Dose-Response Effect of Docosahexaenoic Acid Ethyl Ester on Maze Behavior and Brain Fatty Acid Composition in Adult Mice

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Abstract: The dose-response effect of dietary docosahexaenoic acid (DHA, 22:6 n-3) ethyl ester (EE) on maze-learning ability in mice was studied. Male Crj:CD-1 mice aged three months were fed a) a diet containing 5 g palm oil/100 g diet (control group); b) a diet containing 0.5 g DHA ethyl ester/100 g diet plus 4.5 g palm oil/100 g diet (DHA-EE 0.5% group); c) a diet containing 1g DHA ethyl ester/100 g diet plus 4g palm oil/100 g diet (DHA-EE 1% group); d) a diet containing 2 g DHA ethyl ester/100 g diet plus 3 g palm oil/100 g diet (DHA-EE 2% group) for four months. Maze-learning ability was assessed three months after the start of the experiment. The time required to reach the maze exit and the number of times that a mouse strayed into blind alleys in the maze were measured in three trials, performed every four days. In trial 1, the DHA-EE 0.5%, 1% and 2% groups required less ($p < 0.05$) time to reach the maze exit, and the DHA-EE 2% group strayed ($p < 0.05$) into blind alleys fewer times than the control group. In trial 3 performed four days after the second trial, the DHA-EE 2% group needed less ($p < 0.05$) time to find the exit and spent a fewer ($p < 0.05$) number of times in blind alleys than did the control group. In the total lipids of plasma and brain of mice fed DHA, increasing intakes of DHA resulted in an increase in DHA levels, with a corresponding decrease in arachidonic acid (20:4 n-6). Improved maze-learning ability in mice fed DHA-EE 2% was associated with higher DHA levels in brain. Our results suggest that there are no linear dose-response effects of DHA on maze-learning ability, however, the intake of DHA-EE 2% diet improves learning ability in adult mice as demonstrated by maze performance.

Key words: dose-response, docosahexaenoic acid (DHA), maze-learning ability, brain, fatty acid composition

Abbreviations used: AA, arachidonic acid; DHA, docosahexaenoic acid; DHA-EE, docosahexaenoic acid ethyl ester; HUFA, highly unsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Introduction

Docosahexaenoic acid (DHA, 22:6n-3) is the elongation-desaturation product of the parent fatty acid, α-linolenic acid, and one of the major highly unsaturated fatty acids (HUFAs) of membrane phospholipids in the mammalian central nervous system. Dietary n-3 fatty acid deficiency is associated with biochemical changes such as decreased brain DHA [1, 2] and monoaminergic neurotransmitter levels [3]. In addition, behavioral and physiological alterations occur, such as disturbed electroretinographic measurements and other vision-related parameters [4], reduced learning ability [5–8], increased intake of water [9], and initiated bouts of stereotyped behavior [10]. Such studies have suggested that dietary n-3 polyunsaturated fatty acids (PUFAs) play an important role in normal cerebral development. However, studies on the effects of dietary DHA on learning performance tasks are not consistent. Gamoh *et al* [11] have suggested that chronic administration of DHA is conductive to the improvement of reference memory-related learning ability. Wainwright *et al* [12] observed no improvement in the performance of a working-memory task by feeding DHA and arachidonic acid (AA, 20:4n-6) to rats tested in the Morris water maze. Our previous work showed that intakes of DHA and egg-PC (phosphatidylcholine) improved learning ability in both young and old mice but there was no synergistic effect of DHA and egg-PC [13].

There were several studies to examine the time course of DHA recovery in the nervous system by switching from an n-3 fatty acid deficient to a diet containing a high level of α-linolenic acid [14–17]. They found a slow recovery of DHA in brain synaptosomes, mitochondria, myelin, and microsomes, with nearly complete recovery by 75 days after diet reversal [14]. These studies concentrated on the repletion rates after addition of dietary α-linolenic acid. Our recent work showed a relationship between the intake period of DHA and maze behavior in mice, suggesting that it may take time after the incorporation of DHA into the brain for improvement in learning ability to occur [18]. However, few studies have been conducted on the relationship between doses of DHA and maze-learning ability. In this study, we examined the effect of various doses of DHA ethyl ester on maze-learning ability and fatty acid composition of plasma and brain in adult mice.

