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Combined Deficiency of Iron and (n-3) Fatty Acids in Male Rats Disrupts Brain Monoamine Metabolism and Produces Greater Memory Deficits Than Iron Deficiency or (n-3) Fatty Acid Deficiency Alone[1–3]

Jeannine Baumgartner,[4,7]* Cornelius M. Smuts,[7] Linda Malan,[7] Myrtha Arnold,[5] Benjamin K. Yee,[6,8] Laura E. Bianco,[9] Mark V. Boekschoten,[10] Michael Müller,[10] Wolfgang Langhans,[5] Richard F. Hurrell,[4] and Michael B. Zimmermann[4,11]

[4]Laboratory of Human Nutrition, and [5]Physiology and Behaviour Laboratory, Institute of Food, Nutrition and Health, and [6]Laboratory of Behavioural Neurobiology, ETH, Zurich, Switzerland; [7]Centre of Excellence in Nutrition, North-West University, Potchefstroom, South Africa; [8]Legacy Research Institute, Portland OR; [9]Department of Nutrition Sciences, The Pennsylvania State University, University Park, PA; [10]Nutrition, Metabolism and Genomics Group, Division of Human Nutrition, and [11]Division of Human Nutrition, Wageningen University, Wageningen, The Netherlands

Abstract

Deficiencies of iron (Fe) (ID) and (n-3) fatty acids (FA) [(n-3)FAD] may impair brain development and function through shared mechanisms. However, little is known about the potential interactions between these 2 common deficiencies. We studied the effects of ID and (n-3)FAD, alone and in combination, on brain monoamine pathways (by measuring monoamines and related gene expression) and spatial working and reference memory (by Morris water maze testing). Using a 2 × 2 design, male rats were fed an ID, (n-3)FAD, ID+(n-3)FAD, or control diet for 5 wk postweaning (postnatal d 21–56) after (n-3)FAD had been induced over 2 generations. The (n-3)FAD and ID diets decreased brain (n-3) FA by 70–76% and Fe by 20–32%, respectively. ID and (n-3)FAD significantly increased dopamine (DA) concentrations in the olfactory bulb (OB) and striatum, with an additive 1- to 2-fold increase in ID+(n-3)FAD rats compared with controls ($P < 0.05$). ID decreased serotonin (5-HT) levels in OB, with a significant decrease in ID+(n-3)FAD rats. Furthermore, norepinephrine concentrations were increased 2-fold in the frontal cortex (FC) of (n-3)FAD rats ($P < 0.05$). Dopa decarboxylase was downregulated in the hippocampus of ID and ID+(n-3)FAD rats (fold-change = −1.33; $P < 0.05$). ID and (n-3)FAD significantly impaired working memory performance and the impairment positively correlated with DA concentrations in FC ($r = 0.39$; $P = 0.026$). Reference memory was impaired in the ID+(n-3)FAD rats ($P < 0.05$) and was negatively associated with 5-HT in FC ($r = −0.42$; $P = 0.018$). These results suggest that the combined deficiencies of Fe and (n-3) FA disrupt brain monoamine metabolism and produce greater deficits in reference memory than ID or (n-3)FAD alone. J. Nutr. 142: 1463–1471, 2012.

Introduction

Growth and development in early life impose high nutritional requirements often not met by dietary sources. Iron (Fe) deficiency (ID)[12] is a common nutrient deficiency during childhood, particularly in developing countries (1), and children with low fish and seafood intakes and a high use of vegetable oils rich in (n-6) fatty acids (FA) are at risk of inadequate (n-3) FA intakes (2). Both Fe and (n-3) FA are essential nutrients for normal brain development (3).

Fe is a cofactor for enzymes involved in cell division and the synthesis of neurotransmitters, myelin, and brain eicosanoids (4–8) and therefore plays a central role in neuronal growth, differentiation, and myelination (8,9). Most studies of ID in rats have examined the pre- and early postnatal periods; less is known about the effects of ID in the postweaning phase. However, during this period, myelination continues, particularly in the frontal lobes, so that cognitive impairment due to ID may be caused by alterations in myelination or monoamine metabolism (10).

Similarly, (n-3) FA are essential for neuronal growth and differentiation, myelination, and eicosanoid synthesis (11–13).

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[3] Supplemental Figures 1 and 2 and Tables 1 and 2 are available from the ''Online Supporting Material'' link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
[12] Abbreviations used: ARA, arachidonic acid; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; FA, fatty acids; (n-3)FAD, (n-3) fatty acid deficiency; FC, frontal cortex; 5-HIAA, 5-hydroxyindoleacetic acid; Hip, hippocampus; 5-HT, serotonin; ID, iron deficiency; LCPUF, long-chain PUFA; MWM, Morris water maze; NE, norepinephrine; OB, olfactory bulb; PND, postnatal day; Str, striatum.
* To whom correspondence should be addressed. E-mail: jeannine.baumgartner@gmail.com.

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(n-3) FA deficiency [(n-3)FAD] alters dopaminergic and serotonergic neurotransmission (14), which may cause behavioral dysfunction (15). The long-chain PUFA (LCPUFA) DHA, the most abundant (n-3) FA in the brain, enhances neuronal membrane fluidity, which in turn affects cell signaling (16).

Thus, ID and (n-3)FAD early in life may impair brain development and function through shared mechanisms. Moreover, these deficiencies may directly interact via Fe-dependent hepatic desaturases involved in the conversion of essential FA into LCPUFA and/or via Fe-dependent cyclooxygenase involved in the synthesis of eicosanoids derived from LCPUFA (7,17,18). However, to our knowledge, there are no published data in the study of animal models on the consequences of concurrent ID and (n-3)FAD on brain development and cognitive functioning.

The aim of this study was to investigate the effects of ID and (n-3)FAD, alone and in combination, on the brain of juvenile rats. Our hypothesis was that these 2 deficiencies would interact and a concurrent deficiency would impair brain monoamine metabolism, as well as working and reference memory, to a greater extent than either deficiency alone.

