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Cognitive Deficits in Docosahexaenoic Acid-Deficient Rats

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This study investigated the influence of brain docosahexaenoic acid (DHA) deficiency on simple and complex olfactory-based learning and memory in 2nd generation (F2) adult male rats. Rats raised and maintained on either an n-3-adequate or an n-3-deficient diet were tested for acquisition of an olfactory learning set and an olfactory memory task, and for motivation to obtain a water reward. Despite a 76% decrease in brain DHA, n-3-deficient rats were able to acquire most simple 2-odor discrimination tasks but were deficient in the acquisition of a 20-problem olfactory learning set. This deficit could not be attributed to changes in sensory capacity but, instead, appeared to represent a deficit in higher order learning.

Docosahexaenoic acid (DHA) is a long chain, polyunsaturated fatty acid found in high concentrations within the phospholipid bilayer of cell membranes in the cerebral gray matter, neurons, and in retinal photoreceptors (for reviews, see Salem, 1989; Salem, Kim, & Yergey, 1986). DHA-phospholipid species appear to be optimal for the functional activation of G-protein-coupled receptors such as rhodopsin (Litman & Mitchell, 1996; Mitchell & Litman, 1998). In addition, the rate of coupling of the activated receptor to its G-protein is enhanced in DHA-containing bilayers (Litman, Niu, Polozova, & Mitchell, 2001).

There are functional consequences to diminishing the level of nervous system DHA with diets low in n-3 fat sources (Farquharson, Cockburn, Patrick, Jamieson, & Logan, 1992; Jamieson et al., 1999; Makrides, Neumann, Byard, Simmer, & Gibson, 1994), including diminished visual acuity, abnormal electroretinograms (Benolken, Anderson, & Wheeler, 1973; D. G. Birch, Birch, Hoffman, & Uauy, 1992; E. E. Birch, Birch, Hoffman, & Uauy, 1992; E. E. Birch, Hoffman, Uauy, Birch, & Prestidge, 1998; Carlson & Werkman, 1996; Carlson, Werkman, Rhodes, & Tolley, 1993; Uauy, Birch, Birch, Tyson, & Hoffman, 1990), and learning and behavioral deficits (Agostoni, Trojan, Bellu, Riva, & Giovannini, 1995; Carlson, Werkman, Peeples, & Wilson, 1994; Willatts, Forsyth, DiModugno, Varma, & Colvin, 1998). Formula-fed infants whose diet was supplemented with DHA or DHA plus arachidonic acid during the first 4 months of life had higher Bayley's Mental Developmental Index scores, in both Cognitive and Motor subscales, at 18 months of life than did infants given standard nonsupplemented formula (E. E. Birch, Garfield, Hoffman, Uauy, & Birch, 2000).

Attempts to develop an animal model to examine learning or cognitive deficits resulting from DHA deficiency have met with limited success. Studies of the n-3 fatty acid deficiency syndrome have been criticized because of inadequate controls and numbers of study animals, problems with statistical analyses, and the possibility that behavioral tasks using visual cues may be confounded by deficits in sensory function, as suggested by electroretinographic and visual acuity studies (Wainwright, 1992, 1993).

Recently, we reported that the second generation of rats maintained on an n-3 fatty acid-deficient diet had severe loss of brain DHA (e.g., an 82% decrease of DHA in the olfactory bulb; Greiner, Moriguchi, Hutton, Slotnick, & Salem, 1999). The results of an initial behavioral study indicated that simple odor discrimination and spatial learning tasks may provide useful behavioral assays for DHA deficiency (Greiner et al., 1999; Greiner, Moriguchi, Hutton, Slotnick, & Salem, 2000; Moriguchi, Greiner, & Salem, 2000). Odor-cued learning tasks are of particular interest for assessing cognitive behavior because they do not require visual guidance, and rats rapidly learn both simple discrimination problems and more complex, cognitively based tasks when trained with odors (Slotnick, 1990, 1994; Slotnick, Hanford, & Hodos, 2000).

The purpose of the present study was to determine whether severe brain DHA deficiency would affect cognitive performance and long-term memory as assessed by an olfactory learning set task. Learning set tasks (Harlow, 1949, 1959) involve the presen-

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tation of a series of simple discrimination problems, all of which have a common solution. If subjects acquire a strategy (e.g., win–stay, lose–shift in go/no-go discrimination problems) during this training, then each subsequent problem can be solved with no or few errors. The rate of interproblem transfer and the level of asymptotic performance can be used to measure the extent to which strategy learning has occurred. Because rats acquire a learning set when trained with odors, we used the learning set paradigm devised by Slotnick and Katz (1974) to provide a quantitative measure of cognitive behavior in DHA-sufficient and DHA-deficient rats.

The results of several studies indicate that water intake may be enhanced in DHA-deficient animals (Reisbick, Neuringer, Connor, & Barstad, 1992; Reisbick, Neuringer, Connor, & Iliff-Sizemore, 1991; Reisbick, Neuringer, Hasnain, & Connor, 1990). Because thirsty rats were reinforced with water for correct responses in the learning set task, a second goal of this study was to determine whether there were motivational differences between the two dietary groups.

Method

Subjects

Three-week-old female Long-Evans rats were purchased from Charles River (Portage, MI) and were randomized into one of two dietary groups, with the constraint that the mean body weights matched. The females were maintained on the assigned diets throughout their lifetime. At 11 weeks of age, these females (F1 generation) were mated with chow (NIH 31)-fed males. All rats were maintained in the National Institute on Alcohol Abuse and Alcoholism animal facility under conventional conditions, with controlled temperature (21 ± 1 °C) and an average relative humidity of 50%. The level of fluorescent luminance was about 65 lux. Food and water were supplied on an ad-lib basis.

The litters were culled to 10 pups on Postnatal Day 2, and the male pups (F2 generation) were weaned from their mothers on Postnatal Day 28, as described by Greiner et al. (2000). After weaning, the F2 rats were maintained on the same diet as the dam throughout the entire study period.

Two male littermates, matched for body weight, were selected from each of 15 litters in both dietary groups to participate in the learning set or motivation studies described below. At 7.5 weeks of age, the rats used for Experiment 1 (the learning set and memory study) and Experiment 2 (the motivational study) were individually housed and their water intake was limited to 10 ml/day. The rats were weighed weekly during the deprivation period. All animal procedures were approved by the National Institute on Alcohol Abuse and Alcoholism Animal Care and Use Committee.

