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Recovery of brain docosahexaenoate leads to recovery of spatial task performance

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Abstract

Infants fed vegetable oil-based formulas may have poorer visual function, lower cognitive scores and acquire learning tasks more slowly in comparison with those breast fed or those fed formulas supplemented with docosahexaenoate. The aim of the present study was to determine the reversibility of losses in brain function associated with the loss of brain DHA. Rats were fed very low or adequate levels of n-3 fatty acids through three generations. The n-3 fatty acid deficient animals of the F3 generation were then given an n-3 adequate diet containing alpha-linolenic and docosahexaenoic acids (DHA) at birth, weaning (3 weeks) or young adulthood (7 weeks). The spatial task performance of these animals returned to the n-3 adequate diet was then compared using the Morris water at two different ages, at 9 or 13 weeks. Our results indicate that animals repleted since birth or at weaning were able to achieve nearly the same level of brain DHA and spatial task performance as animals maintained for three generations on an n-3 adequate diet. In the case of young adult animals, the degree of DHA and behavioral performance recovery depended upon the duration of dietary repletion with substantial recovery in animals after 6 weeks but little recovery of function after two weeks. The significance of these findings is that they indicate that at least some of the adverse effects of DHA deficiency during neurodevelopment may be reversible with an n-3 fatty acid supplemented diet.

Keywords: docosahexaenoic acid, functional recovery, infant nutrition, learning ability, Morris water maze, n-3 polyunsaturated fatty acid deficiency.

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Three autopsy studies of infants who died from sudden infant death syndrome have now demonstrated that infants fed vegetable oil-based formulas have lower levels of brain DHA than breast-fed infants (Farquharson *et al*. 1992; Makrides *et al*. 1994; Jamieson *et al*. 1999). Vegetable oil-based formula feeding in early development is associated with poorer cognitive development (Carlson *et al*. 1994; Agostoni *et al*. 1995; Birch *et al*. 2000), learning (Willatts *et al*. 1998), visual acuity (Uauy *et al*. 1990; Birch *et al*. 1992; Carlson *et al*. 1993) and visual recognition memory (Carlson and Werkman 1996). Animal studies support this view as abnormal electroretinograms (Wheeler *et al*. 1975; Neuringer *et al*. 1984, 1986; Pawlosky *et al*. 1997; Weisinger *et al*. 1999), losses in visual acuity (Connor and Neuringer 1988), spatial task performance (Nakashima *et al*. 1993; Frances *et al*. 1996; Wainwright *et al*. 1998; Moriguchi *et al*. 2000), neuromotor performance (Champoux *et al*. 2002), olfactory-based learning (Greiner *et al*. 1999, 2001; Catalan *et al*. 2002) as well as deficits in other associative learning tasks (for reviews, see Okuyama *et al*. 1997; Hamosh and Salem 1998) have been observed when the dietary intake of n-3 fatty acids is limited.

At present, in North America alone, it is estimated that 2–3 million babies per year are fed infant formulas devoid of long-chain polyunsaturates such as DHA. Moreover, the world now contains two generations of adults or juveniles that have been given formulas with low levels of n-3 fatty acids since formula feeding became popular in the 1950s. Thus, it is important to understand whether the effects of suboptimal development due to limitation of n-3 fatty acid sources persist and are reversible once a diet containing adequate levels of n-3 fatty acids are consumed.

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Abbreviations DHA, docosahexaenoic acid, 22:6n3; DPAn-6, docosapentaenoic acid, 22:5n6; n-3 Adq, n-3 adequate; n-3 Def, n-3 deficient.

Several animal studies have indicated that nervous system DHA can be restored, albeit slowly, after severe losses are incurred due to deprivation of dietary n-3 fatty acids during development. Early rat studies indicated that the brain (Bourre *et al*. 1984; Youyou *et al*. 1986) DHA level was restored when alpha-linolenate (LNA) was added to the diet. However, indications are that functional effects of the DHA decline may not be reversible. For example, rhesus monkeys at a juvenile stage could be repleted in brain DHA (Connor *et al*. 1990), however, electroretinographic changes observed during n-3 deficiency remained (Connor and Neuringer 1988). Also, an increased mean arterial blood pressure associated with an n-3 fatty acid deficiency during development did not normalize in adulthood when an n-3 adequate diet was restored (Weisinger *et al*. 2001).

In this study, the issue of the reversibility of losses in spatial task performance associated with n-3 deficiency was explored in relation to the stage of development. Animals were made deficient in brain DHA by severely limiting n-3 dietary fats through three generations. Separate groups were then switched to an n-3 containing diet with both LNA and DHA either at birth, at weaning or in early adulthood (Fig. 1). Animals were allowed to recover for two different periods of time as they were tested at 9 or 13 weeks of age. Two reference groups were also maintained on n-3 adequate and n-3 deficient diets and tested at each time point. A cued version of the water maze was given initially (visible trial) to control for sensory and motor function. The place version of the Morris water maze was then used to assess spatial learning ability. The decrease in escape latency in successive trials over 4 days was used as an index of spatial learning. Subsequently, the platform was removed and a probe trial was performed as an additional measure of spatial learning. Motor activity measures were acquired as a further control related to general level of arousal/motivation. Performance on the Morris water maze task was correlated with the brain level of DHA.

Materials and methods

Study design

The protocol and all animal procedures used in this experiment was approved by the Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism. Female, Long-Evans rats were obtained from Charles River (Portage, MI, USA) at weaning (3 weeks of age). Weanling females were divided into two dietary groups on a pseudorandom basis with the constraint that the two groups had the same mean body weight. One group of 15 females was fed the n-3 adequate (n-3 Adq) diet and the second group of 30 females was fed an n-3 deficient (n-3 Def) diet (diet composition below). The females in both dietary groups were mated with 9-week-old males when they were 8 weeks old. Their litters (F2 generation) were culled to 10 pups/litter and weaned to the same diet as their mothers. The F2 generation females ($n = 100$ for the n-3 Def, $n = 50$ for n-3 Adq, for a total of 150 dams) were then mated and their pups (F3 generation) were randomized into one of 10 different groups. The individual animals within each of these groups were not littermates. Thus, one male individual from each of 12 separate litters was assigned to each experimental group to achieve an $n = 12$/group for the 10 groups employed. Only males were used in these experiments in order to minimize variability that may have been introduced by hormonal factors or estrous cycle. In the group of animals cross-fostered at birth, a total of four animals were lost due to inadequate care by the new dam.

