The Journal of Nutrition
Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

In Male Rats with Concurrent Iron and (n-3) Fatty Acid Deficiency, Provision of Either Iron or (n-3) Fatty Acids Alone Alters Monoamine Metabolism and Exacerbates the Cognitive Deficits Associated with Combined Deficiency[1–3]

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Abstract

Concurrent deficiencies of iron (Fe) (ID) and (n-3) fatty acids [(n-3)FAD)] in rats can alter brain monoamine pathways and impair learning and memory. We examined whether repletion with Fe and DHA/EPA, alone and in combination, corrects the deficits in brain monoamine activity (by measuring monoamines and related gene expression) and spatial working and reference memory [by Morris water maze (MWM) testing] associated with deficiency. Using a 2 × 2 design, male rats with concurrent ID and (n-3) FAD [ID+(n-3)FAD] were fed an Fe+DHA/EPA, Fe+(n-3)FAD, ID+DHA/EPA, or ID+(n-3)FAD diet for 5 wk [postnatal d 56–91]. Biochemical measures and MWM performance after repletion were compared to age-matched control rats. The provision of Fe in combination with DHA/EPA synergistically increased Fe concentrations in the olfactory bulb (OB) (Fe x DHA/EPA interaction). Similarly, provision of DHA/EPA in combination with Fe resulted in higher brain DHA concentrations than provision of DHA alone in the frontal cortex (FC) and OB ($P < 0.05$). Dopamine (DA) receptor D1 was upregulated in the hippocampus of Fe+DHA/EPA rats (fold-change = 1.25; $P < 0.05$) and there were significant Fe x DHA/EPA interactions on serotonin (5-HT) in the OB and on the DA metabolite dihydroxyphenylacetic acid in the FC and striatum. Working memory performance was impaired in ID+DHA/EPA rats compared with controls ($P < 0.05$). In the reference memory task, Fe+DHA/EPA improved learning behavior, but Fe or DHA/EPA alone did not. These findings suggest that feeding either Fe or DHA/EPA alone to adult rats with both ID and (n-3)FAD affects the DA and 5-HT pathways differently than combined repletion and exacerbates the cognitive deficits associated with combined deficiency. J. Nutr. 142: 1472–1478, 2012.

Introduction

Both iron (Fe) and (n-3) fatty acids [(n-3) FA][12] play important roles in monoaminergic neurotransmission and myelination (1–3), and deficiencies of these nutrients may cause abnormalities in brain development and function through shared mechanisms. In animal models, Fe deficiency (ID) causes abnormal neurotransmission and brain hypomyelination by impairing oligodendrocyte synthesis of lipids and cholesterol (4,5). Pre- and postnatal ID can cause sensorimotor deficits, abnormal emotional behavior, and cognitive impairment (6–8) and depending on the timing and severity of ID, these alterations may be at least partially reversible by Fe repletion (9).

Adequate DHA in cell membranes in the brain ensures optimal membrane fluidity and thickness, required for normal cell signaling (10). Both DHA and EPA have been shown to stimulate the expression of specific myelin proteins (2). Brain (n-3) FA deficiency [(n-3)FAD] alters the production and activity of monoaminergic neurotransmitters (11) and this can impair learning and memory (12). Whether the neurodevelopmental

[1] Supported by Unilever Research and Development, Vlaardingen, The Netherlands, and the Swiss Foundation for Nutrition Research.
[2] Author disclosures: J. Baumgartner, C. M. Smuts, L. Malan, M. Arnold, B. K. Yee, L. E. Bianco, M. V. Boekschoten, M. Müller, W. Langhans, R. F. Hurrell, and M. B. Zimmermann, no conflicts of interest.
[3] Supplemental Table 1 and Figures 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: DA, dopamine; DOPAC, dihydroxyphenylacetic acid; (n-3) FA, (n-3) fatty acids; (n-3)FAD, (n-3) fatty acid deficiency; FC, frontal cortex; Fe, iron; 5-HIAA, 5-hydroxyindoleacetic acid; Hip, hippocampus; 5-HT, serotonin; ID, iron deficiency; MWM, Morris water maze; OB, olfactory bulb; PND, postnatal day; Str, striatum.
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Manuscript received December 13, 2011. Initial review completed February 5, 2012. Revision accepted May 26, 2012.
First published online June 27, 2012; doi:10.3945/jn.111.156299.

abnormalities resulting from early (n-3)FAD can be reversed by later repletion remains uncertain (13).

We previously showed additive and interactive effects of concurrent ID and (n-3)FAD on brain monoamine concentrations and on spatial working and reference memory in young rats (14). Whether these effects are lasting or can be reversed by repletion with Fe and (n-3) FA is unclear. Therefore, the aim of this study was to examine, in rats with concurrent ID and (n-3) FAD, whether repletion with Fe and (n-3) FA as DHA/EPA, alone and in combination, corrects disturbed monoamine metabolism and improves learning and memory. We hypothesized that repletion with a combination of Fe and DHA/EPA would be more effective than repletion with either nutrient alone.

