment, a well-known characteristic of AD.

The *right panels* in both figures show the effect of DHA administered to vehicle and Aβ-infused rats. Randomized 2-factor (block and group) ANOVA revealed significant main effects of both blocks of trials ($P < 0.0001$) and groups ($P < 0.0001$) on the number of RMEs (Fig. 1, *right panel*), with a significant block × group interaction ($P < 0.0001$). Similarly, significant main effects of both blocks of trials ($P < 0.0001$) and groups ($P < 0.0001$) were observed, but without a significant block × group interaction ($P = 0.0911$) on the number of WMEs (Fig. 2, *right panel*). Subtest analysis (Table 2) of the number of RMEs showed the effect of DHA on Aβ-infused rats [blocks of trials ($P < 0.0001$) and groups ($P = 0.0027$), with a significant block × group interaction ($P = 0.0051$)]; the effect of DHA on vehicle rats [blocks of trials ($P < 0.0001$) and groups ($P = 0.0008$), without a significant block × group interaction]; and the effect of Aβ on vehicle rats [blocks of trials ($P < 0.0001$) and groups ($P < 0.0001$), with a significant block × group interaction ($P < 0.0001$)], demonstrating that the Aβ + DHA and DHA rats had a lower RME score than the Aβ-infused and vehicle rats (Fig. 1, *right panel*). Similarly, subtest analysis (Table 2) of the number of WMEs showed the effect of DHA on Aβ-infused rats [blocks of trials ($P < 0.0001$) and groups ($P = 0.0003$), without a significant block × group interaction]; the effect of DHA on vehicle rats [blocks of trials ($P < 0.0001$), but not groups ($P = 0.0823$), without a significant block × group interaction]; and the effect of Aβ on vehicle rats [blocks of trials ($P < 0.0001$) and groups ($P < 0.0001$), without a significant block × group interaction ($P = 0.067$)], demonstrating that the Aβ + DHA and DHA rats had a lower WME score than the Aβ-infused and vehicle rats (Fig. 2, *right panel*). These analyses suggest that administration of DHA improved reference and working memory-related spatial cognition of Aβ-infused and vehicle rats.

Effect of DHA administration on fatty acid profiles of plasma and brain. The plasma DHA level was significantly higher in both the DHA and Aβ + DHA groups than in the vehicle and Aβ groups and was accompanied by a significant decrease in arachidonic acid (AA), resulting in a significantly higher DHA/AA ratio (Table 3). The plasma levels of eicosapentaenoic acid [20:5(n-3)] and docosapentaenoic acid [DPA, 22:5(n-3)] were also significantly higher in both the DHA and Aβ + DHA groups than in the vehicle and Aβ groups. The increase in plasma (n-3) PUFA led to a higher unsaturation index (USI) of fatty acids in both the DHA and Aβ + DHA groups than in vehicle and Aβ groups (Table 3).

The administration of DHA significantly increased the DHA level in the hippocampus and hence the DHA/AA

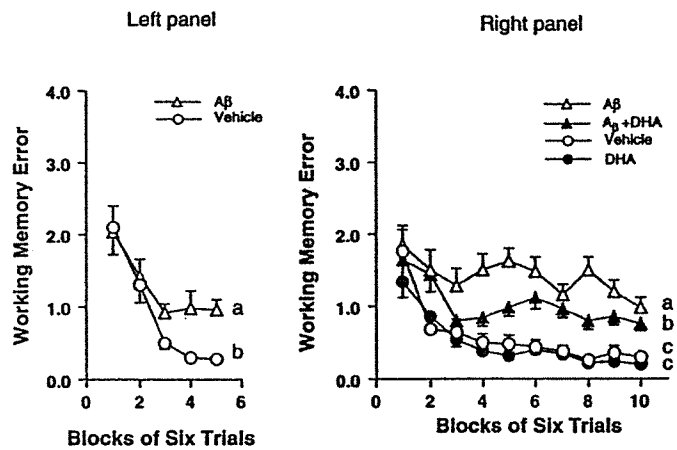

FIGURE 2 Effect of the infusion of Aβ peptide (1–40) into the rat cerebral ventricle (*left panel*) and the effect of DHA administered to the Aβ-infused rats (*right panel*) on working memory–related learning ability in the radial maze task. Each value represents the number of WMEs as the mean ± SEM in each block of 6 trials. Groups without a common letter for the main effects of groups are significantly different at $P < 0.05$. *Left panel*: vehicle rats ($n = 19$), Aβ rats ($n = 19$). The significance of differences between 2 groups was determined by randomized 2-factor (block and group) ANOVA followed by Fisher's PLSD test; significant main effects of blocks of trials ($P < 0.0001$) and groups ($P < 0.0001$) were observed, with a significant block × group interaction ($P = 0.0174$) on the number of WMEs. *Right panel*: Aβ rats ($n = 9$), Aβ + DHA rats ($n = 10$), vehicle rats ($n = 9$), vehicle + DHA ($n = 10$). The significance of differences among the 4 groups was determined by randomized 2-factor (block and group) ANOVA followed by Fisher's PLSD test; the main effects of blocks of trial and groups were both significant ($P < 0.0001$), but without a significant block × group interaction ($P = 0.0911$) on the number of WMEs. Details of subtest analysis between 2 groups of main effects of blocks of trials and groups, and between 2 groups of block × group interaction are shown in Table 2.

