Phospholipid supplementation reverses behavioral and biochemical alterations induced by n–3 polyunsaturated fatty acid deficiency in mice

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Abstract **This study investigated the effects of a diet deficient in α-linolenic acid followed or not by supplementation with phospholipids rich in n–3 polyunsaturated fatty acids (PUFA) on behavior and phospholipid fatty acid composition in selected brain regions. Three weeks before mating, two groups of mice were fed a semisynthetic diet containing both linoleic and α-linolenic acid or a diet deficient in α-linolenic acid. Pups were fed the same diet as their dams. At the age of 7 weeks, a part of the deficient group was supplemented with n–3 PUFA from either egg yolk or pig brain phospholipids for 2 months. In the open field, rearing activity was significantly reduced in the deficient group. In the elevated plus maze (anxiety protocol), the time spent on open arms was significantly smaller in deficient mice than in controls. Using the learning protocol with the same task, the α-linolenic acid deficiency induced a learning deficit. Rearing activity and learning deficits were completely restored by supplementation with egg yolk or cerebral phospholipids, though the level of anxiety remained significantly higher than that of controls. There were no differences among the 4 diet groups for either the Morris water maze or passive avoidance. In control mice, the level of 22:6 n–3 was significantly higher in the frontal cortex compared to all other regions analysed. The frontal cortex and the striatum were the most markedly affected by the deficiency. Supplementation with phospholipids restored normal fatty acid composition in brain regions except for frontal cortex. Egg yolk or cerebral phospholipids are an effective source of n–3 PUFA for reversing behavioral changes and altered fatty acid composition induced by a diet deficient in n–3 PUFA.**—Carrié, I., M. Clément, D. de Javel, H. Francès, and J-M. Bourre. **Phospholipid supplementation reverses behavioral and biochemical alterations induced by n–3 polyunsaturated fatty acid deficiency in mice.** ***J. Lipid Res.*** **2000.** 41: **473–480.**

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The central nervous system has the second greatest concentration of lipids, immediately after adipose tissue. These brain lipids contain a very high amount of long-chain polyunsaturated fatty acids (LCPUFA), particularly arachidonic acid (AA; 20:4 n–6) and docosahexaenoic acid (DHA; 22:6 n–3). These two LCPUFA, which are the major constituents of neural cell membrane phospholipids, are derived, respectively, from two dietary precursors: linoleic acid (18:2 n–6) and α-linolenic acid (18:3 n–3). Linoleic and α-linolenic acids are the only sources for LCPUFA. Vertebrates are unable to synthesize these two essential polyunsaturated fatty acids (PUFA), they must be provided by the diet. Large amounts of AA and DHA are incorporated into the central nervous system during pre- and postnatal development (1–3). A diet deficient in α-linolenic acid modifies the fatty acid composition of cell membranes and organelles in the brain (4, 5). It induces a dramatic loss of DHA compensated for by an increase in docosapentaenoic acid (22:5 n–6). These alterations lead to changes in physical properties of cerebral membranes, and alterations in enzyme activities, receptors, transport, and cellular interactions (3, 6). These physiological modifications are accompanied by learning and behavioral defects in animals.

Lamptey and Walker (7) were the first to report behavioral effects of feeding an n–3 PUFA-deficient diet in rats. More than 10 years later, other studies reported learning deficits in n–3 PUFA-deficient animals. For example, deficiency alters learning in brightness-discrimination and active avoidance in rats (6, 8). Our own studies have shown that young mice (7-weeks-old) fed a diet deficient in α-linolenic acid exhibit learning impairments on habituation, elevated plus-maze (learning protocol), Morris water maze, and passive avoidance (9–12). A deficit in spatial learning on the Morris water maze has also been observed by Nakashima et al. (13) in deficient mice, in disagreement with Wainwright et al. (14). Conversely, spatial learning was improved

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; CTL, control diet; DEF, α-linolenic acid-deficient diet; LCPUFA, long-chain polyunsaturated fatty acids; B-PL, brain phospholipids; E-PL, egg yolk phospholipids.

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by supplementation with n–3 PUFA (15, 16). Recently, assessment of neurotransmission processes has shown that chronic dietary α-linolenic acid deficiency alters dopaminergic and serotoninergic systems in rats (17). These neurochemical modifications have been related to a reduction of distractibility (18). Thus, animal studies of n–3 PUFA deficiency established the importance of n–3 PUFA in behavior.

Currently, the need to supplement infant formulas with LCPUFA has been questioned as the human milk contains both precursors and LCPUFA. Studies of brain fatty acid composition have demonstrated that breast-fed infants have greater levels of cerebral cortex DHA than formula-fed infants (19, 20). More recently, studies have investigated the effect of enrichment of infant formula with DHA or DHA and AA on cognitive development. Some results suggest a beneficial effect of LCPUFA-supplemented formula on visual attention in preterm infants (21, 22) and on cognitive performance in term infants compared to those not supplemented (23). However, other studies have found no differences (24, 25). In piglets, formula enriched with both n–3 and n–6 LCPUFA from phospholipids has been shown to be an effective source for supplying brain DHA rather than fish oil which provides only n–3 LCPUFA (26).

