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Olfactory discrimination deficits in $n-3$ fatty acid-deficient rats

Rebecca Sheaff Greiner[a], Toru Moriguchi[a], Burton M. Slotnick[b], Ana Hutton[a], Norman Salem, Jr.[a,*]

[a]Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, Division on Intramural Clinical and Biological Research, National Institutes of Health, 12420 Parklawn Drive, Park V Building/Room 158, Rockville, MD 20852, USA
[b]Department of Psychology, The American University, Washington, DC, USA

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Abstract

Docosahexaenoic acid (DHA), a long chain $n-3$ fatty acid, is present in high concentrations in the central nervous system. Although the role that DHA may play in neural function is not well understood, infants fed formulas containing low levels of $n-3$ fatty acids have decreased visual acuity and neurodevelopmental test scores. The present experiment assessed whether dietary manipulations that decrease the concentration of DHA in the brain interfered with olfactory-based learning. We fed rats a diet that provided adequate $n-3$ fatty acids or a diet that was deficient in $n-3$ fatty acids for two generations. The second generation $n-3$-deficient group had 81% less brain DHA (82% less in olfactory bulb) compared to the $n-3$-adequate group and made significantly more errors in a series of olfactory-cued, 2-odor discrimination tasks compared to the adequate group. These results suggest that lower levels of central nervous system DHA lead to poorer performance in a series of simple odor discrimination tasks. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: DHA; docosahexaenoic acid; Omega-3 fatty acids; Olfaction; Odor discrimination; Essential fatty acids; Behavior; $n-3$ fatty acid deficiency

1. Introduction

The high concentration of docosahexaenoic acid (DHA; 22:6$n-3$) in the brain and retina and their resistance to dietary challenges indicates that this $n-3$ polyunsaturated fatty acid is of functional importance in these organs [9,10,15,27,28]. Abnormal electroretinograms and diminished visual acuity have been reported in animals and humans fed diets deficient in $n-3$ fatty acids during development [1–5,8,11,12,21,22,34,39–41]. Several rodent behavioral studies have also examined the role of DHA in simple associative learning processes with mixed results [13,16,19,35,42]. Rodents are macrosmatic and their high olfactory acuity allows them to learn about their environment and ultimately, to increase their chances of survival. Recently, improved rodent behavioral methods based on olfaction have become available [18,30–32]. In fact, when odors are used as the salient cues, rats are able to demonstrate the acquisition of a learning set or near errorless learning [18], a feat previously only ascribed to primates.

The present study used an improved diet for reducing tissue levels of DHA and provides an initial assessment of olfactory discrimination learning in DHA-deficient rats. The olfactory task used consists of odor detection and discrimination problems [18,30–32] and, for future studies, this task can be expanded to assess more complex learning including matching to sample, short- and long-term memory and learning sets [18]. These and similar tests of brain function may provide sensitive tests of learning and memory-related performance in the $n-3$-deficient rat.

2. Methods

2.1. Subjects

Twelve 21-day-old Long–Evans female rats (Charles River, Portage, MI) were delivered to our animal facility, randomized into two groups and began consuming one of two experimental diets ($n=6$ per dietary group). Animals were maintained on a 12-h light–dark cycle at a temperature

* Corresponding author. Tel.: +1-301-443-2393; fax: +1-301-594-0035.
E-mail address: nsalem@niaaa.nih.gov (N. Salem).

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Table 1
Diet composition

	g/100 g diet	
Casein, vitamin-free	20	
Carbohydrate	60	
Cornstarch	15	
Sucrose	10	
Dextrose	20	
Maltose–dextrin	15	
Cellulose	5	
Salt mix[a]	3.5	
Vitamin mix[a]	1	
L-cystine	0.3	
Choline bitartrate	0.25	
TBHQ	0.002	
Fat	10	
Fat sources	$n-3$ deficient	$n-3$ adequate
Coconut oil (hydrogenated)	8.1	7.75
Safflower oil	1.9	1.77
Flaxseed oil	–	0.48

[a] As detailed in Ref. [26].