The overall scheme was to switch n-3 Def pups to the n-3 Adq diet at birth, weaning or at young adulthood (7 weeks) in order to determine the reversibility of n-3 deficiency at various stages of neurodevelopment (study design schematically presented in Fig. 1). Two sets of animals were produced for each condition as behavioral testing was planned at two ages, 9 and 13 weeks. Two groups of n-3 Adq pups were maintained to serve as reference points at the two time points for behavioral testing. The n-3 Def pups were divided into eight separate groups. Two of the groups were cross-fostered to F2 generation dams that had been maintained on an n-3 Adq diet. The six additional groups of n-3 Def rats were allowed to feed from their n-3 Def mothers. At weaning (21 days), two of the groups were switched to an n-3 Adq diet. Two of the n-3 Def groups were switched to the n-3 Adq diet when they were 7 weeks of age. The remaining two groups were maintained on the n-3 Def diet to serve as deficient reference points at the two time points of behavioral

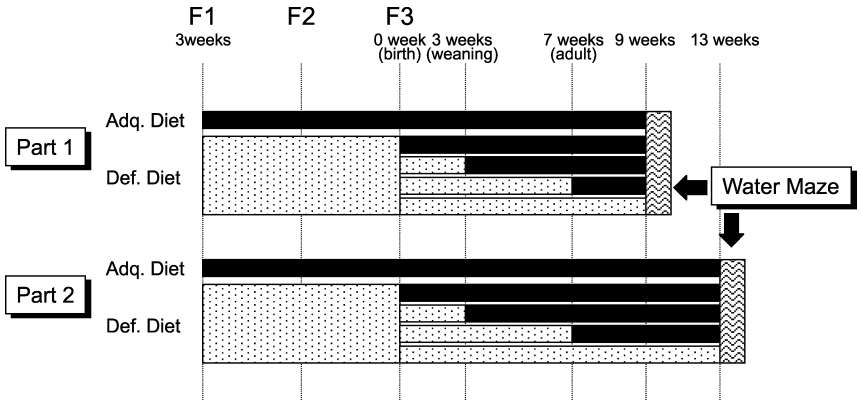

Fig. 1 A flow diagram illustrating study design. The n-3 Def diet was switched to the n-3 Adq diet at birth (0 week), weaning (3 weeks) or at young adulthood (7 weeks) in order to determine the reversibility of n-3 deficiency at various stages of neurodevelopment.

testing. All rats were maintained under conventional conditions and at 23 ± 1°C and with a 12-h light-dark cycle (06.00–18.00 h).

Diet composition

The diets used were patterned after those of the American Institute of Nutrition (AIN-93 Reeves *et al.* 1993) with fat source modifications in order to provide for low or adequate levels of n-3 fatty acids. Both diets (Table 1) had the same basal macronutrients, vitamins, minerals and basal fats (hydrogenated coconut and safflower oils). However, the n-3 adequate diet also contained flaxseed oil and DHASCO (Martek Biosciences, Columbia, MD, USA), fats that supply LNA and DHA, respectively, as their principal component. The fatty acid composition of the two diets was balanced for saturates, monounsaturates and linoleic acid (LA) with the small quantity of n-3 fatty acids substituting for hydrogenated coconut oil in the n-3 adequate diet (Table 1).

Table 1 Composition of experimental diets*

	Amount (g/100 g diet)	
Ingredient	n-3 Def.	n-3 Adq.
Casein, vitamin free	20	20
Carbohydrate:	60	60
Cornstarch	15	15
Sucrose	10	10
Dextrose	19.9	19.9
Maltose-dextrin	15	15
Cellulose	5	5
Mineral & salt mix	3.5	3.5
Vitamin mix	1	1
L-cystine	0.3	0.3
Choline bitartrate	0.25	0.25
TBHQ	0.002	0.002
Fat:	10	10
Hydrogenated coconut oil	8.1	7.45
Safflower oil	1.9	1.77
Flaxseed oil	none	0.48
DHASCO	none	0.3
Fatty acid composition	(Percentage)	
Saturates	80.9	75.6
Mono-unsaturates	4.0	4.8
18:2n-6	15.1	15.7
18:3n-3	0.04	2.6
20:4n-6	nd	0.02
22:6n-3	nd	1.3
n-6/n-3	378	4.1
18:2n-6/18:3n-3	377	6.2

*The two experimental diets, an n-3 fatty acid adequate diet (n-3 Adq) and an n-3 fatty acid deficient diet (n-3 Def), were based on the AIN-93 (Reeves *et al.* 1993) formulation with several modifications to obtain the extremely low basal level of n-3 fatty acid required in this study. TBHQ, *t*-butylhydroquinone. The 20:5n-3 and 22:5n-3 fatty acids were less than 0.01%. nd, not detected.

Behavioral methods

Motor activity test

Each rat was individually placed into a cage (25 cm × 45 cm × 20 cm) and the ambulatory time and the moving distance were measured for 30 min using a video image analyzer (Videomax V, Columbus Instruments, Columbus, OH, USA). Only one motor activity session was used in this experiment.

Morris water maze

The methods used for the Morris water maze task were taken from Moriguchi *et al*. (2000). A circular pool (four feet in diameter and two feet deep) containing tap water, filled to within 10 cm from the top, is placed in a setting where various prominent cues (e.g. a metal board on the wall, a prominent door) were arranged. Water temperature was maintained at 20 ± 1°C. The swimming area was arbitrarily divided into four quadrant regions (regions A–D), and two starting points were located at the two boundaries of the quadrant located diagonally across from the platform position. In order to acclimate the rats to the swimming task, they were allowed to swim in the water for 1 min on the day prior to starting the water maze test. The next day, the rats were submitted to a visible trial to test eyesight and swimming ability. A black (visible) escape platform (circular, 10 cm diameter) was placed in quadrant region A in the circular pool. Its top surface was higher than the water level by 1.0 cm. Each rat was allowed to stay on the platform for 30 s after reaching it (successful rat). If the rat failed to find the platform within 90 s, it was gently placed on the platform for 30 s. On the second day, a transparent platform was used in place of the black platform and the height was 1.0 cm below the surface of the water (learning trial). This hidden platform was round, 10 cm in diameter, and placed at a 15-cm distance from the outer wall. As a result of these two changes, the swimming rat could not see the platform. At the start of the testing period, the rat was placed into the pool from one of the two starting points used in the visible trial. Each rat received two trials per day (session) and was randomly placed at two different starting points in successive trials so as to insure that sensory cues available in the testing room were used to find the platform (rather than swimming at a particular angle from a set starting position). Only two trials per day were used rather than four, as in previous experiments it was observed that this task had an appropriate level of difficulty for the type of intervention described in this work (Moriguchi *et al*. 2000). An inter-trial interval of 5 min was used. The time required for a rat to reach the hidden platform (escape latency), swimming time, swimming speed, the duration of the immobilized state (resting time) and swimming path were automatically digitized and recorded by computer. Sessions were repeated for four consecutive days. 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 analyses

At the end of the behavioral analyses, the 10 and 14-week-old animals were killed by decapitation with a guillotine and the brains rapidly removed, weighed and stored in an ultracold freezer at −80°C. Brains were later thawed, cut along the midline and half of the brain used for

fatty acid analysis. The one-step lipid extraction and transmethylation procedure of Lepage and Roy was used (Lepage and Roy 1986). Analysis of the fatty acid methyl esters was performed by capillary GLC as previously described (Salem *et al*. 1996).

Statistical methods

All data were analyzed using the Statistica program (Statsoft, Tulsa, OK, USA). Analysis of the number of successful rats that reached the hidden platform in the visible trial was performed using the Fisher exact probability test. Other parameters in the behavioral experiments were analyzed by one-way or two-way ANOVA. Where ANOVA analyses found significant differences, Duncan's multiple range test was then performed. The Duncan test was considered an appropriate post hoc test for an $n = 9$–12 animals and with the level of variability inherent in behavioral measures so as not to miss biologically significant effects; however, it is noted that the Duncan test does not adjust for family wise errors. Lipid compositional differences were analyzed using the Tukey HSD test after one-way ANOVA. For the correlation of brain DHA with Morris water maze parameters, the use of the regression analysis routine in the Sigma Plot program (SPSS Science, Inc., Chicago, IL, USA) was used.

