One generation of n-3 polyunsaturated fatty acid deprivation increases depression and aggression test scores in rats

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Abstract Male rat pups at weaning (21 days of age) were subjected to a diet deficient or adequate in n-3 polyunsaturated fatty acids (n-3 PUFAs) for 15 weeks. Performance on tests of locomotor activity, depression, and aggression was measured in that order during the ensuing 3 weeks, after which brain lipid composition was determined. In the n-3 PUFA-deprived rats, compared with n-3 PUFA-adequate rats, docosahexaenoic acid (22:6n-3) in brain phospholipid was reduced by 36% and docosapentaenoic acid (22:5n-6) was elevated by 90%, whereas brain phospholipid concentrations were unchanged. N-3 PUFA-deprived rats had a significantly increased ($P = 0.03$) score on the Porsolt forced-swim test for depression, and increased blocking time ($P = 0.03$) and blocking number ($P = 0.04$) scores (uncorrected for multiple comparisons) on the isolation-induced resident-intruder test for aggression. Large effect sizes ($d > 0.8$) were found on the depression score and on the blocking time score of the aggression test. Scores on the open-field test for locomotor activity did not differ significantly between groups, and had only small to medium effect sizes. This single-generational n-3 PUFA-deprived rat model, which demonstrated significant changes in brain lipid composition and in test scores for depression and aggression, may be useful for elucidating the contribution of disturbed brain PUFA metabolism to human depression, aggression, and bipolar disorder.—DeMar, J. C., Jr., K. Ma, J. M. Bell, M. Igarashi, D. Greenstein, and S. I. Rapoport. **One generation of n-3 polyunsaturated fatty acid deprivation increases depression and aggression test scores in rats.** *J. Lipid Res.* 2006. **47:** 172–180.

Supplementary key words docosahexaenoic • locomotor • rat • activity • behavior • diet

Docosahexaenoic acid (DHA, 22:6n-3), a nutritionally essential n-3 polyunsaturated fatty acid (n-3 PUFA) enriched in brain phospholipids (1), is important for maintaining normal brain structure and function (2–4). DHA cannot be synthesized de novo in mammalian tissue, but must be obtained directly from the diet or by elongation from its shorter chain nutritionally essential precursors, including α-linolenic acid (α-LNA, 18:3n-3) (5). Because these precursors can undergo significant β-oxidation in the presence of dietary DHA (6, 7), the most efficient way of providing DHA to the brain is through dietary DHA (8).

In response to dietary n-3 PUFA deprivation, the brain resists DHA loss by reducing DHA turnover within phospholipids (9–11), while not changing arachidonic acid (ARA, 20:4n-6) turnover. Severe depletion of brain DHA, approaching 80%, can be achieved by subjecting rats to n-3 PUFA deprivation for two or more consecutive generations (4, 9, 10), or by artificially rearing newborn pups on a formula free of n-3 PUFA (12). In addition, single-generational n-3 PUFA deprivation in rodents, starting after weaning at 21 days, reduces brain DHA by about 40% (11, 13).

Severe brain DHA depletion by multigenerational n-3 PUFA deprivation in rats is reported to reduce neuronal size in the hippocampus, hypothalamus, and parietal cortex (14). Also reduced are brain glucose metabolism (15), evoked acetylcholine release and cholinergic muscarinic receptor density in the hippocampus (16), norepinephrine in the cerebral cortex, hippocampus, and striatum (17), and dopamine release and vesicular monoamine transporter density in the cerebral cortex and nucleus accumbens (18, 19). Changes with less severe single-generational dietary n-3 PUFA deprivation include defective serotonergic and dopaminergic signaling (20, 21). Dietary DHA

Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; LA, linoleic acid; α-LNA, α-linolenic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SSRI, selective serotonin reuptake inhibitor; TLC, thin-layer chromatography.

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supplementation is reported to increase rat brain acetylcholine, norepinephrine, serotonin, and dopamine (16, 17, 22).

Several epidemiological studies suggest that a low dietary n-3 PUFA intake can increase human locomotor activity, aggression, and depression, as well as the prevalence of bipolar disorder and other psychiatric and neurodegenerative diseases in which these symptoms may be prominent (23–35). However, to date, most of these epidemiological correlations have not been tested or confirmed in controlled clinical trials (36, 37).

To further consider the potential role of low dietary n-3 PUFAs in behavioral disturbances, we thought it of interest to determine whether one-generational nutritional n-3 PUFA deprivation in rats would increase locomotor activity, depression, or aggression. Accordingly, we subjected 21-day-old rat pups to 15 weeks of either an n-3 PUFA-deprived or an n-3 PUFA-adequate diet (11) and in the ensuing 3 weeks administered tests of each of the three behaviors in a fixed order that, as reported previously, does not produce cumulative or interactive effects between tests (38–42). We also separated each test by at least 3 days to minimize interactive effects, and administered the aggression test, in which an animal might be injured, as the last of the three tests. The tests have been validated as clinically relevant in a number of ways, including showing in their outcomes the effects of drugs known to inhibit or stimulate the corresponding behaviors in humans (42–45).