The speed of recovery after deficiency is very slow; many weeks are required for brain cells and organelles to recover in rats (27). The dietary α-linolenic acid requirements for obtaining and maintaining a physiological level of DHA have been determined in developing and adult rats (6, 28). Bourre, Dumont, and Durand (29) demonstrated that brain phospholipids providing both n–3 and n–6 LCPUFA are useful as a source of n–3 polyunsaturated fatty acids. The requirement is 50–60 mg of n–3 PUFA /100 g of diet from brain phospholipids to restore normal brain fatty acid composition in α-linolenic acid-deficient developing rats.

To evaluate any link between n–3 fatty acids and behavior, we speculated whether behavioral deficits induced by deficiency in our strain of mice could be reversed by DHA supplementation. In this study, we investigated the effects of a diet deficient in α-linolenic acid, with or without supplementation with n–3 LCPUFA, on behavioral and learning abilities in adult mice. The effectiveness of different sources of phospholipids rich in DHA was examined by comparing purified phospholipids: those from hen egg yolks or pig brains. The effect of diets on the phospholipid fatty acid composition of selected brain regions is also reported.

MATERIALS AND METHODS

Animals and diets

Female OF1 mice originating from IFFA-CREDO (L'Arbresle, France) and bred in our laboratory were divided into two groups 3 weeks before mating. The two groups were fed purified diets (INRA, Jouy en Josas) containing 6% lipids that were similar except for fatty acids. Control diet lipids (CTL group) were a mixture of peanut oil and rapeseed oil containing ~1200 mg of linoleic acid and ~200 mg of α-linolenic acid per 100 g of diet. α--Linolenic acid-deficient diet lipids (DEF group) were peanut oil containing ~1200 mg of linoleic acid per 100 g of diet and traces of α-linolenic acid (<6 mg per 100 g of diet). Pups were fed the same diet as their dams. At weaning, pups were separated according to sex and were housed in such a way that each home cage contained 6 pups from 6 different dams. At 7 weeks, the adulthood, some of the deficient group received a diet supplemented with ~50 mg of n–3 PUFA per 100 g of diet for 2 months. This supplemented diet contained 40 mg of docosahexaenoic acid per 100 g of diet provided by either egg yolk phospholipids (E-PL group) or pig brain phospholipids (B-PL group). Hens were fed a special diet designed to increase DHA level in egg yolk phospholipid from 3% to 7%. Cerebral phospholipids were purified from pig brains and adsorbed onto an aerosyl (silica powder) matrix according to the method previously described (29). These phospholipids were prepared by Laboratoires Ponroy (Les Clayes sous Bois, France). The composition of diets and the fatty acid contents are reported in **Table 1** and **Table 2**. Diets were prepared monthly and stored at 4°C.

Mice were housed in an air-conditioned animal room illuminated from 8 am to 8 pm and maintained at 21 ± 1°C. They were given free access to their respective diets and water. Male mice aged 4 months were used for behavioral tests.

Experimental protocols were approved and met government guidelines (Ministry of Agriculture, Authorization no. 03007; June 4, 1991).

Open-field

A wooden white-painted open-field (50 × 50 × 25 cm) in a light- and sound-attenuated chamber was used. The floor was divided by black lines into 25 squares (10 × 10 cm). A mouse placed in the middle of the open-field was observed for 5 min. The number of squares crossed (motor activity) and rearings against the walls (exploratory activity) were counted.

Elevated plus-maze test

Apparatus: a grey elevated plus-maze was used. Two open arms (25 × 5 cm) and two (25 × 5 cm) closed arms were attached at

TABLE 1. Diet composition

	Control	n–3 Deficient	Egg-PL[a]	Brain-PL[b]
		g/kg		
Casein	220	220	220	220
Corn starch	432.3	432.3	432.3	432.3
Saccharose	216.1	216.1	216.1	216.1
Cellulose	20	20	20	20
Mineral mixture[c]	40	40	40	40
Vitamin mixture[d]	10	10	10	10
dl methionine	1.6	1.6	1.6	1.6
Peanut oil	30	60	48.7	49.9
Rapeseed oil	30	—	—	—
Egg PL	—	—	16.2	—
Brain PL	—	—	—	20.3

