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N-3 Fatty Acid Deficiency Induced by a Modified Artificial Rearing Method Leads to Poorer Performance in Spatial Learning Tasks

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ABSTRACT

Docosahexaenoic acid (DHA) is a major structural component of the nervous system, and depletion may lead to losses in neural function. Our objective was to demonstrate a deficit in spatial task performance in rats with low brain DHA due to a low n-3 fatty acid intake using a first-generational artificial rearing technique. Newborn rat pups were separated on d 2 and assigned to two artificial rearing groups or a dam-reared control group. Pups were hand fed artificial milk *via* custom-designed nursing bottles containing either 0.02% (n-3 Def) or 3.1% (n-3 Adq) of total fatty acids as LNA. At d 21, rats were weaned to either n-3 Def or n-3 Adq pelleted diets and several behavioral tasks were evaluated at 9 wk of age. Brain DHA was lower (58% and 61%, $p < 0.001$) in n-3 Def in comparison to n-3 Adq and dam-reared rats, respectively. At adulthood, the n-3 fatty acid–deficient rats had a significantly greater moving time than the dam-reared group ($p < 0.05$), but there were no differences among the three groups in the elevated plus maze test. The n-3 fatty acid deficient rats exhibited a longer escape latency ($p < 0.05$) and poorer memory retention in the Morris water maze compared with n-3 fatty acid adequate and dam-reared rats. We concluded that artificial rearing can be used to produce n-3 fatty acid deficiency in the first generation. This deficiency was associated with significantly reduced spatial learning. Adequate brain DHA levels are required for optimal spatial learning. **(*Pediatr Res* 58: 741–748, 2005)**

Abbreviations

AA, arachidonic acid
AR, artificial rearing
DHA, docosahexaenoic acid, 22:6n-3
DPAn-6, docosapentaenoic acid, n-6, 22:5n-6
EPA, eicosapentaenoic acid
LA, linoleic acid
LNA, alpha-linolenic acid, 18:3n-3
n-3 Adq, n-3 fatty acid adequate group
n-3 Def, n-3 fatty acid deficient group

Many studies have now demonstrated that prolonged consumption of an n-3 fatty acid–deficient diet resulted in a loss in brain and retinal DHA leading to losses in neural function (1–5). For example, the decrease in brain DHA was associated with poorer performance on simple associative learning tasks (6–8), alterations in electroretinograms (9–13), and spatial task performance in rodents (14–16). Our previous work showed that rats on an n-3 fatty acid deficient diet throughout two generations had a defect in two-odor olfactory discrimination tasks (17,18) and olfactory-cued set learning (19). In these studies, brain DHA decreased by 83% with compensation by an increase in brain DPAn-6. Rhesus monkey infants fed n-3 fatty acid–deficient diets had increased look duration (20) whereas monkeys supplemented with 1% DHA and 1% AA during early development exhibited stronger orienting and motor skills (21).

The general paradigm used in these studies was a two-generation model in which dams are fed a diet nearly devoid of sources of n-3 fatty acids and their offspring studied while maintained on the n-3 fatty acid deficient diet. This has proven to be a most useful approach to induce the loss of brain and retinal DHA and enable study of functional losses. It was remarkable that the diet-induced substitution of the 22-carbon n-6 polyunsaturate DPAn-6 for neural DHA was associated with these losses in brain and retinal function. Studies of subsequent DHA repletion has shown in a fairly conclusive manner that the functional gain was indeed related to the increase in DHA and the decline in DPAn-6. In particular, when function returns together with brain or retinal DHA in a repletion experiment, this was strong evidence that the gain in function was indeed due to the gain in neural DHA (22–24),

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although there has been one suggestion that not only the levels of DHA but also that of AA must normalize to restore proper brain function (25). Kodas *et al.* (26) found that altered dopaminergic neurotransmission induced by chronic n-3 fatty acid deficiency was reversed with supply of an LNA diet when provided from birth or during the first 2 wk of life, suggesting that critical periods exist for n-3 fatty acid supply for neural development.

One limitation of the two-generational deficiency approach is that it poorly mimics the human situation. Although the intake of the longer chain n-3 fatty acids, EPA and DHA are quite low in the western world, particularly in North America (27), where there is a mean intake of the n-3 precursor LNA of about 1.5 g/d (28–30). This is reflected in circulating pools of n-3 fatty acids as, for example, the percentage of serum total n-3 fatty acids in Americans was only 2.5% whereas the percentage in Japanese was 9–11.8% (31). These low levels are the result of the low intake of preformed EPA and DHA together with the low rate of metabolic conversion of LNA to EPA and DHA *in vivo* (32). Nevertheless, a moderate level of n-3 fatty acid intake is able to support, to some extent, brain and retinal DHA (33,34) as it appears to be accreted against a concentration gradient. This is not to say that the brain levels in the case of low n-3 fatty acid intake are the same as those with higher levels of intake of preformed DHA (33,35,36), an observation that may have relevance to the growing psychiatric literature that associates n-3 fatty acid intake with the propensity toward the development of various psychiatric ailments (37–39).

For many animal studies, a significant decrease in brain DHA is required to produce a measurable decline in organ or organism function (22). More specifically, it appears that a 50% or greater loss of DHA may be required to observe such differences (22,40). Thus, a model of n-3 fatty acid deficiency that better mimics the human situation but that still produces a substantial decline in brain and retinal DHA would be very valuable for the development of this field.

