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DHA Depletion in Rat Brain Is Associated With Impairment on Spatial Learning and Memory

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Objective To examine the effect of docosahexaenoic acid (DHA) deficiency in brain on spatial learning and memory in rats. **Methods** Sprague Dawley rats were fed with an n-3 fatty acid deficient diet for two generations to induce DHA depletion in brain. DHA in seven brain regions was analyzed using the gas-liquid chromatography. Morris water maze (MWM) was employed as an assessing index of spatial learning and memory in the n-3 fatty acid deficient adult rats of second generation. **Results** Feeding an n-3 deficient diet for two generations depleted DHA differently by 39%-63% in the seven brain regions including cerebellum, medulla, hypothalamus, striatum, hippocampus, cortex and midbrain. The MWM test showed that the n-3 deficient rats took a longer time and swam a longer distance to find the escape platform than the n-3 Adq group. **Conclusion** The spatial learning and memory in adult rats are partially impaired by brain DHA depletion.

Key words: Docosahexaenoic acid; Memory; Morris water maze; Spatial learning

INTRODUCTION

Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) are highly concentrated in the mammalian nervous system and rapidly accreted during the brain growth spurt in both prenatal and neonatal periods[1-2]. DHA and AA can be synthesized from their respective short-chain precursors, alpha-linolenic acid (LNA, 18:3n-3) and linoleic acid (LA, 18:2n-6), via the desaturation and elongation pathway. LNA and LA are regarded as essential fatty acids, since mammals cannot synthesize them and have to obtain them from diets. Unlike DHA and AA, LNA and LA are quantitatively minor in mammalian brains[3].

Depletion of brain DHA can be induced by a diet that is deficient in LNA. Previous studies showed that DHA depletion in brain can lead rodents to have a poor performance in the Morris water maze, shock avoidance, olfactory discrimination, and exploratory behavior[4-7]. However, other studies reported no difference in performance for spatial learning tasks between the normal control and DHA-depleted mice[8-9]. Previous studies have found that hippocampus, striatum, basal forebrain, cerebellum and neocortical areas are the most important regions for spatial learning and memory. The present study was to investigate if a LNA deficiency in diet depletes DHA differently in brain regions (including cerebellum, medulla, hypothalamus, striatum, hippocampus, cortex, and midbrain) and to examine how brain DHA depletion affects the spatial learning and memory in rats.

MATERIALS AND METHODS

Animals

Twenty weaning Sprague Dawley (SD) female rats were randomly divided into two groups and fed either with a diet that was adequate in LNA (n-3 Adq) or with a diet that was deficient in LNA (n-3 Def).

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The rats were allowed to have free access to the experimental diet and tap water. When the rats reached 10 weeks of age, they were mated with male rats (12 weeks of age). The pups (F2, second generation) were placed on their corresponding maternal diet through out the entire experimental period. At week 17, only F2 male rats from different litters were chosen to perform the Morris Water Maze (MWM) test.

Diets

The composition of n-3 Adq diet and n-3 Def diet was similar except for their fat content (Table 1). All diet ingredients were purchased from Harlan Teklad (Madison, WI, USA) except for cornstarch, oils and sucrose that were obtained from a local supermarket. Each diet had 6% fat (by weight). The n-3 Adq diet contained (per kg) 2.3 g of coconut oil, 8.4 g of canola oil, 6.8 g of sunflower oil and 2.5 g of flaxseed oil, while the n-3 Def diet had only 44.2 g of coconut oil and 15.8 g of sunflower oil. Fatty acid composition of the two diets was similar except for LNA, which accounted for 3.49% in the n-3 Adq diet but it was less than 0.04% of total fatty acids in the n-3 Def diet (Table 2).

TABLE 1

Composition (g/kg diet) of n-3 Adequate and Deficient Diets

	n-3 Adequate Diet	n-3 Deficient Diet
Corn Starch	571.0	571.0
Casein	235.0	235.0
Sucrose	50.0	50.0
Mineral Mix AIN 76	35.0	35.0
Cellulose	32.0	32.0
Vitamin Mix AIN 76A	10.0	10.0
Choline Bitatrate	4.0	4.0
DL-Methionine	3.0	3.0
Total Fat	60.0	60.0
Coconut Oil	42.3	44.2
Canola Oil	8.4	0
Sunflower Oil	6.8	15.8
Flaxseed Oil	2.5	0

TABLE 2

Fatty Acid Composition (% of Total Fatty Acids) of n-3 Adequate and Deficient Diets