[a] Egg yolk phospholipids.
[b] Brain phospholipids.
[c] Composition of the mineral mixture (g/kg of diet): $CaHPO_4$, $2H_2O$, 15.2; K_2HPO_4, 9.6; $CaCO_3$, 7.2; NaCl, 2.76; MgO, 0.8; $MgSO_4$, $7H_2O$, 3.6; $FeSO_4$, $7H_2O$, 0.344; $ZnSO_4$, 7 H_2O, 0.2; $MnSO_4$, H_2O, 0.2; $CuSO_4$, $5H_2O$, 0.04; NaF, 0.032; KI, 0.0016; $CoCO_3$, 0.0008; Na_2SeO_3, $5H_2O$, 0.0008; $(NH_4)6Mo7O_{24},4H_2O$, 0.0008; $CrK(SO_4)2$, $12H_2O$, 0.02.
[d] Composition of vitamin supplements triturated in dextrose (mg/kg of diet), United States Biochemicals Corp., Cleveland, OH, USA: l-ascorbic acid, 100; choline chlorhydrate, 750; d-calcium pantothenate, 30; inositol, 50; menadione, 1; nicotinic acid, 45; para-aminobenzoic acid, 50; pyridoxine HCl, 10; riboflavin, 10; thiamine HCl, 10; retinyl acetate, 10; cholecalciferol, 0.0625; d-biotin, 0.2; folic acid, 2; cyanocobalamin, 0.0135; *dl*-alpha-tocopherol acetate, 50.

TABLE 2. Fatty acid composition of dietary lipids

Fatty Acids	Control	n–3 Deficient	Egg-PL	Brain-PL
		mg/100 mg fatty acids		
14:0	0.5	0.4	0.4	0.3
16:0	9.0	12.5	14.0	12.2
18:0	3.2	4.0	5.6	5.4
20:0	0.9	1.5	0.7	1.3
22:0	1.3	2.5	1.9	2.2
24:0	—	—	—	0.8
Σ SFA	15.9	20.9	22.6	22.2
16:1 n–7	—	—	0.6	1.0
18:1 n–9	56.7	50.4	51.3	50.7
20:1 n–9	1.2	1.1	1.1	1.2
Σ MFA	57.9	51.5	53.0	52.9
18:2 n–6	23.6	27.8	23.5	23.5
20:4 n–6	—	—	0.3	0.7
Σ n–6 PUFA	23.6	27.8	23.8	24.2
18:3 n–3	3.6	<0.1	<0.1	<0.1
20:5 n–3	—	—	<0.1	<0.1
22:5 n–3	—	—	<0.1	<0.1
22:6 n–3	—	—	0.7	0.7
Σ n–3 PUFA	3.6	—	0.7	0.7
n–6 + n–3	27.2	27.8	24.5	24.9
n–6/n–3	6.5	—	34.0	34.6

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; Egg-PL, egg yolk phospholipids; Brain-PL, brain phospholipids.

right angles to a central platform (5 × 5 cm). The open arms and the central platform were covered with white plastic-coated paper. The apparatus was 40 cm above the floor.

Anxiety protocol. The mouse was placed on the central platform with its head towards an open arm. The frequency of entries onto the open and closed arms was noted and time spent on the open arms was recorded over 5 min. In this test, entry onto either arm was counted when the mouse had its body and four paws on the arm.

Learning protocol. The mouse was placed at the end of an open arm with its back to the central platform. The time for the mouse to cross a line half way along one of the closed arms was measured (transfer latency) on day 1 and day 2. The mouse had to have its body and four paws on the other side of the line. If the mouse had not crossed the line after 90 sec, it was placed beyond it. After crossing the line, the mouse had 30 sec for exploring the apparatus. Learning was defined as a reduced transfer latency on day 2 compared to day 1.

Morris water maze

A white circular platform (6 cm in diameter) was placed on a pedestal 19 cm above the floor of a grey plastic tank, 80 cm in diameter and 30 cm high. The tank was filled with water (21 ± 1°C) to a level of 20 cm. The submerged platform was made invisible by adding a white opacifier: Lytron 631 (Norton International, distributed by Brenntag France, Sartrouville, France).

Place learning. Each mouse underwent four trials a day for 4 consecutive days. For each trial, the mouse was placed in the water facing the pool wall at one of eight possible starting locations, which were regularly distributed around the tank. There were visual cues in the room including posters on the walls, a light, and the experimenter, who always stood in the same position. Latency to finding the hidden platform was recorded (Escape Latency). If a mouse did not find the platform after 120 s of swimming, it was gently put on it. Once the mouse located the platform (or was put on it) it was permitted to remain there for 30 s. At the end of the four trials the mouse was dried with paper towels and returned to a holding cage positioned 40 cm under a lamp.

Probe trial. On the fifth day of the learning test, the platform was withdrawn. The circular tank was divided in four quadrants, delimited by two perpendicular yarns placed 15 cm above the water. The time the mouse swam in each of the four quadrants of the tank was recorded for 100 s. Learning was defined as a mouse spending a time significantly longer than 25 s in the quadrant where the platform was located (training quadrant).

Passive Avoidance

Mice were placed in the dark in the laboratory 17 h before the test. A mouse was placed in the automated apparatus (Gemini Avoidance System; San Diego Instruments) and allowed to explore for 3 min, the guillotine-type door being open. The door was then closed and the mouse was placed in the right compartment (26 × 20 × 15). After 30 s of adaptation, the compartment lighted up with an intensity of 543 lux, and the door opened. When the mouse entered the dark compartment (left), the door closed and an electric footshock of 0.3 mA was delivered for 10 sec. Each mouse underwent one trial, a cut-off time of 300 s was assigned. Latency to entering the dark compartment was recorded. The retention test (day 2) was performed 24 h after the acquisition test (day 1). Learning was considered as a significant increase in the latency on day 2 compared to day 1. Twelve male mice per group were tested.