Recently, efforts have been made in our laboratory (41,42) to develop a first-generation model of n-3 fatty acid deficiency using an artificial rearing approach pioneered by Hoshiba (43,44). This method employs a feeding rack containing six silicon nipples from which the animals can be taught to feed. However, in our hands, pup survival was poor due to difficulties with the adjustment of the nipple resistance, which was critical for a proper flow rate upon pup suckling. Hoshiba has improved upon this method with the introduction of a new type of nursing bottle for hand feeding that leads to much better pup survival (45). These methods are unique in that they allow for the artificial rearing (AR) of rat pups from around the first day of life. The hand-feeding technique with the newly developed bottles was thus used for the present work.

In this experiment, the dams were fed a diet containing 3 wt% LNA, an "n-3 adequate" diet. Their pups were fed artificial rat milk beginning on the second day of life. In this manner, total control of the nutrient and essential fatty acid intake, in particular, was obtained. Artificial rat milk diets were formulated after the method of Kanno *et al.* (46) but were modified so as to provide very low levels of n-3 fatty acids, or a diet incorporating 3 wt% LNA. The loss in brain DHA associated with an n-3 fatty acid deficient diet and the performance of pups on several behavioral tasks were characterized. Measures of locomotor activity and activity on the elevated plus maze were made as controls for the key endpoint of spatial task performance in the Morris water maze. Spatial task performance consisted of measures of escape latency over a 4-d period, as previously established for a two-generation model of n-3 fatty acid deficiency, as well as a probe trial in which the platform was removed in the swim test (16,22). Our hypothesis was that feeding the n-3 fatty acid deficient diet from shortly after birth through adulthood would lead to a marked decrease in brain DHA and that this would result in losses in spatial task performance.

MATERIALS AND METHODS

Animals and study design. This experimental protocol was approved by the Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health. Time-pregnant, female Long-Evans rats on d3 of gestation were obtained from Charles River (Portage, MI) and immediately started on an "n-3 adequate" pelleted diet (maternal diet) containing 3.1% α-linolenic acid (Table 1). They were maintained in our animal facility under conventional conditions with controlled temperature (23 $\pm$ 1°C) and illumination (12 h, 0600–1800 h) and water was provided *ad libitum*. At postnatal d 2, three male pups were selected from each litter (n = 12 litters) based upon body weight and age so that they were born within a 12-h time window and a similar group mean body weight. They were then randomly allocated into each of the three groups. In this manner, each of the groups was

Table 1. *Nutrient and fatty acid composition of pelleted diets given to dams and pups at weaning*

Ingredient	Amount (g/100 g)		
Casein, Vitamin free*	20		
Carbohydrates:			
Corn starch	15		
Sucrose	10		
Dextrose	19.9		
Maltose-dextrin	15		
Cellulose	5		
Mineral and salt mix†	3.5		
Vitamin mix‡	1		
L-Cystine	0.3		
Choline bitartrate	0.25		
TBHQ§	0.002		
Fat:	Maternal	n-3 Def	n-3 Adq
Hydrogenated coconut oil	7.75	2.7	2.7
Safflower oil	1.77	—	—
Flaxseed oil	0.48	—	—
Medium chain triglyceride	—	1.3	1.3
18:1n-9 ethyl ester	—	4.5	4.2
18:2n-6 ethyl ester	—	1.5	1.5
18:3n-3 ethyl ester	—	—	0.3
Fatty acid composition (wt %)			
Total Saturated	77.2	28.0	29.2
Total monounsaturated	4.3	52.2	48.2
18:2n-6	15.3	17.8	17.6
18:3n-3	3.1	0.06	3.2
n-6/n-3	4.9	296.7	5.5

* Dyets Inc. catalogue #400625 for maternal diet and ALACID casein (NZMP North America Inc) for n-3 Def and n-3 Adq diet.

† Dyets Inc. catalogue #210025.

‡ Dyets Inc. catalogue #310025.

§ TBHQ is tert-butyl hydroquinone.

composed of 12 male pups from each of the 12 different dams and each dietary group was composed of siblings of individuals in the other groups. The first group was the control group and was allowed to suckle from dams fed on an n-3 fatty acid adequate diet (dam's milk group). The second and third groups were artificially fed one of the two experimental milks (n-3 fatty acid deficient milk and n-3 fatty acid adequate milk group, respectively, Table 2).

Artificial rearing system. The artificial rearing procedure used was the hand-feeding technique with newly developed nursing bottles as described by Hoshiba (45). The artificial rearing system consisted of a custom made nursing bottle, cage, stainless steel sieve, cover lid, and electronic hot pad. The nursing bottles, custom made by Dr Junji Hoshiba, were composed of nipples, a milk inflow tube, a milk overflow tube, and a refill syringe (Fig. 1). The cage was placed on an electronically thermostated hot pad initially set at 33.5°C. The temperature was slowly decreased over the next several days until it was 30°C by d 6, where it remained until d 15; the pad was then maintained at 28°C until d 21. A stainless steel sieve was placed on the top of the cage with some chopped up paper towels for bedding. Fresh artificial milk was loading into the nursing bottle by a refill syringe. There were separate cages for each of the two dietary groups with one receiving the n-3 fatty acid–deficient milk and the other the n-3 fatty acid–adequate milk. On postnatal d 2, pups were capable of suckling from silicon nipples connected to the nursing bottles. They were separated from their dams and fed artificial milk from a nursing bottle with a silicon nipple every 3 h by hand for a total of 13 h per day. The nursing bottles with silicon nipples and fresh milk were kept in a refrigerator after feeding to minimize bacterial growth. The hand feeding was performed until postnatal d 14–15 when they opened the eyes. Milk was then provided twice per day in 40 mL aliquots in 50 mL conical tubes that were fitted with water-bottle tops so that rat pups could drink *ad libitum*. These pups were weaned to pelleted diets with a fat composition similar to that fed during the artificial rearing period (Table 1). The dam's milk group was weaned onto the same diet as their dams. When these artificially reared animals and dam-rearing animals were 8 wk old, behavioral experiments were begun.