Methods

Animals and housing. All experimental procedures were approved by the Veterinary Office of the Department of Health of the Canton of Zürich. At a commercial animal breeder (RCC), female Wistar rats (3 wk old) were fed a standard (Teklad Global Diet 2018S) or an (n-3)FAD diet (detailed below). At 11 wk of age, they were mated with 12-wk-old male breeders of the same strain. After the mating period, the females continued to consume their respective diet and the rat pups were kept with their dams until weaning. Twenty male rat pups born to the (n-3)FAD dams and 19 born to the dams fed a standard diet were brought to our animal facility (Schwerzenbach) and were included into the study at 21 ± 3 d of age [postnatal d (PND) 21]. Rats were individually housed in stainless steel cages, kept under a 12-h reversed light/-dark-cycle (lights off 0800–2000 h) at 21 ± 2°C and 55 ± 5% relative humidity, and had free access to their respective rat diet and Millipore water (Milli-Q UF Plus, Millipore). The rats were handled daily to acclimatize them to human touch and body weights were recorded 3 times/wk. Individual food intake was recorded daily by subtracting the amount of food left in the food bowl from the amount of food provided the previous day, considering the amount of food spilled on a paper lined underneath the wired cage floor.

Study design and diets. Over the depletion period of 5 wk, rats born to dams receiving the standard diet (n = 19) were randomly divided into 2 groups receiving either a control (n = 9) or an Fe-deficient (ID; n = 10) diet (**Supplemental Fig. 1**). The rats born to dams receiving the (n-3)FAD diet (n = 20) were also randomly allocated into 2 groups receiving an (n-3)FAD (n = 10) or an ID+(n-3)FAD diet (n = 10).

The purified experimental diets (**Supplemental Table 1**) were obtained commercially (Dyets) and were based on the AIN-93G (19) formulation with modifications in Fe content and fat source. All diets contained 10% fat. The basal AIN-93G formulation contained soybean oil at 70 g/kg diet and hydrogenated coconut oil at 30 g/kg diet. Based on the reported approximate FA composition of these oils, the basal diet was estimated to provide ~35 g/kg diet linoleic acid and 5 g/kg diet α-linolenic acid. The (n-3)FAD diets contained hydrogenated coconut oil at 81 g/kg diet and safflower oil at 19 g/kg diet, providing ~15 g/kg diet linoleic acid and ~0.04 g/kg diet α-linolenic acid (based on estimates). The Fe-sufficient diets contained 35 mg Fe/kg and the ID diets during the depletion period contained 3.0 mg Fe/kg. The Fe concentrations in diets were confirmed in spot samples from each batch of diets in our laboratory by using atomic absorption spectrometry.

All diets were custom prepared in powdered form to avoid lipid oxidation during pelletization, vacuum packed in bags of 2 kg, and stored at −20°C until use.

Tissue collection. At the end of the depletion period (PND 56 and 57), rats were exposed to CO_2 to introduce unconsciousness for blood collection by cardiac puncture and then killed by decapitation. The brains were rapidly removed and separated into 2 hemispheres along the midline on an ice-cool glass Petri dish. The cerebellum, olfactory bulb (OB), frontal cortex (FC), striatum (Str), and hippocampus (Hip) were isolated from both hemispheres by freehand dissection. Brain regions were placed into preweighed Eppendorf tubes, their wet weight was recorded, and then they were snap-frozen in liquid nitrogen and stored at −80°C. Per brain area, tissues obtained from the left and right hemispheres were alternately used for the analysis of brain Fe and FA contents.

Brain Fe analysis. Brain regions were homogenized and digested with nitric acid according to Erikson et al. (20) and total Fe concentrations measured using graphite furnace atomic absorption spectrometry (Perkin Elmer AA400).

Total phospholipid FA analysis. Lipids were extracted from each brain region with chloroform:methanol (2:1, v:v; containing 0.01% BHT) by a modification of the method of Folch et al. (21). The lipid extracts were concentrated and the neutral lipids separated from the phospholipids by TLC (Silica gel 60 plates, 10 × 20 cm, Merck) and eluted with diethyl ether:petroleum ether:acetic acid (30:90:1, v:v:v). The lipid band containing phospholipids was removed from the TLC plate and transmethylated with methanol:sulphuric acid (95:5, v:v) at 70°C for 2 h to yield FAME. The resulting FAME were extracted with water and hexane. The organic layer was evaporated, redissolved in hexane, and analyzed by quadrupole (QP) gas chromatography electron ionization mass spectrometry (GC-EI-MS) on an Agilent Technologies 7890A GC system equipped with an Agilent Technologies 5975C VL mass selective detector. The GC separation of FAME was carried out on an HB-5MS capillary column (30 m × 0.250 mm × 0.25 μm; Agilent J&W) using helium as the carrier gas at a flow rate of 0.9 mL/min. The GC injector was held at a temperature of 250°C and the MS source and QP were maintained at temperatures of 230°C and 150°C, respectively. The injection volume of the sample solution was 1 μL, using a split ratio of 1:25 for the brain samples. The oven temperature started at 140°C and was programmed at +3°C/min from 140 to 220°C, held at 220°C for 2 min, then programmed at +3°C/min to 230°C and held at 230°C for 10 min. The total analysis time was 45 min. MS with 70 eV EI was carried out in full scan acquisition mode and all mass spectra were acquired over the m/z range of 50–750. Quantification of FAME was done using the selected ion extraction method.

For the calibration of FAME, a standard reference mixture of 26 FAME (Nu-Check-Prep) and 3 single FAME standards (Larodan Fine Chemicals) were injected at equal concentrations ranging between 1 and 400 ng in 1 μL and calibration plots for each FAME were obtained using peak areas from mass chromatograms, using C17:0 as an external standard. Data analysis was performed using MSD ChemStation software (Agilent G1701EA version E.02.00.493). Relative percentages of FA were calculated by taking the concentration of a given FA derivative as a percentage of the total concentration of all FA identified in the sample.