Materials and Methods

Animals and diets

Male Crj: CD-1 mice were obtained from Charles River Japan Inc. (Atsugi, Kanagawa, Japan). DHA (DHA-95E, ethyl ester derivative of all cis-4,7,10,13,16,19-docosahexaenoic acid, 95% pure) was obtained from Harima Chemicals Inc. (Tsukuba, Japan). Thirty-six three-month-old mice were randomly divided into four groups of nine: a) a group fed 5 g palm oil/100 g diet (control group); b) a group fed 0.5 g DHA ethyl ester/100 g diet plus 4.5 g palm oil /100 g diet (DHA-EE 0.5% group); c) a group fed 1 g DHA ethyl ester/100 g diet plus 4 g palm oil/100 g diet (DHA-EE 1% group); d) a group fed 2 g DHA eth-

Table I: A Diet Composition

	g/kg diet			
Corn Starch	488			
Casein	200			
Sucrose	150			
Cellulose	50			
Mineral mixture[1]	40			
Vitamin mixture[2]	20			
L-Methionine	2			
Fat	50			
Fat sources	Control (Palm oil)	DHA-EE 0.5%	DHA-EE 1%	DHA-EE 2%
Palm oil	50	45	40	30
DHA-EE[3]	0	5	10	20

[1] The mineral and vitamin mixtures were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan) and the compositions have been described previously by Kohashi *et al* [34]. The mineral mixtures contained per 100 g: $CaHPO_4 \cdot 2H_2O$, 14.56 g; KH_2PO_4, 25.72 g; NaH_2PO_4, 9.35 g; NaCl, 4.66 g; Ca-lactate, 35.09 g; Fe-citrate, 3.18 g; $MgSO_4$, 7.17 g; $ZnCO_3$, 0.11 g; $MnSO_4 \cdot 4H_2O$, 0.12 g; $CuSO_4 \cdot 5H_2O$, 0.03 g; KI, 0.01 g.

[2] The vitamin mixtures contained per 100 g: retinyl acetate, 0.1 g; cholecalciferol, 0.00025 g; isomer α-tocopheryl acetate, 0.5 g; menadione, 0.52 g; thiamine. HCl, 0.12 g; riboflavin, 0.4 g; pyridoxine. HCl, 0.08 g; cyanocobalamin, 0.00005 g; ascorbic acid, 3 g; biotin, 0.002 g; folic acid, 0.02 g; calcium pantothenate, 0.5 g; ρ-aminobenzoic acid, 0.5 g; niacin, 0.6 g; inositol, 0.6 g; choline chloride, 20 g; cellulose powder, 73.1 g.

[3] DHA-EE, docosahexaenoic acid ethyl ester.

Table II: Fatty acid composition of lipids in different diet groups

	Diet group			
Fatty acids	Control (Palm oil)	DHA-EE 0.5%	DHA-EE 1%	DHA-EE 2%
		g/100 g total fatty acids		
16:0	46.4	45.4	44.2	38.6
18:0	5.3	3.7	3.4	2.9
20:0	0.2	0.3	0.2	0.2
Total Sat.	**51.9**	**49.4**	**47.8**	**41.7**
18:1(n-9)	38.3	35.2	31.2	26.5
18:1(n-7)	0.7	0.6	0.6	0.5
20:1(n-9)	0.1	–	–	–
Total Mono.	**39.1**	**35.8**	**31.8**	**27.0**
18:2(n-6)	9.0	8.1	7.5	6.2
20:3(n-6)	–	–	–	–
20:4(n-6)	–	–	–	–
22:4(n-6)	–	–	–	–
22:5(n-6)	–	–	–	–
Total n-6	**9.0**	**8.1**	**7.5**	**6.2**
18:3(n-3)	–	–	–	–
20:5(n-3)	–	0.4	0.7	1.4
22:6(n-3)	–	6.3	12.2	23.7
Total n-3	**–**	**6.7**	**12.9**	**25.1**

yl ester/100 g diet plus 3 g palm oil /100 g diet (DHA-EE 2% group) for four months. Each diet contained 5 g/100 g fat sources (Table I). The main fatty acid composition of lipids in each diet group is presented in Table II. In the present study, the control diet was n-3 fatty acid-deficient and all diets were deficient in α-linolenic acid. The control and DHA-EE 0.5%, 1% and 2% diets contained 5 g/100 g, 4.5 g/100 g, 4 g/100 g, and 3 g/100 g triglyceride, respectively. The diets were powdered and stored at –25°C, and fresh supplies were given to the mice once every two days. All diets were handled so as to minimize oxidation of the fatty acids. The diet and water were given *ad libitum.* All mice were housed in a standard environment, in which temperature was maintained at 24 ± 0.5°C, and the relative humidity was kept at 65 ± 5% with 12-hour periods of light and dark. Body weights were measured once a week. All mice were housed 4 to 5 per one cage and maintained according to the guidelines for experimental animals of National Food Research Institute, Japan.

