n-3 Polyunsaturated fatty acid supplementation reverses stress-induced modifications on brain monoamine levels in mice

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Abstract The aim of this study was to examine the effects of supplementation with n-3 polyunsaturated fatty acids (PUFAs) on stress responses in mice subjected to an unpredictable chronic mild stress (UCMS) procedure. Stress-induced modifications in coat and aggressiveness were evaluated, and phospholipid PUFA profiles and monoamine levels were analyzed in the frontal cortex, hippocampus, and striatum. The results showed that repeated exposure to mild stressors induced degradation in the physical state of the coat, lowered body weight gain, and increased aggressiveness, without any effect of n-3 PUFA supplementation. The UCMS induced a significant decrease in the levels of norepinephrine in the frontal cortex and striatum, and a nonsignificant decrease in the hippocampus. The tissue levels of serotonin (5-HT) were 40% to 65% decreased in the three brain regions studied. Interestingly, the n-3 PUFA supplementation reversed this stress-induced reduction in 5-HT levels. These findings showed that supplementation in n-3 long-chain PUFAs might reverse certain effects of UCMS in cerebral structures involved in stress-related behaviors.—Vancassel, S., S. Leman, L. Hanonick, S. Denis, J. Roger, M. Nollet, S. Bodard, I. Kousignian, C. Belzung, and S. Chalon. **n-3 Polyunsaturated fatty acid supplementation reverses stress-induced modifications on brain monoamine levels in mice. *J. Lipid Res.* 2008.** 49: **340–348.**

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The mammalian brain is particularly rich in docosahexaenoic acid (22:6n-3, DHA), the main n-3 polyunsaturated fatty acid (PUFA). DHA is provided directly by the diet from aquatic sources and, after endogenous synthesis in the liver, from its vegetable dietary precursor α-linolenic acid (18:3n-3, ALA) by successive desaturation and elongation. ALA is present in the brain at very low concentrations, whereas DHA can represent half of the total PUFAs inserted into phospholipids that constitute the structure of neuronal membranes. Accumulation of DHA in brain membranes is particularly high during the perinatal period, coinciding with the formation of synapses (1). It has been shown that the accumulation of DHA in the human infant brain during the first 6 months of life is half that of the total amount in the body, around 5 mg per day (2).

A diet deficient in ALA results in changes in the composition of cells, organelles, and synaptic membranes in the central nervous system and leads to reduced learning ability. Several animal studies have therefore focused on the effects of dietary n-3 PUFA deficiency on behavioral functions, showing reduced performance in spatial memory and discrimination tasks (as reviewed in Ref. 3). Moreover, rodents subjected to diets deficient in DHA or its precursor show reduced attention and reduction in locomotor responses to novelty, habituation, and anxiety (4–6). n-3 PUFA-deficient animals also exhibit aggressive behavior and increased symptoms of depression in a forced swim test (7). However, most of these effects were reversed by dietary supplementation with long-chain n-3 PUFAs (8–10).

It has been shown that these behavioral impairments may be the result of changes in the release of neurotrans-

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Abbreviations: ALA, α-linolenic acid; DA, dopamine; DHA, docosahexaenoic acid; DOPAC, dihydroxyphenyl acetic acid; EPA, eicosapentaenoic acid; 5-HIAA, 5-hydroxyindole acetic acid; 5-HT, serotonin; HVA, homovanillic acid; NE, norepinephrine; NSF, novelty suppression of feeding; PE, phosphatidylethanolamine; SFA, saturated fatty acid; TFA, total fatty acid; UCMS, unpredictable chronic mild stress.