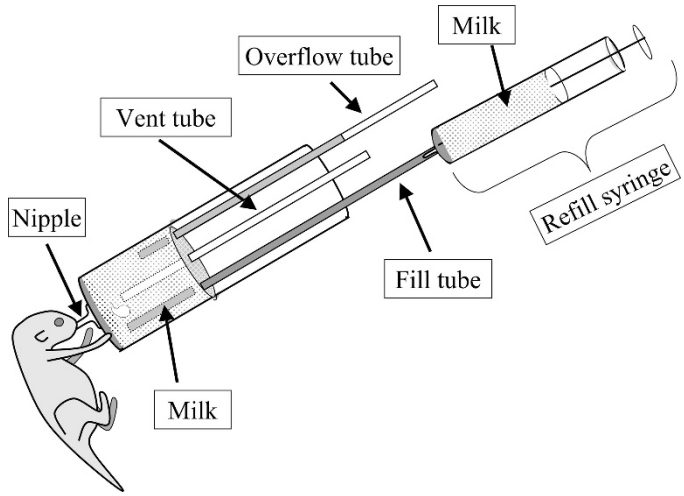

Figure 1. Feeding apparatus developed by Hoshiba (45).

Table 2. *Nutrient composition of artificial rat milk diets*

Ingredient	Amount (mg/100 ml milk)	
Casein (ALACID, acid casein)*	6275	
Whey protein isolate (ALACEN895)*	4000	
Carbohydrate (alpha-lactose)†	1893	
Serine†	28.8	
Cystine†	22.5	
Tryptophan†	27.0	
Minerals:		
NaOH†	2100	
KOH†	170	
$GlyCaPO_4$†	800	
$MgCl_2$ $6H_2O$‡	183	
$CaCl_2$ $2H_2O$‡	210	
Ca_3 $4H_2O$-Citrate†	250	
Na_2HPO_4‡	114	
KH_2PO_4‡	51.0	
$FeSO_4$†	3.0	
$ZnSO_4$†	6.0	
$CuSO_4$†	1.6	
$MnSO_4$†	0.07	
NaF†	0.16	
KI†	0.18	
Carnitine†	4.0	
Picolinate†	2.0	
Ethanolamine†	3.4	
Taurine†	15.0	
Vitamin mix (dextrose)§	500	
Tricholine citrate†	370	
Cholesterol†	40	
Fat Sources	**n-3 Def**	**n-3 Adq**
	g/100 ml milk	
MCT oil¶	1.56	1.56
Coconut oil (hydrogenated)‖	3.24	3.24
18:1n-9 ethyl ester**	5.04	4.68
18:2n-6 ethyl ester**	2.16	2.16
18:3n-3 ethyl ester**	—	0.36

Component sources were as follows: * NZMP (North America) Inc, Santa Rosa, CA; † Sigma-Aldrich Corp. St. Louis, MO; ‡ Malinkrodt, Hazelwood, MO; § RX993666 Harlan, Madison, WI; ¶ Mead Johnson Nutritionals, Evansville, IN; ‖ Dyets, Bethlehem, PA; ** 99% grade, Nu-Chek Prep, Inc. Elysian, MN.

Artificial rat milk. The artificial milk formula was modified from the method of Kanno *et al.* (46). Table 2 shows the ingredients used as well as their commercial sources. Casein and whey protein were used as protein sources and lactose was used as the carbohydrate. The protein sources were carefully chosen so as not to introduce significant levels of n-3 fatty acids, as this is often the major source of introduction of these components into n-3 fatty acid–deficient diets. Casein contained 2.9% methionine and whey protein contained 2.5% methionine. For complete dissolution, all ingredients were mixed in the order described by Kanno *et al.* so as to help prevent precipitation. The first step was that acid casein was slowly dissolved in an alkali solution with NaOH and KOH under continuous stirring at 70°C. Three amino acids, serine, cystine, and tryptophan, were added to the alkali solution before the addition of casein as these protein sources contained lower levels of these amino acids relative to rat milk. In the second step, calcium and magnesium were added to the casein solution to obtain micelles of casein salts. A mixture of calcium and the calcium salt of glycerophosphoric acid ($GlyCaPO_4$), and Mg_2Cl_2-$6H_2O$ in an appropriate amount of water, which had been autoclaved and then homogenized with a hand homogenizer (PGC Scientifics, Gaithersburg, MD), was slowly added to the casein solution with continuous stirring. Thereafter, the calcium citrate solution was slowly added (after autoclaving and homogenization). A lactose solution sterilized in an autoclave was added to the casein solution. A mixture of Na_2HPO_4, KH_2PO_4, $FeSO_4$, $ZnSO_4$, $CuSO_4$, $MnSO_4$, NaF, and KI was added to the casein-micelle solution under continuous stirring. The casein salt solution was cooled to below 40°C and then whey protein in sterilized water was added to the solution with continuous stirring. A solution containing carnitine, picolinate, ethanolamine, taurine, tricholine citrate, the vitamin mixture, and 10N NaOH was then added. Finally, cholesterol and the fat sources were added to the above mixture under continuous stirring. The milk was then homogenized two times under high pressure (120 kg/cm^2), using a homogenizer with a two-stage valve (Model #HP50–250 FES International, Irwindale, CA) that had been cleaned by rinsing with 0.1 M NaOH and then neutralized with sterilized water. The homogenized milk was pasteurized twice at 63°C for 30 min, 6 h apart. The complete milk was poured into 500 mL sterilized bottles and stored at 4°C. The final pH of milk was 6.5. The milks contained 12 wt% lipids composed of saturated fat (medium chain triglycerides and hydrogenated coconut oil) and the ethyl ester form of purified unsaturated fatty acids (Nu-Chek Prep, Inc., Elysian, MN). The n-3 fatty acid deficient milk (n-3 Def) contained 16.3% LA and 0.02% LNA and the n-3 fatty acid adequate (n-3 Adq) milk contained 16.6% LA and 3.1% LNA (Table 3). The addition of the LNA to the n-3 Adq diet was substituted for an equal quantity of ethyl oleate; this introduced only a 7% difference in oleate content between the two diets. Saturated, monounsaturated, and LA were balanced with only the level of 18:3n-3 as a dietary variable.