Fatty acid analyses

Mice were killed by decapitation. Brains were quickly removed and dissected on ice into olfactory bulb (OB), frontal cortex (FC), striatum (ST), hippocampus (HC), and cerebellum (CB). There were 6 samples for each structure per diet group, each sample was pooled from 2 or 5 mice according to weight of the structure. Samples were lyophilized and stored at −70°C until fatty acid analysis was performed.

Lipids were extracted from the brain regions using chloroform–methanol 2:1 according to the method of Folch, Lees, and Sloane Stanley (30). Total phospholipids were separated by thin-layer chromatography using silica gel plates (Durasil-25, Macherey-Nagel, Hoerdt, France). Solvents were hexane–diethyl ether–acetic acid 90:30:1 (by vol). Phospholipid fatty acids were transesterified according to the method of Lepage and Roy (31). Fatty acid methyl esters were analyzed on a Delsi gas chromatograph equipped with a flame ionization detector and a silica capillary column (length 30 m, internal diameter 0.25 mm, stationary phase SPB-PUFA, SUPELCO France). Helium was used as the carrier gas. Analysis was performed in isothermal mode, the oven temperature was maintained at 210°C, the injector and detector temperatures were maintained, respectively, at 230°C and 250°C. Fatty acids were identified by comparison with standard mixtures. Areas were calculated with a Merck-Hitachi 2500 integrator, and fatty acid concentrations were reported as percent of total fatty acid content.

Statistical analyses

One-way ANOVA was used to compare rearing activity and anxiety among the 4 groups. Student's two-tailed, paired *t*-test was used to compare latencies on the elevated plus-maze and passive avoidance between days 1 and 2 for each group. Place learning in the Morris water maze was examined by multivariate analysis (Systat software): two-way ANOVA for repeated measures with two factors (time, diet). Student's two-tailed, unpaired *t*-test was used for the probe trial.

Two-way ANOVA with two factors, brain structure and diet (SigmaStat Software, SPSS), was used to evaluate the differences between brain structures, the effect of diet, and the interaction

between structure and diet for each fatty acid. The Tukey test was performed for multiple comparisons. Only P values less than 0.01 were considered significant. Percentages were compared by one-way ANOVA, the Bonferroni post test was then applied with $P < 0.05$.

RESULTS

Open field

The number of squares crossed did not differ significantly among the 4 diet groups (**Fig. 1**). The number of rearings was significantly decreased in the deficient group compared to control and both supplemented groups ($P < 0.05$).

Anxiety

When a mouse was placed on the center platform with its head toward an open arm, the time spent on the open arms was significantly lower in deficient mice than in the control and supplemented groups ($P < 0.001$; **Fig. 2**). In control mice, the time spent on the open arms was significantly higher than in the supplemented groups ($P < 0.01$). There were no significant differences between the 4 groups in the total number of entries (data not shown).

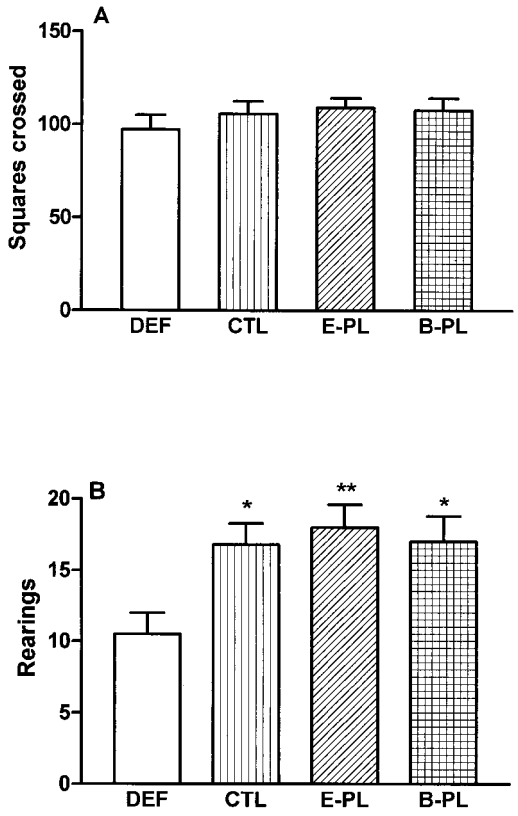

Fig. 1. Open-field. The number of squares crossed and rearings were recorded for 5 min. The motor activity (A) did not differ significantly among the 4 diet groups. The exploratory activity (B) of deficient group DEF was significantly lower than that of control CTL, egg E-PL or cerebral phospholipids B-PL groups. * $P < 0.05$; ** $P < 0.01$ vs. deficient group. Values are means ± SEM; n = 21 mice in each diet group.