TABLE 2

Statistical comparisons among the vehicle, DHA, Aβ, and Aβ + DHA groups in a randomized 2-factor (block and group) ANOVA followed by Fisher's PLSD test[1]

Group	Reference memory error			Working memory error		
	Block	Group	Block × Group interaction	Block	Group	Block × Group interaction
	P-value					
Aβ vs. Aβ + DHA	<0.0001	0.0027	0.0051	<0.0001	0.0003	0.551
Aβ vs. Vehicle	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.067
Aβ vs. DHA	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.079
Aβ + DHA vs. Vehicle	<0.0001	<0.0001	0.0227	<0.0001	<0.0001	0.083
Aβ + DHA vs. DHA	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.794
Vehicle vs. DHA	<0.0001	0.0008	0.367	<0.0001	0.0823	0.470

[1] Data are presented in Figures 1 and 2; $n = 9$ or 10.

molar ratio in the DHA and Aβ + DHA groups compared with the vehicle and Aβ groups. Furthermore, it significantly increased the DHA/AA molar ratio in the DHA and Aβ + DHA groups (Table 4). The DPA levels in both the cerebral cortex and the hippocampus were significantly higher in both the DHA and Aβ + DHA groups than in the vehicle and Aβ groups. The USI of the cerebral cortex was significantly higher in the DHA and Aβ + DHA groups than in the vehicle and Aβ groups, and that of the hippocampus in the DHA group, but not in the Aβ + DHA group, was significantly higher than in the vehicle group.

Significant positive correlations were observed between cortical and plasma DHA levels [(cortex DHA) = 40.5 (plasma DHA) + 142.4; r = 0.42, $P = 0.0085$] and between hippocampal and plasma DHA levels [(hippocampal DHA) = 37.5 (plasma DHA) + 134.6; r = 0.54, $P = 0.0004$]. These results suggest a substantial entry of DHA into the cortico-hippocampal regions of the brain.

Effect of DHA administration on the oxidative status of rat brains. TBARS levels in both the cerebral cortex and the hippocampus were higher in the Aβ group than in the vehicle, DHA, or Aβ + DHA group ($P < 0.05$). Similarly, levels of ROS in both tissues were significantly higher in the Aβ group than in any of the other groups (Table 5).

Significant positive correlations were found between learning ability (both RMEs and WMEs) and cortico-hippocampal ROS (Table 6). Likewise, RMEs correlated positively with cortico-hippocampal TBARS, and WMEs correlated with hippocampal, but not with cortex TBARS. On the other hand, there was a significant negative correlation of RMEs with the hippocampal DHA/AA ratio.

DISCUSSION

The present study describes the effects of DHA administration on the learning impairment of Aβ-infused rats produced by infusing Aβ peptide (1–40) into the brain ventricle. The infusion of Aβ impaired both reference and working memory, indicating a deficit in learning ability, a well-known characteristic of Alzheimer's disease. The administration of DHA improved both the reference and the working memory of Aβ-infused rats, clearly indicating that DHA ameliorated the Aβ-induced impairment of spatial cognitive learning ability in Aβ-infused rats.

In the Aβ-infused rats administered DHA, the increase in the level of DHA in the hippocampus and cerebral cortex was accompanied by a significant decrease in AA, resulting in a significant increase in the DHA/AA ratio. An increased

TABLE 3

Plasma fatty acid profile in vehicle, DHA, Aβ, and Aβ + DHA rats[1]

Fatty acid[2]	Vehicle	DHA	Aβ	Aβ + DHA
	μmol/L			
PA, 16:0	1.815 ± 0.120	1.597 ± 0.155	1.846 ± 0.194	1.627 ± 0.088
SA, 18:0	0.570 ± 0.019^{a}	0.437 ± 0.021^{b}	0.529 ± 0.021^{a}	0.440 ± 0.023^{b}
OA, 18:1(n-9)	0.879 ± 0.089	0.701 ± 0.081	0.952 ± 0.153	0.698 ± 0.047
LA, 18:2(n-6)	2.012 ± 0.111	1.752 ± 0.168	1.944 ± 0.240	1.796 ± 0.103
LLN, 18:3(n-3)	0.040 ± 0.004	0.029 ± 0.005	0.040 ± 0.007	0.032 ± 0.002
AA, 20:4(n-6)	1.490 ± 0.059^{a}	0.554 ± 0.026^{b}	1.386 ± 0.048^{a}	0.590 ± 0.046^{b}
EPA, 20:5(n-3)	0.029 ± 0.003^{b}	0.141 ± 0.024^{a}	0.025 ± 0.004^{b}	0.161 ± 0.019^{a}
DPA, 22:5(n-3)	0.028 ± 0.004^{b}	0.049 ± 0.009^{a}	0.032 ± 0.007^{ab}	0.046 ± 0.005^{a}
DHA, 22:6(n-3)	0.202 ± 0.014^{b}	0.547 ± 0.052^{a}	0.208 ± 0.021^{b}	0.581 ± 0.035^{a}
DHA/AA	0.135 ± 0.005^{b}	0.983 ± 0.079^{a}	0.149 ± 0.011^{b}	1.027 ± 0.095^{a}
USI[3]	177.2 ± 2.456^{b}	185.2 ± 1.587^{a}	174.9 ± 3.395^{b}	188.6 ± 2.592^{a}