of 20–21°C and given free access to food and (initially) water. The F1 females were mated with chow-fed males. Their offspring, the F2 generation, were weaned to the diet of the dam and maintained on these diets throughout the study. One F2 male animal was selected (based on body weight) per litter for the olfactory discrimination task. At 54 days of age, 2 weeks prior to training, the F2 generation male rats were placed on a water deprivation schedule until the end of the experiment. The 2-week water deprivation period prior to training has been used extensively in this lab and is an adjustment period to allow rats to achieve a stable motivational state. Each rat received 8 ml water/day, approximately 1/3 of the free access water intake for rats of this age. Behavioral training of F2 generation males began at 68 days of age, behavioral testing immediately followed, and tissues from these animals were collected for fatty acid analysis at 12 weeks of age. One of the F2 generation males maintained on the deficient diet was excluded due to failure to acquire the initial operant task. Thus, there were five F2 generation rats in the $n-3$-deficient group and six in the $n-3$-adequate group. All animal procedures were approved by the National Institute on Alcohol Abuse and Alcoholism Animal Care and Use Committee.

2.2. Dietary treatments

The diets were based on the AIN93 [26] formulation with several modifications necessary to obtain the extremely low $n-3$ fatty acid level requirements of this study (Table 1). Because rats are very efficient in incorporating $n-3$ fatty acids, all components of the diets were analyzed for the presence of $n-3$ fatty acids. Cornstarch, a source of carbohydrate, contains a significant quantity of 18:3$n-3$ and thus maltose–dextrin was used as a partial carbohydrate substitute. Casein, the protein source, also contains some $n-3$ fatty acids including 18:3$n-3$ and 22:5$n-3$. Therefore, vitamin-free casein, a lower fat product, replaced the unprocessed casein. The difference between the two experimental diets, $n-3$ adequate and $n-3$ deficient, was the ratio of oils used to supply fat. Both diets contained 10% fat and differed only in the addition of a small amount of flaxseed oil in place of an equal amount of hydrogenated coconut oil to supply the $n-3$ fatty acids the $n-3$-adequate diet. The fatty acid composition of the total diets is shown in Table 2.

2.3. Olfactometer

Odor discrimination tests used two identical 8-channel olfactometers similar in design to that described previously [7]. A special feature of the unit was the use of pinch valves to control odor flow in the odor saturator bottles. The valves operate by occluding a short piece of odorless soft tubing (C-flex, Cole Parmer) connecting the saturator to the upstream and downstream air distribution manifolds. When a new odor was used in the system, all glass and Teflon connectors were washed with 95% ethanol and the tubing replaced. This served to completely eliminate any potential contamination from prior odors. The olfactometers and

Table 2
Fatty acid composition of the diets (wt.% of total fatty acids)

Fatty acid[a]	$n-3$ deficient	$n-3$ adequate
8:0	0.8	1.9
10:0	3.8	3.8
12:0	39.7	36.5
14:0	16.7	15.8
16:0	9.8	9.6
18:0	9.7	9.4
20:0	0.2	0.2
22:0	0.1	0.1
24:0	0.1	0.1
Total saturated	80.8	77.2
16:1$n-7$	0.03	0.03
18:1$n-9$	3.5	3.9
18:1$n-7$	0.3	0.3
20:1$n-9$	0.1	0.1
22:1$n-9$	0.01	0.02
Total monounsaturated	3.9	4.3
18:2$n-6$	15.1	15.3
20:2$n-6$	0.05	nd
Total $n-6$	15.1	15.3
18:3$n-3$	0.04	3.1
Total $n-3$	0.04	3.1
$n-6/n-3$	346	5
Total PUFA	15	18

nd = not detected.

[a] The following fatty acids were analyzed and were not present at detectable levels: 12:1, 14:1, 20:3$n-6$, 20:4$n-6$, 20:5$n-3$, 22:5$n-3$, and 22:6$n-3$.

digital interfaces were obtained from Knosys Olfactometers Ltd. (Bethesda, MD). The odor vapor, generated by passing 50 cc/min over the surface of the odorant material, was manifolded with a 1950 cc/min stream of clean air before being introduced to the sampling port. Thus, the concentration of all odor stimuli experienced by the rat at the sampling port of the multiple odor system was 2.5% of the concentration of the headspace above the liquid odorant.