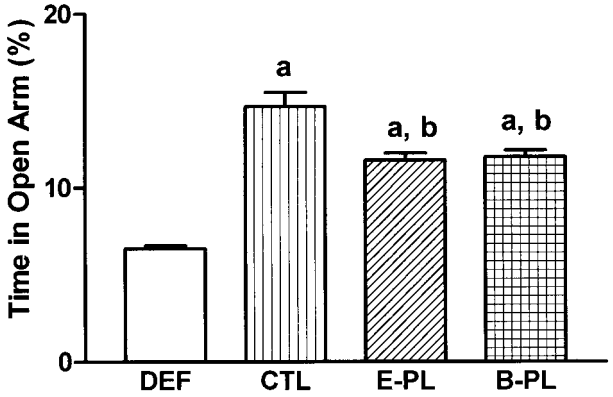

Fig. 2. Anxiety in the elevated plus-maze. The percentage of time (seconds, mean ± SEM) spent in open arms was calculated with 100% = total time spent in either an open or a closed arm. The time spent in open arms was significantly lower in deficient mice (DEF**)** than in control (CTL) and both supplemented groups (E-PL; B-PL). a: $P < 0.001$ vs. deficient group; b: $P < 0.01$ vs. control. Values are means ± SEM; n = 12 mice in each diet group.

Elevated plus maze: learning protocol

In deficient mice, the latency did not differ significantly between day 1 and day 2 (**Fig. 3**). For controls and both supplemented groups, the latency decreased significantly on day 2 compared to day 1 ($P < 0.001$). α-Linolenic acid deficiency induced learning deficits on this test.

Morris water maze

The latency in finding the hidden platform decreased significantly over the successive learning trials in both groups ($P < 0.001$; **Fig. 4**). There was no effect of diet and no interaction between diet and learning. Thus, the deficient diet had no effect on learning in 4-month-old mice after 16 training trials performed over 4 days.

In the probe trial, after the 4 days of training, the time spent swimming in the training quadrant Q1 was significantly longer than 25 sec for all groups, retention occurring in all 4 diet groups.

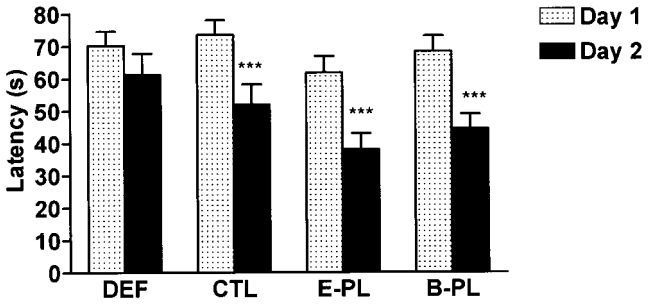

Fig. 3. Learning protocol in the elevated plus-maze. Latency to crossing the midpoint of the closed arms was recorded. The latency on day 2 decreased significantly for control and both supplemented groups (*** $P < 0.001$). Deficient mice did not show any significant difference between day 2 and day 1. Values are means ± SEM; n = 12 mice in each diet group. DEF, CTL, E-PL, B-PL as for Fig. 1.

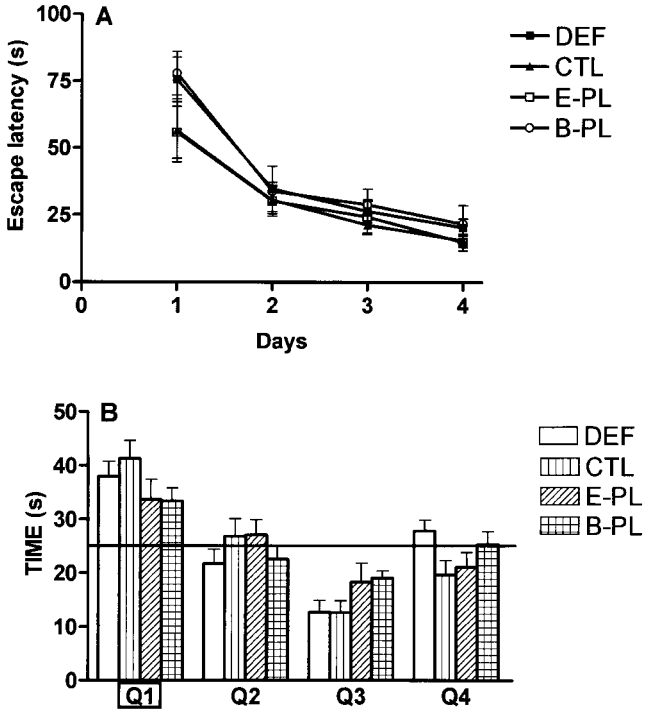

Fig. 4. Performance in the Morris water maze (A) as measured by the time it took mice to find the hidden platform (escape latency). Each point represents mean group performance (± SEM) in four trials from days 1 to 4 (n = 12 mice in each diet group). There was significant spatial learning for the 4 groups. Probe trial (B). The time spent in each of the four quadrants was recorded. All groups spent a time significantly longer than 25 s in the training quadrant Q1. DEF, CTL, E-PL, B-PL as for Fig. 1.