METHODS

The research protocol was approved by the Animal Use and Care Committee of the National Institute on Child Health and Human Development, and followed the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health, Publication 80-23).

Description of animals

Three groups of male 18-day-old Long-Evans rat pups (ten animals each) and their nursing surrogate mothers were purchased from Charles River Laboratories (Portage, MI). The pups were not littermates. The animals were housed in a facility with regulated temperature (24°C) and humidity (40–70%), under a 12 h light/dark cycle with ambient fluorescent lighting of 100 Lux. They were kept in 45 cm (L) × 24 cm (W) × 20 cm (H) transparent plastic cages with a wire-bar food/water hopper and plastic vented lid (Zytem™, Lab Products Inc.; Seaford, DE). The pups were allowed to nurse until 21 days old. Then they were removed from their mothers, mixed without identification, and assigned randomly to an n-3 PUFA-adequate or -deficient diet group (n = 15) (see below). Food and water were provided ad libitum, and food in the cage was replaced with fresh food every 2–3 days. Body weight was recorded daily.

After 15 weeks on a specific diet, each rat was placed in a separate cage and, for the ensuing 3 weeks, administered each of three behavioral tests in the following order: 1) open-field test for locomotor activity (41), 2) Porsolt forced-swim test for depression (42), and 3) the isolation-induced resident-intruder test for aggression (38). The isolation-induced resident-intruder test for aggression was given last, at 18 weeks, because of the possibility of a test-related injury. The rats were placed in individual cages at 15 weeks to bring out their territorial instincts and thus increase their "fighting potential" (40). The cages were adjacent to each other in a rack, so the rats could visually interact. They were provided with paper tubes for play, and were weighed every two days.

To prevent testing bias, a rat was chosen at random from each of the two diet groups, and given the test battery in the order outlined above. However, the open-field test was given to only ten of the fifteen rats in each group, because access to the test equipment was limited. Each test was completed in 2–3 days, and a rest period of at least 3 days was allowed before the next test was administered, making test interactions unlikely (43).

n-3 PUFA-adequate and -deficient diets

The n-3 PUFA-adequate and -deficient diets were prepared by Dyets, Inc. (Bethlehem, PA) and were identical to diets whose ingredients and fatty acid composition have been reported (11). Each diet was designed around a standard American Institute of Nutrition-93 formulation, with carbohydrate, protein, fat, fiber, salt, and vitamin/essential amino acid content set at 60, 20, and 10, 5, 3.5, and 1.5% (by weight), respectively (**Table 1**) (46, 47). Fat came from added hydrogenated coconut, safflower, or flaxseed oils. Hydrogenated coconut oil (~6%) was added to both diets as a saturated fatty acid base. Both diets contained safflower oil (~3%) to provide linoleic acid (LA, 18:2 n-6) at ~26% of total fatty acids. The safflower oil had very little α-LNA (<0.1%) and no measurable quantities of other n-3 PUFAs. Flaxseed oil (~1%) was added only to the n-3 PUFA-adequate diet to provide α-LNA at ~4% of total fatty acids. No other n-3 PUFA was found in the adequate diet. LA and α-LNA were set at 6% and 1%, respectively, of total caloric intake (3,935 kcal/kg), giving a ratio of 6:1 in the n-3 PUFA-adequate diet. Intake and the ratios of LA and α-LNA followed published requirements for optimal ARA and DHA accretion in rodent brain (48, 49).

Behavioral tests

The behavioral tests were conducted in a closed procedure room illuminated by 40W fluorescent ceiling lights (100 Lux), with minimal visual or auditory distractions.

TABLE 1. General composition of n-3 PUFA-adequate and n-3 PUFA-deficient diets

Component	n-3-Adequate	n-3-Deficient
	Weight % (g/100 g diet)	
Protein	20	20
Casein	20	20
Carbohydrate	60	60
Dextrose	20	20
Cornstarch	15	15
Maltodextrin	15	15
Sucrose	10	10
Fat	10	10
Hydrogenated coconut oil	6.0	6.6
Safflower oil	3.2	3.4
Flaxseed oil	0.8	—
Additives	10	10
Cellulose	5.0	5.0
Salts	3.5	3.5
Vitamins	1.0	1.0
Choline chloride	0.25	0.25
L-cysteine	0.25	0.25
THBQ	0.002	0.002

PUFA, polyunsaturated fatty acid; THBQ, tertiary-butylhydroquinone, an antioxidant.

Open-field test for locomotor activity. Because of limited access to test equipment, only ten rats from each of the two diet groups of fifteen, chosen at random, were administered the open-field test for locomotor activity (40, 41) after 15 weeks on the diet. The test was carried out using a Digiscan® locomotor activity monitor (Model RXYZCM, Omnitech Electronics; Columbus, OH), which consists of an open-top 40 cm (L) × 40 cm (W) × 30 cm (H) clear Plexiglas holding box equipped with photocell beam sensor bars (16 infrared sensors per bar, 2.5 cm apart) running horizontally across the walls (50). A horizontal-plane motion sensor bar was fixed to each wall at 4 cm above the floor, and a vertical-plane motion sensor bar was fixed to the right and left walls at 15 cm above the floor. Two holding boxes were used at the same time for separately testing an n-3 PUFA-deficient and an n-3 PUFA-adequate rat. The boxes were separated by >25 cm, and a heavy cardboard sheet served as a visual barrier between them.