The Plexiglas test chamber was similar to that previously described [18]. The front panel of the chamber contained a 25-mm-diameter glass odor sampling tube, a magnetic buzzer, and a 24 VDC lamp. The 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 via the common and normally open ports of a three-way solenoid valve. The top of the tube was connected to a 25-cfm exhaust fan via flexible hose. A 20-mm-diameter hole in the tube and chamber wall served as a sniff port for sampling odor stimuli. Snout insertions into the tube were detected by an infrared photocell unit. A 13-gauge stainless steel tube ending in a 3-mm ball served to record responses and to deliver water reinforcement. The ball end of the tube was located 50 mm to one side and 50 mm above the sniff port. The tube was connected to a water reservoir via a two-way, normally closed solenoid valve. Lick responses on the tube were detected by a sensitive circuit connected between it and the stainless steel floor of the chamber. A 25-cfm intake fan was mounted on the opposite wall. The fan maintained the chamber under positive pressure and insured that odor stimuli introduced to the sampling tube could not escape into the chamber. All training and test procedures for each olfactometer were controlled by separate 486 PC computers via Knosys digital interfaces. Control programs were written in QBASIC.

2.4. Olfactory training and test procedures

Beginning at approximately 9 weeks of age, the F2 generation rats were trained to detect and discriminate odors using procedures that have been described in detail [30]. Briefly, in initial sessions, rats were trained using standard operant conditioning methods to insert their snout in the odor sampling tube for a 0.04-ml water reinforcement. Snout insertion produced a 1.0-s sample of 1% vapor of ethyl acetate. When stable behavior on this task was achieved, all rats were given a series of seven 2-odor discrimination problems.

In each 2-odor discrimination problem, one odor served as the S+ stimulus and the other as the S− stimulus. There was no specific cue that indicated the start of a trial. After the intertrial interval, snout insertion into the odor sampling tube initiated the trail. A response on the water delivery tube after delivery of the S+ stimulus was reinforced with 0.04 ml of water. A response on the water delivery tube after delivery of the S− stimulus was punished by extending the intertrial interval to 10 s from 4 s. Responding on S+ trials (hits) and not responding on S− trials (correct rejections) were scored as correct. Responding on S− trials (false alarms) and not responding on S+ trials (misses) were scored as errors.

Table 3
Odors[a] used in olfactory-based learning task

Problem	S+	S−
1	Peppermint	Orange
2	Butter	Banana
3	Strawberry	Onion
4	Brandy	Butternut
5	Lemon	Vanilla
6	Cherry	Coconut
7	Prune[b]	Tomato[b]

[a] All from McCormick (diluted to 5% by vol. with water) except prune and tomato juice.
[b] Juice — undiluted; Giant Food supermarket.

A different 2-odor discrimination problem was presented each day to all rats. One hundred trials were given to rats on Problems 1–5 and 60 trials were given on Problem 6 and 7. On each problem, each block of 20 trials was scored. Odors were presented in a random order with the constraint that each odor was presented 10 times in each block of 20 trials. Pairs of odors for each problem were generated by random selection from a series of McCormick (Hunt Valley, MD) and Giant Food (Landover, MD) food flavoring materials (Table 3). The odorants, except juices, were diluted to 5% with water shortly before use. Errors in each block of 20 trials were scored and criterion was set at 90% correct responding in a block of 20 trials.

2.5. Fatty acid analysis

At the termination of behavioral tests, rats were euthanized by decapitation, the brains removed and the right olfactory bulb and remainder of the right hemisphere were frozen at −80°C. Lipids were extracted [6] from both tissues, the methyl esters prepared using BF_3-methanol and analyzed by gas chromatography as previously described [29]. The fatty acid composition of the olfactory bulb has been previously published [14]. The fatty acid methyl ester 23:0 was used as an internal standard and peaks were identified by comparison with standard mixtures of fatty acids (Nu Chek Prep, Elysian, MN). Data are expressed as weight percent of total fatty acids.

2.6. Statistical analysis

The fatty acid composition was analyzed by the Student t test. The Bonferroni correction was used to adjust the alpha level due to the large number of fatty acids compared. The P value was set at .002. A two-way analysis of variance (ANOVA) with diet as the between group factor and problem as the repeated measure was used to compare group error scores over the seven problems. This was

Table 4
The fatty acid composition of $n-3$-adequate and $n-3$-deficient rat brains