[1] Values are means ± SEM, $n = 9$ or 10. Means in a row with superscripts without a common letter differ, $P < 0.05$.
[2] Abbreviations used: EPA, eicosapentaenoic acid; LA, linoleic acid; LLN, linolenic acid; OA, oleic acid; PA, palmitic acid; SA, stearic acid.
[3] USI was calculated as a function of the sum of the mole percentages of the unsaturated fatty acids times the number of olefinic double bonds.

TABLE 4

Cortico-hippocampal fatty acid levels in vehicle, DHA, Aβ, and Aβ + DHA rats[1]

	Cerebral cortex				Hippocampus			
Fatty acid[2]	Vehicle	DHA	Aβ	Aβ + DHA	Vehicle	DHA	Aβ	Aβ + DHA
	nmol/mg protein							
PA, 16:0	249.9 ± 8.0	242.8 ± 7.9	253.5 ± 8.6	246.5 ± 10.4	248.3 ± 4.0^{a}	248.0 ± 7.4^{a}	229.1 ± 8.6^{b}	238.4 ± 5.7^{ab}
SA, 18:0	220.7 ± 7.6	216.3 ± 6.9	228.8 ± 9.7	219.3 ± 9.2	223.4 ± 4.0	221.9 ± 7.1	215.7 ± 3.9	216.8 ± 5.5
OA, 18:1(n-9)	135.9 ± 8.6	135.0 ± 5.2	150.4 ± 10.4	138.9 ± 7.7	167.6 ± 4.0	175.8 ± 7.7	160.8 ± 4.4	166.7 ± 6.9
LA, 18:2(n-6)	5.2 ± 0.4^{b}	6.8 ± 0.4^{a}	5.8 ± 0.6^{ab}	7.0 ± 0.3^{a}	4.6 ± 0.1^{b}	5.9 ± 0.4^{a}	4.6 ± 0.3^{b}	5.9 ± 0.2^{a}
LLN, 18:3(n-3)	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	0.2 ± 0.0^{b}
AA, 20:4(n-6)	105.9 ± 3.7^{a}	93.1 ± 3.0^{b}	104.1 ± 2.8^{a}	92.6 ± 4.6^{b}	113.2 ± 2.9^{a}	102.6 ± 3.1^{c}	109.4 ± 2.6^{b}	101.9 ± 2.2^{c}
EPA, 20:5(n-3)	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.0
DPA, 22:5(n-3)	1.0 ± 0.1^{b}	1.6 ± 0.1^{a}	1.1 ± 0.1^{b}	1.6 ± 0.1^{a}	1.1 ± 0.1^{b}	1.6 ± 0.1^{a}	1.2 ± 0.1^{b}	1.6 ± 0.1^{a}
DHA, 22:6(n-3)	146.8 ± 4.5^{b}	161.7 ± 6.4^{ab}	157.3 ± 8.9^{ab}	166.4 ± 6.2^{a}	143.1 ± 2.9^{b}	159.6 ± 5.8^{a}	140.1 ± 3.7^{b}	153.1 ± 3.8^{ab}
DHA/AA	1.39 ± 0.03^{b}	1.74 ± 0.06^{a}	1.52 ± 0.08^{b}	1.81 ± 0.06^{a}	1.27 ± 0.02^{b}	1.56 ± 0.04^{a}	1.29 ± 0.05^{b}	1.51 ± 0.03^{a}
USI[3]	168.5 ± 0.9^{b}	175.0 ± 1.1^{a}	170.0 ± 1.7^{b}	175.7 ± 1.2^{a}	166.0 ± 0.7^{b}	171.0 ± 1.0^{a}	169.1 ± 2.4^{ab}	171.3 ± 0.5^{a}

[1] Values are means ± SEM, n = 9 or 10. Means in a row for each brain area with superscripts without a common letter differ, $P < 0.05$.
[2] Abbreviations used: EPA, eicosapentaenoic acid; LA, linoleic acid; LLN, linolenic acid; OA, oleic acid; PA, palmitic acid; SA, stearic acid.
[3] USI was calculated as a function of the sum of the mole percentages of the unsaturated fatty acids times the number of olefinic double bonds.