A rat was placed in each box for 60 min, and a computer tabulated its movements automatically during each of six 10 min intervals (Accuscan Software, Digipro version 1.5). At the end of the test, the number of fecal pellets on the floor of the holding box was counted as a measure of anxiety (41). The rat was returned to its cage, and then the holding box was cleaned with 70% ethanol before receiving another rat. End points measured by computer during the 60 min period were frequency, distance, and/or duration of horizontal and vertical movements, stereotypies (repetitions), revolutions, and margin occupation (perimeter of holding box).

Porsolt forced-swim test for depression. Rats on n-3 PUFA-adequate and -deficient diets (n = 15) were administered the Porsolt forced-swim test for depression (40, 42). Selection was random from the two dietary groups until all rats in both groups were tested, but each rat was studied only once. The test apparatus consisted of a clear plastic cylindrical tank, 35 cm high and 25 cm in diameter, which was filled to a depth of 25 cm with tap water equilibrated overnight to room temperature (24°C). A glass plate covered the top of the tank to prevent escape while the rat was swimming. The water in the tank was replaced by fresh water at room temperature after three rats had been tested. To habituate the rat, it was forced to swim in the tank for 15 min, then removed and dried by toweling and a heat lamp, and returned to its cage. Twenty-four hours later, the rat was returned to the tank and allowed to swim for 5 min while its swimming activity was recorded by a video camera, mounted above the tank. After this, the rat was dried and returned to its cage. End points of the test were total time spent floating in an immobile position, without paddling of limbs (swimming).

Isolation-induced resident-intruder test for aggression. Rats in each of the two diet groups (n = 15) were administered the isolation-induced resident-intruder test (38, 44). Selection order of the "resident" test rat was at random from the two dietary groups until all rats in both groups were tested, but each rat was studied only once. During the test, the housing cage (missing hopper and lid) containing the "resident" rat was placed on the floor and covered with a glass plate, which was not weighted down, to allow for ventilation. The glass cover was pretreated with anti-fog eyeglass cleaner to prevent loss of visibility from moisture condensation (Visaclean®, Pfizer; New York, NY). A fully naive, two-month-old male Fischer-344 rat (Charles River Laboratories) weighing <200 g ("intruder" rat) was introduced into the cage, and was allowed to freely interact with the "resident" rat for 10 min before it was removed. Animal interactions were recorded by a tripod-mounted video camera placed directly above the cage. End points of the test were frequency and duration of aggressive

allogrooming and blocking of the "intruder" rat (51). Aggressive allogrooming was defined to occur when the "resident" rat vigorously preened the fur of the "intruder" along its back, sides, chest, neck, or face, and bracing the "intruder" with the forepaws often accompanied this. Preening of the "intruder's" anal-genital area or tail was not counted. Blocking was defined as the "resident" rat forcibly pushing the "intruder" into the cage walls or onto its back and holding it in position to prevent escape. Shoving and pinning the "intruder" with the forepaws or nose was common. Blocking and aggressive allogrooming interactions were deemed to be over when the "resident" rat broke contact and the "intruder" regained freedom of movement.

Analysis of brain lipids

Brain lipids were measured as reported previously (11), after the rats had been 18 weeks on a diet and when all behavioral tests had been completed. A rat was killed by intracardiac injection of sodium pentobarbital, and the head was immediately subjected to focused-head microwave irradiation (5.5 kW, 4.0 s) with an industrial microwave generator (Model S6F, Cober Electronics; Stamford, CT), and the brain was removed. Lipids from whole brain were extracted using a partition system of chloroform-methanol-0.5 M KCl (2:1:0.75; v/v/v) (52). The extracts were separated into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) classes, by thin-layer chromatography (TLC) on Silica gel 60 plates (EM Separation Technologies; Gibbstown, NJ) using a solvent system of chloroform-methanol-water-glacial acetic acid (60:50:4:1; v/v/v/v) (53). The TLC plates were sprayed with 0.03% TNS (6-p-toluidine-2-napthalene-sulfonic acid) (Acros Organics; Fair Lawn, NJ) in 50 mM Tris buffer (pH 7.4) (w/v) and lipid bands were visualized under ultraviolet light. Positions of brain phospholipid bands were identified using phospholipid standards run on the plates. Concentrations of phospholipid classes were determined by assaying the lipid phosphorus content of the TLC scrapes (54). To determine fatty acid concentrations, TLC scrapes containing brain phospholipids were converted to fatty acid methyl esters (FAMEs) using 1% H_2SO_4 in methanol (55). Prior to methylation, di-17:0 PC (Sigma-Aldrich; St. Louis, MO) was added to the phospholipids as an internal standard. FAMEs were separated on a 30 m × 0.25 mm i.d. capillary column (SP-2330, Supelco; Bellefonte, PA) using gas chromatography (GC) with a flame ionization detector (Model 6890N, Agilent Technologies; Palo Alto, CA). Runs were initiated at 80°C, with a temperature gradient to 160°C (10°C/min) and 230°C (3°C/min) in 31 min, and held at 230°C for 10 min. Peaks were identified by retention times of FAME standards. Fatty acid concentrations (μmol/g brain) were calculated by proportional comparison of GC peak areas to that of the 17:0 internal standard.