Results

Body weight and brain fatty acid analysis

Table 1 shows the ingredients used to construct a diet that is sufficient in all nutrients including n-6 fatty acids such as linoleate, but the experimental diet (n-3 Def) is nearly devoid of n-3 fatty acids (level of about 0.04% of total fatty acids). The n-3 Adq diet contains both short- (LNA) and long-chain (DHA) n-3 fatty acids; the n-3 fatty acid content is the independent variable in this dietary study. These diets produced no significant differences in body weights between the n-3 Adq and the n-3 Def groups ($F_{1,44} = 1.577$, $p = 0.216$) at either the 9- or 13-week time points. The mean body weights of the n-3 Adq and n-3 Def groups of the F3 generation agreed to within about 3%.

These diets led to a marked difference in brain DHA and docosapentaenoate n-6 (DPAn-6) composition, e.g. at the 10-week time point (Table 2). In the n-3 Adq group, the reference level of brain DHA was 12.6% of total fatty acids, whereas the n-3 Def group contained only 2.1% DHA. As expected, the DHA was largely replaced in the n-3 Def group brains with DPAn-6; its level rose from 0.2% in the adequate group to 9.9% in the deficient group. Clearly, these variables are inversely linked and are not independent. The adult group, switched to the n-3 Adq diet after 7 weeks and killed at 10 weeks, resembles the alterations observed in the n-3 Def group in many respects (Table 2), that is, little change in saturates, lower levels of monounsaturates such as 18:1n9, a significantly higher level of nearly every n-6 polyunsaturate, and a significantly lower level of DHA. The weaning group has some of these features, although to a generally lesser extent (Table 2). The group of animals cross-fostered to dams fed an n-3 Adq diet at birth exhibited virtually the same fatty acid profile as the n-3 Adq reference group.

Another interesting feature of this experiment is that groups can be compared with respect to their brain fatty acyl compositional profiles when the groups had the same length of n-3 Adq diet repletion but starting at different ages. For examples, the weaning group tested at 9 weeks (and killed after 10 weeks) had 7 weeks of n-3 dietary repletion, just as did the adult group that was tested after 13 weeks (and killed at 14 weeks). However, the brain DHA percentage in the weaning group was higher (11.68 ± 0.09) than that of the adult group (10.62 ± 0.15). This difference was highly significant [$t(22) = 5.89$, $p < 0.00001$, students *t*-test]. Conversely, the DPAn-6-value had risen from 0.98 ± 0.03 in the weaning group to 1.77 ± 0.04 in the adult group [$t(22) = 15.47$, $p < 0.00001$].

Motor activity

Prior to beginning the spatial task measurements, the 9- and 13-week-old rats were subjected to a single 30-min motor activity trial in which their moving time and distances were recorded with the aid of an image analyzer. There were no significant differences between any of the dietary groups at both the 9- and 13-week time points in motor activity using these measures ($F_{4,106} = 2.040$, $p = 0.094$; for moving time, Table 3; $F_{4,106} = 2.263$, $p = 0.067$ for moving distance, data not shown).

Spatial tasks

Visible trial

The n-3 Def group had a faster mean swimming speed than the n-3 Adq group, but this difference between groups was not statistically significant at either age (Table 4). The groups switched to the adequate diet at adulthood had mean swimming speeds that were close to those of the n-3 Def reference group. The number of rats that were successful in the water maze task where the platform was visible was not significantly different in all groups (Table 4). Subsequently, nearly all of the rats were successful in the first learning trial where the platform was placed in the same location but beneath the water surface, and there was no difference between groups in this measure.

Escape latency

The escape latencies of the reference groups as well as the initially deficient groups that had been switched to an n-3 adequate diet at various times in development are presented in Fig. 2 at two different measurement points (at 9 and 13 weeks). There were significant differences between the n-3 Adq and n-3 Def groups at both the 9- ($p < 0.001$) and

Table 2 Adult rat brain fatty acyl composition after three generations of an n-3 Adequate or n-3 deficient diet[a]

Fatty Acid	Adq ($n = 12$)	Birth ($n = 11$)	Weaning ($n = 12$)	Adult ($n = 12$)	Def ($n = 12$)
10:0	nd	nd	nd	nd	nd
12:0	0.02 ± 0.001	0.02 ± 0.002	0.02 ± 0.001	0.02 ± 0.001	0.02 ± 0.002
14:0	0.16 ± 0.003	0.16 ± 0.003	0.16 ± 0.002	0.16 ± 0.002	0.17 ± 0.003*
16:0 DMA	2.12 ± 0.02	2.17 ± 0.02	2.18 ± 0.02	2.21 ± 0.02*	2.14 ± 0.02
16:0	15.64 ± 0.10	15.57 ± 0.11	15.51 ± 0.08	15.85 ± 0.10	15.97 ± 0.06**
18:0 DMA	4.01 ± 0.02	4.12 ± 0.04*	4.05 ± 0.02	3.99 ± 0.02*	3.90 ± 0.03
18:0	19.16 ± 0.05	18.92 ± 0.06*	18.99 ± 0.05	18.95 ± 0.06	18.90 ± 0.05**
20:0	0.74 ± 0.02	0.74 ± 0.01	0.78 ± 0.01	0.76 ± 0.01	0.76 ± 0.01
22:0	0.71 ± 0.02	0.74 ± 0.01	0.77 ± 0.01*	0.75 ± 0.02	0.77 ± 0.01*
24:0	1.44 ± 0.03	1.50 ± 0.03	1.55 ± 0.03	1.57 ± 0.04	1.58 ± 0.04*
Total saturates	44.00 ± 0.09	43.94 ± 0.09	44.00 ± 0.06	44.26 ± 0.09	44.22 ± 0.08
16:1n-7	0.33 ± 0.005	0.35 ± 0.01	0.33 ± 0.005	0.33 ± 0.005	0.33 ± 0.01
18:1 DMA	1.55 ± 0.02	1.61 ± 0.02	1.60 ± 0.02	1.58 ± 0.02	1.55 ± 0.02
18:1n-9	15.70 ± 0.07	15.60 ± 0.09	15.35 ± 0.05*	14.37 ± 0.07***	14.11 ± 0.09***
18:1n-7	3.16 ± 0.01	3.22 ± 0.01	3.18 ± 0.02	3.30 ± 0.02***	3.38 ± 0.02***
20:1n-9	1.81 ± 0.04	1.81 ± 0.03	1.83 ± 0.02	1.68 ± 0.03*	1.67 ± 0.02*
22:1n-9	0.18 ± 0.01	0.19 ± 0.004	0.19 ± 0.003	0.19 ± 0.005	0.19 ± 0.004
24:1n-9	2.38 ± 0.04	2.46 ± 0.04	2.45 ± 0.04	2.43 ± 0.05	2.43 ± 0.03
Total monounsaturates	25.11 ± 0.16	25.23 ± 0.15	24.93 ± 0.11	22.29 ± 0.15***	23.66 ± 0.14***
18:2n-6	0.61 ± 0.02	0.64 ± 0.01	0.58 ± 0.02	0.49 ± 0.01***	0.42 ± 0.01***
18:3n-6	nd	nd	nd	nd	nd
20:2n-6	0.16 ± 0.004	0.16 ± 0.004	0.16 ± 0.004	0.15 ± 0.003	0.14 ± 0.003*
20:3n-6	0.50 ± 0.01	0.50 ± 0.01	0.43 ± 0.01**	0.35 ± 0.01***	0.30 ± 0.005***
20:4n-6	7.84 ± 0.06	7.80 ± 0.08	7.89 ± 0.04	8.59 ± 0.09***	9.16 ± 0.07***
22:4n-6	2.14 ± 0.02	2.14 ± 0.02	2.31 ± 0.02***	2.84 ± 0.03***	3.38 ± 0.04***
22:5n-6	0.17 ± 0.002	0.19 ± 0.01	0.98 ± 0.03***	5.19 ± 0.13***	9.90 ± 0.07***
Total n-6 polyunsaturates	11.43 ± 0.06	11.45 ± 0.09	12.35 ± 0.06***	17.61 ± 0.18***	23.31 ± 0.11***
18:3n-3	nd	nd	nd	nd	nd
22:5n-3	0.30 ± 0.004	0.31 ± 0.01	0.31 ± 0.01	0.23 ± 0.01***	0.13 ± 0.002***
22:6n-3	12.58 ± 0.13	12.44 ± 0.05	11.68 ± 0.09***	7.55 ± 0.11***	2.13 ± 0.03***
Total n-3 polyunsaturates	12.88 ± 0.13	12.75 ± 0.06	11.99 ± 0.09***	7.78 ± 0.11***	2.26 ± 0.03***
22:5n-6/22:6n-3	0.01 ± 0.0002	0.02 ± 0.001	0.08 ± 0.003	0.69 ± 0.03***	4.67 ± 0.08***
22:5n-6 + 22:6n-3	12.75 ± 0.13	12.64 ± 0.06	12.66 ± 0.10	12.74 ± 0.09	12.03 ± 0.08***
n-6 + n-3	24.31 ± 0.17	24.20 ± 0.14	24.34 ± 0.09	25.39 ± 0.16***	25.57 ± 0.12***
Total fatty acids(mg/g wet weight)	42.75 ± 0.40	42.65 ± 0.27	43.15 ± 0.15	42.78 ± 0.30	42.37 ± 0.22