Brain monoamine analysis. Dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid, serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), and norepinephrine (NE) were measured in the Str, FC, and OB using reverse-phase HPLC with electrochemical detection. The regions were prepared and analyzed as described elsewhere with modifications (22,23). Briefly, 1 g of tissue was diluted into 10 mL PBS spiked with complete protease inhibitor tablets (Roche Diagnostics). Samples were homogenized on ice with a PT 1200E Polytron tissue homogenizer using acid-washed, plastic dispersing aggregates (single use; Kinematic). Homogenates were then passed 10 times through a 1-mL insulin syringe with a 29G × 12.7 mm needle (Beckton Dickinson) to further homogenize the tissue. Homogenates were sonicated with 5 up and down strokes. Then 50 μL of the homogenate was added to 50 μL of 0.24 mol/L $HClO_4$ and 10 μL of internal standard (dihydroxybenzylamine, 1.1 mg/L) was added to track the efficiency of extraction in all samples. The spiked solution was sonicated for 10 s, filtered using Costar Spin-X 0.2-μm filter tubes (Corning), and 10 μL was injected onto the HPLC by an ESA 542 refrigerated auto sampler, as described by Bianco et al. (23).

Affymetrix microarray analysis. Hip samples of 5 rats/group were randomly selected for microarray analysis. Rats that were included in the microarray analysis were killed during similar times of the day (1530–1800 h) to avoid variation in gene expression due to differences in circadian rhythm and only rats that completed the Morris water maze (MWM) testing were included.

Rat total RNA was isolated from Hip and individual microarrays (20 arrays in total) were performed using Affymetrix GeneChip Rat Gene 1.0 ST arrays as described by de Wilde et al. (24), using 100 ng of RNA for Whole Transcription cDNA synthesis (Affymetrix). In this paper, only genes involved in monoaminergic neurotransmission are discussed.

MWM test. The MWM testing was carried out in the dark phase of the cycle in all the experimental groups (n = 10 in each group) and the control group (n = 6). The maze consisted of a black, circular, fiberglass tank (2 m in diameter) that was positioned in the middle of a well-lit testing room enriched with visual stimuli and was filled daily with fresh tap water (22 ± 1°C) to a depth of 30 cm. A black, circular platform (11 cm in diameter) submerged 1.5 cm below the water surface was used as an escape platform invisible to the rats. The swimming paths of the trials were tracked by Ethovision software (version 3.1) via a video camera. The rats underwent 4 phases of testing in the water maze over 10 consecutive days: *1*) cued task (1 d, PND 45); *2*) working memory task (4 d, PND 46–49); *3*) reference memory task (4 d, PND 50–53); and *4*) probe test (1 d, PND 54).

The cued task served as a test for nonspecific sensory motor differences and to familiarize the rats with the general procedures. The platform was made visible with a local cue and was positioned in the center of the maze. Each rat underwent 4 consecutive trials, with the starting position varying randomly among the 4 possible release points (N, W, S, and E). Each rat was allowed a maximum of 90 s to escape onto the platform. If a rat failed to do so, it was guided to the platform and was left on it for an inter-trial interval of 15 s before the next trial.

The next day, in the working memory task, the platform position (randomized across rats) varied from one day to the next in a nonrepetitive manner but remained submerged at the same position across the 4 trials within a day. The 4 platform positions were 16 cm off the wall in the NW, NE, SE, and SW directions of the tank. The starting positions varied randomly across the trials among the 4 possible release points (N, W, S, and E). The rats were allowed 90 s to find the platform and the inter-trial interval was 15 s. This task taxes the flexible use of day-dependent, short-term (working) memory (25).

The reference memory task began the day after completion of the working memory task. This task assessed long-term spatial memory by applying a procedure in which the platform remained in a fixed location across trials and across training days, whereas the starting positions randomly changed across trials. As described above, the rats were allowed to search for the platform for 90 s, with an inter-trial interval of 15 s. At 24 h after the final reference trial, a probe test was conducted to assess memory retention. To this end, the platform was removed from the tank and the rats were allowed to freely swim in the water maze for 60 s (one trial only). The search pattern during the probe test provided an index of the persistence in searching the platform, where it had been located throughout the reference memory task.

Statistical analysis. Data were analyzed and expressed using IBM SPSS Statistics software (version 19.0) and Microsoft Excel 2010. Data were examined for normality of distribution by a Shapiro-Wilk test and the presence of outliers (±3 SD from the mean, boxplots). Homogeneity of variance was examined by the Levene's test. Datasets that significantly deviated from normality and/or variance homogeneity were transformed prior to inferential statistical analysis using ANOVA. Two-way [Fe and (n-3) FA sufficient vs. deficient] repeated-measures ANOVA was conducted on the latency, distance moved, and mean swimming speed to find the platform obtained from the different MWM tasks. Trials and days were used as repeated measurement variables. Fisher's least significant difference tests were used for appropriate pair-wise comparison following the emergence of significant effects from the overall ANOVA. For all other measurements, treatment effects and their interactions were analyzed by 2-way ANOVA, followed by 1-way ANOVA for diet as the between-subject variable and Tukey's test for multiple pair-wise comparisons. Significant treatment effects in the absence of a significant interaction effect indicate additive effects of the treatments, whereas a significant interaction implies synergism or antagonism. Pearson's correlations were performed between brain monoamines and MWM performance indices. To determine if significant associations were influenced by experimental diets, multiple linear regression analyses were performed controlling for diet group. Results were expressed as means ± SEM and differences were considered significant at $P < 0.05$. From the microarray analysis, differentially expressed genes were obtained by 2-tailed, paired, intensity-based, moderated, paired *t*-statistics (26). Comparisons were performed between the gene expression in the control group and the deficiency groups [ID, (n-3)FAD, and ID+(n-3)FAD]. Probe sets that met the criterion of $P < 0.05$ and a fold-change >1.2 were considered to be significantly regulated.

Results

Brain weight, food intake, and body weight gain. The pooled weight of 5 selected brain regions (Hip, cerebellum, OB, FC, and Str) was decreased by (n-3)FAD ($P < 0.05$) (**Table 1**). Both ID and (n-3)FAD decreased food intake and body weight gain ($P < 0.05$). However, ID increased the relative food intake (g/g body weight), which was greater in the ID+(n-3)FAD group than in the control, (n-3)FAD, and ID groups ($P < 0.05$). The ID group in turn had a greater relative food intake than the (n-3)FAD and control groups ($P < 0.05$).