Methods

Rats and diets. All experimental procedures were approved by the Veterinary Office of the Department of Health of the Canton of Zürich. Wistar rats were first depleted in (n-3) FA over 2 generations as previously described (14) and after weaning at postnatal day (PND) 21, male (n-3)FAD rats received a concurrent ID and (n-3)FAD [ID+(n-3) FAD] diet for a depletion period of 5 wk. At PND 56, the now ID+(n-3) FAD rats (n = 39) were randomly divided into 4 groups (**Supplemental Fig. 1**). The repletion study was designed as a 2 × 2 factorial experiment and the 4 groups received the following diets for a period of 5 wk: *1*) a continued ID and (n-3)FAD [ID+(n-3)FAD, n = 10]; *2*) Fe sufficient and (n-3)FAD [Fe+(n-3)FAD, n = 10]; *3*) Fe and DHA/EPA sufficient (Fe+DHA/EPA, n = 10); and *4*) ID and DHA/EPA sufficient (ID+DHA/EPA, n = 10). Furthermore, we included an age-matched (positive) control group from (n-3) FA-sufficient dams that received the basal AIN-93G diet.

The purified experimental diets were obtained commercially (Dyets) and were based on the AIN-93G (15) formulation with modifications in Fe content and fat source (**Supplemental Table 1**). All diets contained 10% fat. The (n-3)FAD diets contained hydrogenated coconut oil at 81 g/kg diet, and safflower oil at 19 g/kg diet. The DHA/EPA sufficient diets contained hydrogenated coconut oil at 70 g/kg diet, safflower oil at 27 g/kg diet, and commercially obtained Incromega DHA 500 TG SR (minimum 6% of fatty acids as EPA; minimum 58% of fatty acids as DHA) (Croda Chemicals Europe) at 3 g/kg diet. All diets included (n-6) FA in the form of linoleic acid. The DHA/EPA-sufficient diets did not contain the (n-3) FA precursor α-linolenic acid. The Fe-sufficient diets contained 35 mg Fe/kg and the ID diets during the preceding depletion period of 5 wk contained 3 mg Fe/kg. For the repletion period, the Fe dose in the ID diets was increased to 10 mg Fe/kg diet to avoid potential deaths from prolonged severe ID anemia. All diets were custom prepared as previously described (14).

Tissue collection. At the end of the repletion period (PND 91 and 92), rats were exposed to CO_2 to introduce unconsciousness for blood collection by cardiac puncture and were then killed by decapitation. The brains were rapidly removed and the cerebellum, olfactory bulb (OB), frontal cortex (FC), striatum (Str), and hippocampus (Hip) were dissected and prepared for analysis as previously described (14).

Brain Fe analysis. Brain regions were homogenized and digested with nitric acid according to Erikson et al. (16) and total Fe concentrations were measured using graphite furnace atomic absorption spectrometry (Perkin Elmer AA400).

Total phospholipid fatty acid analysis. Lipids were extracted from each brain region with chloroform:methanol (2:1, v:v) by a modification of the method of Folch et al. (17). Total phospholipid FA fractions were isolated by TLC, trans-methylated to yield FAME, and analyzed by quadrupole GC-EI-MS on an Agilent Technologies 7890 A GC system equipped with an Agilent Technologies 5975C VL mass selective detector as previously described (14).

Brain monoamine analysis. Dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid, serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), and norepinephrine 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 minimal modifications (18,19).

Affymetrix microarray analysis. Hip samples of 5 rats/group were randomly selected for microarray analysis and microarray analysis was performed as previously described (14).

Morris water maze test. The Morris water maze (MWM) was used to evaluate spatial learning and memory performance. Eight randomly selected rats from the experimental groups and 8 rats from the control group were included. The rats underwent 4 phases of testing in the water maze on 10 consecutive days: *1*) cued task (1 d, PND 80); *2*) working memory task (4 d, PND 81–84); *3*) reference memory task (4 d, PND 85–88); and *4*) probe test (1 d, PND 89). The procedures of the conducted tasks were previously described (14).