Pelleted diets. The three pelleted diets used were based on the AIN-93 (47) formulation with several modifications to obtain the extremely low n-3 fatty acid level required in this study (Table 1). The custom pelleted diets were obtained commercially and used a cold pelleting process to preserve unsatur-

Table 3. *Fatty acid compositions of artificial rat milk*

Fatty acids*	Dietary group	
	n-3 Def	n-3 Adq
	g/100g	
Σ Saturated	33.8	32.6
Σ Monounsaturated	48.4	47.6
18:2n-6	16.3	16.6
18:3n-3	0.02	3.1
n-6/n-3	815	5.5

* Only trace quantities of long the chain polyunsaturated fatty acids, 20:4n-6, 20:5n-3, 22:5n-3 and 22:6n-3 were detected, i.e. less than 0.01%. Other minor peaks were not included.

ated fats (Dyets, Bethlehem, PA). The diets were designed to contain a fatty acid profile very similar to that in the artificial rat milk (or dam's milk) for each group. The dependent variable in the two artificially reared groups was the substitution of α-linolenate ethyl ester for a portion of the oleate ethyl ester in the n-3 Adq diet. The maternal diet contained a fatty acyl composition similar to that of the n-3 Adq diet but was made up with a mixture of safflower and flax oils (rather than purified ethyl esters) due to the greater quantity of diet to be consumed. All three diets contained 10 wt% as fat and had a similar content of LA. The rats were maintained on the appropriate pelleted diet after weaning until they were killed.

Motor activity test. The motor activity of the animals was recorded using a video image analyzer (Videomax V, Columbus Instruments, Columbus, OH). Each rat was individually placed at 0930–1300 h into a cage (25 cm × 45 cm × 20 cm) and the moving time and the moving distance were measured for 30 min.

Elevated plus-maze. To order to measure anxiety-related behavior, the elevated plus-maze was used (48,49). The plus maze was elevated 70 cm above the floor and consisted of two opposite open-arms (45 cm × 15 cm) and two opposite enclosed-arms. The path of each animal was observed for 5 min after a resting period of 2 min in the central square of the maze. These measurements were repeated for 2 d. The number of entries to the open arms and the visiting time of the open arm were recorded.

Morris water maze test. To evaluate performance in a spatial task, the Morris water maze test was performed. This method has been previously described in detail by Moriguchi *et al.* (16,22). Briefly, the swimming area was arbitrarily divided into four quadrant (regions A–D), and two starting points were arranged at the corners of a quadrant rim. After swimming training, the rats were submitted to a visible trial where a black (visible) escape platform was placed in quadrant region A. If a rat failed to find the platform within 90 s, it was gently placed on the platform for 30 s. On the second day, a hidden platform was used in place of the black platform and each rat received two trials per day after being randomly placed at the two different starting points. The maximal trial length was 90 s, with a maximal intertrial interval of 9 min. The escape latency was then defined as the sum of the two trials on a given day. The escape latency, swimming time, swimming speed, the duration of the floating state (resting time), and swimming path were automatically digitized and recorded by computer. Sessions were repeated for four consecutive days for a total of eight trials. On the day following the last session, the platform was removed and the rat was allowed to search for the platform for 90 s (probe trial). The number of crossings of the position where the platform had been placed (quadrant region A) and the number of crossings in the corresponding imaginary positions in the other quadrant regions (regions B–D) were recorded.

Lipid composition. After the behavioral experiments at 15 wk of age, the rats were killed by decapitation. Tissue samples including brain and retina were removed and stored at −80°C. The total lipid extracts of tissues were prepared according to the method of Folch *et al.* (50). The lipid extracts were transmethylated with 14% BF_3-methanol at 100°C for 60 min by a modification of the method of Morrison and Smith (51) with hexane addition (52). The total lipids in artificial milk and pelleted diets were extracted by the method of Lepage and Roy (53). Fatty acid methyl esters were then analyzed by gas chromatography as previously described (52). The fatty acid methyl esters were identified by comparison to the retention times of a standard mixture (Nu-Check Prep 462). The concentrations of individual and total fatty acids were obtained using an internal standard (22:3n-3 as methyl ester, 432 μg/brain and 64.8 μg/retina).

Statistical analysis. All data were expressed as mean ± SEM and significance was determined using one-way ANOVA using Statistica (StatSoft, Tulsa, OK). Behavioral experiments were analyzed using Duncan's multiple range test when the initial repeated-measures ANOVA analyses resulted in significant differences. Body weight and fatty acid compositional differences were analyzed using Tukey's honest significant difference test.