[a]Rats were 10 weeks of age at the time of killing. Statistically significant changes are represented as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus n-3 Adq group (one-way ANOVA followed by Tukey HSD test). nd, not detected or at a level below 0.01% of total fatty acids. Data are presented as the mean ± SEM.

13- ($p < 0.005$) week measurement points, with the n-3 Def group taking considerably longer to reach the platform. The group (termed 'Birth') that were cross-fostered to n-3 Adq dams at birth and reared to the n-3 Adq diet were virtually identical to the n-3 Adq group and thus were significantly different (9 weeks, $p < 0.005$; 13 weeks, $p < 0.05$) than the n-3 Def group at both the 9- and 13-week time points. Both of the groups switched at the time of weaning (3 weeks of age) to the n-3 Adq diet were also significantly different from the n-3 Def group (both $p < 0.05$). However, it is noted that in the 9-week trial, where the brain DHA had only been allowed to recover for 2 weeks prior to the onset of behavioral testing (the adult group), that the escape latency was greater than the n-3 Adq group ($p < 0.05$). It was noteworthy that when the diet was switched at 7 weeks of age and the measurements begun after only 2 weeks of diet repletion (9 weeks), that the escape latency was then significantly different than the n-3 Adq ($p < 0.05$), though the mean values were lower than those of the n-3 Def group). However, when the 'Adult' group was allowed to recover for 6 weeks, there was no longer a significant difference between the performances of these rats versus the n-3 Adq (Fig. 2 and 13-week time point). Two-way ANOVA analysis of the effects of group–session interactions indicated no significant difference at the 9- ($F_{12,162} = 1.43$, $p = 0.16$) or 13-week ($F_{12,156} = 1.22$, $p = 0.27$) time points.

Table 3 Motor activity measured at two ages in n-3 adequate and deficient rats*

	Moving time (s/30 min)	
Groups	9 weeks	13 weeks
Adq.	317.9 ± 33.0 (12)	471.7 ± 59.8 (12)
Birth	324.7 ± 29.9 (11)	411.1 ± 30.5 (9)
Weaning	291.1 ± 29.9 (12)	278.0 ± 37.4 (12)
Adult	382.4 ± 57.8 (12)	315.2 ± 37.9 (12)
Def.	338.6 ± 41.4 (12)	375.7 ± 30.8 (12)

*Data are presented as the mean ± SEM. $F_{(4,106)} = 2.040$, $p = 0.094$.

More detailed analyses of the learning trials were made. The swimming time and swimming distance measurements showed exactly the same statistically significant findings at both measurement points, as did the escape latencies (Fig. 3). An exception was that there was one additional significant difference in the swimming distance when measured at the 9-week point; the swimming distances were greater in the 'Adult' versus 'Birth' group ($p < 0.05$). That is, the swimming times and distances were longer for the n-3 Def groups and this effect diminished with the same pattern as that noted above for the various groups upon diet repletion.

The ratio of inside versus outside swimming was determined from the swimming patterns during the first two sessions; outside swimming was defined as the amount of time spent within the outer 12-cm region of the pool. Again, the differences between the n-3 Adq and n-3 Def groups as well as those on repletion diets exhibited the same pattern of significant differences as that described above for escape latencies, with a greater percentage of inside swimming time for the n-3 Adq group (data not shown). The resting time for all groups was very similar.

Table 4 Visible trial and first learning trial in the water maze test*

			No. of successful rats	
Groups	No. of rats	Swimming speed (cm/s)	Visible test	1st learning trial
9 weeks of age				
Adq.	12	19.4 ± 1.1	7/12	12/12
Birth	11	19.8 ± 1.6	5/11	11/11
Weaning	12	20.5 ± 1.0	6/12	12/12
Adult	12	22.4 ± 2.1	5/12	11/12
Def.	12	22.9 ± 2.6	4/12	10/12
13 weeks of age				
Adq.	12	20.7 ± 1.8	7/12	12/12
Birth	9	23.7 ± 2.5	7/9	9/9
Weaning	12	24.4 ± 3.3	7/12	11/12
Adult	12	28.1 ± 2.7	4/12	11/12
Def.	12	28.7 ± 3.1	7/12	9/12

*Data are presented as the mean ± SEM. Swimming speed, $F_{(4,106)} = 2.263$, $p = 0.067$.

Probe trial

After 4 days of learning trials, the platform was removed and the rats subjected to a probe trial. The number of crossings of the former platform position (Quadrant A) was compared with the number of crossings of the other three quadrants (Quadrants B–D). Figure 4 shows that there was a robust difference between the n-3 Adq and n-3 Def groups in this measure. When measured at both the 9- and 13-week time points, the n-3 Def group appears to be swimming in a random fashion (Quadrant C is the one bounded by the two starting points for the trial and is usually crossed less than other quadrants). In contrast, the n-3 Adq groups cross position A, the former position of the hidden platform, more often than any other position ($p < 0.0005$).