Dietary Treatments

Composition of the two diets (Dyets, Bethlehem, PA; see Table 1), n-3-deficient and n-3-adequate, was based on the AIN-93 (Reeves, Neilsen, & Fahey, 1993) diet formulation. Several modifications were necessary to reduce the n-3 fatty acids in the basal diet components. These modifications and diet compositions were similar to those previously described by Greiner et al. (1999), except that DHASCO (Martek Corp., Columbia, MD) was added to supply DHA to the n-3-adequate diet. The control diet (n-3-adequate) was identical to the experimental diet (n-3-deficient) except for the addition of a small amount of flaxseed oil and DHASCO, which contained 46% DHA. The fatty acid composition of the diets is listed in Table 2.

Table 1
Diet Composition

Component	g/100 g diet	n-3 adequate	n-3 deficient
Casein, vitamin free	20		
Carbohydrate	60		
Cellulose	5		
Salt mix	3.5		
Vitamin mix	1		
L-cystine	0.3		
Choline bitartrate	0.25		
Fat	10		
Fat sources			
Hydrogenated coconut oil		7.43	8.1
Safflower oil		1.77	1.9
Flaxseed oil		0.5	—
DHASCO[a]		0.3	—

Note. Dashes indicate that component was not added.
[a] Docosahexaenoic acid (DHA) single cell oil, contains 46% DHA (from Martek Corp., Columbia, MD).

Experiment 1: Olfactory Learning Set and Memory Task

Apparatus

Olfactometer. Rats were trained and tested in one of four identical eight-channel olfactometers (see Figure 1) similar in design to that described by Bodyak and Slotnick (1999). The training and testing procedures were controlled by 486 PC computers by means of a digital interface. Control programs were written in QBASIC. The olfactometers, digital interfaces, and control programs were obtained from Knosys Olfactometers Ltd. (Bethesda, MD; http://www.chemsenses.com). Pinch valves, a special feature of the olfactometers, were used to control odor flow in the odor saturator bottles. These valves operated by pinching a short piece of odorless soft tubing used to connect the saturator tubes to the upstream and downstream air distribution manifolds (C-flex, Cole Parmer, Vernon Hills, IL).

Test chamber. The Plexiglas test chamber was similar to that described by Lu and Slotnick (1998). The front panel of the chamber contained a 25-mm-diameter glass odor sampling tube, a magnetic buzzer, and a 13-gauge stainless steel tube for water reinforcement. The stainless steel tube was located 50 mm to one side and 50 mm above the opening of the odor sampling tube and was connected to a water reservoir by a two-way, normally closed solenoid valve. The glass odor sampling tube was mounted vertically on the outside wall of the chamber. The bottom of the tube tapered to 4 mm and was connected to the olfactometer by the common and normally open ports of a three-way solenoid pinch valve. A 20-mm-diameter hole in the glass tube and chamber wall served as a sniff port for sampling the odor stimuli. An infrared photocell unit detected snout insertions into the glass tube. A 25 cfm intake fan mounted on the back panel maintained the chamber under positive pressure and ensured that odor stimuli introduced into the sampling tube did not escape into the chamber. Lick responses on the steel water-delivery tube were detected by a touch-sensitive circuit connected between the tube and the stainless steel floor of the chamber.

Odor and Odor Concentrations

Odors were obtained by random selection without replacement from a stock of approximately 100 common grocery store items, food flavorings (McCormick, Hunt Valley, MD), and perfumes (kindly provided by the Givaudan Fragrances Corp., Mount Olive, NJ). The odor stimulus was

Table 2
Fatty Acid Composition of the Diets as Percentage of Total Fatty Acids

Fatty acid	n-3 adequate	n-3 deficient
Total saturated	73.0	80.0
Total monounsaturated	4.6	3.5
18:2n-6	15.2	15.5
n-6 total	15.2	15.5
18:3n-3	3.0	0.05
22:6n-3	1.5	—
n-3 total	4.5	0.05
18:2n-6/18:3n-3	5.0	310.0
n-6/n-3	3.4	310.0

Note. Dash indicates not detected.

generated by passing 50 cc/min of air over the surface of 5 ml of undiluted liquid odorant material and mixing it with a 1,950 cc/min stream of clean air. Thus, the odor concentration of all odor stimuli sampled by the rat was 2.5% of the headspace concentration above the liquid odorant. Each of the odors was clearly detectable and recognizable by human observers. When new odors were used in the system, all glass and Teflon connectors were washed with 95% (vol/vol) ethanol and new saturator tubes and tubing were used. This served to eliminate completely any potential contamination from prior odors.

Procedure

Olfactory training and testing. Two weeks after initiation of their water deprivation schedule, 15 n-3-deficient and 15 n-3-adequate F2 generation rats were trained to detect and discriminate odors, by means of procedures that have been previously described in detail by Slotnick (1990). Briefly, during the initial sessions, the rats were trained by standard operant conditioning methods to insert their snouts into the odor sampling tube, sample the S+ stimulus (generated from a 5% aqueous solution of ethyl acetate), and then respond by licking the stainless steel water delivery tube for a 0.04-ml water reinforcement. Snout insertion produced a 1-s sample of the ethyl acetate vapor. Upon completion of this initial training, the S− stimulus (clean air) was introduced. Responding on the water delivery tube after delivery of the S+ odor (ethyl acetate) was reinforced with the water reward. Responding after delivery of the S− stimulus (clean air) was punished by an extension of the intertrial interval from 4 s to 15 s. When stable behavior on this task was achieved, the learning set task was initiated.