Brain Fe. ID lowered Fe concentrations in all 4 brain regions ($P < 0.05$) (**Table 2**). In ID+(n-3)FAD rats, Fe concentrations were reduced by 21.1% in the Str, 29.4% in the FC, 38.1% in the OB, and 38.9% in the Hip. In the Hip, (n-3)FAD lowered brain Fe concentrations ($P < 0.05$).

TABLE 1 Weight gain, food intake, and brain weight of male rats fed an ID, (n-3)FAD, ID+(n-3)FAD, or control diet for 5 wk[1]

					P value[2]		
	Control	ID	(n-3)FAD	ID+(n-3)FAD	ID	(n-3)FAD	ID x (n-3)FAD
Total body weight gain, *g/35 d*	222 ± 9.7[a]	149 ± 3.6[b]	200 ± 7.6[a]	131 ± 6.5[b]	<0.001	0.043	0.51
Total food intake, *g/35 d*	564 ± 15.4[a]	423 ± 9.6[b]	512 ± 15.4[a]	402 ± 11.3[b]	<0.001	0.050	0.17
Relative food intake, *g/g body weight*	2.6 ± 0.1[c]	2.8 ± 0.0[b]	2.6 ± 0.1[c]	3.1 ± 0.1[a]	<0.001	0.13	0.16
Brain weight,[3] *mg*	617 ± 32.7[a]	596 ± 7.9[a,b]	550 ± 16.4[a,b]	535 ± 11.0[b]	0.30	0.003	0.99

[1] Values are means ± SEM, n = 8–10/group. Means in a row with superscripts without a common letter differ, $P < 0.05$. (n-3)FAD, (n-3) fatty acid deficient; FC, frontal cortex; Hip, hippocampus; ID, iron deficient; OB, olfactory bulb; Str, striatum.
[2] Two-way ANOVA was used to test effects of dietary Fe (deficient vs. sufficient) and dietary n-3 FA (deficient vs. sufficient), and Fe × n-3 FA interactions, $P < 0.05$.
[3] Brain weight represents pooled weight of 5 different brain regions (FC, cerebellum, Hip, Str, and OB).

Brain total phospholipid FA. The (n-3)FAD diet provided over 2 generations markedly altered the brain (n-3) and (n-6) total phospholipid FA compositions (Table 2). DHA concentrations in the FC, OB, Str, and Hip were significantly reduced by 75.6, 70.8, 68.8, and 67.6%, respectively, in the (n-3)FAD groups. In the OB, there was an ID × (n-3)FAD interaction on DHA ($P < 0.05$) and a trend toward an interaction on total (n-3) FA ($P = 0.05$). In the Hip, ID tended to lower DHA concentrations ($P = 0.05$). Brain EPA concentrations were not influenced by (n-3)FAD, but ID lowered EPA in the Hip ($P < 0.05$). In contrast, (n-3)FAD increased arachidonic acid (ARA) concentrations in the OB and Str ($P < 0.05$). In the OB, ARA was higher in the (n-3)FAD than in ID rats ($P < 0.05$). Furthermore, there was a significant ID × (n-3) FAD interaction on ARA concentrations in the Str; ARA was higher in (n-3)FAD than in ID+(n-3)FAD, ID, and control rats ($P < 0.05$).

Brain monoamines. In both the Str and OB, ID and (n-3)FAD increased DA concentrations (**Table 3**), with an additive increase in ID+(n-3)FAD rats compared with controls ($P < 0.05$) (**Fig. 1***A*, *B*). In the Str, (n-3)FAD decreased DOPAC concentrations (Fig. 1*B*), which were lower in ID+(n-3)FAD rats than in controls ($P < 0.05$). In the FC, there was a significant ID × (n-3)FAD interaction on DOPAC concentrations. In the OB, ID decreased 5-HT concentrations, which were lower in the ID+(n-3)FAD compared with the (n-3)FAD and control groups ($P < 0.05$) (Fig. 1*A*). In the Str, ID decreased 5-HIAA concentrations, which were lower in the ID+(n-3)FAD group than in the (n-3)FAD and control groups ($P < 0.05$) (Fig. 1*B*). In the FC, (n-3)FAD elevated NE concentrations ($P < 0.05$) (**Fig. 2**). Although no significant ID × (n-3)FAD interaction was found ($P = 0.11$), this effect of (n-3) FAD was reduced in combination with ID. However, a similar pattern was found in the OB with a trend toward an antagonistic ID × (n-3)FAD interaction ($P = 0.07$).

Microarray analysis. Compared with the control group, expression of the dopa decarboxylase (*DDC*) gene was downregulated in the Hip of ID and ID+(n-3)FAD rats with a fold-change of −1.31 and −1.33 ($P < 0.05$), respectively (**Supplemental Table 2**). The monoamine oxidase B (*MAOB*) gene tended to be downregulated in the ID+(n-3)FAD rats ($P = 0.044$; fold-change = −1.09). The DA receptor D2 (*DRD2*) gene tended to be downregulated in

TABLE 2 Fe concentration and major phospholipid FA composition in different brain regions of male rats fed an ID, (n-3)FAD, ID+(n-3)FAD, or a control diet for 5 wk[1]