Statistical analysis. Data were analyzed and expressed using IBM SPSS Statistics software (version 19.0) and Microsoft Excel 2010. Data were treated and analyzed using the same statistical procedures as described in our previous study (14). The main effects of Fe and DHA/EPA repletion (and their interactions) on all the variables were analyzed by 2-way factorial ANOVA, followed by 1-way ANOVA for diet as between-subject variable and Tukey's test (Fisher's least significant difference test for MWM data) for multiple pair-wise comparisons. This 2-way factorial ANOVA excluded the control group. 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. To investigate differences between the experimental (repletion) groups and the control group, 1-way ANOVA with diet as the between-subject variable was conducted followed by post hoc Dunnett's test. For the MWM probe trial, a 2 × 2 × 4 (Fe × DHA/EPA × quadrant) and a 5 × 4 × 4 (diet × quadrant) ANOVA on percent distance swum per quadrant during the 60-s trial were performed. Furthermore, the differences in percent distance swum between the 4 quadrants were examined by 1-way ANOVA within each diet group. The results were expressed as means ± SEM and differences were considered significant at $P < 0.05$. In the microarray analysis, 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. At PND 92, the body weight of ID+(n-3)FAD rats was reduced by 48.8% compared with the age-matched controls ($P < 0.001$) (**Table 1**). Both Fe and DHA/EPA repletion increased total body weight gain and the Fe+DHA/EPA rats had a higher body weight gain than the ID+DHA/EPA and ID+(n-3)FAD rats ($P < 0.05$). However, body weight at PND 92 remained higher in the control group than in the experimental groups ($P < 0.05$ for all groups). There was a significant Fe × DHA/EPA interaction on relative weight gain (g/g food intake), which was greater in the Fe+DHA/EPA group than in the ID+DHA/EPA, Fe+(n-3)FAD, and ID+(n-3)FAD groups ($P < 0.05$). However, all experimental groups had a greater relative food intake than the control groups ($P < 0.001$ for all groups). The pooled weight of 5 brain regions (Hip, cerebellum, OB, FC, and Str) did not differ between the ID+(n-3) FAD and control groups ($P = 0.22$).

Brain Fe. Fe repletion increased Fe concentrations in all 4 brain regions ($P < 0.05$) (**Table 2**). In the OB, there was an Fe × DHA/EPA interaction on brain Fe concentrations; the Fe+DHA/EPA group had greater Fe concentrations than the ID+DHA/EPA and

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

						P value[2]		
	Control	Fe+DHA/EPA	ID+DHA/EPA	Fe+(n-3)FAD	ID+(n-3)FAD	Fe	DHA/EPA	Fe x DHA/EPA
Total body weight, *g*	410 ± 8.0	340 ± 6.3^{a,*}	303 ± 7.8^{b,c,*}	320 ± 7.8^{a,b,*}	291 ± 7.1^{c*}	<0.001	0.040	0.67
Total body weight gain, *g/35 d*	124 ± 4.4	170 ± 4.5^{a,*}	144 ± 3.6^{b,c,*}	155 ± 4.0^{a,b,*}	138 ± 4.3^{c*}	<0.001	0.012	0.30
Total food intake, *g/35 d*	711 ± 14.3	685 ± 13.6^{a}	643 ± 8.1^{b,c,*}	666 ± 12.2^{a,b,*}	602 ± 9.0^{c*}	<0.000	0.010	0.32
Relative weight gain, *g/g food intake*	0.17 ± 0.01	0.25 ± 0.01^{a,*}	0.22 ± 0.00^{b,*}	0.23 ± 0.00^{b,*}	0.23 ± 0.00^{b*}	0.017	0.09	0.013
Brain weight,[3] *mg*	670 ± 26.3	631 ± 19.3	606 ± 20.2	653 ± 34.7	603 ± 18.2	0.14	0.70	0.61

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

ID+(n-3)FAD groups ($P < 0.05$), whereas the Fe concentrations in the OB of Fe+(n-3)FAD rats did not differ from the groups remaining ID.

Brain total phospholipid fatty acids. Repletion of the ID+(n-3)FAD rats with DHA/EPA, alone or in combination with Fe, resulted in higher DHA and total (n-3) FA concentrations in all 4 brain regions ($P < 0.05$) (Table 2), whereas brain EPA was not affected by DHA/EPA repletion. There were Fe × DHA/EPA interactions on DHA concentrations in the FC ($P < 0.05$) and OB ($P = 0.06$), respectively. In the Fe+DHA/EPA group, the DHA concentrations in the Hip did not differ from controls ($P = 0.08$).

Brain monoamines. ID+(n-3)FAD rats had higher DA concentrations in the FC than those in controls ($P < 0.05$) (**Table 3**), whereas repletion with Fe and/or DHA/EPA reduced DA concentrations to a level not different from that of the controls. Fe decreased DA concentrations in the Str ($P < 0.05$). Significant Fe × DHA/EPA interactions were found on DOPAC concentrations in the OB and Str. In the OB, there was an Fe × DHA interaction on 5-HT concentrations; the Fe+(n-3)FAD group had higher OB 5-HT concentrations than the ID+(n-3)FAD group ($P < 0.05$), but this increase was attenuated when Fe was fed in combination with DHA/EPA. In the Str, there was an effect of DHA/EPA and a nearly significant Fe × DHA/EPA interaction ($P = 0.05$) on 5-HT concentrations, which were higher in the Fe+DHA/EPA than in the Fe+(n-3)FAD group ($P < 0.05$). Additionally, there was an Fe × DHA/EPA interaction on 5-HIAA concentrations in the Str; the ID+DHA/EPA group had lower 5-HIAA concentrations in the Str than the ID+(n-3)FAD group ($P < 0.05$) and this decrease was attenuated when DHA/EPA was fed in combination with Fe. In the FC, DHA/EPA decreased norepinephrine concentrations,