RESULTS

Body weight. The artificially reared pups had a significantly lower body weight than that of dam reared pups from day 2 to 14, but there were no significant differences between n-3 fatty acid deficient and n-3 fatty acid adequate milk groups [$F(2,31) = 58.594$, $p < 0.001$; n-3 Def, $p < 0.001$; n-3 Adq, $p < 0.001$ *versus* dam reared; n-3 Def, $p = 0.124$ *versus* n-3 Adq (Fig. 2)]. After 15 d of age, the artificially reared pups gained weight rapidly so as to catch up to the dam-reared pups and there were no significant differences between the three dietary groups by 24 d of age. At 8 wk old, at the beginning of the behavioral testing, the artificially reared rats showed significantly larger mean body weight than the dam reared group [$F(2,31) = 5.035$, $p < 0.05$; n-3 Def, $p < 0.05$; n-3 Adq, $p < 0.05$ *versus* dam reared; n-3 Def, $p = 0.992$ *versus* n-3 Adq]. The mean body weights were 319 ± 9 g for the n-3 Def milk group, 318 ± 8 g for the n-3 Adq milk group, and 286 ± 11 g for the dams' milk group. The only difference observed between dam- and artificially reared pups was the greater body weight from d 2 of age to d 14 of the dam-reared pups. There were no other differences apparent between dam- and artificially reared pups.

Brain and retinal fatty acid composition. The artificial rearing method was useful in obtaining low levels of brain and retina DHA in rats fed on the n-3 Def milk. The pelleted and artificial rat milk diets had very low n-3 fatty acids in the n-3 Def case with n-6/n-3 ratios of 297 (Table 1) and 815 (Table 3), respectively. The very high ratio was achieved in the latter case due to the use of purified fatty acid ethyl esters for the supply of the unsaturated fatty acids. This treatment was successful in inducing a loss in brain DHA. At 15 wk of age, rats in the n-3 Def group exhibited a 58% and 60% decrease in brain DHA compared with the n-3 Adq and dams' milk groups, respectively [$F(2,13) = 286.000$, $p < 0.001$], which was

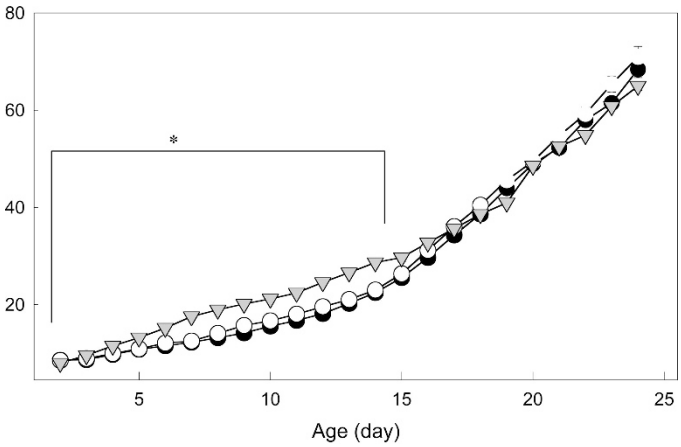

Figure 2. Body weights over the first 24 d of artificial rearing in comparison to dam-reared rat pups. The body weight is presented as the mean ± SEM, with an $n = 11$ or 12. There were no significant differences between n-3 fatty acid–deficient (black circle) and n-3 fatty acid–adequate milk (white circle) groups. $F(2,31) = 58.594$, $p < 0.001$; n-3 Def, $p < 0.001$; n-3 Adq, $p < 0.001$ *vs* dam reared (inverted triangle); n-3 Def, $p = 0.124$ *vs* n-3 Adq. *indicates $p < 0.001$ for Dam's group vs. the artifically reared groups fron d2–d4.

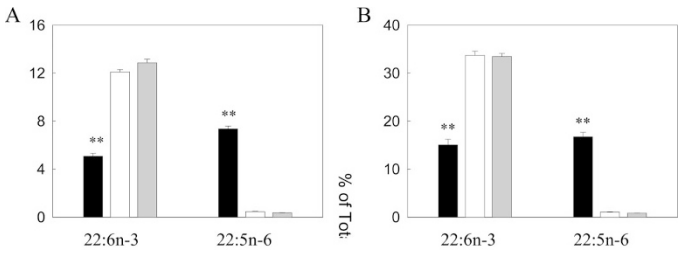

Figure 3. Effect of artificial rearing on an n-3 fatty acid–deficient or –adequate diet on (*A*) brain and (*B*) retina fatty acid composition ($n = 13$) at 15 wk of age. The differences among the three groups in brain and retina fatty acid composition are significantly different in a one-way ANOVA [$F(2,13) = 286.000$, $p < 0.001$; n-3 Adq, $p < 0.001$; dam's, $p < 0.001$ *vs* n-3 Def in brain DHA; $F(2,13) = 966.536$, $p < 0.001$; n-3 Adq, $p < 0.001$; dam's, $p < 0.001$ *vs* n-3 Def in brain DPAn-6; $F(2,13) = 134.012$, $p < 0.001$; n-3 Adq, $p < 0.001$; dam's, $p < 0.001$ *vs* n-3 Def in retina DHA; $F(2,13) = 321.350$, $p < 0.001$; n-3 Adq, $p < 0.001$; dam's, $p < 0.001$ *vs* n-3 Def in retina DPAn-6]. There was no significant difference between the n-3 Adq and dam's milk groups. ** Indicates $p < 0.001$ for the n-3 Def vs. the n-3 Adq and Dam's groups. ■ n−3 Def Milk; □ n−3 Adq Milk; ▨ Dam's Milk.