Lipid composition of n-3 PUFA-adequate and -deficient diets

Food pellets from the two diets were subjected to fatty acid analysis, following the methods described above for the brain. Briefly, six random ∼0.6 g samples (n = 3) of each diet were extracted using chloroform-methanol-0.5M KCl to obtain total lipids. Following the addition of di 17:0 PC as an internal standard, associated fatty acids were methylated with 1% H_2SO_4 in methanol, and the resulting FAMEs were separated by GC. Analysis of each total lipid extract was done in duplicate. Fatty acid concentrations were quantified by comparing GC peak areas to the peak area of the 17:0 internal standard.

Table 2 shows that the total fatty acid content of both diets equaled 154–156 mmol/g diet, or about 4g/100 g diet. LA in n-3 PUFA-adequate and -deficient diets equaled 36 ± 3 (SD) and

TABLE 2. Fatty acid composition of n-3 PUFA-adequate and n-3 PUFA-deficient diets

Fatty Acid	n-3 PUFA-Adequate Diet		n-3 PUFA-Deficient Diet	
	μmol/g Diet	% of total	μmol/g Diet	% of Total
12:0	54.6. ± 3.7	35.0	58.9 ± 3.7	38.3
14:0	22.3 ± 1.7	14.3	23.6 ± 1.6	15.3
14:1n-9	0.028 ± 0.020	0.018	0.034 ± 0.014	0.022
16:0	14.2 ± 1.1	9.1	14.3 ± 1.1	9.3
16:1n-9	0.061 ± 0.004	0.039	0.058 ± 0.011	0.038
18:0	11.5 ± 0.9	7.4	11.3 ± 0.8	7.3
18:1n-9	10.2 ± 0.9	6.5	8.2 ± 0.8	5.3
18:2n-6	36.2 ± 3.1	23.2	37.3 ± 3.7	24.3
18:3n-3	7.1 ± 0.6	4.5	0.099 ± 0.014	0.064
20:4n-6	0.020 ± 0.005	0.013	0.016 ± 0.014	0.011
22:6n-3	ND	ND	ND	ND
Total	156.2 ± 11.8	100	153.7 ± 11.7	100
Saturated	102.6 ± 7.3	65.7	108.1 ± 7.2	70.3
Monounsaturated	10.3 ± 0.9	6.6	8.3 ± 0.8	5.4
n-3 PUFA	7.1 ± 0.6	4.5	0.10 ± 0.01	0.064
n-6 PUFA	36.3 ± 3.1	23.2	37.3 ± 3.7	24.3

Data are mean ± SD; n = 6; ND, not detected.

37 ± 4 mmol/g diet, respectively, corresponding to 23% and 24% of total fatty acid. In both diets, LA was the primary source of n-6 PUFA, inasmuch as only a trace (0.01%) of ARA was present in each. The α-LNA content of the n-3 PUFA-adequate diet was 7 ± 1 μmol/g diet, 5% of total fatty acid, with only a trace (0.06%) found in the n-3 PUFA-deficient diet. Other common dietary n-3 PUFAs [EPA (20:5n-3), DPA (22:5n-3), or DHA] were not detected in either diet. The ratio of LA to α-LNA was 5 and 377 for the n-3 PUFA-adequate and -deficient diets, respectively. The saturated fatty acids, 12:0, 14:0, 16:0, and 18:0 were found in both diets ($\sim$106 μmol/g diet) and comprised 66–70% of their total fatty acid. Monounsaturated fatty acids in both diets consisted mainly of 18:1n-9, 5–7% of total fatty acids ($\sim$9 μmol/g diet).

Data analysis and statistics

Data from the open-field test were analyzed by converting the Digiscan activity monitor computer files into Excel 2000 format (Microsoft; Seattle, WA). The six 10 min recording intervals for each activity parameter were summed before comparisons were made between groups. For the Porsolt forced-swim and the isolation-induced resident-intruder tests, a single person (not the test administrator) scored the frequency and duration of activities from coded videotapes, which did not indicate whether the rat had been on an n-3 PUFA-deprived or -adequate diet. A rat was identified on the videotape before the test began, by alphabetical letter, test type, and date. The code was never revealed to the scorer.