Passive Avoidance

The latency in entering the dark compartment was significantly increased from day 1 to day 2 in all groups ($P < 0.001$; **Fig. 5**). The results of this experiment conducted with a 1-day interval between acquisition and the retention test showed good retention for all 4 diet groups.

Fatty acid analysis

None of the 4 diets induced significant variations of the saturated and monounsaturated fatty acid composition in brain regions (data not shown). The principal long-chain polyunsaturated fatty acid contents are shown in **Table 3**. Two-way ANOVA showed significant differences in brain regions, a diet effect and interaction between regions and diet ($P < 0.001$). In control mice, the cerebellum had a significant lower level of 20:4 n–6 (AA) and 22:4 n–6 than the other regions ($P < 0.01$). The level of 22:6 n–3 (DHA) was significantly the highest in frontal cortex and the lowest in striatum ($P < 0.01$). α-Linolenic acid deficiency induced a decrease in 22:6 n–3 that was compensated by an increase in 22:5 n–6 in the 5 regions. **Figure 6** shows that the regions most marked by α-linolenic acid deficiency were the frontal cortex and striatum ($P < 0.05$). Supplementation with egg yolk or cerebral phospholipids restored the level of 22:6 n–3 in brain regions except for the frontal cortex where the difference from control remained significant.

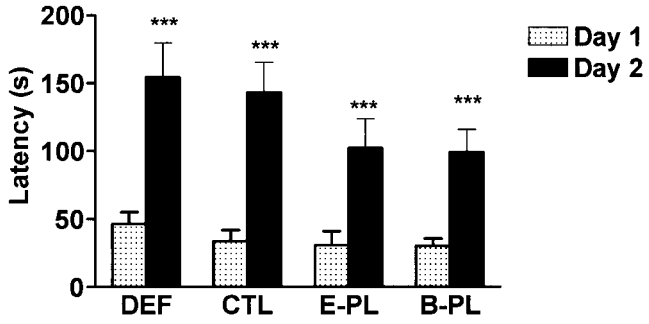

Fig. 5. Passive avoidance. Latency to entering the dark compartment was measured on days 1 and 2. Latency increased significantly on day 2 in all diet groups; *** $P < 0.001$. There were no between-group differences. Values are means ± SEM; n = 12 mice in each diet group. DEF, CTL, E-PL, B-PL as for Fig. 1.

DISCUSSION

This study examined the effects of a supplementation or no supplementation with phospholipids rich in n–3 PUFA on behavior, learning, and phospholipid fatty acid composition of brain regions in α-linolenic acid-deficient mice.

The open field test was used as a test of exploration. The number of squares crossed reflects the general motor activity, and rearings are associated to exploratory behaviors (32). In this study, there was no significant difference among diet groups for the number of squares crossed. The number of rearings was significantly lower in deficient mice than in controls. Thus, exploratory activity (rearings) was reduced by n–3 PUFA deficiency without changes in motor activity (crossings). These results are consistent with those of Lamptey and Walker (7) and Enslen, Milon, and Malnoë (33) who also observed a decrease in rearing activity in deficient rats. Supplementation with egg yolk or cerebral phospholipids was significantly effective at restoring normal activity in deficient mice. Exploratory behavior is believed to reflect the emotional reactivity to a novel environment, but also the fear and the curiosity, or the arousal level. Thus, it is difficult to interpret this behavior because it is the result of combined states (34). The percentage of time on the open arms of the elevated plus maze is an index of anxiety (35). In deficient mice, anxiety was significantly increased compared to controls, whereas it was significantly reduced by supplementation. However, the level of anxiety in supplemented groups remained significantly higher than that of controls. Nakashima et al. (13) have reported that n–3 PUFA-deficient mice tended to be more anxious than controls. These results did not agree with those observed in deficient rats (18).

A learning protocol has been developed and validated in rats and mice using the elevated plus maze (36, 37). On this test, latency significantly decreased between day 1 and day 2 in control and supplemented groups in contrast to deficient mice. This indicates that the deficiency-induced learning impairment is reversed by supplementation with phospholipids. In the Morris water maze, mice are trained