Fatty Acid	n-3 Adequate Diet	n-3 Deficient Diet
12:0	33.32	34.57
14:0	13.52	14.58
16:0	9.75	8.73
18:0	3.47	3.20
18:1n-9	14.97	17.14
18:2n-6	17.45	17.68
18:3n-3	3.49	<0.04
Others	3.85	4.09

Morris Water Maze

A large blue circular tank with 182 cm in diameter and 75 cm in depth was employed as a swimming pool in the MWM test (QT 502 Aquatic Ecosystems Inc, USA)[10]. The pool was filled with the tap water and the water temperature was maintained around 23℃±2℃. Non-dairy creamer (100 g) was added into the pool to opaque the water. The pool was divided into four quadrants, named as zones 1, 2, 3, and 4, respectively.

The first test was conducted to assess the ability to learn and remember a constant location of the escape platform by rats. A transparent escape platform (12 cm by 12 cm^2) was centrally fixed in zone 4 and submerged 1.5 cm below the water surface. Prominent extra maze cues were placed around the testing room to enable the rats to learn the location of platform. A digital video camera (Sony Digital Handycam DCR-PC101E, Sony Corporation, Tokyo, Japan) was mounted over the pool and operated by a remote control to record rat's behavior. The data were analyzed on a computer installed with Noldus analytical software (Noldus Information technology, The Netherlands). Sixteen F2 adult male rats (n=8 each group) from the n-3 Def and the n-3 Adq groups performed the MWM test. The rats were allowed to swim freely in the pool for 2 minutes on the day before the test to adapt the water environment. All rats were given six trials a day for four consecutive days. The starting points were at the middle of each quadrant rim. To avoid accidental hit on the platform due to the short distance, zone 4 (containing platform) was not used as the starting one. The rats were released from each starting point facing the inner wall of the tank in a random manner during each day of test and allowed to swim for 120 s to find the platform. If the rat failed to find the escape platform within 120 s, it would be manually guided onto the platform. Once the rat found the platform, it was allowed to stay on it for 20 s for localization of the platform and then removed to a warm place for 30 s.

A probe trial was then carried out to evaluate how well the rats learned the task. In this task, the hidden escape platform was removed from the water pool. After the last trial on day 4, the rats were allowed to swim for 120 s in the pool without a platform. The distribution of time spent and distance swum by each rat in each zone was recorded and analyzed.

On day 8 after a 3-day rest, a long-term memory test was then carried out. In brief, the rats were given six trials as described elsewhere above for the first 4-day tests. The long-term memory would lead the rats to locate the platform in zone 4. A trial was completed when the rats found successfully the

hidden platform. On the same day after completing the long-term memory test, the rats were killed under a light carbon dioxide anesthesia. The brain was removed and dissected into cerebellum, medulla, hypothalamus, striatum, hippocampus, cerebral cortex and midbrain for the fatty acid analysis according to the procedure previously described by Glowinski and Iversen[11].

Fatty Acid Analysis

Total lipids of regional brain samples were extracted using chloroform-methanol (2:1, vol/vol) containing 2% butyrated hydroxytoluene (Sigma Chemical, St Louis, MO, USA) as an antioxidant. L-phosphatidylcholine diheptadecanoyl (Sigma Chemical, St Louis, MO, USA) was added as an internal standard to quantify total phospholipids (PL). Neutral lipid thin-layer chromatography (TLC, 20 cm by 20 cm plates pre-coated with 250 μm silica gel 60A, Macherey-Nagel Gmbh & Co. KG, Düren, Germany) was performed to separate total PL in a developing solvent system of hexane-diethyl ether-acetic acid (80:20:1; by volume). Total PL was recovered from the TLC plate, and their fatty acids were converted to the corresponding methyl esters using 14% boron trifluoride in methanol (Sigma Chemical, St Louis, MO, USA) under nitrogen gas at 90℃ for 60 min.

The fatty acid methyl esters were analyzed on a flexible silica capillary column (Innowax 19091N-213, 30 m × 0.32 mm, i.d., J & W Scientific, Folsom, CA, USA) in a HP 5980 Series II gas-liquid chromatograph equipped with a flame-ionization detector (Palo Alto, CA, USA). The column temperature was programmed from 180℃-230℃ at a rate of 2℃/min and then held for 5 min. Injector and detector temperatures were set at 250℃ and 300 ℃, respectively. Helium was used as the carrier gas at a head pressure of 15 psi. Identification of each fatty acid methyl ester was made by comparison of retention time of authentic standards (Sigma Chemical Co., St. Louis, MO, USA).