Scored data were decoded and analyzed by the investigator who carried out the behavioral tests but who had no prior knowledge of each rat's classification, and were expressed as

mean ± SD. Data were screened for outliers, and t-test assumptions were assessed. For the two measures that violated the normality assumption (number of rotations and blocking events), Mann-Whitney U test results are reported. For the two violations of the homogeneity of variance assumption (horizontal time and number of stereotypies), Aspin-Welch results are reported (56). For all other measures, group comparisons were conducted using two-sample t-tests. For the behavioral measures, we first used the conventional α of 0.05. We also considered a more conservative approach and calculated separate Bonferroni corrections for each behavioral realm: locomotion α = 0.005 (0.05/10); depression α = 0.05 (0.05/1); aggression α = 0.012 (0.05/4) (57). We did not use a highly conservative overall Bonferroni correction (0.05/15 = 0.01), because of the large numbers of comparisons and the exploratory nature of the study (i.e., type I errors are preferred to type II/false-negative errors) (58). For the same reason, we did not use a Bonferroni correction for the lipid analytical data.

Cohen's d was calculated as a measure of effect size (59). An effect size is an index of the magnitude of an effect, with which to index what can be alternately called the degree of departure from the null hypothesis. Small, medium, and large effect sizes are equivalent to d values of 0.30, 0.50, and 0.80, respectively. Effect sizes are not influenced by low statistical power and are useful when conceptualizing the meaningfulness of a group difference in a behavioral study. An effect size, in the context of this study, also can be understood as the average percentile of the average deprived rat relative to the average control rat.

RESULTS

Body and brain weights

Body weight did not differ significantly between the n-3 PUFA-adequate (n = 15) and -deprived rats (n = 15) during the 15 weeks on their respective diets ($P > 0.05$). Initial body weights of the 21-day-old pups equaled 50.0 ± 5.9 g and 50.3 ± 4.7 g, respectively, whereas after 15 weeks, the weights equaled 664 ± 99 g and 687 ± 95 g, respectively. At 18 weeks, mean brain weights in the n-3 PUFA-adequate and -deficient groups also did not differ significantly, equaling 1.85 ± 0.16 g and 1.82 ± 0.18 g, respectively.

Brain lipid composition

Table 3 shows that n-3 PUFA deprivation did not significantly change the absolute concentration of any of the four brain phospholipid classes tested (PC, PE, PI, and PS), whereas **Table 4** shows that fatty acid concentrations within these classes were significantly altered. Docosapentaenoic acid (DPAn-6, 22:5n-6) largely replaced DHA. The n-3 PUFA-deprived rats had 36% less DHA in phospholipids than did the dietary-adequate animals ($P < 0.001$).

TABLE 3. Phospholipid concentrations in n-3 PUFA-deprived and n-3 PUFA-adequate rats

Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylinositol		Phosphatidylserine	
Adequate	Deprived	Adequate	Deprived	Adequate	Deprived	Adequate	Deprived
			Concentration (μmol/g brain)				
25.3 ± 0.6	24.7 ± 1.1	25.5 ± 2.2	25.0 ± 0.8	2.6 ± 0.2	2.6 ± 0.2	8.1 ± 0.2	7.9 ± 0.5

Data are mean ± SD (n = 5). There was no significant group difference in any phospholipid concentration ($P > 0.05$).

TABLE 4. Brain esterified fatty acid composition in n-3 PUFA-deprived and n-3 PUFA-adequate rats

Fatty Acid	PC Adequate	PC Deprived	PE Adequate	PE Deprived	PI Adequate	PI Deprived	PS Adequate	PS Deprived	Total PL Adequate	Total PL Deprived
					Concentration (μmol/g brain)					
16:0	22.2 ± 0.6	20.4 ± 1.4[a]	2.8 ± 0.2	2.8 ± 0.2	0.42 ± 0.07	0.42 ± 0.06	0.33 ± 0.05	0.15 ± 0.12[c]	25.7 ± 0.5	23.8 ± 1.7
16:1n-9	0.28 ± 0.01	0.24 ± 0.02[b]	0.14 ± 0.02	0.13 ± 0.02	0.011 ± 0004	0.011 ± 0.001	0.017 ± 0.002	0.006 ± 0.001[c]	0.44 ± 0.02	0.38 ± 0.04[a]
18:0	7.2 ± 0.2	6.1 ± 0.4[b]	7.7 ± 0.5	7.3 ± 0.5	1.3 ± 0.2	1.4 ± 0.1	6.8 ± 1.2	3.2 ± 0.4[c]	23.0 ± 1.5	18.0 ± 1.2[c]
18:1n-9	11.4 ± 0.3	9.9 ± 0.7[b]	8.3 ± 0.9	7.4 ± 0.6	0.31 ± 0.05	0.31 ± 0.05	3.7 ± 0.5	1.7 ± 0.2[c]	23.6 ± 1.3	19.3 ± 1.5[b]
18:2n-6	0.37 ± 0.01	0.27 ± 0.02[c]	0.18 ± 0.05	0.11 ± 0.02[b]	0.013 ± 0.002	0.013 ± 0.003	0.029 ± 0.004	0.010 ± 0.001[c]	0.59 ± 0.05	0.40 ± 0.04[c]
18:3n-3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
20:4n-6	2.3 ± 0.05	2.2 ± 0.1	4.7 ± 0.4	4.8 ± 0.4	1.2 ± 0.2	1.3 ± 0.1	0.60 ± 0.10	0.28 ± 0.03c	8.8 ± 0.6	8.7 ± 0.6
22:5n-6	0.036 ± 0.004	0.45 ± 0.10[c]	0.11 ± 0.04	1.6 ± 0.4[c]	0.015 ± 0.006	0.030 ± 0.010[a]	0.073 ± 0.014	0.40 ± 0.09[c]	0.23 ± 0.04	2.5 ± 0.6[c]
22:6n-3	1.6 ± 0.1	1.0 ± 0.1[c]	6.2 ± 0.6	4.6 ± 0.5[b]	0.14 ± 0.03	0.10 ± 0.01[a]	2.6 ± 0.5	0.94 ± 0.16[c]	10.6 ± 0.1	6.7 ± 0.8[c]
22:5 / 22:6	0.023 ± 0.002	0.44 ± 0.13[c]	0.017 ± 0.006	0.35 ± 0.10[c]	0.12 ± 0.07	0.29 ± 0.10[a]	0.028 ± 0.001	0.44 ± 0.12[c]	0.019 ± 0.006	0.38 ± 0.11[c]
n-6 / n-3	1.7 ± 0.07	2.9 ± 0.3[c]	0.80 ± 0.05	1.4 ± 0.2[c]	9.2 ± 0.2	13.7 ± 0.8[c]	0.27 ± 0.02	0.76 ± 0.14[c]	0.90 ± 0.03	1.7 ± 0.2[c]