The probe trial data for rats in the 'Birth', 'Weaning' and 'Adult' groups that were switched to the n-3 Adq diets at 0, 3 and 7 weeks of age, respectively, is shown in Fig. 5. Animals switched at birth are similar to the n-3 Adq group with a significantly greater number of crossings of quadrant A when measured at 9 ($p < 0.005$) and 13 ($p < 0.05$) weeks of age. Similarly, the animals switched to the n-3 Adq diet at weaning were similar to the n-3 Adq reference group on the retention trial including both those groups tested at 9 weeks ($p < 0.05$) and those tested after 13 weeks ($p < 0.01$). When animals were switched to the n-3 Adq diet at early adulthood, at 7 weeks of age, they were able to perform as well as an n-3 Adq animals, providing that the period of diet repletion was of a sufficient duration. That is, the 'Adult' animals tested after 6 weeks of dietary repletion (13-week measurement) showed a highly significant differential with respect to their crossings in quadrant A versus the other positions ($p < 0.005$). However, 'Adult' animals allowed to recover for only 2 weeks prior to the onset of behavioral testing (9-week measurement) were very similar to the n-3 Def group (Fig. 4). The only significant difference in this group was between quadrant A and C, the latter being the position bounded by the two starting positions, and thus one where little time was spent in most experiments.

Correlation of spatial task performance and brain fatty acid composition

Fatty acid analysis by gas chromatography was performed for each animal at the end of behavioral testing (at 10 or 14 weeks of age) and the group mean values of DHA and DPAn-6 for each of the 10 experimental groups were calculated. These values were plotted against the mean total latencies (the sum of the four learning session values) and are presented in Fig. 6. As the DHA value increased, the total escape latency decreased, corresponding to finding the

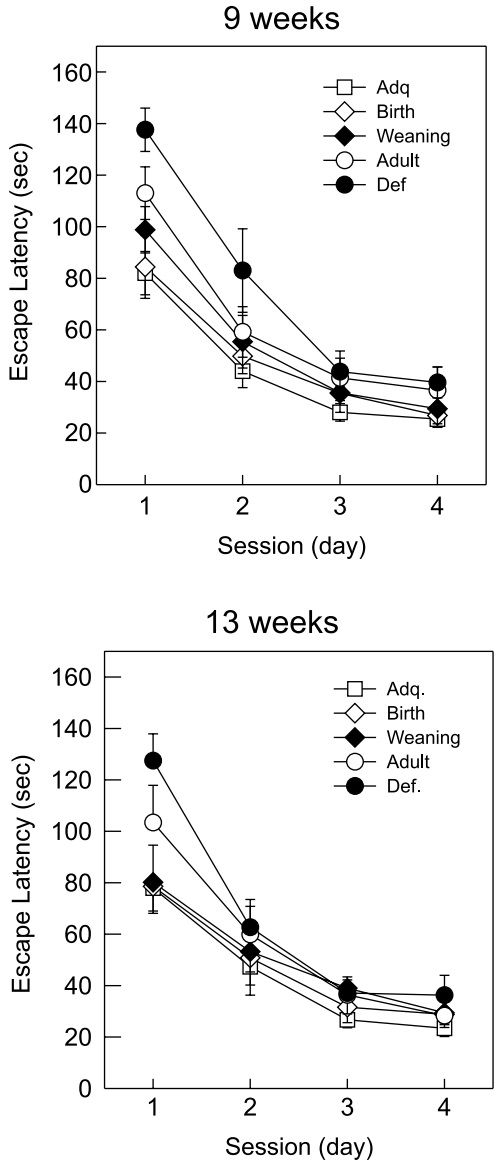

Fig. 2 The reversibility of n-3 deficiency on escape latency in the Morris water maze. The time required for a rat to find and climb onto the hidden platform (escape latency) at 9 and 13 weeks of age are presented as the mean ± SEM for 9–12 rats/group. The differences were statistically significant in a two-way ANOVA (9 week, $F_{4,54} = 4.738$, $p < 0.005$. Duncan's multiple range test indicated the following significant differences: Adult, $p < 0.05$ compared with Adq group; also Adq, $p < 0.001$; Birth, $p < 0.005$; Weaning, $p < 0.05$ compared with Def group. At 13 weeks, ANOVA indicated $F_{4,54} = 3.688$, $p < 0.05$. Duncan's multiple range test indicated the following significant differences: Adq, $p < 0.005$; Birth, $p < 0.05$; Weaning, $p < 0.05$ compared with Def group.

hidden platform more quickly. The relationship to brain DPAn-6 showed a direct relationship with total latency, so as the brain DPAn-6-value increased; the animals took longer to find the platform. In both cases, a good fit was not obtained by a single linear correlation due to the tailing-off behavior at high DHA and low DPAn-6-values. The data plotted in Fig. 6 are group mean values as these allow the visualization of the interesting 'tailing-off' behavior at higher DHA and low DPAn-6-values. When these correlations are run with the individual data points, for which there is of course much greater scatter, these correlations remain statistically significant, but are weaker (DHA, $r = 0.46$, $p < 0.0001$; DPAn-6, $r = 0.48$, $p < 0.0001$). Similarly, the percentage of brain DHA was related to the percentage of time spent in Region A in the probe trail (data not shown).

Discussion

As DHA is tenaciously retained by the brain (for review, see Salem *et al*. 1986), in order to significantly deplete the brain of DHA it has been necessary to deprive rats of dietary sources of n-3 fatty acids for two or more generations (Ward *et al*. 1996). In order to accentuate this difference in brain biochemistry, three generations of dietary deficiency were used in this experiment. It is necessary to use a carefully constructed dietary regime so as not to inadvertently introduce n-3 fatty acids in other dietary components such as protein or carbohydrate sources. Thus, a marked difference was obtained in the brain DHA level between n-3 Adq and n-3 Def and the repletion groups had intermediate values. Our previous results indicate that the brain would recover its DHA with a half time of 2.9 weeks (Moriguchi *et al*. 2001). This then allowed for a study of the relationship of brain DHA and behavior. A second goal of the study was to determine if there was a critical period in which a dietary DHA supply was needed for proper brain function, using spatial task performance as the principal measure.