Data Analysis

Data from the fatty acid analysis were expressed as mean ± standard deviation (SD) while for the MWM test they were expressed as mean ± standard error of the mean (SEM). Where applicable, one-way or two-way analysis of variance (ANOVA) was used followed by multiple post hoc comparisons to statistically evaluate significant differences between the n-3 Adq and the n-3 Def groups using SPSS 13.0. The results of MWM test were expressed as the mean escape latency, the distance swum to find the hidden platform and the time spent in zone 4 (target zone). In the probe trial test, the duration and distance swum in the target zone was calculated as an index for rats to remember the location of platform. Differences were considered significant when $P<0.05$.

RESULTS

There was no significant difference in body weight gain between the two groups. The final body weight was 500 ± 35 g for the n-3 Adq and 475 ± 45 g for the n-3 Def rats, respectively. The fatty acid analysis showed that feeding an n-3 fatty acid deficient diet caused depletion of DHA in brain. As shown in Fig.1, DHA was reduced in various regions of brain

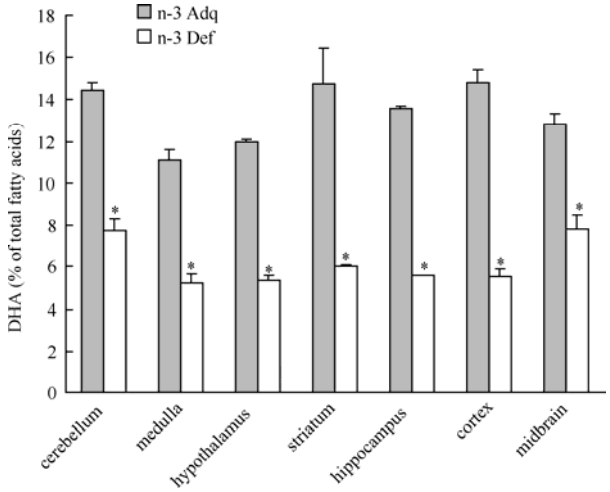

FIG. 1. Depletion in brain docosahexaenoic acid (DHA, % of total fatty acids) of seven brain sub-regions caused by feeding an n-3 fatty acid deficient diet for two generations in rats. Data are expressed as $\bar{x} \pm SEM$; $n=8$. *Means at the same region differ significantly, $P<0.001$. The solid bars represent the male SD rats fed with the n-3 fatty acid adequate diet (n-3 Adq) while the open bars represent the male SD rat fed with the n-3 fatty acid deficient diet (n-3 Def) for two generations.

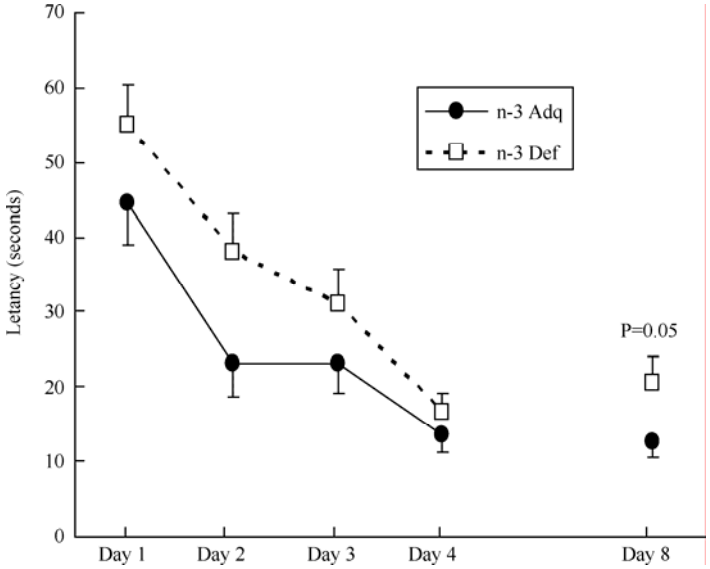

FIG. 2. Escape latency (seconds) to find the hidden platform in rats fed with an n-3 fatty acid adequate diet (n-3 Adq) or an n-3 fatty acid deficient diet (n-3 Def) for two generations. In the Morris Water Maze test, the rats (n=8 each group) were given six trials on days 1, 2, 3, 4, and 8. Data were expressed as $\bar{x} \pm$SEM; n=8. The differences in escape latency on days 1-4 between the n-3 Def group and the n-3 Adq were statistically significant in a two-way ANOVA (F=6.994, P=0.008). The difference in performance on day 8 was statistically analyzed in a one-way ANOVA (*P =0.05).