In this phase, the rats were given a series of 20 two-odor discrimination problems (see Table 3). The odors were randomly paired and ordered prior to the beginning of the testing period. Subgroups were formed within the dietary groups to counterbalance the order of odor pair presentation. Individual rats were tested in the same olfactometer, in the same numerical order, and at similar times of the day (0800–1500) to minimize any effects these variables may introduce on performance. In each odor pair, one odor served as the S+ stimulus and the other as the S− stimulus, and these assignments were counterbalanced across rats in each group. The stimuli were presented in a random sequence within the session, with the restriction that there was an equal number of S+ and S− trials in every block of 20 trials. Each daily session consisted of 61 trials, and a different pair of odors was used each day. The first trial in each session served to provide information on whether a response to the first odor presented was reinforced. Outcomes of these trials were noted but were not scored. In the remaining 60 trials, responding on the S+ odor trials (hits) and not responding on the S− odor trials (correct rejections) were scored as correct. Responding on the S− odor trials (false alarms) and not responding on the S+ odor trials (misses) were scored as errors. Measured endpoints were the total percent correct responses in each block of 20 trials, the number of errors to reach criterion performance of 95% correct responding in a block of 20 trials, and the stimulus sampling time (the time the rat kept its snout in the sampling tube during the 1-s stimulus presentation).

Problem order was partly counterbalanced as follows: 15 rats in each dietary group were divided in Subgroups A (n = 7) and B (n = 8). As shown in Table 3, each set of four problems was given in one order for Subgroup A and the reverse order for Subgroup B.

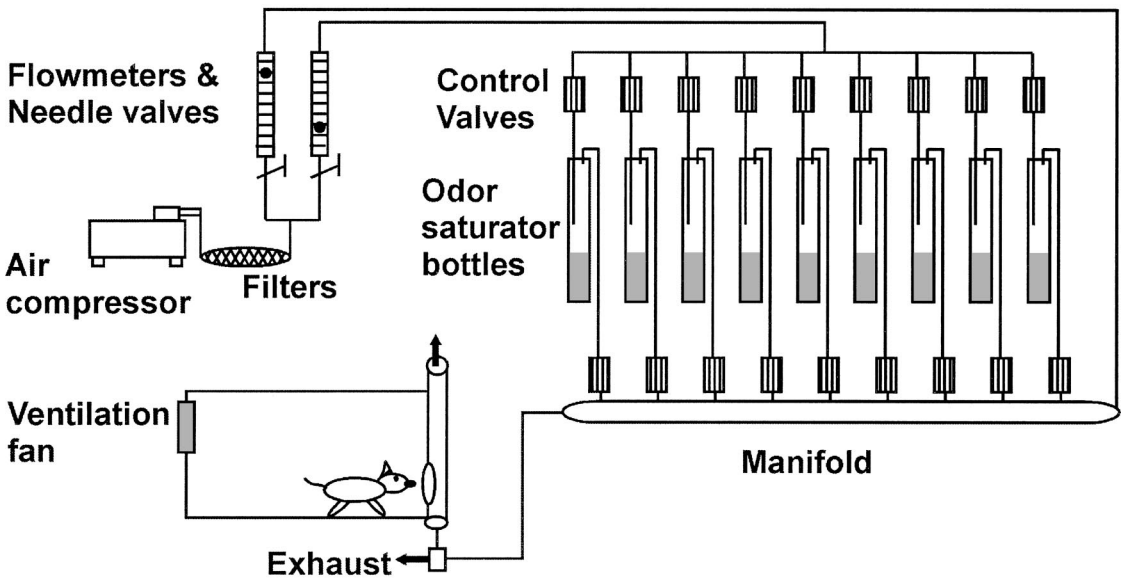

Figure 1. A schematic drawing of the principal components of the olfactometer used in the two-odor discrimination task.

Table 3
Odor Pairs Used in the Olfactory Learning Set Task and Memory Test

Session order			
Group A	Group B	S+ odor	S− odor
		Learning set task	
1	4	Vanilla	Root beer
2	3	Maple	Brandy
3	2	Lemon	Butternut
4	1	Banana	Almond
5	8	Orange	Butter
6	7	Black walnut	Pineapple
7	6	Rum	Anise
8	5	Peppermint	Strawberry
9	12	Cherry	Coconut
10	11	Roasted peanut	Oregano
11	10	Lilac	Caramel
12	9	Sage	Watermelon
13	16	Rose	Peach
14	15	Mulberry	Magnolia
15	14	Apricot	Baby powder
16	13	Prune juice	Apple juice
17	20	Mustard	Coffee
18	19	Olive juice	Mango
19	18	Ginger	Cinnamon
20	17	V8 juice	Worcestershire sauce
		Memory test	
1	1	Grape	Lime juice
2	2	Beets	Spearmint
3	3	Soy sauce	Raspberry
4	4	Onion	Eucalyptus

Note. The order of presentation of odor pairs was partially counterbalanced within four blocks. Group A and Group B consisted of an equal number of rats from each dietary group. S = stimulus.

Memory test. Three weeks after the completion of the discrimination task (at approximately 17 weeks of age), the rats were trained on four additional novel two-odor discrimination tasks (see Table 3; bottom). Training was continued on each odor pair until the rat achieved criterion performance of more than 90% correct responses in 40 consecutive trials. Once criterion was reached, the four odor pairs were presented (four S+ and four S− odors) within the same 160-trial session. After two sessions, the probability of reinforcement for correctly responding to the S+ odor was reduced to 0.5 (for three sessions) and to 0.33 (for three sessions). The partial reinforcement schedule was used to increase resistance to extinction in preparation for the memory test. The rats received three additional such sessions, and on the last training day, the reinforcement probability was returned to 1.0.

The rats were then allowed to rest in their home cages for 33 days and were maintained on the same water-deprivation schedule. On Day 34, the rats were given a single 80-trial session on the multiple odor problem (memory test). In this session, responding to three of the four S+ odors was not reinforced, and responses to the S− odors were not punished by an extended intertrial interval. However, to minimize response extinction, responses to one of the S+ stimuli (grape) was reinforced. Thus, in this memory test, the rat was given no feedback concerning response accuracy for seven of the eight odors. Endpoint values were the total number of errors made (not responding to S+ odor or responding to the S− odor) and the odor sample time.