					P value[2]		
	Control	ID	(n-3)FAD	ID+(n-3)FAD	ID	(n-3)FAD	Fe x (n-3)FAD
Brain Fe, *nmol/g tissue*							
FC	229 ± 12.6[a]	155 ± 5.1[b]	215 ± 12.0[a]	162 ± 8.6[b]	<0.001	0.76	0.32
OB	255 ± 12.3[a]	172 ± 10.0[b]	260 ± 15.6[a]	158 ± 8.2[b]	<0.001	0.51	0.39
Str	230 ± 11.3[a]	187 ± 8.0[b]	244 ± 15.9[a]	181 ± 8.0[b]	<0.001	0.84	0.46
Hip	220 ± 10.4[a]	148 ± 5.3[b]	195 ± 9.0[a]	135 ± 2.8[b]	<0.001	0.011	0.63
Tissue FA, *% of total FA*							
20:5(n-3) (EPA)							
FC	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.79	0.15	0.57
OB	0.11 ± 0.03	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.02	0.34	0.47	0.38
Str	0.07 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.05	0.44	0.96
Hip	0.18 ± 0.05[a]	0.07 ± 0.01[b]	0.12 ± 0.02[ab]	0.10 ± 0.02[ab]	0.009	0.47	0.08
22:6(n-3) (DHA)							
FC	14.6 ± 0.4[a]	14.1 ± 0.6[a]	3.6 ± 0.4[b]	4.3 ± 0.3[b]	0.23	<0.001	0.08
OB	18.1 ± 0.6[a]	16.5 ± 0.4[a]	5.3 ± 0.3[b]	5.6 ± 0.2[b]	0.59	<0.001	0.046
Str	13.3 ± 0.4[a]	12.9 ± 0.5[a]	4.1 ± 0.2[b]	4.1 ± 0.2[b]	0.58	<0.001	0.81
Hip	14.9 ± 0.8[a]	13.8 ± 0.4[a]	4.8 ± 0.2[b]	4.0 ± 0.4[b]	0.05	<0.001	0.39
20:4(n-6) (ARA)							
FC	16.5 ± 0.2	15.8 ± 0.3	17.1 ± 0.6	16.9 ± 0.4	0.33	0.06	0.51
OB	13.6 ± 0.5[ab]	13.4 ± 0.3[b]	14.7 ± 0.2[a]	13.9 ± 0.2[ab]	0.21	0.013	0.36
Str	14.4 ± 0.3[b]	14.9 ± 0.5[b]	16.6 ± 0.4[a]	14.9 ± 0.3[b]	0.14	0.005	0.007
Hip	19.6 ± 0.8	19.3 ± 0.5	19.6 ± 0.4	19.7 ± 0.5	0.81	0.64	0.74
Total (n-3) FA							
FC	14.9 ± 0.4[a]	14.6 ± 0.6[a]	3.8 ± 0.4[b]	4.6 ± 0.3[b]	0.20	<0.001	0.10
OB	18.8 ± 0.6[a]	17.1 ± 0.3[a]	5.7 ± 0.3[b]	6.0 ± 0.3[b]	0.58	<0.001	0.05
Str	13.7 ± 0.4[a]	13.3 ± 0.5[a]	4.5 ± 0.2[b]	4.5 ± 0.2[b]	0.71	<0.001	0.62
Hip	15.8 ± 0.9[a]	14.5 ± 0.5[a]	5.3 ± 0.3[b]	4.6 ± 0.4[b]	0.10	<0.001	0.70
Total (n-6) FA							
FC	21.3 ± 0.3[b]	21.5 ± 0.5[b]	30.0 ± 1.9[a]	29.6 ± 1.3[a]	0.95	<0.001	0.91
OB	20.6 ± 0.3[b]	20.4 ± 0.4[b]	29.9 ± 0.4[a]	29.4 ± 0.4[a]	0.39	<0.001	0.76
Str	19.6 ± 0.3[b]	20.5 ± 0.6[b]	27.3 ± 0.8[a]	27.3 ± 0.8[a]	0.50	<0.001	0.50
Hip	27.8 ± 1.6[b]	26.1 ± 0.7[b]	33.7 ± 0.9[a]	34.0 ± 1.5[a]	0.54	<0.001	0.45

[1] Values are means ± SEM, $n = 8$–10/group. Means in a row with superscripts without a common letter differ, $P < 0.05$. ARA, arachidonic acid; FA, fatty acids; (n-3)FAD, (n-3) fatty acid deficient; FC, frontal cortex; Hip, hippocampus; ID, iron deficient; OB, olfactory bulb; Str, striatum.

[2] Two-way ANOVA was used to test effects of dietary Fe (deficient vs. sufficient) and dietary (n-3) FA (deficient vs. sufficient), and Fe × (n-3) FA interactions, $P < 0.05$.

TABLE 3 Monoamine concentrations in 3 selected brain regions of male rats fed an ID, (n-3)FAD, ID+(n-3)FAD, or a control diet for 5 wk[1]

					P value[2]		
	Control	ID	(n-3)FAD	ID+(n-3)FAD	ID	(n-3)FAD	ID x (n-3)FAD
DA			*pmol/mg tissue*				
FC	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.82	0.25	0.49
OB	1.5 ± 0.3^{b}	2.1 ± 0.5^{ab}	1.9 ± 0.2^{ab}	3.4 ± 0.7^{a}	0.027	0.039	0.44
Str	4.4 ± 0.9^{b}	11.7 ± 3.2^{ab}	7.2 ± 1.1^{ab}	14.5 ± 2.7^{a}	0.001	0.044	0.80
DOPAC							
FC	2.1 ± 0.5	3.0 ± 0.2	2.7 ± 0.3	1.8 ± 0.3	0.71	0.71	0.011
OB	3.5 ± 0.3	2.7 ± 0.4	2.8 ± 0.5	2.6 ± 0.3	0.30	0.31	0.29
Str	12.6 ± 1.3^{a}	11.0 ± 0.7^{ab}	10.8 ± 1.1^{ab}	8.7 ± 0.8^{b}	0.09	0.028	0.64
Homovanillic acid							
FC	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	0.18	0.52	0.07
OB	1.7 ± 0.1^{ab}	1.4 ± 0.1^{b}	1.9 ± 0.1^{a}	1.8 ± 0.2^{ab}	0.10	0.018	0.66
Str	16.1 ± 1.1	14.9 ± 0.9	15.4 ± 1.4	13.0 ± 0.7	0.14	0.19	0.69
5-HT							
FC	3.3 ± 0.6	2.8 ± 0.5	2.8 ± 0.5	2.5 ± 0.5	0.54	0.42	0.83
OB	8.3 ± 1.3^{a}	6.7 ± 1.8^{ab}	8.2 ± 1.6^{a}	3.1 ± 0.4^{b}	0.003	0.07	0.21
Str	2.4 ± 0.5	2.5 ± 0.3	1.9 ± 0.2	2.4 ± 0.5	0.47	0.35	0.93
5-HIAA							
FC	2.1 ± 0.4	2.1 ± 0.2	2.6 ± 0.4	1.9 ± 0.3	0.47	0.65	0.20
OB	1.1 ± 0.2	1.6 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	0.33	0.25	0.16
Str	4.2 ± 0.9^{a}	3.7 ± 0.8^{ab}	4.2 ± 1.0^{a}	1.3 ± 0.2^{b}	0.020	0.06	0.09
NE							
FC	2.1 ± 0.4^{b}	2.0 ± 0.2^{b}	6.0 ± 1.3^{a}	3.3 ± 0.9^{ab}	0.12	0.002	0.11
OB	2.9 ± 0.8	3.3 ± 0.7	5.1 ± 0.9	2.9 ± 0.9	0.26	0.33	0.07
Str	1.9 ± 0.4	1.8 ± 0.3	1.3 ± 0.2	1.4 ± 0.2	0.92	0.12	0.66