Determination of maze-learning ability

To determine maze-learning ability in mice, a video tracking and motion analysis system (EMTEC Co., Ltd., Tama, Tokyo, Japan) was used. The motion analysis system, measuring rapid real-time picture acquisition, has previously been described by Lim and Suzuki [13]. The apparatus consisted of a maze (36 × 50 cm) with eight blind alleys from entrance to exit, a shot camera (Artist G120), image processing equipment (microprocessor for rapid information processing), personal computer, and a printer. Mice were put into the entrance door and were allowed to explore the maze to find water, which was placed into touch with outside the opened maze exit. A program for monitoring the pattern of animal movement and the time spent getting from the maze entrance to exit was adopted. This program allowed direct recording of the X-Y coordinates of mouse movement onto a computer disk file. Maze learning ability was assessed three months after the start of the feeding trial. Before the learning test, all mice were conditioned using a simple maze of three partition walls; this conditioning also served as training for mice to seek water. The first maze trial was done after 24 hours of water deprivation so that the thirsty mice sought water. Trial 2 was performed under the same conditions on day 4 following the first trial; trial 3 was conducted four days after the second trial. The time required to reach the exit from the entrance, the number of times that a mouse strayed into blind alleys, and the behavior of the mouse in the maze were measured.

Preparation of plasma samples and brain homogenates

After four months of feeding trials, all mice were fasted for 24 hours before being anesthetized with diethyl ether. Blood was then collected from the inferior vena cava and the mice were euthanized by decapitation. The blood plasma was separated by centrifugation at 900 × g for 20 minutes at 4°C. The whole brain of each mouse was rapidly removed and homogenized in ice-cold 0.32 M sucrose

(9 mL/g tissue) using a Teflon-glass homogenizer. The blood plasma samples and brain homogenates were kept at –25°C until required for fatty acid analysis.

Fatty acid analysis

Total lipid was extracted by the method of Bligh and Dyer [19]. Samples of the diet, brain homogenates and plasma were subjected to lipid extraction with chloroform: methanol: water (1:2:0.8, v/v/v) and the lipids in the methanol/-water phase were again extracted with chloroform. KCl (0.88%) was added to the combined extracts and sufficient time allowed to separate the lipid phase from the aqueous phase. The water remaining in the lipid phase was removed by the addition of anhydrous Na_2SO_4 and the lipid phase was then dried under nitrogen. Methylation of fatty acids was carried out according to the official AOCS method [20]. 0.5N NaOH was added to the lipid fraction and heated at 100°C for 5 minutes. After cooling, the lipid fraction was heated with 14% boron trifluoride-methanol reagent in a sealed vial at 100°C for 30 minutes. The fatty acid methyl esters were extracted with n-hexane, dried under a stream of nitrogen, re-dissolved in n-hexane and stored at 4°C until analysis. The fatty acid methyl esters were separated by gas liquid chromatography using a 30 m × 0.25 mm i.d. capillary column (Supelcowax 10, Supelco, Bellefonte, USA) and detected by flame ionization (Shimadzu Co., Kyoto, Japan). The column temperature was programmed from 175°C to 225°C at a rate of 3°C/minute and the carrier gas was helium. The injector temperature was 250°C and the detector temperature was 270°C. The chromatograms were recorded and the percentage composition of individual peaks was calculated with a Chromatopac C-R6A (Shimadzu Co., Kyoto, Japan). The fatty acid esters were identified by comparison of their retention times with authenticated standards.

Statistics

All results were expressed as means ± SE, and statistical significance was determined by one- way analysis of variance using the SIGMASTAT statistical program package (Jandel Co., Erkrath, Germany). When the F-test was significant, comparisons among the dietary groups were done using Tukey's HSD test at $\alpha = 0.05$.