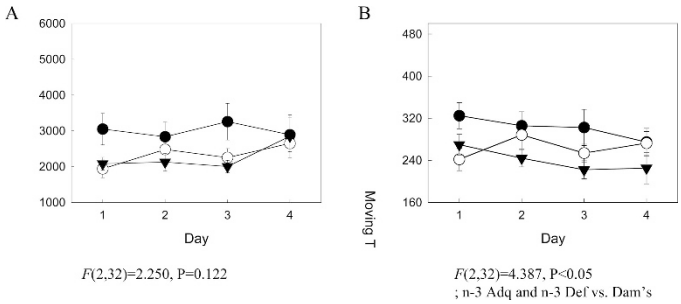

Figure 4. Effect of n-3 fatty acid deficiency on (*A*) moving distance and (*B*) moving time in the motor activity test. The moving distance and moving time is presented as the mean ± SEM, with $n = 11$ or 12. The differences among the three groups in the moving time are significantly different in a repeated measures, one-way ANOVA [$F(2,32) = 4.387$, $p < 0.05$; n-3 Def, $p < 0.01$ *vs* dam's]. —●— n−3 Def Milk; —○—- n−3 Adq Milk; —▼— Dam's Milk.

largely compensated for by a marked increase in brain DPAn-6 [$F(2,13) = 966.536$, $p < 0.001$ (Fig. 3)]. In addition, retinal DHA was decreased by 55% in the n-3–deficient group compared with both the n-3–adequate and dam-reared groups [$F(2,13) = 134.012$, $p < 0.001$]. The decrease was again largely replaced by an increased percentage of DPAn-6 [$F(2,13) = 321.350$, $p < 0.001$].

Behavioral tests. In the motor activity test, there were no statistically significant differences in moving distance [$F(2,32) = 2.250$, $p = 0.122$] among the three groups (Fig. 4). However, the n-3 Def group showed a significantly greater moving time than the dam-reared group [$F(2,32) = 4.387$, $p < 0.05$; n-3 Def, $p < 0.01$ *versus* dam reared; Adq, $p = 0.191$ *versus* dam reared].

In the elevated plus-maze test, the three groups showed significant differences between d 1 and d 2 in the visiting time in the open arm [$F(1,28) = 36.877$, $p < 0.001$] and number of visits to the open arm [$F(1,28) = 13.930$, $p < 0.001$ (Fig. 5)]. However, there was no significant difference among the three groups in the visiting time to the open arm [$F(2,28) = 1.885$, $p = 0.171$] and the number of visits to the open arm [$F(2,28) = 1.439$, $p = 0.254$ (Fig. 5)].

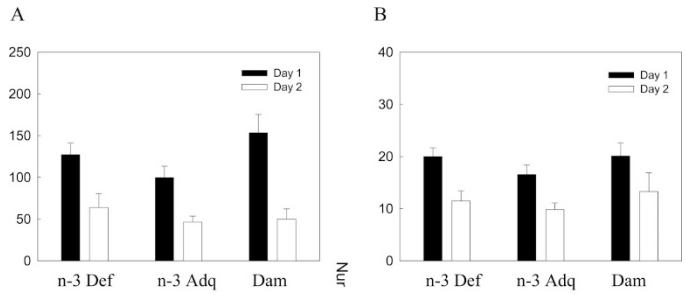

Figure 5. Effect of n-3 fatty acid deficiency on (*A*) visiting time and (*B*) number of visits in the open arm in the elevated plus maze. The visiting time and number of visits in the open arm is presented as the mean ± SEM, with $n = 10$ or 11. The difference between days is significantly different in a repeated measures, one-way ANOVA [$F(1,28) = 36.877$, $p < 0.001$ for visiting time; $F(1,28) = 13.930$, $p < 0.001$ for number of visits].

In the Morris water maze test, the escape latency was not different among the dietary groups in the visible trial [$F(2,31) = 0.080$, $p = 0.923$ (Table 4)]. In the visible trial, 3 rats out of 11 in the n-3 Def group reached the platform, whereas 5 of 12 in the n-3 Adq group and 5 out of 11 in the dam-reared group reached the platform. In the learning trials, the escape latency of the n-3 Adq and dams' milk groups gradually decreased over the testing period (Fig. 6). However, the n-3 Def rats showed a significantly longer escape latency than in either the n-3 Adq or the dams' milk group [$F(2,31) = 4.546$, $p < 0.05$; n-3 Adq, $p < 0.05$; dam reared, $p < 0.05$ *versus* n-3 Def]. There were no significant differences between the n-3 Adq and dams' milk groups.

The escape latency values were subdivided into swimming and resting time. The resting times in all groups were similar and there were no significant differences [$F(2,31) = 1.184$, $p = 0.320$] between the three groups (Fig. 7). Where the escape latency was increased in the n-3 Def group, the swimming time was also significantly increased [$F(2,31) = 4.360$, $p < 0.05$; n-3 Adq, $p < 0.05$; dam reared, $p < 0.05$ *versus* n -3 Def (Fig. 7)] but again there was no difference between the n-3 Adq and dams' milk groups. During the learning trials, increases in swimming distance were associated with increased escape latency, and this difference was significant for the n-3 Def group in comparison to the other two dietary groups [$F(2,31) = 6.205$, $p < 0.01$; n-3 Adq, $p < 0.05$; dam reared, $p < 0.01$ *versus* n-3 Def (Fig. 8)]. During the learning trials, the artificially reared rats displayed a significantly higher swimming speed compared with the dam-reared groups [$F(2,31) = 4.927$, $p < 0.05$; n-3 Def, $p < 0.01$; n-3 Adq, $p < 0.05$ *versus* dam reared (Fig. 8)].

In the memory retention trial, the number of crossings of the platform position (region A) was significantly greater than those of other regions for the n-3 Adq and dams' milk groups [$F(3, 44) = 6.021$, $p < 0.005$ for n-3 Adq milk; $F(3, 40) = 9.390$, $p < 0.001$ for dams' milk (Fig. 9)]. However, the n-3 Def milk group swam randomly without preference for the previous platform location [$F(3, 40) = 1.594$, $p = 0.206$].