TABLE 3. Long chain polyunsaturated fatty acid composition

Group	Olfactory Bulb	Frontal Cortex	Striatum	Hippocampus	Cerebellum
			% total fatty acids		
20:4 n−6					
CTL	9.8 ± 0.1[a]	8.7 ± 0.1[a]	8.7 ± 0.9[a]	10.5 ± 0.8[a]	5.7 ± 0.3[b]
DEF	10.6 ± 0.5	8.9 ± 1.3	8.9 ± 0.6	11.6 ± 0.7	7.1 ± 1.0
E-PL	9.8 ± 0.7	9.4 ± 0.5	9.3 ± 0.5	10.6 ± 0.7	5.8 ± 0.7
B-PL	10.1 ± 0.3	9.0 ± 0.4	8.9 ± 0.3	11.4 ± 0.6	5.9 ± 0.4
22:4 n−6					
CTL	3.0 ± 0.1[a]	2.0 ± 0.2[a]	2.7 ± 0.2[a]	2.3 ± 0.5[a]	1.4 ± 0.3[b]
DEF	3.4 ± 0.3	2.5 ± 0.3	3.2 ± 0.5	2.8 ± 0.4	2.4 ± 0.1
E-PL	2.8 ± 0.1	2.4 ± 0.2	2.8 ± 0.1	2.6 ± 0.3	1.6 ± 0.2
B-PL	2.9 ± 0.3	2.2 ± 0.2	2.7 ± 0.3	2.4 ± 0.2	1.9 ± 0.1
22:5 n−6					
CTL	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.2
DEF	4.3 ± 0.4*	6.7 ± 0.6*	3.7 ± 0.4*	4.8 ± 0.5*	2.9 ± 0.4*
E-PL	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.7 ± 0.5	0.2 ± 0.1
B-PL	0.5 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.2 ± 0.03
22:6 n−3					
CTL	18.1 ± 0.1[b]	22.1 ± 0.8[a]	13.9 ± 0.9[c]	16.8 ± 0.5[b]	17.0 ± 0.7[b]
DEF	12.0 ± 1.2*	13.0 ± 1.0*	8.4 ± 0.9*	11.1 ± 1.1*	12.6 ± 0.5*
E-PL	17.4 ± 0.7	19.5 ± 0.7**	14.0 ± 0.5	15.9 ± 1.5	16.8 ± 0.6
B-PL	16.6 ± 1.1	20.6 ± 0.8**	13.7 ± 0.6	15.9 ± 1.3	16.3 ± 0.7

Composition of brain regions in four diet groups: control group (CTL), n−3 PUFA-deficient group (DEF), egg yolk phospholipid-supplemented group (E-PL), cerebral phospholipid-supplemented group (B-PL). Values are mean ± SD.

* $P < 0.01$ vs. all other diet groups; ** $P < 0.01$ vs. control.

[a,b,c] Letters indicate that the means differed significantly among regions with $P < 0.01$ in control group.

to escape from water by learning the spatial position of the platform relative to distal cues (38). All diet groups acquired place learning and showed good retention for the probe trial. Thus, performance in the spatial learning task was not affected by a diet deficient in α-linolenic acid. Wainwright et al. (14) also reported no spatial learning deficits in deficient mice. Passive avoidance procedures are widely used to investigate the effects on learning/acquisition or memory processes. It is an associative learning evaluating the memory trace for an aversive event (electric shock) (39). In this test, all 4 diet groups increased the latency on day 2 significantly . The deficient group performed as well as the control and supplemented groups.

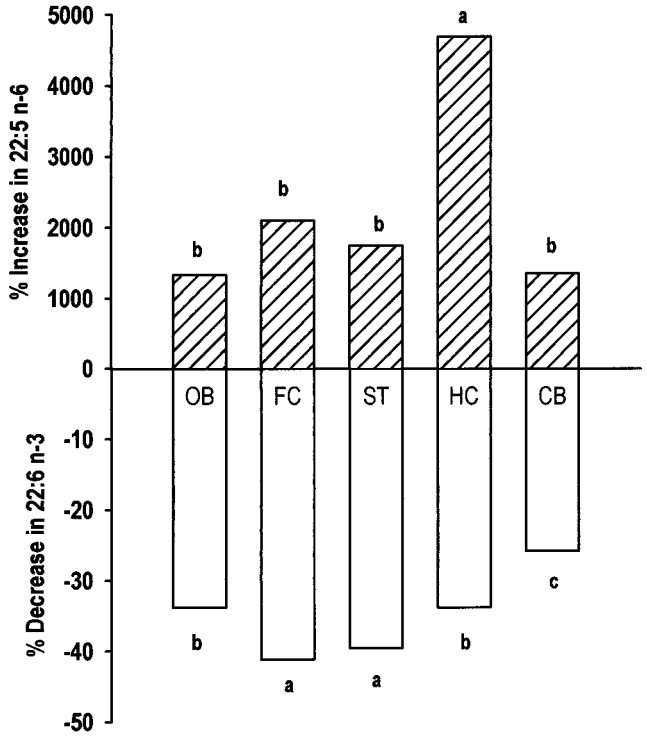

Fig. 6. Percentage increase in 22:5 n−6 and decrease in 22:6 n−3 in n−3 PUFA-deficient mice compared with control. Different letters were assigned when percentages differed significantly with $P < 0.05$ between regions. OB, olfactory bulb; FC, frontal cortex; ST, striatum; HC, hippocampus; CB, cerebellum.