TABLE 2 Fe concentration and major phospholipid fatty acid composition in different brain regions of male control rats and ID+(n-3)FAD rats repleted with an Fe+DHA/EPA, ID+DHA/EPA, Fe+(n-3)FAD, or an ID+(n-3)FAD diet for 5 wk[1]

						P value[2]		
	Control	(Fe)+DHA/EPA	(ID)+DHA/EPA	Fe+(n-3)FAD	ID+(n-3)FAD	Fe	DHA/EPA	Fe x DHA/EPA
Brain Fe, *nmol/g tissue*								
FC	235 ± 11.2	221 ± 9.7^{a}	174 ± 7.2^{b*}	208 ± 10.5^{a,b}	191 ± 14.7^{a,b,*}	0.004	0.81	0.21
OB	300 ± 21.5	290 ± 16.1^{a}	217 ± 13.1^{b*}	234 ± 12.1^{a,b,*}	222 ± 18.7^{b,*}	0.008	0.07	0.037
Str	263 ± 13.6	236 ± 14.1	193 ± 10.8*	237 ± 14.2	204 ± 17.3*	0.011	0.94	0.57
Hip	213 ± 6.7	248 ± 11.2^{a}	182 ± 16.8^{b}	198 ± 19.4^{a,b}	161 ± 11.9^{b,*}	0.006	0.11	0.75
Tissue fatty acids, *% of total FA*								
20:5(n-3) (EPA)								
FC	0.05 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.92	0.41	0.022
OB	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.42	0.56	0.81
Str	0.07 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.19	0.91	0.15
Hip	0.20 ± 0.10	0.14 ± 0.03	0.15 ± 0.05	0.14 ± 0.03	0.09 ± 0.03	0.54	0.50	0.43
22:6(n-3) (DHA)								
FC	13.5 ± 0.2	10.9 ± 0.3^{a,*}	9.6 ± 0.6^{a*}	3.7 ± 0.2^{b,*}	4.0 ± 0.2^{b,*}	0.47	<0.001	0.021
OB	16.0 ± 0.3	13.3 ± 0.4^{a,*}	12.8 ± 0.3^{a*}	4.5 ± 0.3^{b,*}	5.2 ± 0.3^{b,*}	0.26	<0.001	0.06
Str	14.1 ± 0.3	10.9 ± 0.4^{a,*}	10.2 ± 0.5^{a*}	4.5 ± 0.3^{b,*}	4.3 ± 0.3^{b,*}	0.30	<0.001	0.67
Hip	14.5 ± 0.6	11.5 ± 0.5^{a}	10.5 ± 0.8^{a*}	4.2 ± 0.3^{b,*}	4.7 ± 0.5^{b,*}	0.92	<0.001	0.17
Total (n-3) FA								
FC	14.0 ± 0.2	11.3 ± 0.3^{a,*}	10.1 ± 0.6^{a*}	4.0 ± 0.2^{b,*}	4.2 ± 0.2^{b,*}	0.41	<0.001	0.06
OB	16.5 ± 0.3	13.8 ± 0.3^{a,*}	13.3 ± 0.3^{a*}	4.9 ± 0.3^{b,*}	5.6 ± 0.3^{b,*}	0.30	<0.001	0.07
Str	14.6 ± 0.3	11.4 ± 0.4^{a,*}	10.5 ± 0.5^{a*}	4.8 ± 0.3^{b,*}	4.7 ± 0.3^{b,*}	0.25	<0.001	0.59
Hip	15.5 ± 1.0	12.1 ± 0.5^{a,*}	11.4 ± 0.8^{a*}	4.7 ± 0.3^{b,*}	5.2 ± 0.5^{b,*}	0.92	<0.001	0.25

[1] Values are means ± SEM, n = 8–10/group. Means in a row with superscripts without a common letter differ, $P < 0.05$. *Different from control, $P < 0.05$. (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 DHA/EPA (deficient vs. sufficient), and Fe × DHA/EPA interactions, $P < 0.05$.

TABLE 3 Monoamine concentration in 3 selected brain regions of male control rats and ID+(n-3)FAD rats repleted with an Fe+DHA/EPA, ID+DHA/EPA, Fe+(n-3)FAD, or ID+(n-3)FAD diet for 5 wk[1]