DISCUSSION

This study demonstrates that pups born to dams fed a relatively high LNA intake, an "n-3 adequate diet," during

Table 4. *Effect of n-3 fatty acid deficiency on the visible trial in the Morris water maze test*

Group	No. of rats	Escape latency (sec)	No. of successful rats
n-3 Def	11	74.2 ± 8.8	3
n-3 Adq	12	70.0 ± 7.1	5
Dam's	11	73.5 ± 8.0	5

gestation can be made quite deficient in brain and retinal DHA by feeding a diet very low in n-3 fatty acids from the first days of life through adulthood. A first-generation model is presented that has more relevance to human nutrition than does the two-generational model that is most commonly used in this field. When the brain growth spurt (54) occurs in the absence of dietary n-3 fatty acids, the lack of DHA supply results in a marked loss of brain DHA. Recent studies have shown that most of the brain DHA is supplied by preformed DHA when available in the diet (55). In this study, biosynthesis from LNA is an insignificant source of brain DHA because it has been largely eliminated from the diet (Tables 1 and 3) and circulation (56). At adulthood (3 mo of age), the DHA is reciprocally replaced with the 22-carbon n-6 fatty acid, DPAn-6. However, it is now known that there is a lag in the replacement of the DHA by DPAn-6 in the first several weeks of life in the rat (57), apparently due to the limited rate of synthesis and transport of the DPAn-6 to neural membrane during very active neurogenesis.

The hand-feeding method used here employing the nursing bottles newly developed by Hoshiba (45) improves upon his previous systems (43,44). In our hands, the mortality of pups has fallen dramatically with this new method as only 1 pup died out of 24 artificially reared in the present experiment. This pup died after aspirating milk into the lungs. There is also an

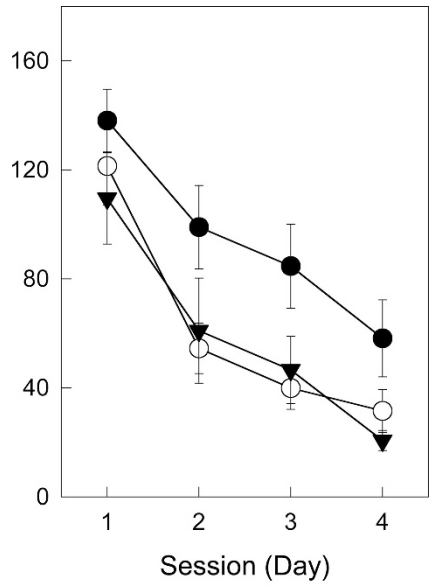

Figure 6. Effect of n-3 fatty acid deficiency on escape latency in the Morris water maze. The escape latency is presented as the mean ± SEM, with $n = 11$ or 12. The differences among three groups ("black circle symbol" n-3 Def Milk; "white circle" n-3 Adq Milk; "black inverted triangle" Dam's Milk) are significantly different in a repeated measures, one-way ANOVA [$F(2,31) = 4.546$, $p < 0.05$; n-3 Adq, $p < 0.05$; dam's, $p < 0.05$ *vs* n-3 Def]. —●—n−3 Def Milk; - —○—- n−3 Adq Milk; —▼— Dam's Milk.

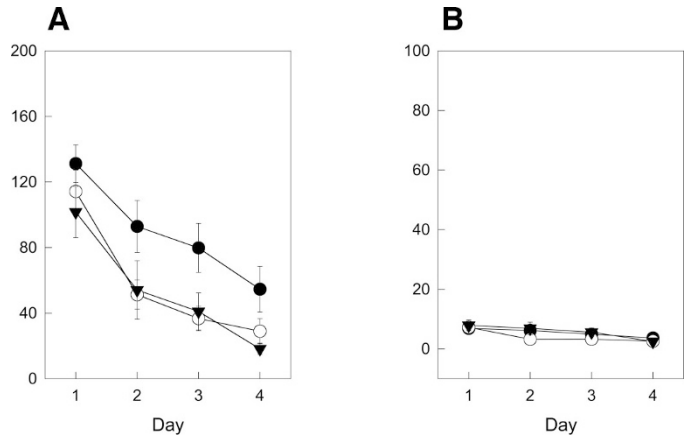

Figure 7. Fractional analysis of the escape latency in the Morris water maze. The swimming (A) and resting (B) times are presented as the mean ± SEM, with $n = 11$ or 12. The differences in swimming time among the three groups ("black circle symbol" n-3 Def Milk; "white circle" n-3 Adq Milk; "black inverted triangle" Dam's Milk) are significantly different in a repeated measures, one-way ANOVA [$F(2,31) = 4.360$, $p < 0.05$; n-3 Adq, $p < 0.05$; dam's, $p < 0.05$ *vs* n-3 Def]. The resting time was not significantly different among the three different groups [$F(2,31) = 1.184$, $p = 0.320$]. —●—n−3 Def Milk; - —○—- n−3 Adq Milk; —▼— Dam's Milk.

improvement in body weight growth with the AR pups catching up to their dam-reared siblings by about d 15 and in adulthood exceeding the dam-reared body weight. From the latter observation, it appears that the AR procedure may have induced some difference in *ad libitum* feeding behavior. A decrease in stomach bloating was also noted with the present method, perhaps due to a decrease in the sucking in of air as the present system uses only the sucking power of the pup to regulate milk flow. Larger nipples are used that better fit the mouths of the pups. It should also be noted that this feeding system can be used to manipulate the intake of any dietary nutrient whose level can be controlled in the artificial rat milk.