DHA/AA ratio in the hippocampus is involved in the acquisition of higher reference memory–related learning ability. These observations are in agreement with the results of previous studies demonstrating that the DHA/AA ratio in the rat cortico-hippocampal region is inversely related to RMEs in radial maze tasks (4) and that an increased DHA/AA molar ratio in the cortico-hippocampal region of Aβ-infused rats is associated with increased active avoidance response–related learning ability (8). The DHA/AA ratio in the cerebrum is an indicator of the antioxidative action of DHA in aged rats (16) and the increased cortico-hippocampal DHA/AA ratio in Aβ-infused rats correlates negatively with corresponding levels of apoptotic products (8). Dietary DHA reduces the amount of AA in phospholipids by jointly decreasing its synthesis and simply replacing it physically (17). AA is considered to be an essential precursor of biologically active molecules, as well as a contributor to increased production of lipid peroxide through the cyclooxygenase pathway. This is because some of the AA-cascade products of endoperoxides themselves have free radical characteristics (18). DHA can modulate the inflammation and oxidative stress in which AA and its metabolites participate directly or indirectly (19). Thus, an increase in the DHA/AA ratio might contribute to decreased TBARS levels, because the higher the DHA level in the brain, the lower the AA level and the higher the DHA/AA ratio. An increased DHA/AA ratio in the cortico-hippocampal regions may therefore play an enhanced role against oxidative neuronal damage and impairment of learning and memory after the infusion of Aβ.

The free-radical hypothesis of AD suggests that increased production of lipid peroxide causes deterioration of a wide variety of cellular enzymes, subsequently exacerbating the neurodegenerative processes (20). Chronic treatment with antioxidants, such as α-tocopherol, improves cognitive functions in aging (21), a process frequently associated with increased oxidative damage and neurodegenerative diseases including AD. In the present study, DHA administration reduced the increased cortico-hippocampal TBARS and ROS levels in Aβ rats to the levels in vehicle rats. DHA protects the brain against ischemic and excitotoxic damage in rats (22,23); it also acts as an antioxidant in brain tissue under certain circumstances because of the intrinsic potential of brain tissue to generate free radicals (16,24). Thus, DHA likely was more effective against Aβ-induced oxidative stress at the neuronal level.

Epidemiologic studies show a relation between dietary (n-3) fatty acids and AD. High fish consumption, especially fish rich in n-3 fatty acid, is associated with a reduced risk for cognitive decline during aging and AD (9,25). Humans with senile dementia, treated for 6 mo with fish oil capsules (1400

TABLE 5

Oxidative status of cerebral cortex and hippocampus in rats administered vehicle, DHA, Aβ, and Aβ + DHA[1]

	Cerebral cortex		Hippocampus	
	TBARS	Reactive oxygen species	TBARS	Reactive oxygen species
	nmol/mg protein	*pmol/(min · mg protein)*	*nmol/mg protein*	*pmol/(min · mg protein)*
Vehicle	1.055 ± 0.018^{b}	0.657 ± 0.052^{b}	0.970 ± 0.042^{b}	0.647 ± 0.056^{b}
DHA	1.035 ± 0.070^{b}	0.629 ± 0.050^{b}	0.864 ± 0.108^{b}	0.623 ± 0.062^{b}
Aβ	1.390 ± 0.105^{a}	1.023 ± 0.114^{a}	1.204 ± 0.075^{a}	1.045 ± 0.100^{a}
Aβ + DHA	1.055 ± 0.050^{b}	0.727 ± 0.038^{b}	0.988 ± 0.047^{b}	0.648 ± 0.078^{b}

[1] Values are means ± SEM, n = 9 or 10. Means in a column with superscripts without a common letter differ, $P < 0.05$.

TABLE 6

Correlation coefficients between learning ability and the ratio of DHA/AA and oxidative stress in rat brain[1]

	Cerebral cortex			Hippocampus		
	DHA/AA	ROS	TBARS	DHA/AA	ROS	TBARS
RME	NS	+0.414	+0.439	−0.332	+0.410	+0.396
P-value		0.010	0.006	0.041	0.011	0.014
WME	NS	+0.354	NS	NS	+0.562	+0.401
P-value		0.029			<0.001	0.013

[1] The number of reference memory errors (RMEs) and working memory errors (WMEs) in block 10 shown in Figures 1 and 2 was used as an indicator of learning ability. Differences with $P < 0.05$ were considered significant. NS, not significant, $P \geq 0.05$.

mg DHA/d) in addition to established drugs, showed improvement in intellectual function (10,26). Although DHA was proposed to play a crucial role in the amelioration of learning impairment in AD, the mechanism of its activity in the brain is not clear. It is assumed, however, that DHA acts primarily as a structural fatty acid of the brain synaptic plasma membrane, resulting in alterations of neuronal functions (27,28). The infusion of Aβ into the rat hippocampus evidently induces deficits in long-term potentiation (LTP) and in working memory (29). In addition, the acetylcholine level decreases in those experiencing memory impairment such as in AD (30). Dietary supplementation with DHA restores neurotransmitter release and reverses impairment in the expression of LTP (31). DHA is crucial for the induction of LTP, and DHA released endogenously during titanic stimulation is sufficient to trigger the expression of LTP (32). Dietary DHA increases cortical acetylcholine levels and concurrently improves avoidance performance (33); this was seen in rat hippocampus during aging (34–36). It improves radial maze-learning ability in aged rats (5,37) and increases the density of dendritic spines (37). These observations suggest that DHA-supplementation increases neuronal cell functions by being incorporated into neural membrane phospholipids.