						P value[2]		
	Control	Fe+DHA/EPA	ID+DHA/EPA	Fe+(n-3)FAD	ID+(n-3)FAD	Fe	DHA/EPA	Fe x DHA/EPA
DA			*pmol/mg tissue*					
FC	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.1*	0.12	0.25	0.85
OB	1.3 ± 0.6	0.9 ± 0.2	1.3 ± 0.2	1.0 ± 0.2	0.7 ± 0.2	0.78	0.37	0.05
Str	6.9 ± 1.6	4.2 ± 1.2	9.8 ± 2.8	6.9 ± 2.7	7.7 ± 1.6	0.047	0.62	0.35
DOPAC								
FC	2.2 ± 0.2	2.8 ± 0.4	2.2 ± 0.4	2.3 ± 0.3	3.3 ± 0.5	0.74	0.70	0.037
OB	3.0 ± 0.4	3.3 ± 0.4	2.4 ± 0.3	2.8 ± 0.2	3.5 ± 0.4	0.74	0.19	0.043
Str	12.8 ± 1.0	11.7 ± 1.7	13.7 ± 1.2	13.7 ± 2.4	11.9 ± 0.8	0.50	0.79	0.60
Homovanillic acid								
FC	1.1 ± 0.1	1.2 ± 0.2	1.1 ± 0.1	1.3 ± 0.2	1.3 ± 0.2	0.82	0.72	0.93
OB	2.2 ± 0.2	1.9 ± 0.2	2.3 ± 0.3	2.1 ± 0.1	2.3 ± 0.3	0.95	0.88	0.51
Str	16.3 ± 1.2	13.6 ± 1.4	17.6 ± 0.9	15.1 ± 1.3	14.4 ± 1.0	0.14	0.56	0.08
5-HT								
FC	3.3 ± 0.7	4.1 ± 1.0	4.5 ± 0.9	4.7 ± 0.8	3.4 ± 0.6	0.54	0.71	0.14
OB	2.4 ± 0.8	2.5 ± 0.5^{ab}	3.4 ± 0.7^{ab}	4.3 ± 0.6^{a}*	2.2 ± 0.6^{b}	0.16	0.85	0.018
Str	2.6 ± 0.6	5.3 ± 1.3^{a}	2.3 ± 0.3^{ab}	1.8 ± 0.3^{b}	2.3 ± 0.5^{ab}	0.33	0.033	0.05
5-HIAA								
FC	2.3 ± 0.3	1.7 ± 0.3	1.9 ± 0.3	2.3 ± 0.3	2.2 ± 0.3	0.92	0.14	0.76
OB	2.2 ± 0.4	2.0 ± 0.4	1.9 ± 0.2	1.8 ± 0.4	1.7 ± 0.3	0.85	0.84	0.62
Str	2.5 ± 0.5	4.9 ± 1.1^{ab}	1.7 ± 0.4^{b}	4.1 ± 1.2^{ab}	5.8 ± 1.2^{a}	0.36	0.14	0.008
Norepinephrine								
FC	1.6 ± 0.6	0.7 ± 0.2^{b}	1.1 ± 0.3^{ab}	2.2 ± 0.6^{a}	1.4 ± 0.3^{ab}	0.55	0.014	0.22
OB	2.8 ± 0.8	2.3 ± 0.4	3.5 ± 0.9	2.0 ± 0.3	2.4 ± 0.4	0.17	0.38	0.40
Str	2.7 ± 0.6	2.2 ± 0.6	2.6 ± 0.4	3.3 ± 0.7	2.2 ± 0.4	0.85	0.55	0.17

[1] Values are means ± SEM, n = 8–10/group. Means in a row with superscripts without a common letter differ, $P < 0.05$. *Different from control, $P < 0.05$. 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; OB, olfactory bulb; Str, striatum.
[2] Two-way ANOVA was used to test effects of dietary Fe (deficient vs. sufficient) and dietary DHA/EPA (deficient vs. sufficient), and Fe × DHA/EPA interactions, $P < 0.05$.

which were lower in the Fe+DHA/EPA group than in the Fe+(n-3)FAD group ($P < 0.05$).

Microarray analysis. Compared with the control group, expression of the DA receptor D1A (*DRD1A*) gene was upregulated in the Hip of Fe+DHA/EPA rats (fold-change = 1.25; $P = 0.002$) and tended to be upregulated in Fe+(n-3)FAD rats (fold-change = 1.20; $P = 0.012$) (**Supplemental Table 2**). Furthermore, the *DRD2* gene tended to be upregulated in ID+(n-3)FAD rats with a fold-change of 1.19 ($P = 0.009$) and in Fe+(n-3)FAD rats with a fold-change of 1.18 ($P = 0.012$). The dopa decarboxylase (*DDC*) gene tended to be downregulated in the Hip of ID+DHA/EPA-repleted rats with a fold-change of −1.17 ($P = 0.039$). There was a trend toward upregulation of *HTR5A* in the Fe+(n-3)FAD group (fold-change = 1.16; $P = 0.014$).

MWM. During the cued task, all groups improved the distance moved to reach the visible platform from trial 1 to trial 4 (main effect for trials; $P < 0.001$). There was no significant effect of diet or diet × trials interaction, indicating normal learning behavior in all diet groups (data not shown). Diet had no significant effect on swimming speed across trials and days (data not shown).