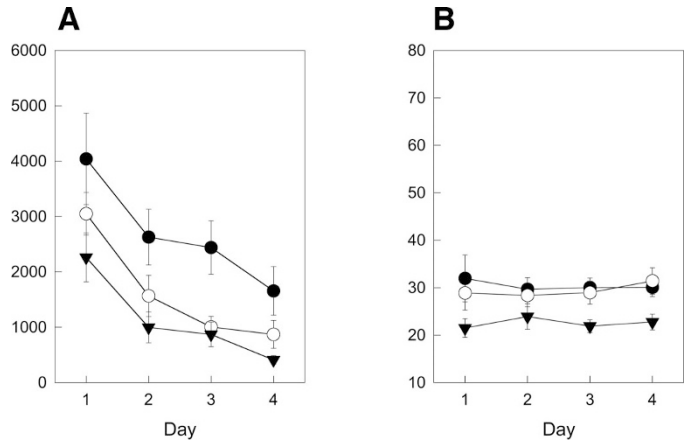

Figure 8. Effect of n-3 fatty acid deficiency on swimming distance and speed. The swimming distance (A) and speed (B) are presented as the mean ± SEM, with $n = 11$ or 12. The differences in swimming distance among the three groups ("black circle symbol" n-3 Def Milk; "white circle" n-3 Adq Milk; "black inverted triangle" Dam's Milk) are significantly different in a repeated measures, one-way ANOVA [$F(2,31) = 6.205$, $p < 0.01$; n-3 Adq, $p < 0.05$; dam's, $p < 0.01$ *vs* n-3 Def]. The swimming speed was significantly different among the three different groups [$F(2,31) = 4.927$, $p < 0.05$; n-3 Def, $p < 0.01$; n-3 Adq, $p < 0.05$ *vs* dam's]. —●—n−3 Def Milk; - —○—- n−3 Adq Milk; —▼— Dam's Milk.

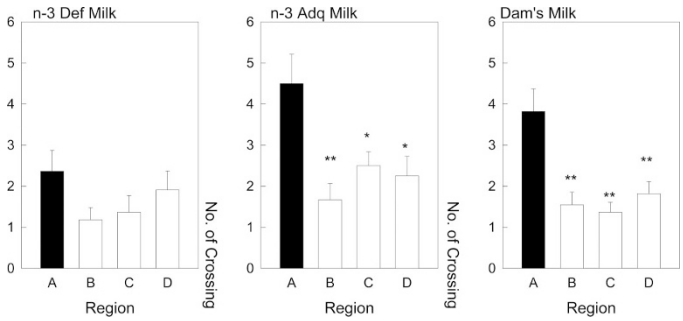

Figure 9. Effect of n-3 fatty acid deficiency on the probe trial. The number of crossings of the platform position (region A; *closed column*) and the corresponding imaginary positions (region B–D; *open column*) are presented as the mean ± SEM, with $n = 11$ or 12. One-way ANOVA [$F(3,44) = 6.021$, $p < 0.005$ for n-3 Adq milk; $F(3,40) = 9.390$, $p < 0.001$ for dam's milk]. *$p < 0.01$, **$p < 0.001$ compared with region A (Duncan's multiple range test).

This study clearly demonstrates that the replacement of DHA with DPAn-6 leads to a loss in behavioral performance on spatial tasks. The escape latency and the spatial retention as assessed by the probe trial were impaired in the n-3 fatty acid–deficient rats whereas the rats fed an artificial rat milk containing LNA performed just as well as dam-reared rats. The behavioral performance correlated well with the DHA level in the brain as both the n-3 Adq and dam-reared groups had similar levels of brain DHA (12–13% range) and low levels of DPAn-6 (about 0.4%) whereas the n-3 Def group had only about 5% brain DHA and about 7% DPAn-6. In addition to the loss of DHA, the lag in replacement of DHA by DPAn-6 in early development (5–20 d postnatal) may also be a factor leading to losses in behavioral performance (57).

The mechanisms underlying loss of function in the n-3 fatty acid–deficient groups are not fully understood, but several lines of evidence now exist that can help to explain the critical role of DHA in the nervous system (1). It has been established that DHA positively modulates the biosynthesis and accumulation of phosphatidylserine in neuronal membranes (58,59) and that this effect of DHA is closely related to signaling events supporting cell functions such as Raf-1 kinase translocation (60,61). Kim *et al.* (60) also found that DHA enrichment prevents apoptotic cell death induced by serum starvation when an appropriate antioxidant is present. Apoptotic cell death plays an important role in neural development and its alteration as a result of DHA deficiency may have adverse consequences. Mitchell *et al.* (62) found that n-3 fatty acid deficiency led to desensitization in visual signaling as evaluated by rhodopsin activation, rhodopsin-G protein coupling, and integrated phosphodiesterase activity. Niu *et al.* (63) extended these findings to the *in vivo* situation with a demonstration of a loss in rhodopsin signaling in rod outer segment membranes prepared from n-3 Def or n-3 Adq animals. This effect of DHA on G-protein signaling system both in the retina and the brain could provide a general underlying mechanism for DHA function in the nervous system. Also, Katajka *et al.* (64) recently found that provision of n-3 polyunsaturated fatty acids altered brain gene expression including those involved in synaptic plasticity and learning, suggesting an independent mechanism for the beneficial effects of n-3 fatty acids on the nervous system.

In summary, this study suggested that feeding an n-3 fatty acid–deficient diet from postnatal d 2 through 15 wk of age induced a 60% reduction in brain DHA and produced a defect in spatial learning-related brain function. Compared with Asian countries, Western women's breast milk contains low levels of n-3 polyunsaturated fatty acids (65) and, until recently, Western milk formulas contained no long-chain (C20 or C22) polyunsaturates but essential fatty acids were supplied predominantly by vegetable oils rich in linoleic acid (66,67). It is clear that the fatty acid composition of infant brain and retina is influenced by the mother's dietary habits and that infants who receive vegetable oil–based formulas have a decrease in brain DHA (68,69). Whether the more moderate n-3 fatty acid deficiencies in the adult human population would lead to a loss in brain DHA is as yet unclear.

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