We found recently that dietary DHA-induced increases in synaptic plasma membrane fluidity contribute to synaptic plasma membrane–bound functions that constitute avoidance learning–related memory (unpublished data). Reduced membrane fluidity correlates with diminished release of acetylcholine from the synaptosomes in vitro (38). Because Aβ infusion into the rat brain reduces acetylcholine levels (30) and DHA administration prevents learning deficits (8), we speculate that presynaptic vesicular fusion and subsequent postsynaptic release of neurotransmitters is facilitated by DHA-induced increases in synaptic plasma membrane fluidity. These results suggest that dietary DHA, by being incorporated into neuronal membranes, affects cholinergic neurotransmission and subsequently exerts positive effects on behavior and brain function.

AD is an age-related disorder characterized by progressive cognitive decline and neurodegeneration (39). Senile plaques are composed predominantly of Aβ peptide. The development of AD pathology was proposed to be the result of Aβ deposition in association with membrane structure. It was demonstrated that plaque formation may be initiated in a plasma membrane form (40), suggesting that lipid composition in different compartments plays a role in Aβ aggregation. Cholesterol modulates Aβ-lipid interactions by decreasing the fluidity of the membrane bilayer and induces the aggregated formation of Aβ (41). Aβ entry into the membrane bilayer may result from an elevated cholesterol to phospholipid ratio. Chronic administration of DHA lowers the cholesterol to phospholipid molar ratio of the cerebral cortex synaptic plasma membrane in rats (42). In a mouse model of AD, a DHA-enriched diet produced a 40–50% reduction in the deposition of cortico-hippocampal Aβ (43). Dietary supplementation with DHA can modulate the gene expression of many enzyme proteins involved in signal transduction processes (36,44). DHA administration stimulates the synthesis of transthyretin, a protein involved in the transport of thyroxin, suggesting that its administration to rats counteracts the formation of insoluble amyloid aggregates by stimulating the transcription of transthyretin. This protein also acts as an Aβ-peptide scavenger, suggesting its role in preventing the formation of Aβ aggregates (45). These data suggest that a DHA-enriched diet may prevent brain atrophy, senile plaque, and neurofibrillary tangle. Further studies are required to clarify the mechanisms of the DHA-mediated ameliorative effects on Alzheimer's disease.

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IMPROVEMENT OF SPATIAL COGNITION WITH DIETARY DOCOSAHEXAENOIC ACID IS ASSOCIATED WITH AN INCREASE IN FOS EXPRESSION IN RAT CA1 HIPPOCAMPUS

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SUMMARY

1. Twenty 5-week-old male Wistar rats were divided into two groups: one group was fed a fish oil-deficient diet and the other group was fed the same diet supplemented with per orally administered docosahexaenoic acid (DHA) for 12 weeks.

2. Six weeks after the start of the administration of DHA, rats were trained for 6 weeks to acquire a reward at the end of each of four arms of an eight-arm radial maze. On completion of the radial maze task, the Fos expression in the hippocampus was examined immunohistochemically.

3. Chronic DHA administration significantly reduced the number of reference and working memory errors. The number of Fos-positive neurons in the CA1 hippocampus significantly increased in DHA-treated rats compared with control rats, demonstrating a statistically significant negative correlation with the number of reference memory errors.

4. These results suggest that the DHA-induced improvement in spatial cognition is associated with increased Fos expression in the CA1 hippocampus.

Key words: *c-fos*, docosahexaenoic acid, hippocampus, radial arm maze, rats, spatial working memory.

INTRODUCTION

The Fos protein, encoded by the immediately early gene *c-fos*, is known to be a transcription factor and a functional marker of neuronal activity. It has been reported that expression of *c-fos* is rapidly induced by a number of extracellular stimuli,[1] including stress[2] and seizures,[3] in various brain regions, including the hippocampus, which plays a crucial role in spatial memory. Dietary deficiency of α-linolenic acid, a precursor of n-3 polyunsaturated fatty acids, such as docosahexaenoic acid (DHA; C22 : 6, n-3), affects synaptic vesicle turnover in the CA1 hippocampus and induces loss of learning ability.[4] Docosahexaenoic acid is one of the main structural lipids in the mammalian brain. Chronic administration of DHA enhances spatial cognition ability in both young[5] and old[6] rats and dietary DHA administration protects against the impairment of learning ability in Alzheimer's disease model rats.[7] Docosahexaenoic acid is essential for normal neurological development and vision.[8] However, the mechanisms of DHA in memory processing are not clearly understood. In the present study, we hypothesized that dietary DHA administration induces an alteration in the hippocampal intracellular signalling cascade, which results in long-term modifications in cell functions affecting genomic events through immediate early genes (IEG), such as *c-fos*. Therefore, in the present study we examined whether *c-fos* expression in the hippocampus is related to the improvement in spatial memory with dietary DHA in rats fed dietary DHA.