Our findings indicated that the performance in spatial tasks was closely related to the level of brain DHA. When animals were switched at birth to an n-3 Adq diet via lactation from a dam fed an n-3 Adq diet, performance on the spatial learning and memory task was very similar to that of the n-3 Adq group. When animals were switched at weaning and allowed to recover brain DHA for 6 or 10 weeks, brain DHA had substantially, though not completely, recovered. Performance of the weaning group in the spatial tasks was significantly different from the n-3 Def group although mean values were, in some cases, intermediate between n-3 Adq and n-3 Def. Perhaps the most interesting group is the young adult group, the animals switched to the n-3 Adq diet at 7 weeks of age. Although only partial recovery of brain DHA and spatial performance had occurred after 2 weeks of diet repletion, nearly full recovery had occurred on the probe trial and partial recovery for the spatial learning task after 6 weeks of diet repletion. This is important as it indicates that brain function can recover from a severe and extended n-3 fatty acid deficiency in the nearly fully developed brain. Thus,

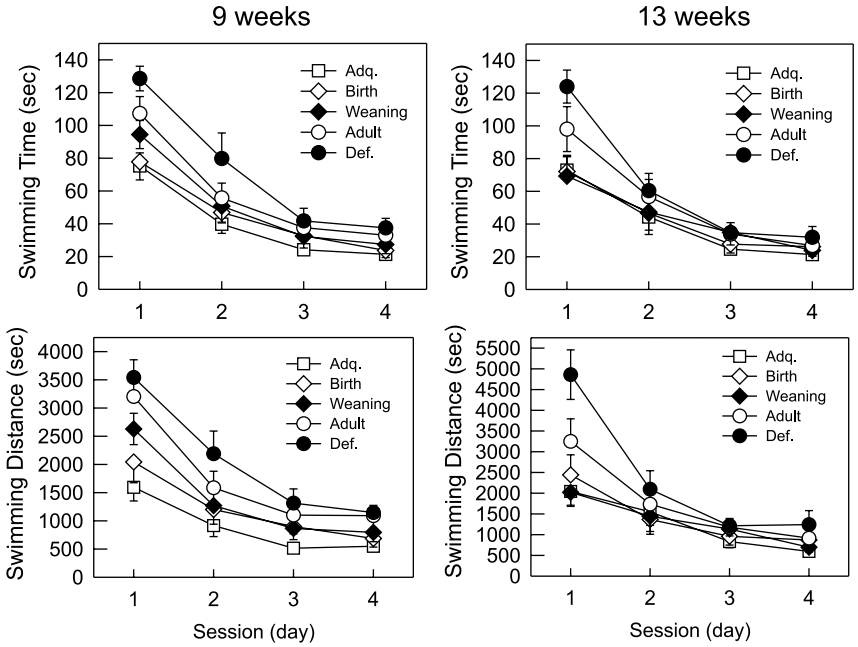

Fig. 3 The reversibility of n-3 deficiency on swimming time and distance in the Morris water maze. The swimming time and distance in learning trials at 9 and 13 weeks of age are presented as the mean ± SEM for 9–12 rats/group. Statistical analysis by using a two-way ANOVA gave the following results at 9 weeks for swimming time: $F_{4,54} = 5.721$, $p < 0.001$. Duncan's multiple range test indicated the following significant differences: Adult, $p < 0.05$ compared with Adq group; also Adq, $p < 0.0005$; Birth, $p < 0.005$; Weaning, $p < 0.01$ compared with Def group. For swimming distance, two-way ANOVA gave the following results at 9 weeks: $F_{4,54} = 7.193$, $p < 0.0005$. Duncan's multiple range test indicated the following significant differences in swimming distance: Adult, $p < 0.005$ compared with Adq group; also Adq, $p < 0.0001$; Birth, $p < 0.005$; Weaning, $p < 0.05$ compared with Def group; also $p < 0.05$ between birth and adult groups. At 13 weeks, two-way ANOVA gave the following results for swimming time, $F_{4,54} = 4.599$, $p < 0.005$. Duncan's multiple range test indicated the following significant differences in swimming time: Adq, $p < 0.005$; Birth, $p < 0.005$; Weaning, $p < 0.01$ compared with Def group. For swimming distance, two-way ANOVA gave the following results at 13 weeks: $F_{4,54} = 4.982$, $p < 0.005$. Duncan's multiple range test indicated the following significant differences: Adq, $p < 0.005$; Birth, $p < 0.005$; Weaning, $p < 0.005$ compared with Def group.

juveniles and young adults who were deprived of adequate n-3 fatty acid sources in early development, for example due to formula feeding, may be expected to regain brain DHA and improve at least some aspects of brain function once adequate sources of dietary DHA are provided.

There are many possible factors that could explain the loss in spatial task performance associated with brain DHA loss. These include motivational, sensory, motor and cognitive factors that are often difficult to separate. The observations here that there were no changes in measures of locomotor activity between dietary groups mitigates against a change in general level of arousal. Also, although swimming speed was not significantly different, there was a trend suggesting that DHA-deficient animals swam *faster* than DHA-adequate animals, as has been observed in a previous Morris water maze study (Moriguchi *et al*. 2000). These observations then indicate that motivational factors are unlikely to explain changes in escape latency. They also indicate, when taken together with the lack of any differences in the visible trial, that there were not any gross changes in motor ability. A loss in sensory functions associated with a loss in DHA may be a factor in explaining the poorer performance. It is known, for example, that there are changes in electroretinograms (Wheeler *et al*. 1975; Neuringer *et al*. 1984; Neuringer *et al*. 1986; Uauy *et al*. 1990; Pawlosky *et al*. 1997; Weisinger *et al*. 1999), visual acuity (Connor and Neuringer 1988; Birch *et al*. 1992; Carlson *et al*. 1993), olfactory discrimination (Greiner *et al*. 1999; Greiner *et al*. 2001; Catalan *et al*. 2002) and auditory conduction velocity (Stockard *et al*. 2000) associated with changes in n-3 fatty acid status. Indeed, the loss of DHA species of phospholipids is known to lead to suboptimal activation of the rhodopsin signaling system in the retina (Mitchell *et al*. 2001), thus providing a mechanistic basis for electroretinographic alterations. However, changes in sensory function associated with DHA loss are small in magnitude and generally can only be detected in a laboratory research setting. Although it is clear that spatial task performance does depend upon visual acuity (Prusky *et al*. 2000a; Carman and Mactutus 2001), rats with only 0.04% of their photoreceptors are capable of learning the place version of the spatial navigation task (O'Stein *et al.* 1995). Also, increased acuity due to environmental enrich-

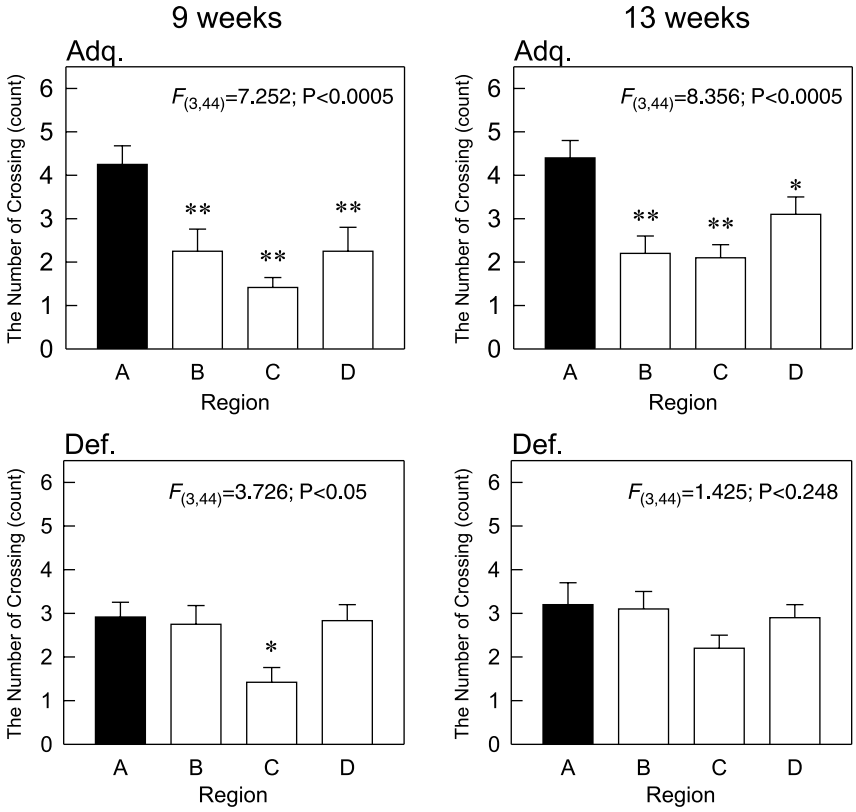

Fig. 4 Effects of n-3 fatty acid deficiency on the probe trial in the Morris water maze. The number of crossings of the platform position (region A, solid bar) and the corresponding imaginary positions (Regions B–D, open bar) on separate groups of 9 and 13 weeks of age are presented as the mean ± SEM for 12 rats/group. $*p < 0.05$, $**p < 0.01$ compared with region A (Duncan's multiple range test after one-way ANOVA).