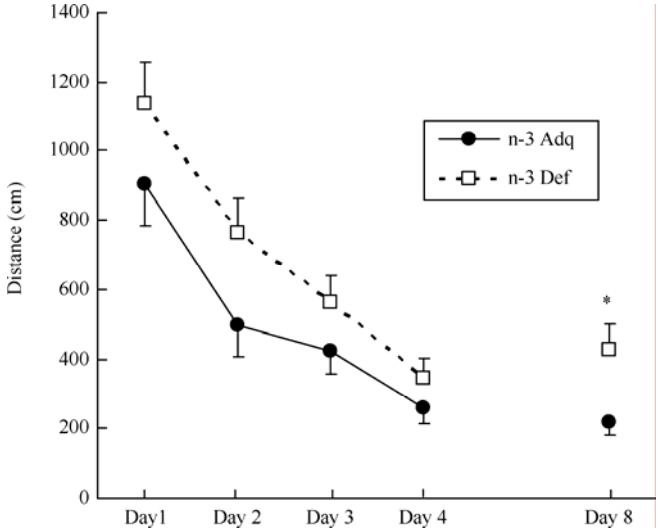

FIG. 3. Distance (cm) swum to find the hidden platform by male SD rats fed with an n-3 fatty acid adequate diet (n-3 Adq) or an n-3 fatty acid deficient diet (n-3 Def) for two generations. In the Morris Water Maze test, the rats (n=8 each group) were given six trials on days 1, 2, 3, 4, and 8. Data were expressed as $\bar{x} \pm$SEM; n=8. The differences on days 1-4 between the n-3 Def group and the n-3 Adq were statistically significant in a two-way ANOVA (F=6.844, P=0.009). The difference in performance on day 8 was statistically analyzed in a one-way ANOVA (*P=0.015).

by 39%-63% in the n-3 Def group compared with that in the n-3 Adq group. Among various regions, cerebral cortex (63%), hippocampus (59%), and striatum (59%) had remarkable DHA depletion followed by hypothalamus (55%), medulla (53%), cerebellum (47%), and midbrain (39%).

In the MWM test, the escape latency of both groups gradually decreased over the testing period, indicating an improvement in performance for both groups. In general, the n-3 Adq group had shorter latencies to locate the escape platform than the n-3-Def rats (P<0.008), suggesting that the n-3 Def group had a relatively poorer spatial learning and memory (Fig. 2). However, the difference in latencies between the two groups became less significant after the first two days of testing (Fig. 2). A similar trend

was seen when the data were expressed as a distance swum by rats to locate the platform (Fig. 3). The n-3 Adq rats swam a shorter distance than the n-3 Def rats to reach the platform (P<0.009). With the progress of trials, the difference in distance between the two groups became less (Fig. 3). When the data were expressed as % swimming distance in zone 4, the n-3 Def rats had a shorter path than the n-3 Adq group in the target zone. Statistically, the difference in % swimming distance in the target zone between the two groups was significant only for the first two days of testing and thereafter became insignificant (Fig. 4).

The long-term memory test was carried out on day 8. The results demonstrated that the n-3 Def group had a greater latency than the n-3 Adq rats (P=0.05). Similarly, the n-3 Def rats swam a longer distance than the n-3 Adq group to find the hidden platform (P<0.05) (Figs. 2 and 3). The present data indicated that the brain DHA-depletion had a long-term memory impairment in water maze performance.

The probe trial was conducted with the platform removed and the rats allowed to swim for 120 s. No significant differences were observed between the two groups in the times spent (Fig. 5) and the distance swum in each zone (Fig. 6).