Data are mean ± SD (n = 5). Fatty acid abbreviations: 16:0, palmitic; 16:1n-9, palmitoleic; 18:0, stearic; 18:1n-9, oleic; 18:2n-6, linoleic; 18:3n-3, linolenic; 20:4n-6, arachidonic; 22:5n-6, docosapentaenoic; 22:6n-3, docosahexaenoic. ND, not detected (<0.001 μmol/g). PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PL, phospholipid.

[a] P < 0.05.
[b] P < 0.01.
[c] P < 0.001, significant difference between n-3 PUFA-deprived and n-3 PUFA-adequate.

The largest reduction (64%) was in PS ($P < 0.001$), and PC, PE, and PI had 34, 25, and 26% reductions, respectively. DPAn-6 was increased by 92, 94, 48, and 82% in PC, PE, PI, and PS ($P < 0.001$), respectively. LA was reduced in PC, PE, and PS by 28, 35, and 64%, respectively ($P < 0.05$), but not in PI. ARA was reduced significantly only in PS (by 52%; $P < 0.001$). Saturated and monounsaturated fatty acids were unchanged in PE and PI, but were significantly reduced in PC and PS.

Behavioral test scores

Table 5 presents means and standard deviations for all behavioral measures administered to n-3 PUFA-adequate and -deprived rats, as well as P values corresponding to two-tailed tests. The last column in the table gives the effect size in terms of Cohen's d (see Methods) (59).

Open-field test for locomotor activity. Means of ten separate scores in each of the two groups were determined for the open-field test for locomotor activity after 15 weeks on each of the two diets. There was no significant difference for any test score (Table 5). Scores of the ten rats in each diet group given the open-field test did not differ from those of the whole group of fifteen rats in the other two behavioral tests ($P > 0.05$).

With regard to Cohen's d, a small-to-medium effect size was associated with marginal distance ($d = 0.42$), and small effect sizes were associated with number of rotations and stereotypies ($d = 0.38, 0.32$, respectively), with deprived rats tending to have greater mean scores. The remaining locomotion measures suggested inconsequential effects.

Porsolt forced-swim test for depression. Mean immobility time on the Porsolt forced-swim test for depression was significantly longer ($P = 0.03$) in the n-3 PUFA-deprived (2.26 ± 0.95 min) than -adequate rats (1.55 ± 0.75 min) (Table 5). Cohen's d for immobility time was 0.83, indicating a large effect size.

Isolation-induced resident-intruder test for aggression. The four individual scores on the isolation-induced resident-intruder test for aggression were highly correlated, with correlation coefficients ranging from 0.75 to 0.94 (data not shown). The n-3 PUFA-deprived rats demonstrated significantly more ($P = 0.04$) mean blocking events (6.13 ± 4.29) relative to controls (3.33 ± 4.48), and a longer ($P = 0.03$) mean blocking time (0.69 ± 0.51 min) relative to controls (0.34 ± 34 min). These differences did not remain significant following the Bonferroni correction for multiple comparisons. Notably, however, effect sizes associated with the four correlated aggression measures ranged from medium to large, $d = 0.55–0.82$ (blocking time).