These present experiments suggest that a diet deficient in α-linolenic acid affects exploratory activity, the level of anxiety, and learning in the elevated-plus maze in 4-month-old male mice. DHA supplementation with egg yolk or cerebral phospholipids reversed the deficits induced by the deficiency. Our previous studies, performed in 7-week-old deficient mice of the same strain, showed learning deficits on place learning in the Morris water maze and passive avoidance, but no changes for the exploratory activity and anxiety (9–12). Only learning deficits on the elevated plus maze were common to both age groups (9). The differences between these two experiments could be explained by an age effect. For example, rats fed a deficient diet show an effect on the distractibility at 6 months but not at 2 months (18). Our early studies were performed in young adult mice (7 weeks) whereas the present study was performed in adult mice (4–5 months). Thus, the impact of the deficiency may differ according to age. In this case, it seems that in young deficient mice learning abilities were particularly affected, whereas in adults exploration and anxiety were affected. However, for the earlier spatial learning study, young mice were female, whereas in the present study male mice were used. Sex differences have

been described for spatial abilities showing better performance for males (40). Another example of an age effect of deficiency is in the retina; we have observed that the effect of an α-linolenic acid-deficient diet on the electroretinogram disappears with age in this strain (12). In addition, in the tests where the aversive motivation is very strong (Morris water maze, passive avoidance), deficient adult mice have been able to learn. The less stressful models such as open field or elevated plus maze enabled behavioral alterations to be observed. However, it is remarkable that these alterations induced by α-linolenic acid deficiency were reversed by supplementation with egg yolk or cerebral phospholipids.

The fatty acid composition of total phospholipids in control mice showed that the region with the highest DHA level was the frontal cortex. These results agree with those observed in rats by Delion et al. (17) who found more DHA in frontal cortex than in striatum. In all brain regions studied in the α-linolenic acid-deficient mice, our results showed a reduced 22:6 n–3 (DHA) level and a compensatory higher 22:5 n–6 level. There were no variations of 20:4 n–6 or 22:4 n–6. These results are in agreement with those for whole brain in this mouse strain (9). Nevertheless, the α-linolenic acid deficiency did not affect the DHA levels in brain regions to the same extent. The most affected regions were the frontal cortex and striatum with, respectively, a reduction of 41.2% and 39.6%. Chronic α-linolenic acid deficiency in rats led to a greater decrease in the level of DHA in plasmenylethanolamine in frontal cortex than in striatum (41). Supplementation with egg yolk or cerebral phospholipids in α-linolenic acid-deficient mice increased DHA concentration to control levels in all brain regions except frontal cortex, where the difference remained significant. Thus, a 2-month diet with phospholipids as source of n–3 PUFA restored normal fatty acid composition. Levels of 22:5 n–6 were decreased in all brain regions. Although cerebral phospholipids provided 2-fold more arachidonic acid than egg yolk, arachidonic levels did not differ between the two supplemented diet groups.

Searching for an explanation for these behavioral effects of the n–3 PUFA deficiency is difficult as the number of known neurochemical modifications induced by the deficiency is small. However, it has been reported that there is a relationship between the dopaminergic system and the PUFA profile in the brain. In chronic α-linolenic acid-deficient rats, endogenous dopamine levels and D2 receptor binding are decreased in the frontal cortex but not in the striatum (17). Zimmer et al. (42) reported later that the deficiency may modify the internalization of dopamine in the storage pool in the frontal cortex. In addition, in rats depleted in dopamine, Yehuda, Rabinovitz, and Mastofsky (43) observed a decrease in motor (crossing) and rearing activity in the open field. In the same rats, administration of a mixture of free essential fatty acids restored normal motor activity, in contrast to rearing activity which was partially restored. Therefore, dopamine may be the link between the n–3 PUFA deficiency and its effects on exploration.

This is the first time that an increase in anxiety was observed in α-linolenic acid-deficient animals. The postsynaptic stimulation of dopaminergic receptors in prefrontal cortex of rats led to a reduction of exploration of the open arms in the elevated plus maze (44). In chronic deficient rats, an increase in 5HT2 serotoninergic receptors in frontal cortex has been reported, however, no modification of anxiety was observed (17, 18). Thus, the apparent learning deficit in the elevated plus maze of deficient mice could be due to a state of anxiety.

In fact, data on the neurochemical modifications induced by n–3 PUFA deficiency are not sufficient to establish an exact correlation between the region-specific neurotransmitter and specific behavior. For example, in a model of transgenic mice deficient in catechol-O-methyltransferase with a marked reduction of dopamine in the frontal cortex, no locomotor deficits were observed but an increase in anxiety was observed (45). On the other hand, a novel environment activates the cortical cholinergic system and there is an increase in acetylcholine release in the frontal cortex during exploratory activity of rats (46). In rodents, olfactory bulbectomy induces behavioral alterations in the open field and elevated plus maze accompanied by neurotransmission changes (47, 48). N–3 PUFA deficiency may affect the function of this brain region.

In conclusion, phospholipid supplementation is effective for reversing behavioral and biochemical changes induced by α-linolenic acid deficiency. The frontal cortex is the region with the highest level of DHA and seems particularly sensitive to n–3 PUFA deficiency. The optimal dose of 50–60 mg/100 g diet of n–3 PUFA in the form of phospholipids determined by Bourre, Dumant, and Durand (29) is sufficient to restore the fatty acid composition of brain membranes. This is 4-fold less than the dose of α-linolenic acid required to achieve the same effect. Thus, dietary phospholipids appear to be of particular interest for providing very long chain polyunsaturated fatty acids.

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