[1] Values are means ± SEM, n = 8–10/group. Means in a row with superscripts without a common letter differ, $P < 0.05$. DA, dopamine; (n-3)FAD, (n-3) fatty acid deficient; 5-HIAA, hydroxyindoleacetic acid; 5-HT, serotonin; DOPAC, dihydroxyphenylacetic acid; FC, frontal cortex; ID, iron deficient; NE, norepinephrine; OB, olfactory bulb; Str, striatum.
[2] Two-way ANOVA was used to test effects of dietary Fe (deficient vs. sufficient) and dietary (n-3) FA (deficient vs. sufficient), and Fe × (n-3) FA interactions, $P < 0.05$.

the ID rats with a fold-change of −1.15 (P = 0.024). Furthermore, there was a trend toward upregulation of the 5-HT receptor 2C (*HTR2C*) gene in the ID and ID+(n-3)FAD rats with a fold-change of 1.32 (P = 0.09) and 1.36 (P = 0.06), respectively.

MWM. During the cued task, all groups had a reduction in distance moved to reach the visible platform from trial 1 to 4 (main effect for trial; $P < 0.001$). During the working memory task, ID decreased swimming speed ($P < 0.05$) (data not shown). Thus, we analyzed working memory by using distance moved (in cm) to find the hidden platform and not by escape latency (in s), which could be confounded by between-group differences in swimming speed. An effect of trial ($P < 0.001$) to find the hidden platform was found, indicating that learning and memory took place across trials (**Fig. 3***A*). Because working memory is typically reflected in a rapid reduction of distance moved from trial 1 (when platform position was unknown) to trial 2, we calculated mean difference in distance moved between trial 1 and trial 2 across all 4 d (Fig. 3*B*). The 3 deficient groups had impaired working memory, reflected by a significantly lower mean difference in distance moved between trial 1 and trial 2 compared with controls. Working memory performance was impaired by (n-3)FAD ($P < 0.05$) and ID tended to impair working memory (P = 0.05). Additionally, there was an ID × (n-3)FAD interaction (P = 0.013), because combined deficiency did not further exacerbate the performance deficits.

Reference memory was evaluated by distance moved to find the hidden platform over the 4 consecutive days (**Fig. 4***A*). All groups showed the expected reduction in distance moved across training days ($P < 0.001$). The mean distance moved across training days during the reference memory task was increased by (n-3)FAD ($P < 0.05$), but no significant effect of ID was found (P = 0.12). The mean distance moved was longer in the ID+(n-3)FAD group than in the controls (P = 0.05) (Fig 4*B*). During the probe test, all 4 groups had an overall preference for the target quadrant where the platform was positioned during the reference memory task (data not shown).

Correlations between brain monoamines and MWM performance. Difference in distance moved between trial 1 and trial 2 during the working memory task (working memory performance) correlated with FC DA concentrations (r = 0.39; P = 0.026) (**Supplemental Fig. 2*A*,*B***). Furthermore, mean distance moved during the reference memory task was negatively associated with FC 5-HT (r = −0.42; P = 0.018). In the regression analysis, diet group was a significant predictor of the relationship between working memory performance and FC DA (ID: $r_{partial}$ = −0.45, P = 0.013; (n-3)FAD: $r_{partial}$ = −0.45, P = 0.012; ID+(n-3)FAD: $r_{partial}$ = −0.40, P = 0.031) and when controlling for diet group, the correlation between working memory performance and FC DA was no longer significant.

Discussion

This is the first study to our knowledge to examine interactions between ID and (n-3)FAD in rats using a 2 × 2 factorial design.

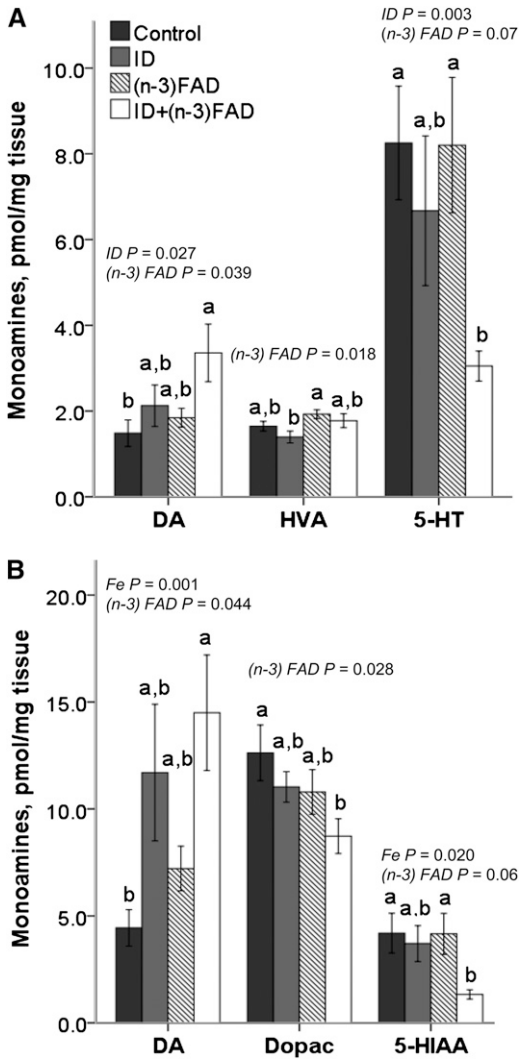

FIGURE 1 Concentrations of selected monoamines in OB (*A*) and Str (*B*) of male rats fed an ID, (n-3)FAD, ID+(n-3)FAD, or control diet for 5 wk. Values are means ± SEM, $n = 8$–10. Labeled means without a common letter differ, $P < 0.05$. DA, dopamine; DOPAC, 3,4-Dihydroxyphenylacetic acid; (n-3)FAD, (n-3) fatty acid deficient; 5-HIAA, 5-hydroxyindolaecetic acid; 5-HT, serotonin; HVA, homovanillic acid; ID, iron deficient; OB, olfactory bulb.