DISCUSSION

This study demonstrates meaningful and large effects on measures of two aspects of behavior in rats deprived of n-3 PUFAs in their diets for 15–18 weeks, commencing at

TABLE 5. Body weight and scores on each of three behavioral tests in n-3 PUFA-deprived and n-3 PUFA-adequate rats

	n-3 PUFA-Adequate		n-3 PUFA-Deprived			
	Mean	SD	Mean	SD	P	Effect Size (Cohen's d)
Body weight (g)	663.70	102.98	687.34	87.84	0.50	0.25
Open-field test (locomotor activity, n = 10)						
Horizontal distance (cm)	1,733.40	1,075.62	1,885.10	529.49	0.69	0.18
Horizontal time (cm)[b]	3.17	1.92	3.27	0.79	0.92	0.06
Marginal distance (min)	961.80	582.84	1,172.80	411.12	0.36	0.42
Marginal time (min)	52.71	4.86	53.00	2.69	0.91	0.07
Vertical stands (number)	72.90	51.73	70.50	36.03	0.91	0.05
Vertical time (min)	3.14	2.26	3.65	2.14	0.60	0.23
Rotations (number)[a]	9.00	7.73	11.50	5.30	0.15	0.38
Stereotypy (number)[b]	223.80	75.56	242.40	31.73	0.50	0.32
Stereotypy time (min)	4.27	1.42	4.60	1.04	0.56	0.26
Fecal pellets (number)	3.80	2.82	3.00	3.83	0.60	0.24
Porsolt forced-swim test (depression, n = 15)						
Immobility time (min)	1.55	0.75	2.26	0.95	0.03	0.83
Isolation-induced resident-intruder test (aggression, n = 15)						
Allogrooming events (number)	5.33	3.74	7.93	5.59	0.14	0.55
Allogrooming time (min)	0.41	0.28	0.68	0.50	0.08	0.66
Blocking events (number)[a]	3.33	4.48	6.13	4.29	0.04	0.64
Blocking time (min)	0.34	0.34	0.69	0.51	0.03	0.82

P values for differences between means by t-tests are uncorrected for multiple comparisons.

[a] Mann-Whitney P value.

[b] Aspin-Welch P value. All other results are two-sample t-test values.

21 days of age (weaning). On the Porsolt forced-swim test for depression, deprived rats demonstrated significantly longer immobility time relative to controls, with a correspondingly large effect size. A large effect size also was evident on the isolation-induced resident-intruder test for aggression, with deprived rats demonstrating a longer blocking time. However, this difference, as well as the group difference in number of blocking events, while significant at α = 0.05, did not survive the Bonferroni correction (α = 0.05/4 = 0.012). Measures of locomotor activity on the open-field test did not differ significantly between n-3 PUFA-deprived and -adequate animals, nor did they have associated large effect sizes. The behavioral changes were accompanied by a 36% reduction in brain DHA with a roughly compensatory increase in DPAn-6 (46) and a reduction in ARA within PS, without any significant change in individual brain phospholipid concentrations. LA also was decreased in brain phospholipid.

To our knowledge, this is the first study in which rats deprived of n-3 PUFAs postweaning (for one generation) were subjected to tests to evaluate three aspects of behavior—locomotor activity, depression, and aggression—and in which analysis confirmed a significant reduction in brain DHA concentration. This single-generational deprivation model reduced brain DHA concentration by 36%, to a lesser extent than the 70–80% reductions caused by multigenerational deprivation (9). Thus, it probably is more clinically relevant than the multigenerational model. Single-generational human n-3 PUFA deprivation occurs with low seafood consumption, use of cooking oils low in n-3 PUFAs (60) and, in infants, with poor maternal nutrition or use of an n-3 PUFA-deficient milk formula (61–63).

The 15–18 weeks of n-3 PUFA deprivation significantly increased ($P = 0.03$) immobility time by about 30% on the Porsolt forced-swim test for depression, and this increase represented a large effect size ($d = 0.83$). With regard to the isolation-induced resident-intruder test for aggression, mean blocking time ($P = 0.03$) and number of blocking events ($P = 0.04$) were significantly increased, although statistical significance did not survive a Bonferroni correction for four comparisons. The effect size associated with blocking time was large ($d = 0.82$), whereas the effect size for each of the other three measures fell in the medium range ($d = 0.55$–0.66), but in all cases, it was higher than effect sizes for measures of locomotor activity (Table 5). The four measures of aggression in each group were highly correlated ($r = 0.75$ to 0.94).

Our findings agree generally with prior reports on single-generational n-3 PUFA deprivation in rodents, but less well with results from multigenerational deprivation or deprivation during gestation, suggesting a dose-behavioral response relation. Locomotor activity was increased in adult mice deprived of n-3 PUFAs over several generations (64), and in adult rats and mice whose mothers were deprived at conception or during gestation (65–67). Deprivation in a single generation did not alter locomotor activity (68), agreeing with our results. N-3 PUFA deprivation initiated during pregnancy and accompanied by excess dietary LA did not increase depression scores in mice in one study (67), nor did three consecutive generations of deprivation in another study (64). We found a significant difference and large effect size on the Porsolt forced-swim test for depression. Finally, our increased scores and medium-to-large effect sizes on the isolation-induced resident-intruder test are consistent with a report that increasing dietary α-LNA lowered aggression in mice (67), and with reports that Wistar rats with a genetic predisposition for killing mice placed in their cages have reduced plasma levels of DHA compared with controls (69–71). With regard to changes in brain lipid composition, our findings agree with our prior report involving more rats given identical n-3 PUFA-deficient and -adequate diets

for 15 weeks (11). The interpretation of these lipid changes is discussed in that report.