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METHODS

Rats were provided for and killed in accordance with the procedures outlined in the *Guidelines for Animal Experimentation of Shimane Medical University* (Shimane, Japan), complied from the *Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science*. Rats were housed in a room at a controlled temperature (23 ± 2°C), relative humidity (50 ± 10%) and light–dark cycles (lights on 08.00–20.00 h; lights off 20.00–08.00 h). Twenty male Wistar rats (5 weeks old; 60–70 g; Jcl : Wistar), purchased from Clea Japan (Osaka, Japan), were provided with a fish oil-deficient diet (F-1®; Funabashi Farm, Funabashi, Japan) and water *ad libitum* for 14 weeks and were then randomly divided into two groups: (i) the DHA group, which was fed DHA-95E orally (300 mg/kg per day; an ethyl-ester all-*cis*-4,7,10,13, 16,19-docosahexaenoate with purity greater than 95%; Harima Chemicals, Tokyo, Japan) gently emulsified in a 5% gum Arabic solution in ice-cold water before administration; and (ii) the control group, which was fed an equal volume of vehicle only.

Learning-related behaviour was assessed using an eight-arm radial maze (Toyo Sangyo, Toyama, Japan), as described previously.[5,6] Briefly, 6 weeks after the start of DHA administration, rats, which were maintained under a food-deprivation schedule, were trained to acquire the reward (a food pellet) at the end of each of four arms of an eight-arm radial maze. The performance in this situation involved two parameters of memory function: (i) reference memory error (RME), entry into unbaited arms; and (ii) working memory error (WME), repeated entry into arms that had already been visited and obtaining the rewards within a trial. Each rat was given two daily trials, 6 days a week for a total of 6 weeks.

On completion of the behavioural performance, rats were anaesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with heparinized phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer (pH 7.2). Brains were excised and immersed in 4% PFA in 0.1 mol/L phosphate buffer for 1 day

and then transferred to 25% sucrose for 3–5 days. Immunohistochemical examination of Fos was performed as described previously.[9] Briefly, each brain was sectioned (40 μm) on a freezing microtome, pre-incubated in 0.1% hydroged peroxide and immersed in an affinity purified polyclonal antibody against the N-peptide (Oncogene Science, Manhasset, NY, USA; dilution 1 : 2500). The avidin–biotin horseradish peroxidase method was used with the Vectastain Elite avidin–biotin–peroxidase complex (ABC) kit (Vector Laboratories, Burlingame, CA, USA). The peroxidase was visualized with diaminobenzidine. Pre-adsorption of the primary antibody with a synthetic Fos peptide (Oncogene Science; 5 μg/mL) eliminated the immunoreactivity completely. Adjacent sections were stained with cresyl violet and used for the histological identification of parts in the immunohistochemical preparations, according to the rat atlas of Paxinos and Watson.[10] For each section, the Fos-positive neurons within the CA1 subfield, the CA3 subfield and the dentate gyrus (DG) of the dorsal hippocampus were identified by lightfield microscopy at ×100. The Fos-positive neurons were analysed on a Macintosh computer with the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Values are expressed as the mean number of Fos-positive neurons/mm^2. The number of Fos-positive neurons was quantified on three to four sections per rat (AP = –4.16 mm).

All data are expressed as the mean±SEM. Behavioural data were analysed by a two-factor (group and block) randomized block factor ANOVA and the between-group differences by one-way ANOVA. The ANOVA was followed by Fisher's protected least significant difference for post hoc comparisons. Correlation was determined by simple regression analysis. Statistical programs used were GB-STAT™ 6.5.4 (Dynamic Microsystems, Silver Spring, MD, USA) and StatView® 4.01 (MindVision Software, Abacus Concepts, Berkeley, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Figure 1 shows the effect of chronic administration of DHA for 6 weeks on reference and working memory related learning ability. The score is expressed as the mean number of RME or WME for each group, with data averaged over blocks of six trials. A two-way ANOVA was conducted based on the scores. The analysis revealed a significant main effect of blocks of trials ($F_{11,99} = 27.80$; $P < 0.0001$) and groups ($F_{1,9} = 38.04$; $P = 0.0002$) on the number of RME (Fig. 1a). Similarly, the analysis revealed a significant main effect of blocks of trials ($F_{11,99} = 12.03$; $P < 0.0001$) and groups ($F_{1,9} = 5.92$; $P = 0.038$) on the number of WME (Fig. 1b). These results suggest that the DHA-treated rats showed a facilitated spatial cognition, although randomized two-factor (block and