Experiment 2: Motivational Study

Procedure

Progressive ratio testing. The second set of water-restricted F2 generation males was used to quantify motivation to obtain the water reward. At 13 weeks of age, these rats were given three training sessions, in the operant chambers, to obtain water by licking at the water delivery tube. Water reinforcement (0.04 ml) was delivered on a variable ratio schedule (mean of 10 licks). The motivational tests were given in Sessions 4 and 5. In these sessions, reinforcement was delivered on a progressive ratio schedule. The first water reinforcement was delivered after two licks, and each subsequent reinforcement was contingent on making two more lick responses than that required to receive the previous reinforcement. The session was terminated when there was a 5-min period of no response. The first progressive ratio motivational test was given after a 24-hr water-deprivation period, and the second test was given 10 weeks later, after 72 hrs of water deprivation. The dependent measures were the total number of responses made and the number of reinforcements received prior to the end of the session.

Lipid analysis. The rats used in Experiment 1 were sacrificed at the end of their designated study period. The brains were removed, transected sagittally along the midline, and immediately frozen at −80 °C prior to lipid extraction. Total lipid extract of the left hemisphere (including the olfactory bulb and the cerebellum) of the brain was obtained by a modification of the Folch lipid extraction method (Schwertner & Mosser, 1993). The total lipid extract was transmethylated with 14% BF_3-methanol at 100 °C for 60 min by a modification of the method by Morrison and Smith (1959). The methyl esters were analyzed by gas chromatography as previously described (Salem, Reyzer, & Karanian, 1996). An internal standard (546 μg per sample) of 22:3n-3 (Nu-Chek-Prep, Elysian, MN) was used to determine the concentrations of each fatty acid within the brain total lipid extract. The data are presented as percentage of total fatty acid weight.

The left frontal cortex of 18 littermates was used to determine the brain phospholipid acyl composition. The phospholipids were separated by two-dimensional thin-layer chromatography on silica gel H plates, with chloroform/methanol/acetic acid/water (50/37.5/3.5/1) as the mobile phase in both directions (Mahadevappa & Holub, 1987). Phospholipid spots were visualized under ultraviolet light after spraying with 2′7′-dichlorofluorescien and scraped into tubes containing 10 μg/sample of the internal standard, 22:3n3. The spots were then transmethylated in the presence of silica gel with 14% BF_3 in methanol for 1 hr at 100 °C and extracted into hexane. The methyl esters were then analyzed by gas liquid chromatography as described above.

Statistical Analysis

All data were analyzed with Statistica software (StatSoft, Tulsa, OK). For analysis, the learning set task was reduced to 10 problem sets by averaging accuracy scores in pairs of successive problems (Problems 1 and 2, 3 and 4, etc.). The results for the first and last block of 20 trials was evaluated by separate analyses of variance in which diet served as the between-group variable and problem set served as the within-group variable. Student's *t* test was used for statistical analysis of the learning set task, memory test, fatty acid analysis, and mean body weight data. Because of the considerable variability in scores, the nonparametric *U* test was used to evaluate group differences on the progressive ratio test. The alpha level for all tests was .05.

Results

Body Weights

All rats in both dietary groups gained weight during the course of the study, and their overall general health was good. There were

no differences in initial body weights for the n-3-adequate and n-3-deficient rats (320 ± 4.0 g and 325 ± 5.0 g, respectively; *t* test: $p = .30$), nor were there any weight differences noted at the time of sacrifice (520 ± 14 g and 500 ± 18 g, respectively; *t* test: $p = .40$). Differences in body weight between groups were not significant at any stage in the behavioral studies. Whole-brain weights were measured at the time of decapitation. The mean (±*SEM*) brain weight for both groups was 1.60 ± 0.03 g.

Olfactory Discrimination Task

All 15 rats in each of the two dietary groups successfully completed the ethyl acetate detection training sessions, and there were no differences in performance between groups on this initial task (data not shown).

There were no differences in performance on the learning set task within subgroups of each dietary group; thus, these subgroups were collapsed to form a single n-3-adequate group and a single n-3-deficient group. Across all problem sets, n-3-adequate rats performed better than n-3-deficient rats on the first block of trials, $F(1, 9) = 4.7$, $p < .04$, and on the last block of trials, $F(1, 9) = 12.8$, $p < .01$. Performance accuracy of both groups on the learning set task improved both within the 60 trials of each set of problems and over the 10 sets of problems (see Figure 2). In Problem Set 1, both groups performed at or near chance on the first block of 20 trials, but the terminal performance accuracy (third block of trials) of n-3-adequate rats was significantly higher than that of n-3-deficient rats, $t(28) = 2.7$, $p < .02$. However, performance accuracy of the two groups was essentially identical in Problem Sets 2–7 (see Figure 2). Differences between groups emerged in the latter half of the series and were characterized by the n-3-adequate rats having, on average, higher accuracy scores in the first block of trials beginning on Problem Set 6 (see Figure 2). Differences between groups in accuracy on the first block of trials were significant in Problem Sets 8, 9, and 10 ($p < .02$, each comparison). Although the n-3-deficient rats were less accurate in the first block of trials in the last three sets, their terminal performance in Problem Sets 2–10 did not differ from that of the n-3-adequate rats.

The inset in Figure 2 shows the percentage of rats in each group that had a mean accuracy score of 90% or higher in the first block of trials in each set of problems. The number of rats in the n-3-adequate group that achieved this near-errorless learning score