During the working memory task, there was an effect of trials ($P < 0.001$) and days ($P < 0.001$) on mean distance moved to find the hidden platform, indicating that overall learning and memory took place across trials and days (**Fig. 1***A*). Additionally, there was an effect of diet ($P < 0.05$) and a trend toward a trial × diet interaction ($P = 0.06$), accompanied by an effect of trials ($P < 0.001$) on mean distance moved to find the hidden platform across trials 1 to 4. The ID+DHA/EPA group moved a longer overall distance to find the hidden platform than the control group ($P < 0.05$). In the 2-way repeated-measures ANOVA excluding the control group, DHA/EPA repletion increased the overall distance moved ($P = 0.028$). Working memory and learning is typically reflected in a rapid reduction of distance moved from trial 1 (when platform position is unknown) to trial 2. Therefore, we calculated the difference in distance moved between trial 1 and trial 2 across all 4 d (Fig. 1*B*). The ID+DHA/EPA rats exhibited impaired working memory, reflected by a lower mean difference in distance moved between trial 1 and trial 2 compared with controls ($P < 0.05$).

During the reference memory task, diet did not affect overall distance moved across all 4 d ($P = 0.32$), but there was a diet × days interaction ($P < 0.05$) (**Fig. 2***A*). Separate analyses of the search pattern from d 1 to 4 for each diet group showed that there was a significant improvement in distance moved from d 1 to 4 ($P < 0.05$) in the Fe+DHA/EPA, ID+(n-3)FAD, and control groups, whereas there was no significant improvement across days in the Fe+(n-3)FAD ($P = 0.40$) and ID+DHA/EPA ($P = 0.24$) groups. On d 4, the ID+DHA/EPA rats moved a longer distance than controls ($P < 0.05$).

After 4 d of reference trials, the platform was removed and the rats were subjected to a probe trial. There was an effect of quadrant ($P < 0.001$) on percent distance swum during the probe trial but no effect of Fe or DHA/EPA and no interaction. The percent distance swum in the target quadrant in experimental

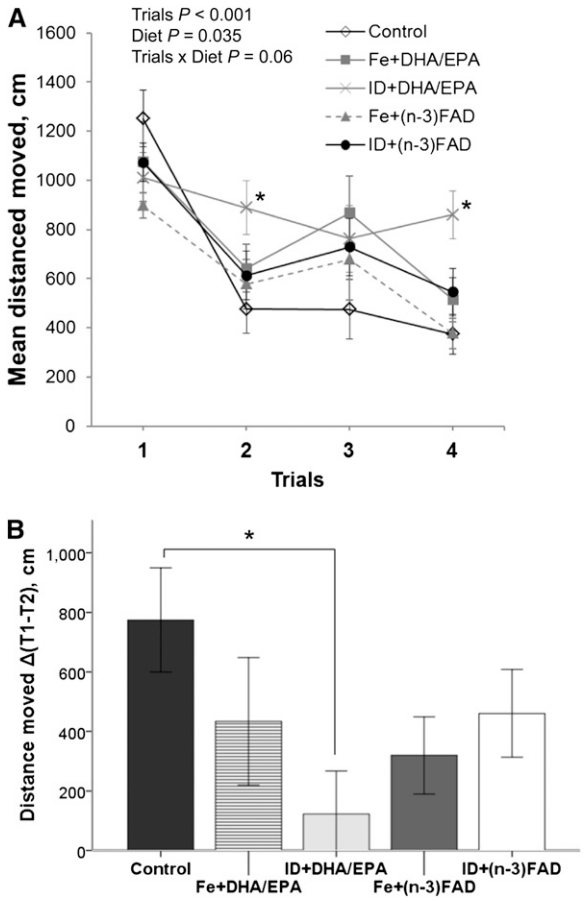

FIGURE 1 Working memory performance in the MWM of male control rats and ID+(n-3)FAD rats repleted with an Fe+DHA/EPA, ID+DHA/EPA, Fe+(n-3)FAD, or ID+(n-3)FAD diet for 5 wk. (*A*) Mean distance moved per trial to reach hidden platform located at different positions across 4 d, with 4 trials/d in the working memory task. (*B*) Working memory performance expressed as difference in distance moved between trial 1 and trial 2. Values are means ± SEM, $n = 7–8$. Labeled means without a common letter differ, $P < 0.05$. *Different from control, $P < 0.05$. (n-3)FAD, (n-3) fatty acid deficient; ID, iron deficient; MWM, Morris water maze.

groups did not differ from those in controls. However, the ID +(n-3)FAD rats showed no significant preference for the target quadrant (Fig. 2*B*), whereas the Fe+DHA/EPA and ID+DHA/EPA rats showed a distinct preference for the target quadrant: within group, the percent distance swum in the target quadrant during a trial of 60 s was significantly higher than percent distance swum in other quadrants.