We chose 2 different depletion regimens for the 2 nutrients. To deplete brain (n-3) FA, an (n-3)FAD diet was provided for 2 generations and the offspring were maintained on the (n-3) FAD diet for 5 wk postweaning. This model represents the human situation where children born to mothers with inadequate (n-3) FA status continue to consume a diet low in (n-3) but high in (n-6) FA throughout childhood into young adulthood. In contrast, the ID diet was introduced only after weaning, representing the human situation where Fe status is generally adequate during fetal life and during the newborn period while breastfeeding but deteriorates during the complementary feeding period and early childhood due to consumption of foods with inadequate Fe.

At PND 21, brain growth spurts (dendritogenesis and synaptogenesis) in rats have ended (27). However, some brain regions, such as the Hip and FC, are not fully developed until PND 35–40 and neurotransmitter systems are only reaching adult levels between PND 30 and 50 (28), thus making the postweaning periods vulnerable to adverse influences.

The depletion experiment reduced brain DHA and Fe levels by 70–76% and 20–32%, respectively. Fe is a cofactor for desaturase enzymes required for the conversion of EFA into EPA, DHA, and ARA; this may explain why DHA concentrations in the Hip and OB were lower in ID rats, and in the Str, the ARA-increasing effect of (n-3)FAD was attenuated in combination with ID (antagonistic effect). Depletion of DHA in cell membranes may adversely affect membrane function and impair receptors involved in Fe uptake (29). This may explain why in the Hip, Fe concentrations were significantly reduced by (n-3) FAD.

The pooled weight of the 5 selected brain regions was reduced by (n-3)FAD. Previous animal studies did not find any changes in total brain weight due to (n-3)FAD (30,31). However, our findings are consistent with a study in human premature infants, showing that breast milk (n-3) PUFA status positively correlated with head circumference at 6 mo (32), a predictor of brain weight (33).

Our data indicate that ID and (n-3)FAD, alone or in combination, alter monoamine and monoamine metabolite concentrations in the OB, FC, and Str. Both ID and (n-3)FAD significantly increased DA concentrations in the OB and Str, resulting in an additive 1- to 2-fold increase in the ID+(n-3)FAD rats. Elevated DA concentrations in different brain regions as a result of ID have been previously reported (22,34–36). The proposed mechanisms for increased DA concentrations caused by ID are altered DA transporter function (37), decreased DA D2 receptor activities (38), and/or decreased activity of MAO (39). Mechanisms explaining the effects of (n-3)FAD on DA and its metabolites may involve altered expression and density of monoamine receptors and transporters, such as variations in DA D2 receptor activity (14,40,41) and/or density of $VMAT_2$, an integral membrane protein that acts to transport monoamines from cellular cytosol into synaptic vesicles (42).

In addition to DA, other neurotransmitters, such as NE and 5-HT (which is metabolized by MAO into 5-HIAA) may also modulate learning and memory (14,43,44). ID decreased 5-HT concentrations in the OB and 5-HIAA concentrations in the Str with a trend for a decreasing effect of (n-3)FAD, suggesting

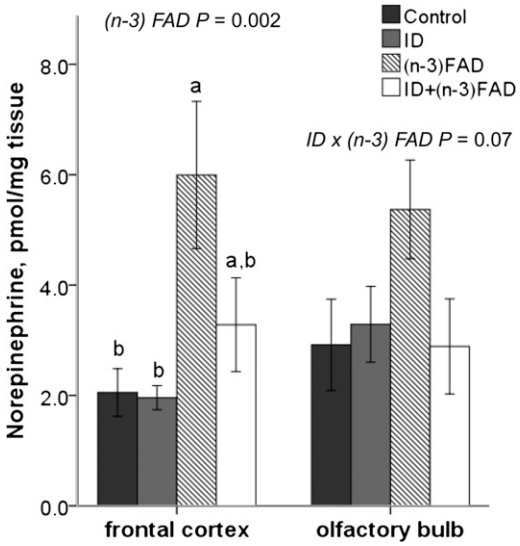

FIGURE 2 NE concentrations in FC and OB of male rats fed an ID, (n-3)FAD, ID+(n-3)FAD, or control diet for 5 wk. Values are means ± SEM, $n = 8$–10. Labeled means without a common letter differ, $P < 0.05$. (n-3)FAD, (n-3) fatty acid deficient; FC, FC, frontal cortex; ID, iron deficient; NE, norepinephrine; OB, olfactory bulb.