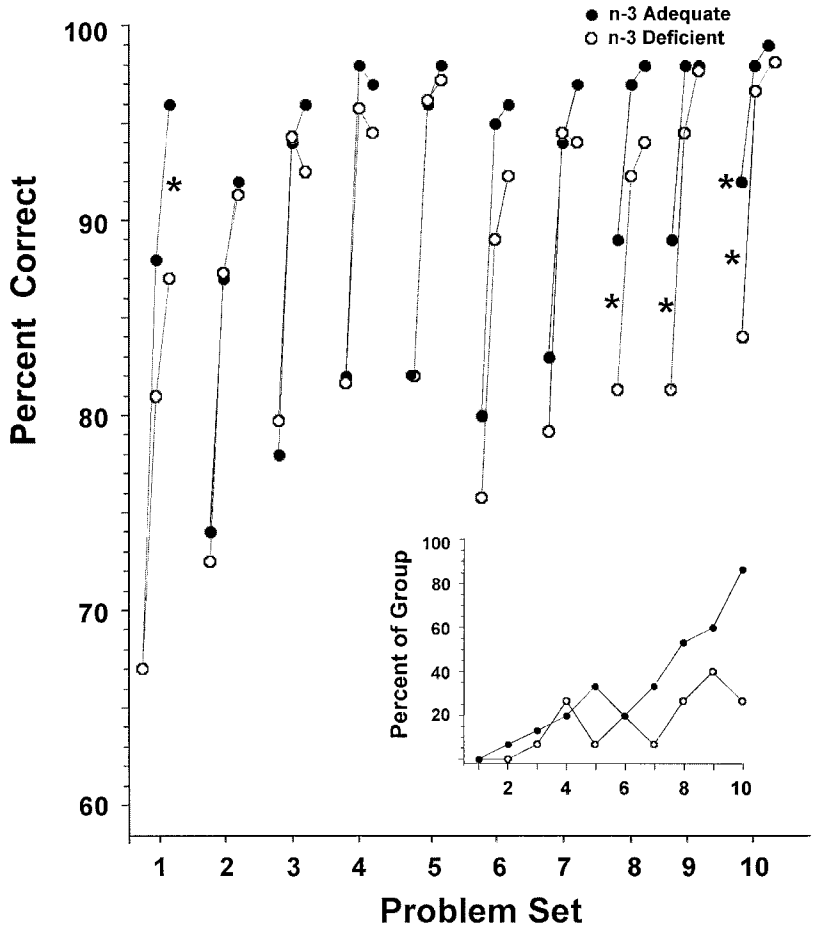

Figure 2. Mean percent correct responding in each block of trials for each set of two-odor discriminations. Asterisks indicate a significant difference between groups for the indicated block of trials ($p < .05$). Inset: Percentage of group having accuracy scores of 90% or higher in the first block of trials in each problem set.

increased gradually in the last five problem sets, and in the last problem set, 87% (13/15) made only 0–2 errors in the first block of 20 trials. In contrast, fewer rats in the n-3-deficient group achieved this near-errorless performance, and in the last problem set only 33% (5/15) had first-block scores of 90% or higher.

The mean sampling time over all trials for all problem sets for the n-3-adequate rats (0.54 s) was slightly but significantly less than that of the n-3-deficient rats (0.57 s; *t* test: $p < .04$). As shown in Figure 3, n-3-deficient rats generally sampled the stimulus slightly longer over all 20 trial blocks (see Figure 3A). For both groups, sampling time increased slightly after Problem Set 3 or 4 and then gradually decreased in the remaining problem sets. As shown in Figures 3B and 3C, this decrease in stimulus sampling time occurred in both the first and last block of trials in the last 5 or 6 problem sets. The decrease in sample time in the last half of the problem series appeared to be closely related to the increase in first-block accuracy for both groups. Indeed, Pearson's product–moment correlation coefficients between accuracy and sampling time on the first block of trials over Problem Sets 6–10 were −0.92 and −0.98 for the n-3-deficient and n-3-adequate groups, respectively.

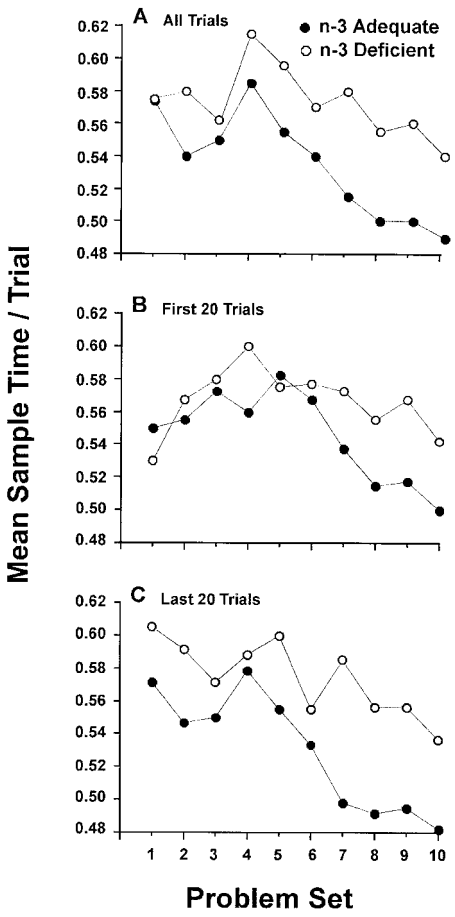

Figure 3. Mean sampling time per trial (in seconds) for each set of problems over all trials (A), on the first block of trials for each set (B), and for the last block of trials for each set (C).

Memory Test

Performance accuracy for the n-3-deficient rats (M = 96%, range = 85.7–98.5%) and the n-3-adequate rats (M = 97%, range = 94.3–98.5%) was nearly perfect, and differences between groups for their memory of these seven odors were not significant. There were also no differences in odor sampling time between the n-3-deficient (0.48 s) and n-3-adequate (0.47 s) groups. Two rats in the deficient diet were excluded from this study because after 2 weeks of training they were unable to meet the 90% correct response criterion on the eight-odor test sessions.

Progressive Ratio Test

All 30 rats completed the preliminary training sessions, but because of time constraints, some rats in each group could not be used in the progressive ratio test. In the first test, given after 24 hr of water deprivation, only 12 rats in the n-3-deficient group and 10 rats in the n-3-adequate group were used. There were no differences in the median number of water reinforcements received by the n-3-deficient rats (68.5) and the n-3-adequate rats (75.5), or in the number of lick responses made (4,955 and 6,050, respectively). Ten weeks later, 12 rats per group were evaluated in a second progressive ratio test that was given after 72 hr of water deprivation. Despite the extreme conditions, there was no significant difference in the number of responses made between dietary groups (n-3-deficient, 7,021 and n-3-adequate, 7,904) or in the reinforcements received (n-3-deficient, 82 and n-3-adequate, 75; Mann–Whitney *U* tests).

Fatty Acid Composition

The brain fatty acid composition was markedly altered by the experimental diets (see Table 4). DHA levels were decreased by 76% in the brain total lipid extracts of the n-3-deficient group as compared with the n-3-adequate group. The 22:5n-6 (docosapentaenoic acid [DPA]) level rose from 0.2% to 9.4% of total fatty acid in the brains of the n-3-deficient group and thus compensated for nearly all of the loss in DHA. The 22:4n-6 and 20:4n-6 levels were also significantly increased in the n-3-deficient group, and they contributed to the replacement of DHA by highly unsaturated n-6 fatty acids.