Fatty acid	$n-3$ deficient ($n=5$)	$n-3$ adequate ($n=6$)
14:0	0.3 (0.2)	0.6 (0.4)
16:0	16.3 (0.2)	17.0 (0.9)
18:0	18.5 (0.2)	18.6 (0.5)
20:0	0.7 (0.09)	0.6 (0.07)
22:0	0.9 (0.06)	0.8 (0.05)
24:0	1.7 (0.2)	1.6 (0.2)
Total saturated	38.5 (0.6)	39.2 (1.2)
$16:1n-7$	0.3 (0.03)	0.4 (0.06)
$18:1n-9$	15.3 (0.5)	16.2 (0.4)
$18:1n-7$	3.7 (0.1)	3.6 (0.1)
$20:1n-9$	2.0 (0.2)	1.9 (0.3)
$22:1n-9$	0.2 (0.02)	0.2 (0.03)
$24:1n-9$	3.3 (0.3)	3.3 (0.3)
Total monounsaturated	24.9 (0.7)	25.5 (0.9)
$18:2n-6$	0.46 (0.0.3)	0.5 (0.04)
$20:3n-6$	0.3 (0.03)	0.3 (0.02)
$20:4n-6$	8.8 (0.3)	8.4 (0.4)
$22:4n-6$	3.6 (0.08)	2.7 (0.09) *
$22:5n-6$	9.0 (0.5)	0.4 (0.05) *
Total $n-6$	22.3 (0.7)	12.4 (0.5) *
$22:5n-3$	0.008 (0.02)	0.13 (0.01) *
$22:6n-3$	2.3 (0.1)	11.8 (0.7) *
Total $n-3$	2.3 (0.1)	11.9 (0.7) *
$20:3n-9$	0.08 (0.003)	0.06 (0.01)
$22:5n-6/22:6n-3$	3.9 (0.2)	0.03 (0.004) *
$22:5n-6+22:6n-3$	11.3 (0.6)	12.2 (0.7)
$n-6/n-3$	9.6 (0.5)	1.0 (0.04) *
$n-6+n-3$	24.6 (0.7)	24.4 (1.1)

Percent of total fatty acids presented as mean (standard deviation).
Values may not equal 100% due to inclusion of unidentified peaks in the total.
$18:3n-6$, $18:3n-3$, and $20:5n-3$ were analyzed but not detected.
* $P<.002$.

followed by preplanned comparisons (t tests) to determine specific differences on each problem. All data were analyzed using Statistica (Statsoft, Tulsa, OK).

3. Results

3.1. Body and organ weight

The experimental diets had no effect on body weight (mean ± S.D.) of $n-3$-adequate (216 ± 20) or $n-3$-deficient (218 ± 26) F1 females at 56 days of age. There were also no differences in body weight (mean ± S.D.) between the two groups of F2 males before ($n-3$ adequate, 265 ± 4; $n-3$ deficient, 256 ± 7) or after ($n-3$ adequate, 356 ± 12; $n-3$ deficient, 345 ± 8) the water deprivation period. Differences between $n-3$-adequate and $n-3$-deficient brain (0.61 ± 0.03 vs. 0.64 ± 0.02) and liver (3.7 ± 0.07 vs. 3.7 ± 0.2) weights when expressed as a percent of body weight were not significant.

3.2. Fatty acid analysis

Brain fatty acid composition was significantly altered by the $n-3$-deficient diet (Table 4). DHA levels were decreased by 81% in the total lipid extract in the $n-3$-deficient brains compared to that of the $n-3$-adequate animals. The DHA decrease was matched by a 21.5-fold increase in docosapentaenoic acid ($22:5n-6$) in the deficient group to maintain 22-carbon polyunsaturated fatty acid levels in the brain. Brain levels of arachidonic acid ($20:4n-6$) and $22:4n-6$ were also significantly higher, 4.8% and 33%, respectively, in the $n-3$-deficient group.

3.3. Behavioral results

As shown in Fig. 1A, $n-3$-adequate rats rapidly acquired each of the seven 2-odor discrimination tasks. Except for Problems 1 and 4, the percent correct on the second block of trials on each problem were 85% or higher. On average, $n-3$-deficient rats performed more poorly