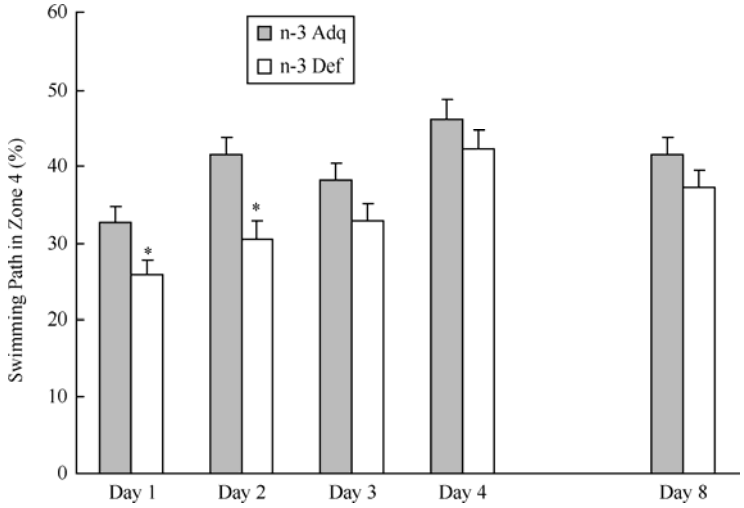

FIG. 4. Swimming distance (%) spent in zone 4 by SD rats fed with an n-3 fatty acid adequate diet (n-3 Adq) or an n-3 fatty acid deficient diet (n-3 Def) for two generations. In the Morris Water Maze test, the rats (n=8 each group) were given six trials on days 1, 2, 3, 4, and 8. Data were expressed as $\bar{x} \pm$SEM; n=8. *Means at the same day differ significantly, P<0.05.

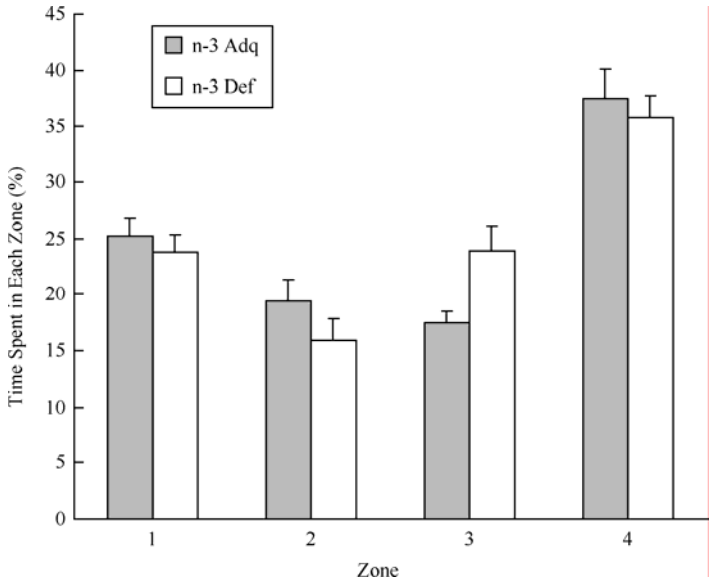

FIG. 5. Time (seconds) spent in each zone by SD rats fed with an n-3 fatty acid adequate diet (n-3 Adq) or an n-3 fatty acid deficient diet (n-3 Def) for two generations when the platform was removed. In the probe trial performance, the rats (n=8 each group) were allowed to swim for 120 s. Data were expressed as $\bar{x} \pm$SEM; n=8.

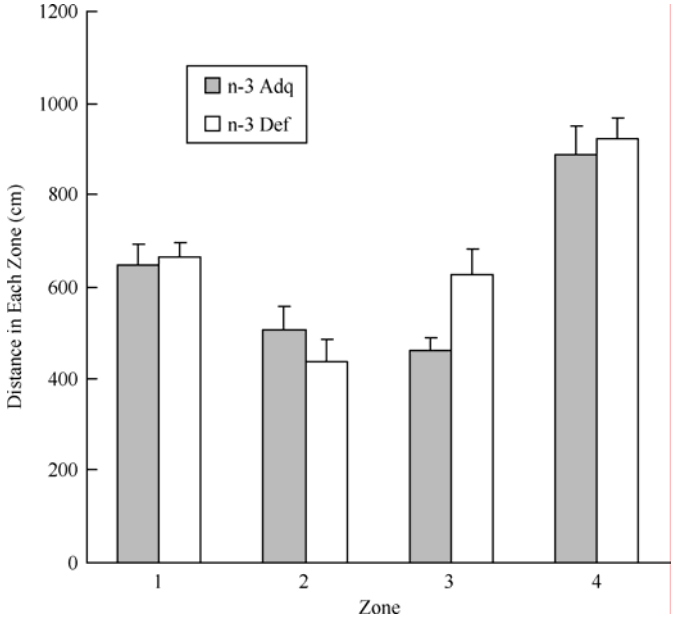

FIG. 6. Distance (cm) traveled in each zone by SD rats fed with an n-3 fatty acid adequate diet (n-3 Adq) or an n-3 fatty acid deficient diet (n-3 Def) for two generations after the platform was removed. In the probe trial performance, the rats (n=8 each group) were allowed to swim for 120 s. Data were expressed as $\bar{x} \pm$ SEM; n=8.