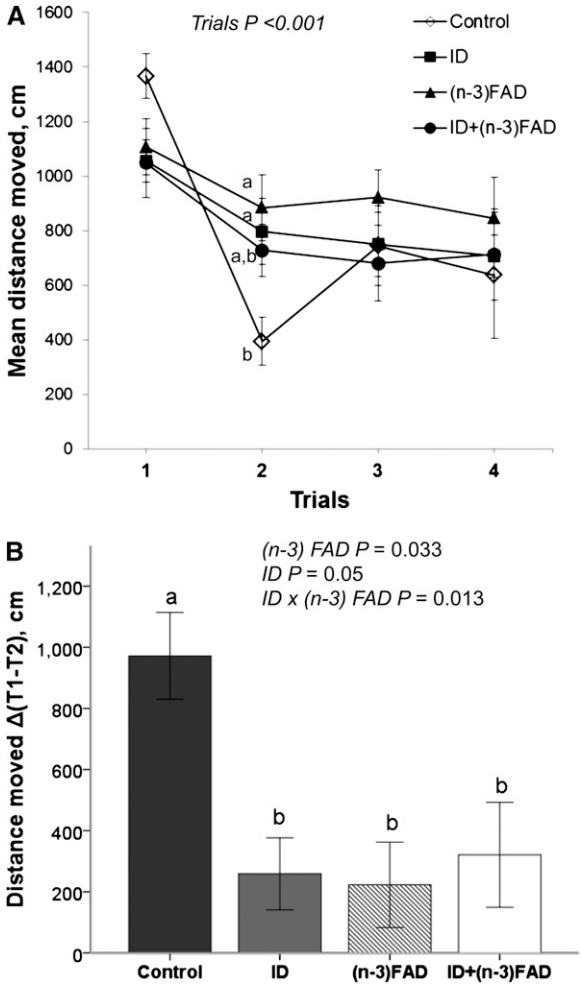

FIGURE 3 Working memory performance in the MWM of male rats fed an ID, (n-3)FAD, ID+(n-3)FAD, or control diet for 5 wk. (*A*) Mean distance moved per trial to reach hidden platform located at different positions across 4 d, with 4 trails/d. (*B*) Mean difference in distance moved between trial 1 and trial 2 across 4 d. Values are means ± SEM, n = 6–10. Labeled means without a common letter differ, $P <$ 0.05. (n-3)FAD, (n-3) fatty acid deficient; ID, iron deficient; MWM, Morris water maze.

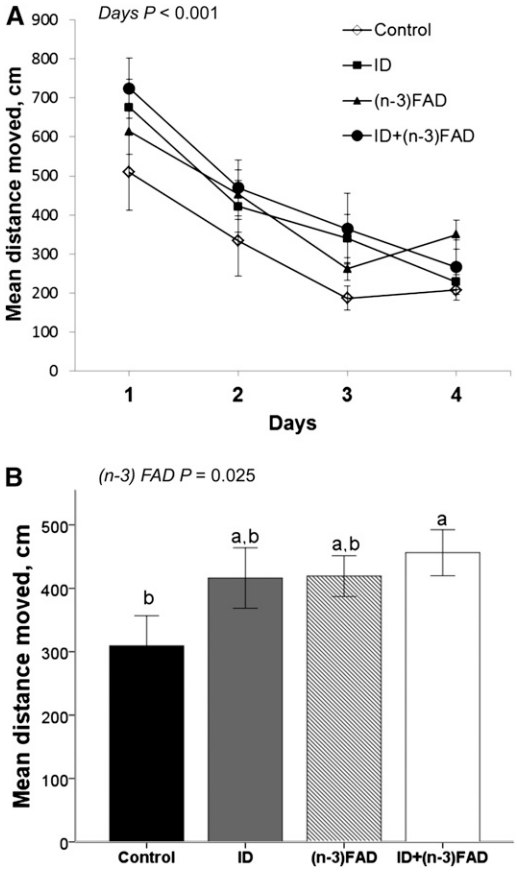

FIGURE 4 Reference memory performance in the MWM of male rats fed an ID, (n-3)FAD, ID+(n-3)FAD, or a control diet for 5 wk. (*A*) Mean distance moved per day to reach hidden platform located at same position across 4 d, with 4 trails/d. (*B*) Mean distance moved across all 4 d (4 trials/d). Values are means ± SEM, n = 6–10. Labeled means without a common letter differ, $P < 0.05$. (n-3)FAD, (n-3) fatty acid deficient; ID, iron deficient; MWM, Morris water maze.

additive reductions (~ −63% 5-HT and −68% 5-HIAA) in ID+(n-3)FAD rats compared with controls. These findings are consistent with previous studies reporting alterations in the serotonergic system caused by ID or (n-3)FAD (34,36,45–48).

Despite Fe being a cofactor of the enzymes tyrosine hydroxylase (synthesis of DA and NE) and tryptophan hydroxylase (synthesis of 5-HT), we did not find alterations in the expression of *TH*, *TPH1*, and *TPH2* genes in ID. However, we did find a significant downregulation of the aromatic L-amino acid decarboxylase (also DOPA decarboxylase) gene *DDC* in ID and ID+(n-3)FAD rats. Because DA concentrations increased in ID, the downregulation of this enzyme may be a compensation to limit the synthesis of DA and to avoid further accumulation. The 5-HT_{2C} receptor gene tended to be upregulated by ID+(n-3)FAD and (n-3)FAD, consistent with previous studies reporting increased 5-HT_2 receptor density in (n-3)FAD (41,49). 5-HT_2 receptors can activate phospholipase A_2 via G-protein coupling, releasing ARA from phospholipids (50), which can modulate signal transduction, transcription, neuronal activity, apoptosis, and other brain processes (51).

Working memory may be modulated by many neurochemical systems, including DA, 5-HT, and NE (43). Working memory performance was impaired by both ID and n-3 FAD, but in combination, they did not further exacerbate impairment (Fig. 3*B*). To our knowledge, these are the first published data using the working memory task in the MWM to assess behavioral deficits in ID rats and are consistent with data from school-aged children in whom ID was associated with diminished working memory by means of event-related potentials (52). Our data showing impaired working memory in (n-3)FAD are consistent with previous data showing that the escape latency during a working memory task was significantly longer in (n-3)FAD rats (53). Furthermore, reference memory performance was impaired by (n-3)FAD and ID+(n-3)FAD rats significantly underperformed compared with controls.

Our study has several limitations. Male rats were used in this study to avoid the potential confounding effects of estrogen that have been reported in previous studies of ID and (n-3)FAD (38,54). However, future studies should be performed to confirm the reported effects in female rats and to evaluate specific gender effects. Additional monoamine data from the Hip would have been potentially valuable for direct correlation with gene expression results and MWM performance. Also, we did not assess other neurotransmitter systems such as glutamate and GABA or other factors that can influence learning and behavior, such as degree of myelination, neuronal inflammation, and

processes of morphogenesis and cell growth. Other behavioral and cognitive tests would have been of additional value. However, this is the first study to our knowledge to show that concurrent ID and (n-3)FAD have significant interactive and additive effects on brain monoamine pathways as well as working and reference memory performance in young rats. Nutrient deficiencies seldom occur in isolation in humans and this study highlights the importance of investigating the interactions of common deficiencies, particularly during critical periods in brain development and growth.

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