Discussion

This is the first study to our knowledge to investigate the effects of repleting ID+(n-3)FAD rats with Fe and DHA/EPA, alone and in combination, during adulthood. The experimental model used may represent the common scenario in low-income countries where children of mothers with poor (n-3) FA status consume a diet low in Fe and (n-3) FA [but high in (n-6) FA] throughout childhood and only begin to consume a diet sufficient in one or both nutrients in early adulthood as dietary quality and variety improves.

The ID+(n-3)FAD rats were successfully depleted in both brain (n-3) FA (by 76–70%) and Fe (by 20–25%). Repletion of the ID+(n-3)FAD rats with DHA/EPA increased brain DHA levels by ~50–80% of control values. There was near complete recovery of brain Fe (~90–100% of controls) after Fe repletion, consistent with previous studies showing that postweaning ID is largely reversible with Fe treatment (9).

Disturbances in brain monoaminergic neurotransmission can impair learning and memory (12). In our previous depletion study, DA concentrations in ID+(n-3)FAD rats were elevated 1- to 2-fold in the OB and Str at PND 56 (14). In the current study, after an additional 5 wk of depletion, at PND 91, the ID+(n-3) FAD rats had significantly higher DA concentrations than controls in the FC but not in the OB or Str. This is consistent with previous studies reporting dramatic increases in DA-mediated neurotransmission in FC during adolescence (20–22), because FC is one of the last areas of the brain to become fully mature. Repletion with Fe and/or DHA/EPA reduced DA concentrations in the FC to levels that did not differ from those of controls. In the Fe+DHA/EPA and Fe+(n-3)FAD groups this reduction may be explained by the upregulation of the DA receptor D1A gene in the Hip and in the ID+DHA/EPA group by the nearly significant downregulation of *DDC*. However, the Fe × DHA/EPA interactions on the DA metabolite DOPAC in the OB and Str indicate that provision of DHA/EPA and Fe in combination to double-deficient rats alters DA metabolism in a different way than provision of either nutrient alone.

In our previous depletion study, ID+(n-3)FAD at PND 56 caused a sharp reduction in 5-HT in the OB and in its metabolite 5-HIAA in the Str compared with controls (14). In the current study, these differences were no longer apparent. However, the antagonistic Fe × DHA/EPA interactions on 5-HT in the OB and on 5-HIAA in the Str suggest that provision of DHA/EPA and Fe in combination to double-deficient rats alters 5-HT metabolism in a different way than provision of either nutrient alone. Neurotransmitter systems can adapt to chronic stressors, such as drug and alcohol exposure. This may also be at least partially true for nutrient deficiencies and could explain why certain monoamines that were altered during the acute depletion period were no longer altered in the rats that remained double-deficient throughout the repletion period. Furthermore, it is possible that repleting double-deficient rats with only one nutrient leads to a disruption of chronic adaptation, whereas repletion with both nutrients may restore the initial state.

The MWM findings indicate that repletion with DHA/EPA alone produced significant deficits in working memory performance compared with age-matched controls. Consistent with the findings from the working memory test, formerly ID+(n-3) FAD rats repleted with DHA/EPA or Fe alone showed little evidence of learning during the reference memory task. In contrast, rats receiving combined Fe+DHA/EPA exhibited a marked improvement across days in the reference memory task. Furthermore, our results from the probe trial suggest that deficits in memory retention were reversible in all repletion groups, except in rats receiving Fe alone (Fig. 2*B*). Several repletion studies, using a rodent model of perinatal ID, have reported irreversible deficits in reference memory performance despite repletion of brain Fe concentrations (6,23–25). In contrast, behavioral alterations associated with perinatal (n-3)FAD (induced over 2–3 generations) have been shown to be reversible (26–29). The repletion diets were provided during adulthood, when the brain is thought to be fully mature. Repleting ID+(n-3)FAD rats during periods of rapid brain development may produce different results.

Several mechanisms may explain why provision of Fe or DHA alone to double-deficient adult rats aggravated deficits in working and reference memory performance. Behavioral deficits can result from impaired neurotransmission, decreased myelin