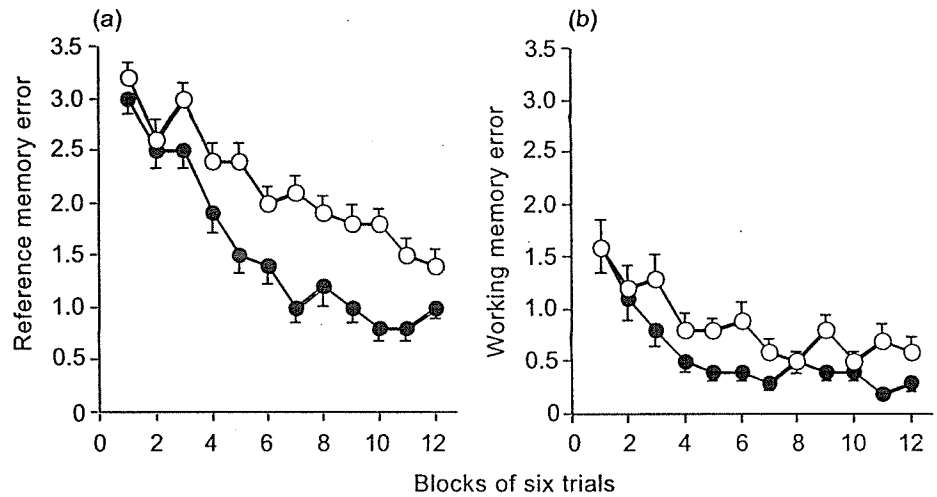

Fig. 1 Effect of chronic administration of docosahexaenoic acid (DHA) on the number of (a) reference memory errors (RME) and (b) working memory errors (WME) of rats in the radial maze task for a period of 6 weeks. (○), control rats ($n = 10$); (●), rats fed 300 mg/kg per day DHA ($n = 10$). Data are the mean±SEM for each block of six trials showing the number of RME and WME made until the rat acquired all the rewards.

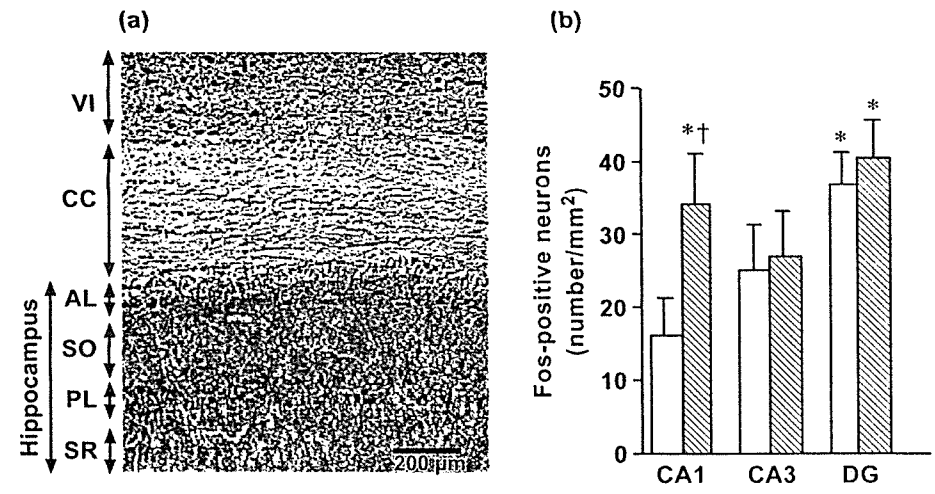

Fig. 2 Effect of chronic administration of docosahexaenoic acid (DHA) on Fos expression in rat hippocampus. (a) A representative photomicrograph of the distribution of Fos-positive neurons in the dorsal hippocampus of DHA-fed rats. Bar, 200 μm. VI, neocortex, Layer VI (multiform layer); CC. corpus callosum; AL, alveus; SO, stratum oriens; PL, pyramidal layer (CA1); SR, Stratum radiatum. (b) Comparison of the number of Fos-positive neurons in the CA1, CA3 and dentate gyrus (DG) of the dorsal hippocampus. (□), control rats ($n = 10$); (▨), rats fed DHA (300 mg/kg per day; $n = 10$). Data are the mean± SEM. *$P < 0.05$ compared with Fos-positive neurons in the CA1 hippocampus of control rats; †$P < 0.05$ compared with Fos-positive neurons in control rats.

group) ANOVA did not reveal a significant block–group interaction for either RME ($F_{11,99} = 1.72$; $P = 0.078$) or WME ($F_{11,99} = 0.774$; $P = 0.665$).

Fos-positive neurons within each hippocampal CA1 subfield of a DHA-treated rat are shown in Fig. 2a. The number of Fos-positive neurons in the CA1 subfield of both the control and DHA-treated rats was lower than that in the cerebral cortex, as suggested from Fig. 2a (data not shown). Quantitative analysis indicated that the number of Fos-positive neurons of control rats was significantly lower in the CA1 than in the DG subfield ($P = 0.0027$), but there were no significant differences between in the CA1 and CA3 subfields of control rats (Fig. 2b). The number of Fos-positive neurons in the CA1 subfield of the dorsal hippocampus was significantly higher in DHA-treated rats than in control rats ($P = 0.047$). The numbers of Fos-positive neurons in the DG and CA3 subfield of the dorsal hippocampus were not different between the two groups.

Figure 3 shows regression analysis of the relationship between the number of CA1 hippocampal Fos-positive neurons and reference memory related learning ability, expressed as the number of RME in the final block. The number of Fos-positive neurons within the CA1 subfield of the dorsal hippocampus demonstrated a significant negative correlation with the number of RME ($r = -0.470$; $P = 0.037$; $n = 20$) and also showed a tendency to be negatively correlated with the number of WME ($r = -0.405$; $P = 0.076$; data not shown).