ment does not lead to better performance on this task (Prusky *et al*. 2000b). Thus, it appears unlikely that a relatively small loss in visual function can explain the poorer spatial task performance in DHA-deficient animals. It is suggested then that cognitive factors may be responsible for the loss in performance in DHA-deficient animals. This conclusion is supported by recent findings from this laboratory that cognitive and not sensory or motivational factors likely underlie the failure of DHA-deficient rats to acquire a learning set in an olfactory discrimination study (Catalan *et al*. 2002). However, other factors including emotional reactivity (Wainwright *et al*. 1994) may also be responsible for the behavioral changes.

There are a few other investigations of the effect of DHA repletion on nervous system function. Our observations agree in principle with those of Weisinger *et al*. (1999) who found that alterations in a- and b-wave amplitudes and flicker responses of n-3 deficient guinea-pigs were reversed after 10 weeks of n-3 repletion. They suggested that the age at which repletion occurs can affect the functional outcome. However, Connor and Neuringer (1988) found that the delay in peak latencies of both rod and cone responses, as well as the impairment in recovery of the dark-adapted response of 22-month-old rhesus monkeys were not normalized, even after the brain DHA level had been fully repleted during 12–28 weeks of a fish oil-containing diet. Also, Weisinger *et al*. (2001) have demonstrated that increased mean arterial blood pressure associated with an n-3 fatty acid deficient diet during development (repletion diet began at 64 post-natal days) was not normalized in the adult after n-3 repletion. It thus appears that some of the effects of n-3 deficiency may be irreversible while others may be reversed by dietary n-3 fatty acids. These studies demonstrate that both the duration of the n-3 deficient diet and therefore the age at which repletion begins, and the length of repletion can be important variables. The degree of repletion will also depend upon the amount and type (DHA vs. LNA) of n-3 fatty acids employed in the repletion diet.

It also appeared that younger animals could restore brain DHA at a faster rate than older animals subsequent to an n-3 fatty acid deficiency, as animals repleted at 3 weeks of age had significantly higher DHA levels than those repleted after 7 weeks, even though both groups had a total repletion time of 7 weeks. This may be due to the more active brain growth and new membrane biosynthesis occurring in the younger animal. The implications of this observation are that nervous system functions that are related to DHA concentration would be expected to be more quickly and easily reversed at a younger age when brain formation is ongoing.

Brain DHA and DPAn-6 levels in the various groups measured at various times after dietary n-3 repletion were related to total escape latency in the Morris water maze task (Fig. 6). The main parts of the curves indicate a linear (or inverse) relationship between these long-chain

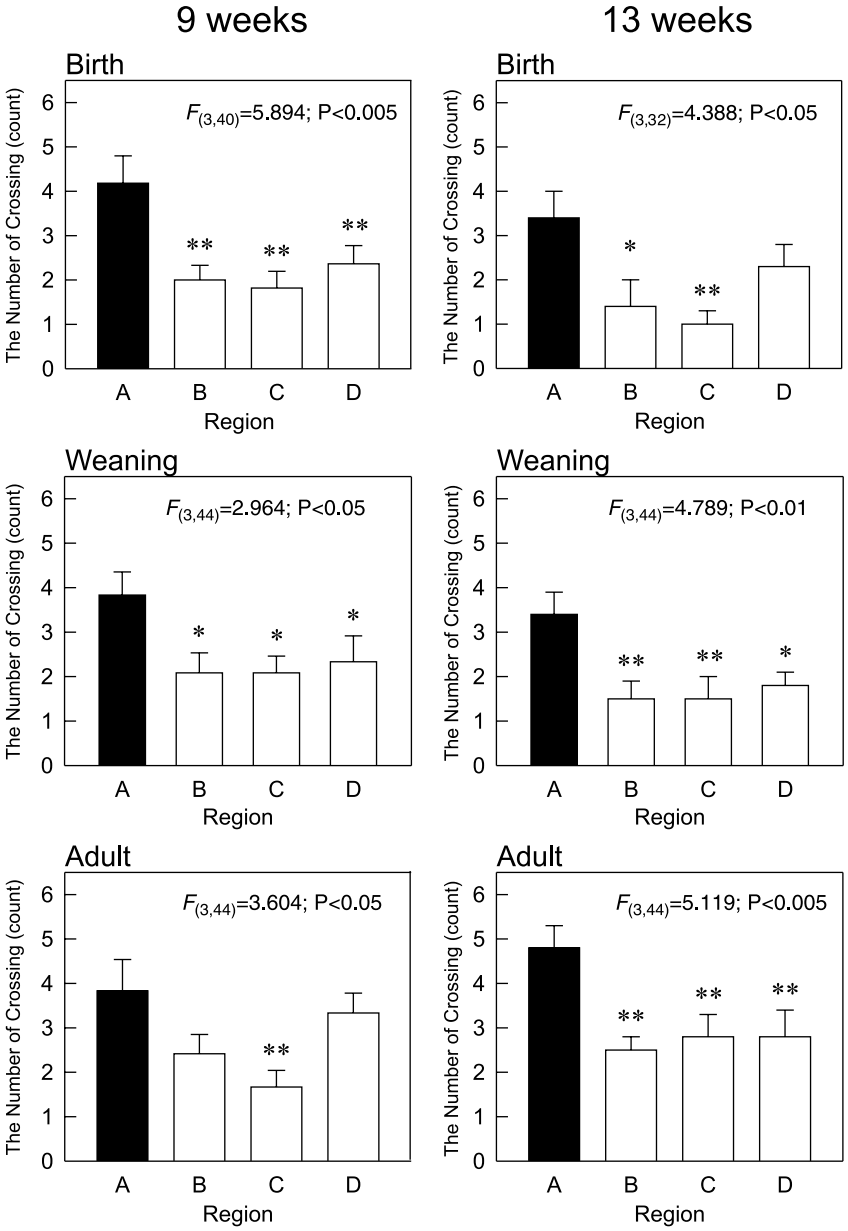

Fig. 5 The reversibility of n-3 deficiency on the probe trial in the Morris water maze. The number of crossings of the platform position (region A, solid bar) and the corresponding imaginary positions (Region B–D, open bar) at 9 and 13 weeks of age are presented as the mean ± SEM for 9–12 rats/group. *$p < 0.05$, **$p < 0.01$ compared with region A (Duncan's multiple range test after one-way ANOVA).