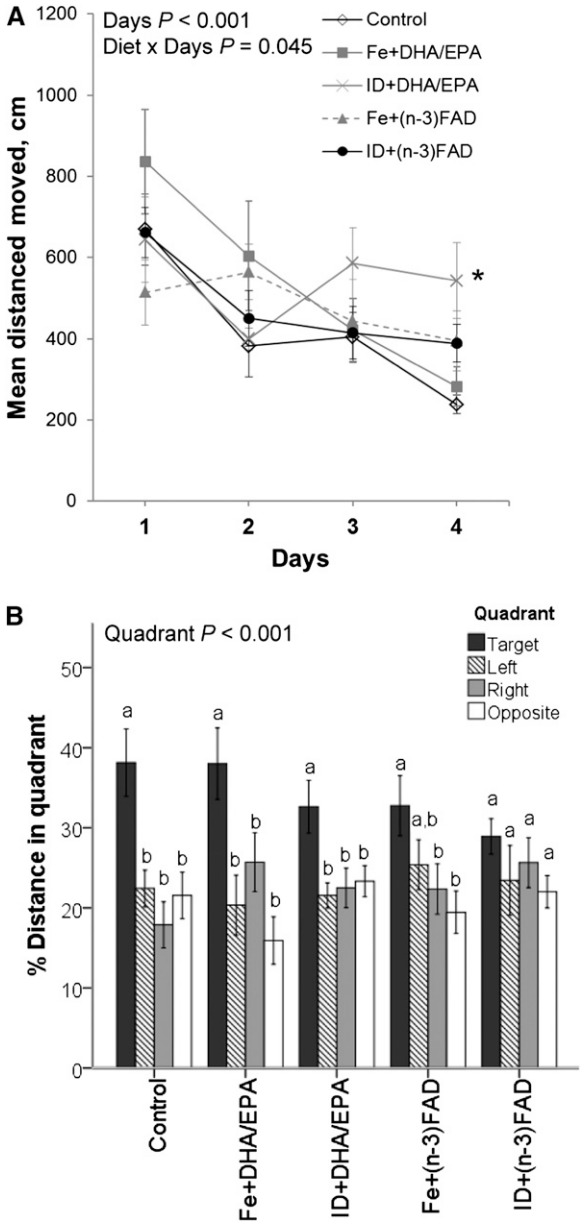

FIGURE 2 Reference memory performance in the MWM of male control and ID+(n-3)FAD rats repleted with an Fe+DHA/EPA, ID+DHA/EPA, Fe+(n-3)FAD, or ID+(n-3)FAD diet for 5 wk. (*A*) Mean distance moved per day to reach hidden platform located at same positions across 4 d, with 4 trials/d in the reference memory task. (*B*) Percentage of total distance spent in each quadrant during the probe trial, when the platform was removed and the rats were allowed to swim freely for 60 s. Values are means ± SEM, n = 7–8. Labeled means without a common letter differ, $P < 0.05$. *Different from control, $P < 0.05$. (n-3)FAD, (n-3) fatty acid deficient; ID, iron deficient; MWM, Morris water maze.

formation, or neuro-inflammation (30,31). The results from an in vitro study in oligodendroglia-like cells suggest that exposure of DHA-enriched cells to moderate levels of Fe may produce adaptation to oxidative stress by triggering a transient scrambling of membrane lipid asymmetry, which stimulates cellular antioxidant systems (32). Because oxidative stress may trigger inflammation that can cause cognitive impairment (30), it can be speculated that provision of either Fe or DHA/EPA alone caused more oxidative stress and inflammation than the combined provision of Fe and DHA/EPA. Similar outcomes emerged in a recent study, which demonstrated improved MWM performance in ID rats after repletion with Fe and a mixture of essential fatty acids, whereas ID rats that were repleted with Fe alone performed worse than controls (33).

Furthermore, we have shown that provision of Fe in combination with DHA/EPA synergistically increased Fe concentrations in the OB (Fe × DHA/EPA interaction). This interaction may be explained by previous studies demonstrating that DHA-enrichment of cell lines in the presence of Fe induces an increase in DMT-1 receptor expression associated with increased intracellular Fe concentrations (32,34).

Conversely, we have shown that DHA concentrations in several brain regions were higher when DHA/EPA was fed in combination with Fe than when fed alone. Provision of DHA/EPA in combination with the prooxidant Fe may have enhanced the incorporation of DHA into membrane phospholipids, where DHA may activate an antioxidant response.

A novel finding in the present study is that provision of Fe or DHA/EPA alone to double-deficient adult rats appeared to affect DA and 5-HT pathways differently than combined repletion and appeared to exacerbate the working and reference memory performance deficits compared with age-matched controls.

These findings may have relevance to human populations, because many children in low-income countries may suffer from both ID and (n-3)FAD due to poor-quality diets (35,36). In these groups, the functional interactions between ID and (n-3)FAD might have been overlooked in previous studies searching for cognitive improvements after provision of only Fe or only (n-3) FA. Furthermore, Fe supplementation and/or fortification programs are expanding in many countries. Our data suggest it may be prudent to consider potential exacerbation of (n-3)FAD (and its functional impairments) when providing Fe supplements to children in low-income countries.

Acknowledgments

The authors thank Anic Ostertag, Florentine Hilty, Lea von Moos, Karin Hotz, Jasmin Tajeri-Foman, Marica Brnić, and Isabelle Baumgartner (ETH Zürich) for their technical assistance; Caroline Remijn (Unilever R & D, Vlaardingen, The Netherlands) for conducting the brain Fe analysis; and Guus Duchateau (Unilever R & D, Vlaardingen, The Netherlands) for constructive inputs into study design. M.B.Z., C.M.S., R.F.H., and J.B. designed research; J.B. and M.A. conducted research; J.B., L.E.B., B.K.Y., L.M., M.M., and M.V.B. analyzed data; J.B. and M.B.Z. wrote the paper; and J.B. had primary responsibility for final content. All authors read and approved the final manuscript.

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