DISCUSSION

The objective of this study was to use an n-3 fatty acid deficient diet to induce DHA depletion in brain and to test the effect of DHA deficiency on the spatial learning and memory in adult F2 male rats. The present study demonstrated that DHA depletion in brain could be achieved by feeding an n-3 fatty acid deficient diet for two generations. Seven brain regions including cortex, hippocampus, striatum, cerebellum, hypothalamus, medulla, and midbrain had DHA depletion by 39%-63% (Fig. 1), suggesting that DHA is not equally reduced in brain sub regions, and its depletion is region-specific. This observation is consistent with previous reports[12-15].

DHA is generally believed to be required for the development of normal brain function in both humans and animals[16]. The present study employed the MWM test to examine a possible link between a decrease in brain concentration of DHA and altered performance in spatial learning and memory in the n-3 deficient rats. Latency in the MWM test is generally considered to reflect the spatial learning and cognitive capacity[10,17-18]. The present study demonstrated that the n-3 Def rats spent more time and swam a longer distance to find the hidden platform compared with the n-3 Adq group, indicating that the n-3 Def rats have a poorer spatial learning ability and memory. In addition, the present study found that the n-3 Def rats had poorer performance than the n-3 Adq rats on day 8, indicating that the long-term memory is partially impaired. The present results suggest that learning and cognitive behaviors are partially related to the brain DHA status. This observation is consistent with the report by Moriguchi *et al.*[7], who found that the n-3 deficient rats of both F2 and F3 generations have the escape latency significantly longer than the corresponding n-3 Adq rats. Carries *et al.*[14] found that not only learning performance in the Morris water maze was significantly impaired but also the a- and b-wave amplitudes in electroretinogram were significantly altered in the n-3 deficient mice. Most interesting is that DHA-rich phospholipid supplementation could restore partially the abnormal electroretinogram in the n-3 deficient mice. However, the present result is in disagreement with the report by Winwright *et al.*[8], who failed to find any difference in the place or cued version of the MWM test between the AA-DHA supplemented and the n-3 deficient rats. We have no explanation for these discrepancies. Perhaps, the difference in spatial learning and memory might be a function of DHA depletion in the brain. Winwright *et al.*[8] showed that DHA level in the brain of the deficient rats was only 46% lower than that in the supplemented rats, whereas in the present study the n-3 deficient rats had DHA depletion by up to 63% in the seven brain sub-regions.

The probe trial assessed the time spent and distance swum by the rats in the target zone where the platform was previously located, and evaluated

the spatial memory in both n-3 Def and n-3 Adq rats. The rats in both groups spent more time and swam a longer distance in zone 4 than they did in the other three zones (Figs. 5 and 6). However, there was no significant difference observed in the time or distance spent or swum by the n-3 Def and the n-3 Adq rats. It has to be pointed out that the probe trial was carried out right after day 4 so that the rats still had short-memory to sense the location of platform. The present result is not consistent with the report by Moriguchi *et al*.[7], who found that an n-3 deficient diet for three generations can impair significantly rats to perform in the probe trial. The plausible explanation for this discrepancy is that DHA deficiency in the study of Moriguchi *et al*.[7] was much more severe than that in the present study. To be more specific, DHA in brain was depleted by 87% in the study of Moriguchi *et al*.[7] whereas it was depleted only by 39%-63% in the present study, suggesting that only severe but not low or moderate DHA depletion in rat brain would impede the spatial performance in the probe trial.

In summary, DHA in brain is significantly depleted in rats fed with an n-3 fatty acid deficient diet for two generations. The rats on the n-3 fatty acid deficient diet perform poorly in learning how to locate the hidden platform. It should be pointed out that the learning ability deficit observed in the n-3 Def group is not attributable to change in growth because no difference in body weight gain was seen between the n-3 Adq and the n-3 Def rats. It is noteworthy that the DHA depletion in this study or other similar studies[7-8,10,13] is too extreme to be achieved by feeding rats with an n-3 deficient diet for two generations. The severe DHA deficiency would not happen under the normal physiological conditions. Therefore, it is of interest to identify a "threshold" brain DHA level below which learning ability and memory would be impaired.

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