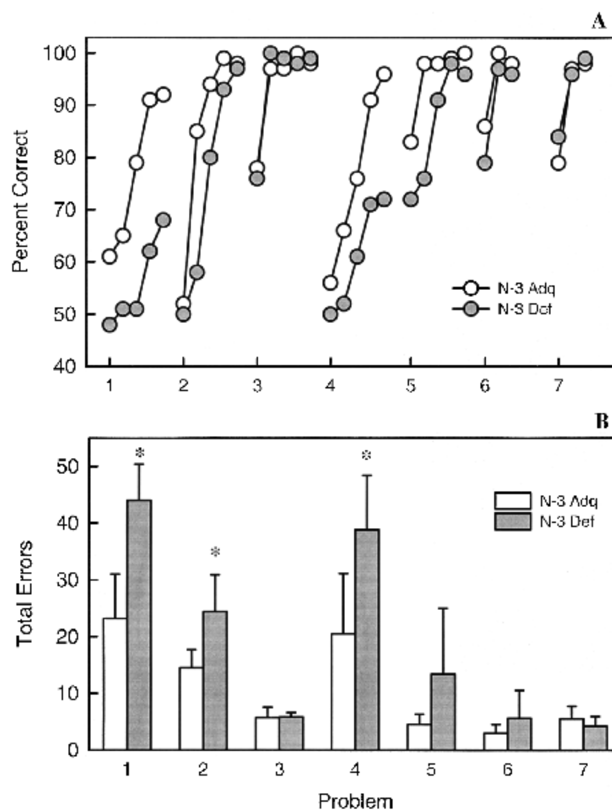

Fig. 1. (A) Mean percent correct on each block of 20 trials for each problem in the $n-3$-deficient and $n-3$-adequate groups. (B) Mean total errors for each group on each problem (1 problem/day). Repeated measures ANOVA indicated a diet effect [$F(1,9)=16.83$, $P=.003$], problem effect [$F(6,54)=55.59$, $P<.001$], and interaction [$F(6,54)=6.81$, $P<.001$]. Preplanned comparisons for each specific problem indicated a significant difference on Problem 1 [$F(1,9)=22.4$, $P=.001$], Problem 2 [$F(1,9)=10.7$, $P=.01$], and Problem 4 [$F(1,9)=8.81$, $P=.02$]. Vertical lines show standard deviations. Asterisks indicate significant differences between groups as stated above.

compared to $n-3$-adequate rats (Fig. 1B). The repeated measures ANOVA analysis indicated a significant effect of diet [$F(1,9)=16.83$, $P=.003$], as well as a significant effect of problem [$F(6,54)=55.59$, $P<.001$] and a significant diet by problem interaction [$F(6,54)=6.81$, $P<.001$]. The $n-3$-deficient animals acquired Problems 1, 2, and 4 more slowly than $n-3$-adequate animals and differences between groups for total errors (Fig. 1B) were significant for Problems 1 [$F(1,9)=22.4$, $P=.001$], 2 [$F(1,9)=10.7$, $P=.01$], and 4 [$F(1,9)=8.81$, $P=.02$]. However, on other problems, notably 3, 5, 6, and 7, acquisition functions and errors to criterion of $n-3$-deficient rats were not statistically significantly different from that of $n-3$-adequate rats.

Because trials were self-initiated, session duration provided one measure of motivational state. Mean session duration was calculated by averaging session durations for each group on each day and then averaging the group means over Days 1–5 or 6–7. Mean duration for the $n-3$-adequate and $n-3$-deficient groups was 23.3 ± 2.7 and 23.9 ± 4.2 min for the 100 trial sessions (Problems 1–5) and 12.6 ± 0.3 and 11.4 ± 0.2 min for the 60 trial sessions (Problems 6 and 7), respectively. None of the differences between groups for session duration were significant.

4. Discussion

It is clear from our results that addition of a small quantity of flaxseed oil to the rat diet when fed over two generations leads to significantly better performance in acquisition of a 2-odor discrimination task. Our interpretation of this finding is that the brain DHA level is the critical variable responsible for this result. The dietary modifications and choice of oils was successful in producing animals with vastly different levels of central nervous system DHA. The $n-3$-deficient brain had only 19% of the DHA found in the $n-3$-adequate brain. This confirms our previous findings reported for the olfactory bulb where an 82% reduction in DHA was observed [14]. This is a striking reduction in DHA after only two generations of animals and is comparable to levels achieved in second generation, artificially reared rats [38]. Previous studies using only dietary manipulation have not been successful in depleting central nervous system DHA to this extent probably because of the intrusion of "hidden" $n-3$ fatty acids in commonly used carbohydrate, protein, and fat sources.