polyunsaturates and performance. It is clear that poorer performance results from brain DHA levels of between 2 and 7% of total fatty acids and the corresponding values of DPAn-6 of 5–10% of fatty acids. However, the tailing portions of each curve falls off of the linear relationship and is better fit by a smooth curve. Although the differences in performances between the groups clustered in this portion of the curve were not significantly different, the steep decline in total latency between values of brain DHA of 11.7–12.5% may be meaningful. One interpretation of these data is that a small decline in brain DHA and a corresponding increment in DPAn-6 may lead to poorer brain function. A small decline in the retinal or brain DHA is observed when LNA only is fed in comparison with that when the diet also contains preformed DHA (Woods *et al*. 1996; Abedin *et al*. 1999; Bowen and Clandinin 2000). It may also occur in infants fed vegetable oil-based formulas devoid of long-chain polyunsaturates (Farquharson *et al*. 1992; Makrides *et al*. 1994; Jamieson *et al*. 1999). Another possibility is that the differences in behavioral performance reflected irreversible structural damage to the brain induced by the period of n-3 deficiency in early development. This concept is supported by the recent observation that adult rat brain CA1 hippocampal neurons (Ahmad *et al*. 2002a, 2002b) as well as those in other brain areas (Ahmad *et al*. 2002b) are smaller after DHA deficiency. However, it is also possible that with a longer period of neural DHA repletion, a more complete behavioral recovery would occur.

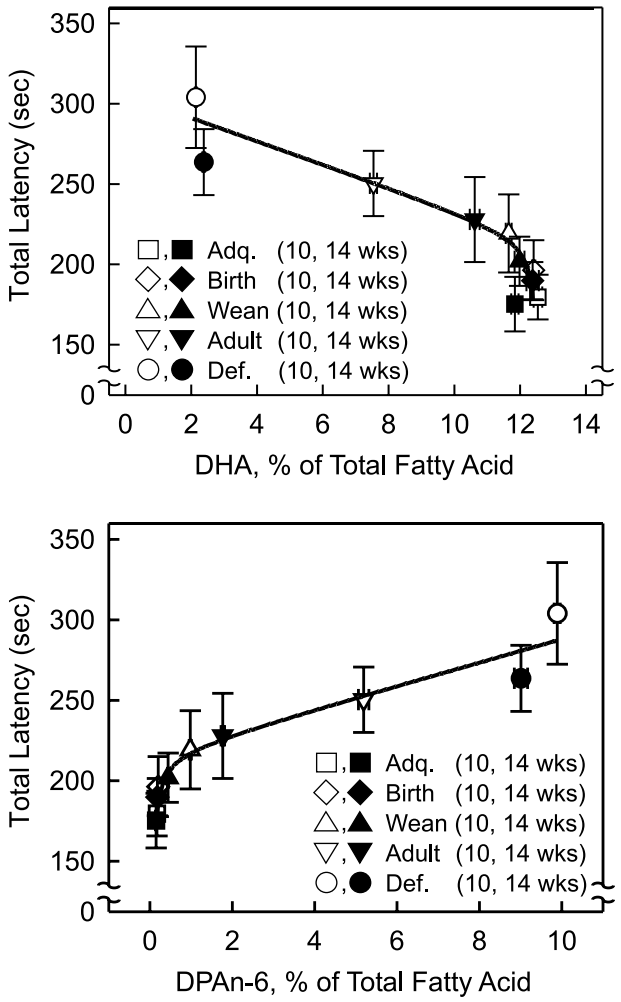

Fig. 6 Correlation of spatial task performance and brain fatty acid composition. Brain DHA and DPAn-6 levels were measured at 10 and 14 weeks of age for the various groups with varying periods of dietary n-3 repletion and these mean values were plotted against the mean total escape latencies (the sum of the four learning session values). Open symbols represent the various groups at 10 weeks and the filled symbols those at 14 weeks. Statistical analysis for the regression curve using the group means gave the following results: DHA, $r = 0.940$, $p < 0.005$; DPAn-6, $r = 0.976$, $p < 0.005$.

The observation that behavioral deficits induced by brain DHA deficiency were reversible has implications for the cellular and molecular mechanisms underlying this deficient state. In terms of relevant cellular level changes, it has been demonstrated that DHA can protect cultured neurons from apoptosis (Kim *et al*. 2000), and it is possible that limited neuronal death can explain the behavioral deficits observed herein. However, Ahmad *et al*. (2002a, 2002b) recently observed that DHA deficiency leads to decreased neuronal size in the CA1 region of the rat hippocampus, but no change in cell density. Although it is not known whether these morphological changes are reversible after DHA repletion, the restoration of cell size is more easily contemplated than growth of a new cell. It is well established that the hippocampus in particular is a critical area for encoding spatial information (Compton *et al*. 1997; de Bruin *et al*. 2001) and spatial task memory (Moser and Moser 1998). Thus, changes in hippocampal neuronal morphology and function may underlie the changes in spatial task performance observed in this and other related studies.

At a molecular level, it is now understood that DHA-containing phospholipids allow a greater degree of rhodopsin activation (Litman and Mitchell 1996; Mitchell and Litman 1998) even when compared with other highly unsaturated phospholipids (e.g. arachidonyl species). Thus, this mechanism may underlie alterations in the electroretinogram and losses in visual acuity observed after retinal DHA loss. The rate of coupling of activated rhodopsin to its G-protein was enhanced in DHA-containing bilayers (Litman *et al*. 2001; Mitchell *et al*. 2001; Niu *et al*. 2001). The activity of the integrated visual transduction pathway as measured by the activity of the cGMP phosphodiesterase was also dependent upon the degree of unsaturation of membrane phospholipids in reconstituted systems (Litman *et al*. 2001). Rhodopsin is a member of the G-protein coupled receptor family; this superfamily of proteins also includes many neurotransmitter receptors that have similar structural motifs and likely have similar dependency on DHA-phospholipids for optimal activity. Thus, DHA deficiency may affect brain function through alteration in the functions of neurotransmitter receptors.

In summary, our observations indicate that lack of n-3 fatty acids in the diet leads to a loss of brain DHA. This loss in DHA leads to a loss in brain function as reflected in this experiment by poorer spatial task performance. This diminution in performance is likely not due to factors related to sensory function, motivation or motor control and therefore may be related to cognitive capacity or emotive factors. It is shown here that the spatial task performance of DHA-deficient rats can be normalized after dietary n-3 fatty acids are supplied for a period long enough to restore brain DHA. Partial restoration of brain DHA leads to intermediate levels of spatial task performance. The correlation of brain DHA with spatial task performance is strong evidence that this one factor is responsible for the poorer behavioral performance. This contention is further strengthened by the reversibility of the behavioral deficit when DHA is added to the diet. No critical period in development was observed in this experiment during which DHA must be supplied. However, earlier intervention appears to be more effective in restoring brain DHA than later ones. The implications of this study are that at least some behavioral deficits associated with low brain DHA as a consequence of a low infant n-3 fatty acid intake during early development may be corrected by addition of DHA to the diet.

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