Tables 5–8 present the fatty acyl composition of the major phospholipids of brain, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI). Brain cortex DHA levels in PC, PE, PS, and PI from the n-3-deficient group were significantly lower than those in the n-3-adequate group (*t* tests: $p < .01$, each comparison). Both PE and PS from the n-3-deficient group contained approximately 5% DHA, compared with approximately 27% in the n-3-adequate group, whereas PC and PI contained only 1.0% and 0.7%, compared with 7.0% and 3.0%, respectively. Low levels of brain DHA in all of these phospholipids in the n-3-deficient group were again compensated for primarily by increases in 22:5n-6 (*t* tests: $p < .01$). Thus, as PE is one of the major phospholipids of rat brain, most of the DHA loss and the gain in 22:5n-6 occurred in this lipid class, with a secondary contribution from PS.

Discussion

This study confirms previous findings by Greiner et al. (1999) that dietary manipulation and control over two generations is

Table 4
Fatty Acid Composition of n-3-Adequate and n-3 Deficient Rat Brain

Fatty acid	n-3 adequate	n-3 deficient
Non essential		
14:0	0.17 ± 0.01	0.18 ± 0.003
16:0 DMA[a]	1.89 ± 0.03	1.85 ± 0.03
16:0	17.32 ± 0.10	17.84 ± 0.10**
18:0 DMA[a]	3.24 ± 0.24	3.12 ± 0.15
18:0	19.89 ± 0.42	19.06 ± 0.89
20:0	0.53 ± 0.01	0.50 ± 0.01
22:0	0.40 ± 0.03	0.41 ± 0.01
23:0	0.23 ± 0.002	0.23 ± 0.01
24:0	0.96 ± 0.02	0.95 ± 0.02
Total saturates	44.70 ± 0.65	44.19 ± 0.93
16:1n-7	0.44 ± 0.03	0.44 ± 0.02
18:1n-7	2.92 ± 0.02	3.11 ± 0.03***
18:1n-9	14.65 ± 0.10	13.41 ± 0.10***
20:1n-9	1.14 ± 0.02	1.05 ± 0.02**
24:1n-9	1.67 ± 0.10	1.53 ± 0.11
Total monounsaturates	20.98 ± 0.15	19.67 ± 0.15***
n-6 PUFAs		
18:2n-6	0.53 ± 0.02	0.40 ± 0.02***
18:3n-6	0.06 ± 0.001	0.05 ± 0.001**
20:2n-6	0.10 ± 0.002	0.08 ± 0.01*
20:3n-6	0.34 ± 0.01	0.20 ± 0.01***
20:4n-6	9.43 ± 0.04	10.50 ± 0.10***
22:4n-6	2.49 ± 0.02	3.38 ± 0.03***
22:5n-6	0.19 ± 0.01	9.40 ± 0.20***
Total n-6	13.13 ± 0.10	24.01 ± 0.12***
n-3 PUFAs		
22:5n-3	0.23 ± 0.01	0.09 ± 0.01***
22:6n-3	12.72 ± 0.20	3.08 ± 0.10***
Total n-3	13.22 ± 0.20	3.41 ± 0.10***
Ratios		
22:5n-6/22:6n-3	0.02 ± 0.001	3.06 ± 0.07***
22:5n-6 + 22:6n-3	12.91 ± 0.20	12.48 ± 0.10
n-6 + n-3	26.35 ± 0.20	27.43 ± 0.14***
Total fatty acid (mg/g brain wet weight)	29.29 ± 0.39	29.68 ± 0.53

Note. Data are presented as the mean (± *SEM*) of the weight percent. PUFAs = polyunsaturated fatty acids. $n = 15$ per diet group.
[a] DMA indicates the dimethyl acetal derivative formed from the alk-l-enyl form of the phospholipid.
* $p < .05$; ** $p < .01$; *** $p < .001$ (Student's t test).

successful in significantly and selectively altering n-3 fatty acid content in the central nervous system. As shown in Table 4, the major effect was in DHA with, as expected, a compensatory increase in DPA levels. The magnitude of this change in n-3 fatty acids is appreciably greater than what had been obtained in many prior studies that maintained rats on an n-3-deficient diet (Bourre, Durand, Pascal, & Youyou, 1989; Tinoco, 1982; Wainwright, Huang, Coscina, Levesque, & McCutcheon, 1994). The aminophospholipids, PE and PS, were the principal fractions responsible for this loss of DHA and increase in DPA.

The performance of n-3-adequate rats in the learning set study is in agreement with prior reports demonstrating that over a series of two-odor discrimination tasks, rats show excellent interproblem transfer and, by the end of training, make few or no errors in acquiring novel odor discrimination tasks (Slotnick et al., 2000; Slotnick & Katz, 1974). This high level of performance indicates that rats are able to acquire a strategy for solving a series of such problems. In the present case, adoption of the win–stay, lose–shift strategy would allow the rat to solve each sequential problem with no or few errors after the first "information" trial of a new problem. Thus, the learning set task provides a measure of higher order learning or, in Harlow's terms, the ability to "learn to learn" (Harlow, 1949, p. 53).

In the present study, the n-3-deficient rats performed about as well as n-3-adequate rats in acquiring most of the two-odor discrimination tasks. However, the terminal performance of n-3-deficient rats on the first problem set was inferior to that of the n-3-adequate rats, and the deficient rats failed to show errorless or near-errorless learning in the last six problems (Problem Sets 8–10) of the series. It is possible that these between-group differences are due to morphological or physiological changes in the olfactory system produced by the n-3-deficient diet. However, for several reasons, it is unlikely that the poorer performance of the n-3-deficient rats was due to changes in sensory capacity. First, the olfactory system is extraordinarily resilient to even gross anatomical insult, as described by Youngentob, Schwob, Sheehe and Youngentob (1997). They reported that rats with experimentally induced loss of more than 95% of their olfactory receptor epithelium had normal or near-normal thresholds for several odors. In related studies, Lu and Slotnick (1998) and Setzer and Slotnick (1998) demonstrated that rats with extensive lesions or toxin-