Results

Body weight and food intake

The average final body weight (mean ± SE, g) showed little variation among the dietary groups (control, 43.3 ± 0.4; DHA-EE 0.5%, 43.0 ± 1.0; DHA-EE 1%, 40.3 ± 0.3; DHA-EE 2%, 44.4 ± 0.3). The food consumption was 4.0 ± 0.1 g/day and the differences among the dietary groups were not significant.

Effect on maze-learning ability in adult mice

During trial 1, the time required to reach the maze exit was significantly less in the DHA-EE 0.5%, 1% and 2% diet groups compared with the control group ($p < 0.05$) (Fig. 1). There were no significant differences in time among diet groups in trial 2. During trial 3, mice fed DHA-EE 2% showed significantly less time needed to reach the maze exit than those fed the control diet ($p < 0.05$).

The numbers of times that a mouse strayed into blind alleys in the maze were significantly fewer in the DHA-EE 2% group than in the control group during trial 1 ($p < 0.05$) (Fig. 2). There were no significant differences in number of times among diet groups in trial 2, in a pattern similar to results of total time required to find the exit. During trial 3, the DHA-EE 2% diet group strayed into blind alleys of the maze fewer times compared with the control group ($p < 0.05$).

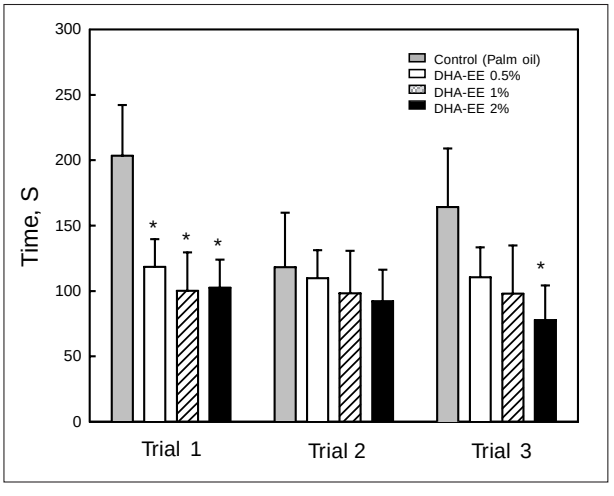

Figure 1: Differences in the time (Time, S) required for mice to reach the maze exit. Mice aged three months fed the control (palm oil) diet, docosahexaenoic acid ethyl ester 0.5% (DHA-EE 0.5%) diet, docosahexaenoic acid ethyl ester 1% (DHA-EE 1%) diet, and docosahexaenoic acid ethyl ester 2% (DHA-EE2%) diet for three months. Results are expressed as means ± SE, n = 9. Differences were analyzed by one-way analysis of variance and comparisons among the dietary groups were done using Tukey's HSD test. Asterisks indicate a statistically significant difference between the control and each experimental diet group at $p < 0.05$.

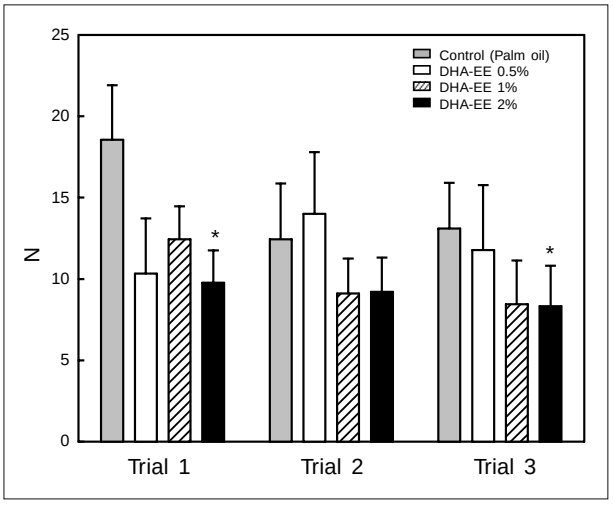

Figure 2: Differences in the number of times (n) that a mouse strayed into blind alleys of the maze. Mice aged three months fed the control (palm oil) diet, docosahexaenoic acid ethyl ester 0.5% (DHA-EE 0.5%) diet, docosahexaenoic acid ethyl ester 1% (DHA-EE 1%) diet and docosahexaenoic acid ethyl ester 2% (DHA-EE 2%) diet for three months. Results are expressed as means ± SE, n = 9. Differences were analyzed by one-way analysis of variance and comparisons among the dietary groups were done using Tukey's HSD test. Asterisks indicate a statistically significant difference between the control and each experimental diet group at $p < 0.05$.