The clear detrimental effect of our single-generational n-3 PUFA deprivation on scores of depression and aggression, but not of locomotor activity, suggests a common brain target affecting the former two behaviors. Multiple neurotransmitter systems are affected by multigenerational n-3 PUFA deprivation, whereas the few studies on single-generational deprivation suggested disturbed serotonergic and dopaminergic neurotransmission (20, 21). Supporting a serotonergic contribution to our behavioral results is evidence that animals treated with selective serotonin reuptake inhibitors (SSRIs) have reduced aggression on appropriate tests (72) and that human depression can be ameliorated by fluoxetine, an SSRI (73). Additionally, low brain serotonin turnover has been correlated with impulsive aggression in nonhuman primates (74), whereas serotonin agonists can reduce aggression in rats (44). Finally, n-3 PUFA dietary supplementation in developing rats or piglets increased brain serotonin concentration (22, 62, 75).

DHA, an n-3 PUFA, competes with ARA, an n-6 PUFA, in multiple brain processes, including the conversion of ARA to eicosanoids via cyclooxygenase-2, membrane excitability, gene transcription, and neuroinflammation (76–78), whereas conversion of α-LNA to DHA is inhibited by excess n-6 PUFAs (5). On this basis, it has been hypothesized that brain functional integrity requires a correct balance of DHA and ARA metabolism, and that if this balance is disrupted, functional integrity will be disturbed (2, 76). Excess n-6 compared with reduced n-3 PUFA metabolism has been postulated to contribute to the symptoms of bipolar disorder (77), which include depression and often aggression and hyperactivity (79). Such an imbalance is consistent with reported negative effects of a low dietary n-6 PUFA in this disorder (80), of a positive effect of dietary n-3 PUFA supplementation (30, 31), of an antistress effect of dietary n-3 PUFA supplementation in rats (17), of evidence of increased depression and aggression in our n-6 PUFA deprivation study, and of the fact that the mood stabilizers lithium, valproic acid, and carbamazepine reduce brain ARA but not DHA metabolism when given chronically to rats at therapeutically relevant doses (53, 77, 81–83).

Final body weight, which did not differ significantly between the two diet groups, was not a significant covariate with any behavioral outcome. This was surprising, because n-3 PUFAs can bind to peroxisome proliferator-activated receptor transcription factors that activate gene expression of enzymes involved in fat oxidation (84). The Zucker diabetic rat has a decreased weight gain when fed fish oil containing DHA and eicosapentaenoic acid (EPA, 20:5n-3) (85), and transgenic mouse models of obesity, when fed excess α-LNA, show reduced weight gain and body fat content, and upregulated oxidative enzymes in the intestine and adipose tissue (86, 87). One possibility is that the high caloric or saturated fat content of our diets (11) negated an effect of n-3 PUFA deprivation on weight reduction.

This study is an exploration of the effects of limited single-generational n-3 PUFA deprivation on three areas of behavior in relation to brain lipid composition. It has limitations, and thus needs to be replicated and extended if the conclusions derived from it are to be further interpreted and applied. One limitation is that although the study was sufficiently powered to detect large effects (at $\alpha = 0.05$), it did not have sufficient power to detect group differences corresponding to medium effect sizes, particularly for the open-field test, in which only ten rats were studied. The study thus suffers from a possible elevated false-negative rate manifested in a failure to statistically detect meaningful effects of n-3 PUFA deprivation. A post hoc power analysis (with $\alpha = 0.05$ and power = 0.80) indicated that sixty-four animals would be required to achieve statistical significance for a medium effect size of 0.50 (59). This analysis should guide future investigations of the effects of n-3 PUFA deprivation on behavior in rats.

Using a multiple-comparison Bonferroni test is excessively conservative if variables are correlated and not independent of each other (58), as in our study. The Bonferroni test also is not always useful, because although it decreases type I error risk (false-positive risk), it increases type II (false-negative) risk, which is not good in an exploratory approach such as ours, with extensive and wide-ranging tests. Supporting this interpretation is the fact that effect sizes d are especially useful when there is insufficient power to achieve statistical significance by conventional t-tests (59). Our effect sizes were quite robust and clearly identified large effect sizes of n-3 PUFA deprivation on depression and aggression scores, but not on locomotor activity scores.

In summary, dietary n-3 PUFA deprivation for 18 weeks caused a 36% reduction in DHA, while increasing the concentration of DPAn-6, in rat brain phospholipids. This imbalance between brain n-3 and n-6 PUFA concentrations was accompanied by evidence, on standard behavioral tests, of increased depression and aggression, but not of increased locomotor activity. To the extent that the increased depression and aggression scores are clinically relevant, our n-6 PUFA deprivation rat model should help to illuminate the brain biochemistry and neurochemistry of human depression, aggression, and bipolar disorder.

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