Prior animal behavioral studies on the effects of fatty acid depletion have been criticized for examining associative learning using techniques that are not relevant to the animal's natural state [36,37]. The majority of these rat studies used visual cues as discriminative stimuli. While vision is certainly a primary sensory modality for primates, macrosmatic animals like rats rely heavily on olfactory cues in learning about their environment [30] and recent studies have demonstrated that rats rapidly acquire a number of relatively complex tasks when provided with odor cues. Thus, olfactory-based learning may provide a more appropriate modality for assessment of sensory and cognitive functions in the $n-3$ fatty acid-deficient rat [30,32].

This study is, in fact, the first to examine olfactory learning in $n-3$-deficient rats. Our results indicate that $n-3$-deficient rats performed more poorly than $n-3$-adequate rats on some problems but not others. The poor performance is not due to an inability to discriminate odors; most $n-3$-deficient rats achieved criterion performance on most of the seven odor discriminations and performed as well as controls on several of these problems. Almost all errors for both groups were false alarms; very few misses were recorded. In general, $n-3$-deficient rat behavioral deficits were characterized by slower acquisition (Problems 1, 2, and 4) and, in Problems 1 and 4, poorer terminal performance relative to that of $n-3$-adequate rats. At present, it is unclear whether the deficits are related to problem difficulty, attentional factors, or delay in acquiring a strategy for solving these 2-odor discrimination tasks. In the first problem, a novel 2-odor task was abruptly introduced after a series of S+ only presentations of the training odor. The problem was the first in which responding to an odor stimulus (S−) was not reinforced and rats had to learn to attend to the qualitative and/or quantitative differences between the two stimuli. The poorer performance of the $n-3$-deficient group on this task may point to a deficit in attention, a decrease in the inhibitory consequences of nonreinforcement, or a deficit in associating reinforcement with the S+ stimulus. Performance on Problem 4 suggests a role for problem difficulty. This discrimination was somewhat more difficult for controls and produced particularly high error scores for the deficient rats. Clearly, additional studies will be required to distinguish in a conclusive manner between these possibilities in explaining the poorer performance of $n-3$-deficient rats.

Another factor that may influence performance of $n-3$-deficient rats is their level of motivation. Since partial water deprivation was employed to increase the motivation to work for a water reward, differences in their responses to water deprivation may lead to differences in their motivation. This concept is supported by work in primates in which $n-3$-deficient rhesus monkeys develop polydipsia [23–25]. However, any differences in water balance between our $n-3$-deficient and $n-3$-adequate groups were not of a magnitude such that it affected their body weights, as body weights of both groups were the same before and after water deprivation. Their general level of arousal was also similar as their siblings showed no differences between groups in mean ambulatory time measured over 4 days or in anxiety as measured in the elevated plus maze [20]. Because trials were self-initiated, the overall session duration provides one measure of motivation. Total session durations were not different between the groups. When the 10-s punishment times were subtracted from the session duration data, the mean session durations for $n-3$-adequate and $n-3$-deficient rats were 21.1 ± 1.4 and 19.6 ± 1.7 on Days 1–5 and

11.9 ± 0.04 and 10.7 ± 0.05 on Days 6–7, respectively. The $n-3$-adequate group had a significantly longer mean session duration on Days 6–7 ($P<.01$). These observations mitigate against an effect of $n-3$ deficiency on the general level of arousal or motivation, but these factors cannot be excluded with the available data.

There are similarities in the sensory transduction process between vision and olfaction [33] and both sensory tissues contain high levels of DHA. Because the $n-3$-deficient diet produced a decrease in DHA in the olfactory bulb, the possibility remains that the deficits observed in the present study may stem from changes in transduction or other sensory as opposed to associative processes. While the present experiment cannot distinguish between these two possibilities, it seems unlikely that the sensory deficit hypothesis could account for the poorer performance of $n-3$-deficient rats in this study. This is because rats with extensive destruction of the olfactory epithelium or olfactory bulb show few, if any, deficits in odor sensitivity [43] or in simple odor discrimination tasks of the type used in the present study [17]. Also, in unpublished studies in this laboratory, we found that $n-3$-deficient rats performed well on a relatively difficult odor mixture discrimination task. That is, in a preliminary experiment, it was observed that $n-3$-deficient rats performed as well as $n-3$-adequate rats in their ability to detect acetic acid at a 0.01% dilution when masked with a 50-fold higher concentration (0.5%) of propionic acid.

In summary, the present results demonstrate our modification of the AIN93 diet fed over two generations produces a profound deficit in the $n-3$ fatty acids in brain and that $n-3$-deficient rats show selective deficits in acquisition of odor discrimination tasks.

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