Fatty acid composition of plasma and brain lipids

There was a striking difference in the mean percentage of both 20:4*n*-6 (AA), 20:5*n*-3 (EPA) and 22:6*n*-3 (DHA) fatty acids in plasma lipids among diet groups (Table III). The mice fed three different amounts of DHA-EE had high levels of DHA and very low levels of AA corresponding to the order of DHA-EE dose ($p < 0.05$). Those mice in the control group had the lowest level of DHA and the highest level of AA in plasma lipids ($p < 0.05$).

Percentage of DHA in brain increased in a dose-response manner in the groups fed various doses of DHA-EE (Table IV). The ratio of DHA/AA in brain of mice fed DHA-EE 0.5%, 1% and 2% diets was significantly higher than in the control group ($p < 0.05$). Conversely, the ratio of n-6/n-3 was significantly higher in mice fed the control diet compared with mice fed various doses of DHA-EE ($p < 0.05$).

Discussion

Our results showed that intake of DHA-EE 2% improved maze-learning ability, in agreement with our previous studies [13, 21]. In mice fed diets containing different concentrations of DHA, increasing dietary DHA resulted in an increasing ratio of DHA/AA in the brain. Gamoh *et al* [11] suggested that chronic administration of DHA had a beneficial effect on the neuronal state by decreasing the level of lipid peroxide, and that the ratio of DHA/AA in the cerebral cortex may be considered as a novel indicator of learning ability. It can be suggested that modifications of HUFA in mouse brain following feeding of DHA, particularly an increase in DHA and a reciprocal decrease in AA levels, are associated with improved learning ability.

The present study showed that intake of DHA-EE 2% was more effective in improving learning ability in adult mice than either DHA-EE 0.5% or 1%. In our maze-learn-

Table III: Selected fatty acid composition (weight %) of plasma lipids in adult mice fed different diets for four months

	Diet group			
Fatty acids	Control (Palm oil)	DHA-EE 0.5%	DHA-EE 1%	DHA-EE 2%
	g/100 g total fatty acids			
16:0	16.88 ± 0.42[a]	22.34 ± 0.59[b]	22.58 ± 0.50[b]	25.32 ± 0.63[c]
18:0	4.79 ± 0.25[a]	4.47 ± 0.26[a]	4.46 ± 0.09[a]	5.59 ± 0.18[b]
18:1(n-9)	25.61 ± 0.54[a]	24.82 ± 0.86[a]	23.10 ± 0.52[a]	19.26 ± 0.49[b]
18:1(n-7)	5.09 ± 0.18[a]	2.33 ± 0.14[b]	1.38 ± 0.04[c]	1.46 ± 0.09[c]
18:2(n-6)	8.17 ± 0.42[a]	13.20 ± 0.74[b]	11.93 ± 0.54[c]	10.32± 0.53[c]
20:1(n-9)	0.50 ± 0.04[a]	0.52 ± 0.04[a]	0.35 ± 0.04[b]	0.29 ± 0.04[b]
20:3(n-6)	0.47 ± 0.03[a]	0.50 ± 0.06[a]	0.37 ± 0.02[b]	0.49 ± 0.04[a]
20:4(n-6)	11.34 ± 0.79[a]	2.51 ± 0.26[b]	1.41 ± 0.12[c]	0.83 ± 0.06[d]
20:5(n-3)	0.27 ± 0.07[a]	4.37 ± 0.25[b]	7.30 ± 0.33[c]	7.02 ± 0.54[c]
22:4(n-6)	0.69 ± 0.05	–	–	–
22:6(n-3)	1.42 ± 0.06[a]	8.84 ± 0.24[b]	11.39 ± 0.45[c]	15.13 ± 0.24[d]
22:6(n-3)/20:4(n-6)	**0.13 ± 0.01[a]**	**3.92 ± 0.56[b]**	**8.64 ± 0.85[c]**	**19.31 ± 1.73[d]**
n-6/n-3	**13.93 ± 0.93[a]**	**1.24 ± 0.10[b]**	**0.73 ± 0.04[c]**	**0.52 ± 0.03[d]**

The values are means ± SE, n = 9. Values for each group with different superscript letters in the same fatty acids and ratio are significantly different at $p < 0.05$ by using Tukey's HSD test.