DISCUSSION

The effect of dietary administration of DHA on Fos expression in various regions of the rat hippocampus was estimated. Expression of Fos in the hippocampal CA1 region was shown to increase with chronic DHA administration, associated with an improvement in spatial memory.

Fos and Jun represent IEG that are induced rapidly by a variety of stimuli, including stimuli involved in regulating synaptic plasticity.[11] It has been reported that age-associated cognitive decline and cognitive impairment are correlated with more potent induction of hippocampal *c-fos* mRNA than a number of other IEG, including the Jun family.[11] Thus, in the present study, we focused on hippocampal *c-fos* expression because it is suggested that Fos immunoreactivity can be used as a marker of neuronal activity that provides information on brain regions underlying learning and memory, presumably associated with neuronal plasticity required for memory processes.[12] However, hippocampal damage is generally known to disrupt radial arm maze performance.[13] Morris *et al.*[14] reported that hippocampal lesions induce impairment of spatial discrimination; therefore, the hippocampus plays an important role in spatial information processing. Moreover, the *c-fos* gene is activated by a number of extracellular stimuli, including stress.[2] In the present study, we observed the relationship between Fos expression in hippocampal regions and the RME and WME obtained using a radial maze because the maze task can estimate two types of memory, reference memory and working memory, without any harmful stress to the rats. Reference memory involves using information that remains constant over time; working memory involves holding information that is pertinent only within a short period of time.[15]

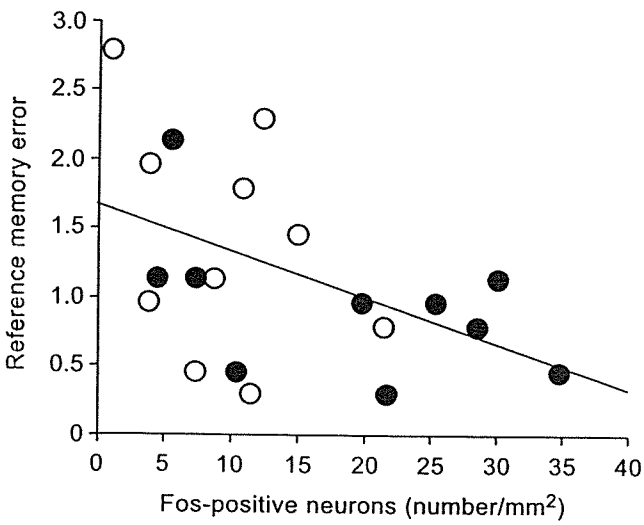

Fig. 3 Relationship between the number of CA1 hippocampal Fos-positive neurons and the number of reference memory errors in the final block of the radial maze task. (○), control rats ($n = 10$); (●), rats fed docosahexaenoic acid (300 mg/kg per day; $n = 20$). $r = -0.470$; $P = 0.037$.

In the present series of experiments, chronic administration of DHA significantly decreased the number of RME and WME, indicating improvement of spatial memory related learning ability in DHA-fed rats. Chronic administration of DHA contributes to protection against neuronal damage in the hippocampal CA1 region and to reduced cognitive deficit caused by transient forebrain ischaemia.[16] The present findings on radial maze performance agree with the results of a previous study[5] demonstrating that chronic administration of DHA improves reference memory related learning ability in young rats, associated with increased hippocampal DHA content. Our recent study also indicates that the increased DHA level in the hippocampus protects against the impairment of learning ability in Alzheimer's disease model rats.[7] Therefore, it is possible that the DHA-induced improvement in spatial cognition seems to be the result of dietary DHA acting, in part, on the hippocampus, although hippocampal DHA content was not measured in the present study.

Long-term potentiation (LTP), a typical and well-known example of synaptic plasticity, is regarded as a neuronal base of learning and memory and is clearly observed in the hippocampus. Long-term potentiation in the hippocampal CA1 region is induced by activation of the *N*-methyl-D-aspartate (NMDA) receptor.[17] Nishikawa *et al.*[18] have observed that DHA potentiates the NMDA-induced response, suggesting that DHA probably plays an important role in the expression of LTP. Long-term potentiation is also thought to be the initial stage of memory formation and indicates an essential role for induced mRNA and protein synthesis during a brief period after the conditioning stimulus.[19] Several genes, especially IEG such as *c-fos*, are induced rapidly in association with LTP in the hippocampus.[11,20] Moreover, the activation of NMDA receptors[21] in the CA1 region of hippocampus[22] is a necessary step involved in the activation of *c-fos* transcription. In the present study, DHA administration to rats increased the number of Fos-positive neurons only in the hippocampal CA1 region and not in the CA3 and DG regions. In addition, the number of Fos-positive neurons in the CA1 subfield was highest in animals that demonstrated the lowest number of RME on the radial arm maze performance. Taking these findings into account, we suggest that

the increased Fos expression in the CA1 hippocampus induced by chronic administration of DHA is conducive to the improvement of spatial cognition.

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