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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, $P \geq 0.05$.

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 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fluidity contribute to synaptic plasma membrane–bound functions that constitute avoidance learning–related memory (unpublished data).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 may play 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

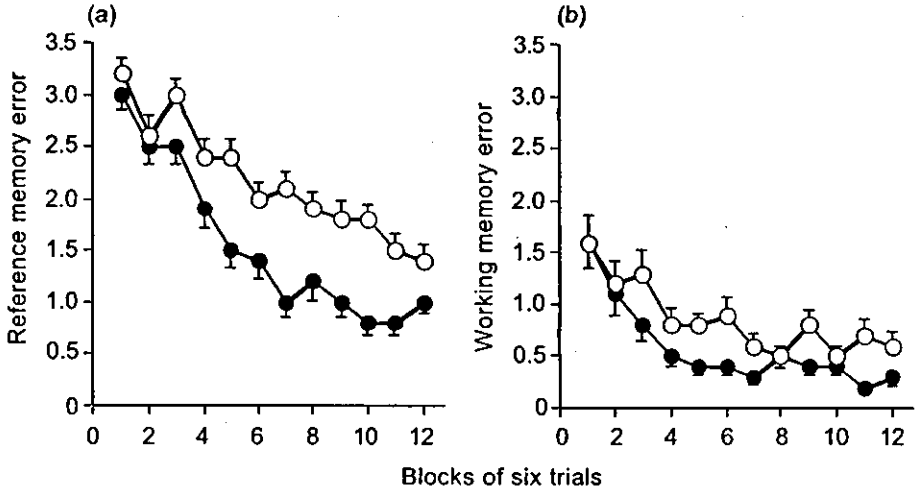

Fig. 1 Effect of chronic administration of docosahexaenoic acid (DHA) on the number of (a) reference memory errors (RME) and (b) working memory errors (WME) of rats in the radial maze task for a period of 6 weeks. (○), control rats ($n = 10$); (●), rats fed 300 mg/kg per day DHA ($n = 10$). Data are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

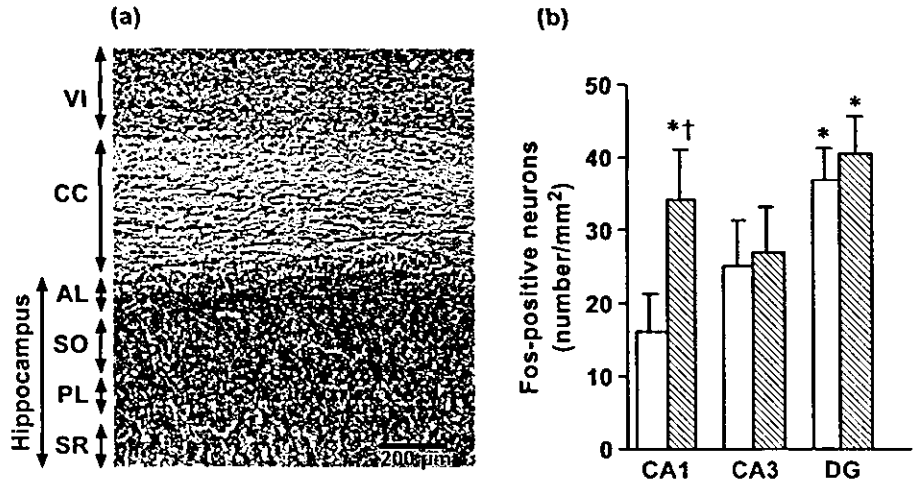

Fig. 2 Effect of chronic administration of docosahexaenoic acid (DHA) on Fos expression in rat hippocampus. (a) A representative photomicrograph of the distribution of Fos-positive neurons in the dorsal hippocampus of DHA-fed rats. Bar, 200 μm. VI, neocortex, Layer VI (multiform layer); CC, corpus callosum; 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]

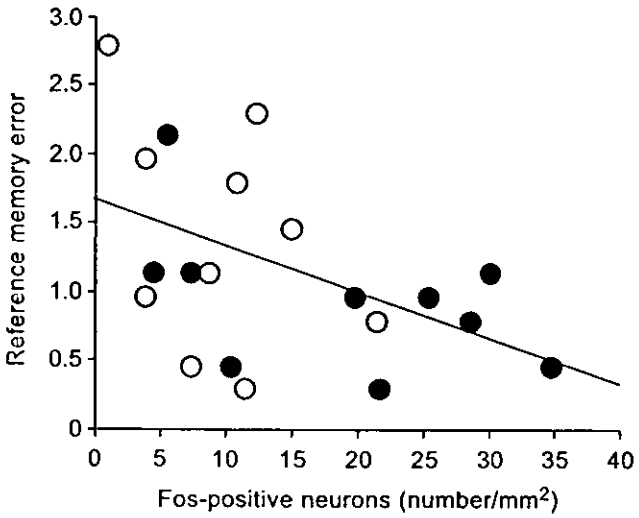

Fig. 3 Relationship between the number of CA1 hippocampal Fos-positive neurons and the number of reference memory errors in the final block of the radial maze task. (○), control rats ($n = 10$); (●), rats fed docosahexaenoic acid (300 mg/kg per day; $n = 20$). $r = -0.470$; $P = 0.037$.

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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