Table IV: Selected fatty acid composition (weight %) of brain total lipids in adult mice fed different diets for four months

	Diet group			
Fatty acids	Control (Palm oil)	DHA-EE 0.5%	DHA-EE 1%	DHA-EE 2%
		g/100 g total fatty acids		
16:0	20.75 ± 0.66	20.57 ± 0.43	21.09 ± 0.34	21.30 ± 0.25
18:0	15.66 ± 0.38	15.82 ± 0.28	15.91 ± 0.17	15.41 ± 0.23
18:1(n-9)	15.68 ± 0.34[a]	16.73 ± 0.31[b]	17.25 ± 0.21[b]	17.46 ± 0.22[b]
18:1(n-7)	3.72 ± 0.09	3.45 ± 0.05	3.39 ± 0.05	3.38 ± 0.04
18:2(n-6)	0.30 ± 0.01[a]	0.43 ± 0.03[b]	0.32 ± 0.02[a]	0.27± 0.01[a]
20:1(n-9)	1.96 ± 0.06	2.13 ± 0.04	2.05 ± 0.04	1.99 ± 0.07
20:1(n-7)	0.46 ± 0.02	0.42 ± 0.03	0.44 ± 0.09	0.43 ± 0.02
20:3(n-6)	0.34 ± 0.03[a]	0.56 ± 0.05[b]	0.56 ± 0.02[b]	0.46 ± 0.03[c]
20:4(n-6)	7.45 ± 0.13[a]	4.15 ± 0.09[b]	3.60 ± 0.07[c]	3.53 ± 0.17[c]
20:5(n-3)	0.26 ± 0.02	0.30 ± 0.03	0.35 ± 0.02	0.34 ± 0.04
22:0	1.95 ± 0.06[a]	1.07 ± 0.04[b]	0.94 ± 0.02[b]	0.76 ± 0.03[c]
22:4(n-6)	0.79 ± 0.05[a]	–	–	–
22:5(n-6)	0.32 ± 0.03	0.30 ± 0.01	0.39 ± 0.01	0.34 ± 0.04
22:6(n-3)	9.60 ± 0.15[a]	11.71 ± 0.24[b]	12.51 ± 0.24[c]	13.28 ± 0.26[d]
22:6(n-3)/20:4(n-6)	**1.29 ± 0.03[a]**	**2.83 ± 0.06[b]**	**3.49 ± 0.09[c]**	**3.82 ± 0.14[c]**
n-6/n-3	**0.93 ± 0.42[a]**	**0.44 ± 0.01[b]**	**0.37 ± 0.01[c]**	**0.34 ± 0.01[c]**

The values are means ± SE, n = 9. Values for each group with different superscript letters in the same fatty acids and ratio are significantly different at $p < 0.05$ by using Tukey's HSD test.

ing method, it may be that the dosing of DHA-EE 2% over three months time in adult mice is a minimum requirement to effect behavioral changes from biochemical alterations. Moreover, our recent study showed that maze-learning ability of mice fed DHA-EE 2% for three months was improved and that DHA levels and the ratio of DHA/AA of brain were 13.2% and 3.77, respectively [18]. In the present work, we observed that DHA levels and ratio of DHA/AA of brain were 13.3% and 3.88, respectively, similar to results of the above study. These results indicate that levels of DHA and ratio of DHA/AA in mouse brain will need to be greater than 13.2% and 3.77, respectively, in order to improve learning ability in our maze-learning system. The average values of brain DHA and AA for each of the 4 experimental groups were plotted against the average time of the three trial values in the maze-learning experiment (Fig. 3). As the DHA value increased, the average time decreased, corresponding to mice finding the exit more quickly. The percentage of DHA was negatively correlated to the average time spent in the maze ($r^2 = 0.99$, $p = 0.001$). The relationship to AA showed a positive correlation with average time ($r^2 = 0.98$, $p = 0.001$), thus as the brain AA value increased, mice took a longer time to find the exit.