Table 5
Fatty Acid Composition of Phosphatidylcholine in the Frontal Cortex

Fatty acid	n-3 adequate	n-3 deficient
Nonessential		
12:0	0.14 ± 0.02	0.11 ± 0.02
14:0	0.32 ± 0.01	0.34 ± 0.02
16:0	40.30 ± 1.00	42.00 ± 0.70
18:0	13.54 ± 0.30	13.30 ± 0.20
20:0	0.23 ± 0.10	0.20 ± 0.02
22:0	0.13 ± 0.01	0.12 ± 0.02
24:0	0.20 ± 0.01	0.20 ± 0.01
Total saturates	54.80 ± 0.01	56.00 ± 0.51
16:1	0.52 ± 0.01	0.52 ± 0.02
18:1n-9	22.20 ± 0.20	21.02 ± 0.30**
18:1n-7	5.42 ± 0.10	5.70 ± 0.10*
20:1n-9	0.82 ± 0.04	0.79 ± 0.04
Total monounsaturates	29.08 ± 0.24	28.15 ± 0.28*
n-6 PUFAs		
18:2n-6	0.80 ± 0.10	0.60 ± 0.01***
18:3n-6	—	—
20:2n-6	0.12 ± 0.01	0.14 ± 0.02
20:3n-6	0.23 ± 0.02	0.22 ± 0.10
20:4n-6	6.40 ± 0.20	7.10 ± 0.12***
22:4n-6	0.80 ± 0.04	1.22 ± 0.10***
22:5n-6	0.10 ± 0.10	5.20 ± 0.20***
Total n-6	8.50 ± 0.30	14.70 ± 0.30***
n-3 PUFAs		
22:6n-3	7.64 ± 0.41	1.20 ± 0.10***
Total n-3	7.64 ± 0.41	1.17 ± 0.07***
Total fatty acid (mg/g brain wet weight)	22.23 ± 0.52	23.33 ± 0.84

Note. Data are presented as the mean (± *SEM*) of the weight percent. PUFAs = polyunsaturated fatty acids. Dashes indicate not detected. $n = 9$ per diet group.
* $p < .05$; ** $p < .01$; *** $p < .001$ (Student's t test).

Table 6
Fatty Acid Composition of Phosphatidylethanolamine in the Frontal Cortex

Fatty acid	n-3 adequate	n-3 deficient
Nonessential		
12:0	0.20 ± 0.03	0.20 ± 0.03
14:0	0.20 ± 0.04	0.20 ± 0.02
16:0 DMA[a]	2.50 ± 0.61	1.50 ± 0.20
16:0	7.60 ± 0.40	8.30 ± 0.20
18:0 DMA[a]	0.33 ± 0.10	0.30 ± 0.10**
18:0	28.00 ± 0.73	30.11 ± 0.30
20:0	0.20 ± 0.03	0.20 ± 0.03
22:0	0.20 ± 0.10	0.20 ± 0.10
24:0	0.22 ± 0.20	0.20 ± 0.04
Total saturates	39.00 ± 0.54	41.10 ± 0.21**
16:1	0.43 ± 0.10	0.30 ± 0.10
18:1n-9	10.40 ± 0.60	10.30 ± 0.23
18:1n-7	1.90 ± 0.13	2.30 ± 0.10**
20:1n-9	0.60 ± 0.12	0.30 ± 0.10*
Total monounsaturates	13.30 ± 0.60	13.20 ± 0.33
n-6 PUFAs		
18:2n-6	1.11 ± 0.21	0.40 ± 0.10**
18:3n-6	0.20 ± 0.04	0.20 ± 0.03
20:2n-6	—	—
20:3n-6	0.51 ± 0.10	—
20:4n-6	11.00 ± 0.30	14.20 ± 0.30***
22:4n-6	4.00 ± 0.30	4.90 ± 0.11**
22:5n-6	4.00 ± 0.51	21.60 ± 0.30***
Total n-6	20.30 ± 0.70	41.20 ± 0.34***
n-3 PUFAs		
22:5n-3	0.22 ± 0.10	0.20 ± 0.04
22:6n-3	27.40 ± 0.54	4.50 ± 0.20***
Total n-3	27.60 ± 0.53	4.62 ± 0.20***
Total fatty acid (mg/g brain wet weight)	22.23 ± 0.52	23.33 ± 0.84

Note. Data are presented as the mean (± *SEM*) of the weight percent. PUFAs = polyunsaturated fatty acids. Dashes indicate not detected. $n = 9$ per diet group.
[a] DMA indicates the dimethyl acetal derivative formed from the alk-l-enyl form of the phospholipid.
* $p < .05$; ** $p < .01$; *** $p < .001$ (Student's t test).

induced deafferentation of the olfactory bulbs showed little or no loss in a variety of odor detection and odor discrimination problems. Second, the n-3-deficient rats performed more poorly than the n-3-adequate rats in the initial and last problem sets of the learning set task but performed as well as n-3-adequate rats on Problem Sets 2–6. Although the order of odor presentations was not completely counterbalanced across problems, it seems unlikely that the first and last odor problem sets were the most difficult of the series.

It is unlikely that differences between groups were due to differences in thirst or motivation to obtain the water reinforcement. Although n-3-deficient rats have been reported to be polydipsic and consume more water than n-3-adequate rats after a period of water deprivation (Reisbick et al., 1990, 1991, 1992), the results of our progressive ratio test fail to demonstrate any difference between the groups in motivation to obtain a water reward.

Consistent differences between groups emerged in the latter half of the problem series and were characterized by lower accuracy scores of the n-3-deficient rats in the first block of 20 trials in the last three problem sets. Over the course of these discrimination tasks, most n-3-adequate rats began to show errorless or near-errorless acquisition, whereas most n-3-deficient rats performed more poorly and showed little or no improvement in their rate of acquisition. Nevertheless, after the first problem set, there were no between-group differences in terminal performance. These outcomes support the contention that n-3-deficient rats are able to learn simple odor discrimination tasks as well as n-3-adequate rats but fail to acquire, or more slowly acquire, a strategy for solving these problems. By increasing the number of problem sets, n-3-deficient rats may be capable of achieving errorless or near-errorless learning. However, their ability to do so is clearly inferior to that of the n-3-adequate rats. Therefore, a reasonable interpretation of these results is that n-3-deficient rats show a mild cognitive deficit in acquiring a higher order learning task.

In this regard, it is important to note that the present index of cognitive behavior (acquisition of a response strategy or the ability to "learn to learn") is qualitatively different from that used in prior behavioral studies of rats maintained on a polyunsaturated fatty acid-deficient diet. In those studies, deficits in learning avoidance or maze tasks were often attributed to changes in cognitive abilities. However, there may be more parsimonious explanations for the deficit in associative learning of simple tasks. In the present study, n-3-deficient rats performed more poorly than n-3-adequate rats in the very first problem set of the learning set task. A similar

Table 7
Fatty Acid Composition of Phosphatidylserine in the Frontal Cortex

Fatty acid	n-3 adequate	n-3 deficient
Nonessential		
12:0	0.20 ± 0.03	0.20 ± 0.02
14:0	0.20 ± 0.02	0.20 ± 0.03
16:0	4.01 ± 0.41	4.42 ± 0.30
18:0	41.33 ± 0.51	38.60 ± 0.60**
20:0	0.14 ± 0.02	0.14 ± 0.02
22:0	0.20 ± 0.03	0.20 ± 0.03
24:0	0.12 ± 0.02	0.14 ± 0.03
Total saturates	46.10 ± 0.63	43.80 ± 0.52**
16:1	0.40 ± 0.10	0.32 ± 0.10
18:1n-9	15.45 ± 0.34	15.11 ± 0.43
18:1n-7	1.72 ± 0.14	2.00 ± 0.10
20:1n-9	—	0.20 ± 0.03
Total monounsaturates	17.54 ± 0.43	17.60 ± 0.44
n-6 PUFAs		
18:2n-6	0.41 ± 0.10	0.52 ± 0.10
18:3n-6	—	—
20:2n-6	—	—
20:3n-6	—	—
20:4n-6	3.20 ± 0.30	2.61 ± 0.10
22:4n-6	4.30 ± 0.22	5.03 ± 0.20*
22:5n-6	1.20 ± 0.10	25.49 ± 0.24***
Total	9.01 ± 0.40	33.70 ± 0.32***
n-3 PUFAs		
22:5n-3	0.12 ± 0.02	0.11 ± 0.02
22:6n-3	27.26 ± 0.40	5.04 ± 0.21***
Total	27.38 ± 0.40	5.20 ± 0.22***
Total fatty acid (mg/g brain wet weight)	22.23 ± 0.52	23.33 ± 0.84

Note. Data are presented as the mean (± *SEM*) of the weight percent. PUFAs = polyunsaturated fatty acids. Dashes indicate not detected. $n = 9$ per diet group.
* $p < .05$; ** $p < .01$; *** $p < .001$ (Student's t test).

Table 8
Fatty Acid Composition of Phosphatidylinositol in the Frontal Cortex

Fatty acid	n-3 adequate	n-3 deficient
Nonessential		
12:0	0.31 ± 0.04	0.24 ± 0.04
14:0	0.11 ± 0.02	0.20 ± 0.02**
16:0	10.10 ± 0.30	9.30 ± 0.34
18:0	36.30 ± 0.24	35.80 ± 0.50
20:0	0.30 ± 0.04	0.51 ± 0.10**
22:0	—	—
24:0	0.23 ± 0.10	0.30 ± 0.03
Total saturates	47.30 ± 0.34	46.30 ± 0.42
16:1	0.81 ± 0.10	1.20 ± 0.11*
18:1n-9	8.51 ± 0.50	8.40 ± 0.60
18:1n-7	1.54 ± 0.04	1.41 ± 0.12
20:1n-9	0.20 ± 0.02	0.31 ± 0.04
Total monounsaturates	11.10 ± 0.52	11.30 ± 0.55
n-6 PUFAs		
18:2n-6	0.70 ± 0.10	0.90 ± 0.20
18:3n-6	—	—
20:2n-6	0.20 ± 0.02	0.30 ± 0.10
20:3n-6	—	—
20:4n-6	36.70 ± 0.70	36.60 ± 0.51
22:4n-6	4.30 ± 0.22	1.80 ± 0.21**
22:5n-6	0.94 ± 0.11	2.31 ± 0.20***
Total	0.30 ± 0.10	41.80 ± 0.42***
n-3 PUFAs		
22:6n-3	3.00 ± 0.10	0.79 ± 0.10***
Total	3.00 ± 0.10	0.79 ± 0.10***
Total fatty acid (mg/g brain wet weight)	22.23 ± 0.52	23.33 ± 0.84

Note. Data are presented as the mean (± *SEM*) of the weight percent. PUFAs = polyunsaturated fatty acids. Dashes indicate not detected. $n = 9$ per group.
* $p < .05$; ** $p < .01$; *** $p < .001$ (Student's t test).

deficit was found by Greiner et al. (2000) in their study of olfactory learning in n-3-deficient rats. This initial study of the deficit in discrimination learning is in agreement with the results of single-problem tasks reported in earlier studies (for reviews, see Bourre et al., 1993; Hamosh & Salem, 1998; Okuyama, Kobayashi, & Wantanabe, 1997). Our results indicate that, with continued training, n-3-deficient rats are able to perform simple discrimination tasks as well as controls.

It is unclear why n-3-deficient rats have a deficit in their initial learning of a discrimination problem. One explanation is a possible decrease in attentiveness to the odor stimuli by the n-3-deficient rats. However, the sampling time data in the learning set task provide no support for this conjecture. In fact, in all problems, the n-3-deficient rats spent at least as much time sampling the odor stimuli as did the n-3-adequate rats. There were no differences between the groups in the olfactory memory test. However, the nearly perfect performance of almost all rats in both groups points to a ceiling effect and suggests that the test did not provide a particularly sensitive measure of memory. Perhaps less extensive pretraining on the odor task or a longer interval between training and testing would provide a more sensitive measure of long-term odor memory. Thus, it may be premature to conclude that memory for odor discrimination recall is not affected by DHA deficiency.

In summary, the present study demonstrates that, through dietary manipulation, the loss of DHA in neuronal aminophospholipids leads to deficits in acquisition of a learning set. Our evidence indicates that these deficits were not due to changes in motivation, sensory capacity, or attentiveness to the discriminative stimuli but, rather, represent a mild retardation in the acquisition of a cognitive task.

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