There have been some reports that supplementation with DHA improves visual function in animals as well as human infants [22–26]. Therefore, it may be postulated that improved learning ability by intake of DHA is associated with an improved visual sensorium. It has been reported that rodents have relatively poor vision [27]. Oakley [28] suggested that decortication did not influence performance in a simple visual discrimination. Since our behavior test was conducted in a black box with black partitions, subtle visual discrimination was not necessary to perform well in the maze. Another factor that may influence performance of mice in DHA-containing diet groups is their level of motivation. Since water deprivation was employed to elicit work for water reward, their level of motivation was not ruled out. There were indeed no differences in behavior when using a simple three-partition maze as a training test between the control and DHA groups. We suggest that differences in visual discrimination and motivation between the control and DHA groups are minimum with regard to their effect on maze performance.

Nishikawa *et al* [29] reported that DHA influenced the activation of N-methyl-D-aspartate receptors, which is an absolute requirement for the induction of long-term potential in the hippocampal region and neocortex. It has been reported that dietary DHA increased cerebral choline and acetylcholine levels in the brain and improved the passive avoidance performance in stroke-prone spontaneously hypertensive rats [30]. In addition, Zimmer *et al* [31] has suggested that DHA deficiency modified several factors of dopaminergic neurotransmission in the nucleus accumbens and that such modifications would be associated with behavioral deficits in DHA-deficient rats. It is known that DHA-containing phospholipids induce greater rhodopsin activation than other highly unsaturated phospholipids [32], and increase the rate of coupling of activated rhodopsin to Gprotein [33]. Rhodopsin is a member of the Gprotein-coupled receptor family, which includes many neurotransmitter receptors that have similar structural characteristics. We assumed that neurotransmitters

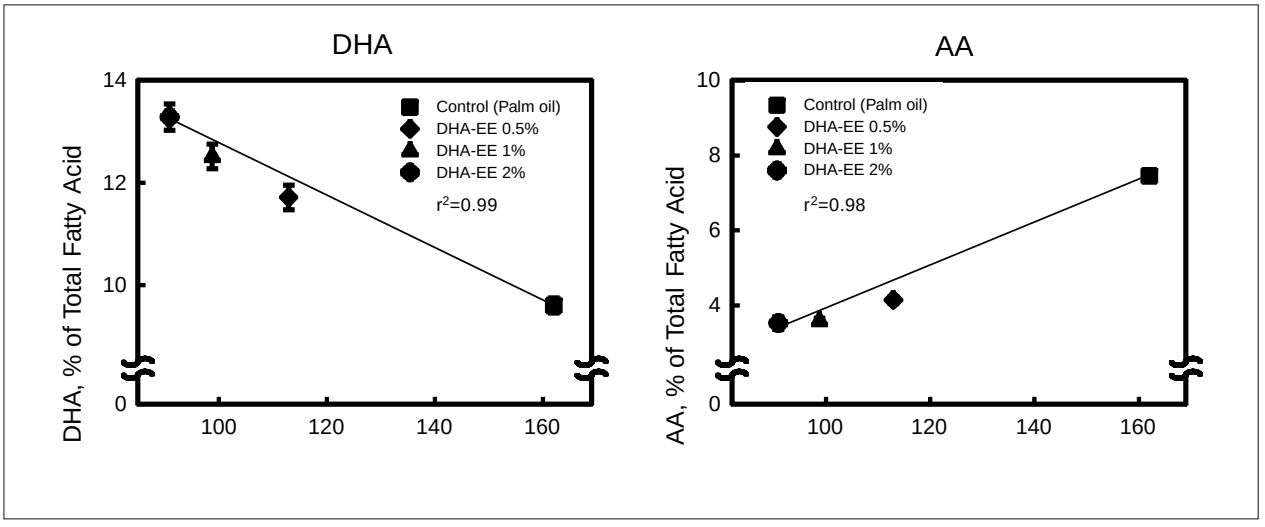

Figure 3: Correlation of maze behavior test and brain fatty acid composition. The percentages of DHA and AA were plotted against the average time of the three trials. Statistical analysis for the regression curve gave the following results: DHA, $r^2 = 0.99$, $p = 0.001$; AA, $r^2 = 0.98$, $p = 0.001$.

were involved in the observed effects of DHA on learning ability, although we have no definitive data. In our previous work, we examined the synergistic effect of dietary DHA and PC on the learning task but found no synergistic effect in the DHA-EE+egg-PC group [21].

In summary, this study suggests that the intake of DHA-EE 2% is more effective on improving maze-learning ability in adult mice than DHA-EE 0.5% and 1%, although we did not find a linear dose-response effect of DHA on maze-learning ability. The improved learning ability in mice fed DHA is associated with higher brain DHA levels.

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