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mitters and in interactions with corresponding receptors (11, 12). In particular, we showed that the presynaptic dopamine (DA) vesicle compartment was reduced in the frontal cortex of deficient rats, resulting in reduced cortical inhibition in ventral areas, particularly in the nucleus accumbens (13–15). These neurochemical changes may be responsible for inattention and inefficient reward processing, contributing to learning impairment and to slowing of extinction (16, 17). Changes in serotonergic neurotransmission were also reported in rats fed a chronic ALA-deficient diet, and these changes were potentially reversible by an adequate diet, depending on when the intervention occurred (18). In particular, weaning seems to be a pivotal period, after which any recovery is impossible, highlighting the importance of adequate supply of n-3 PUFAs during the developmental window (19).

In light of these findings, it can be suggested that early deficiency in n-3 PUFAs, particularly DHA, may result in a cascade of suboptimal development of neurotransmitter systems, especially in limbic structures, leading to impaired emotional and cognitive responses to subsequent environmental challenges. In addition, it has been suggested that DHA may be involved in the regulation of stress responses in rats, inasmuch as DHA intake completely reversed anxiety-like behavior in the elevated plus-maze caused by an n-3 PUFA-deficient diet and attenuated freezing behavior in conditioned fear-stress responses (20). It has also been shown that n-3 fatty acid-deficient mice were more vulnerable to stress, inasmuch as they behaved similarly to mice fed an adequate diet under normal conditions in the elevated-plus maze but were significantly more anxious under stressful conditions (3). In humans, it has previously been reported that administration of fish oil rich in DHA improved resistance to the mental stress of exams in students (21). Moreover, prevention of stress-induced aggression and hostility by DHA supplementation has been demonstrated in clinical trials (22, 23). The authors concluded that DHA influences a possible adaptive mechanism during stress by lowering norepinephrine levels.

There is currently some evidence regarding the involvement of dietary n-3 PUFA in mood disorders, particularly depression. Cross-national epidemiological analyses have suggested that lower n-3 PUFA levels are related to higher prevalence rates of major and postpartum depression (24, 25) and that there is a significant negative correlation between fish consumption and the prevalence of depression (26). In addition, several clinical studies have described abnormally low levels of DHA in the plasma and/or erythrocytes of depressed patients (27, 28).

To understand further the potential relationships between n-3 PUFA and depression, we examined the effects of n-3 PUFA supplementation on various responses induced by chronic stress exposure in mice. Animals were subjected to a chronic mild stress procedure that represents a well-known animal model of depression (29, 30). The consequences of n-3 PUFA supplementation on behavioral parameters and on monoaminergic levels and fatty acid profiles in several brain areas (i.e., the frontal cortex, hippocampus, and striatum) were compared in normal and stress conditions.

MATERIALS AND METHODS

Animals

Fifty-five male BALB/cByJ@Rj mice (Centre d'Elevage Janvier; Le Genest Saint Isle, France) were used in this study. The mice were aged 6 weeks on their arrival and were housed in groups of three to four. They were maintained in a temperature- (22°C) and humidity- (40%) controlled room on an inverted light-dark cycle (light from 20:00 to 8:00) with free access to food (regular chow, Ext M20, SDS; Essex, England) and water. Mice were first acclimatized to the laboratory for 1 week before the start of the experiment. Experiments were conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Mice were initially distributed into four groups. Mice of two groups were subjected to an unpredictable chronic mild stress (UCMS) procedure for 8 weeks, until the end of the behavioral tests. At the start of the experiment, stressed mice were maintained under the same standard conditions, but they were isolated in individual home cages (8.5 × 22 cm) and had no physical contact with the other mice. Animals from the two nonstressed groups were housed in groups of three or four, with a shelter and some tubes placed in their home cages. Several variables were used to assess the stress-induced effects, i.e., condition of coat, body weight, behavior in the novelty suppression of feeding (NSF) test, and behavior in the resident-intruder test. Condition of the coat and body weight were recorded weekly for all mice. The behavioral tests commenced from the seventh week. Mice were euthanized at the end of the behavioral tests (i.e., 8 weeks after the beginning of the UCMS procedure).

UCMS procedure

The stress protocol used was based on the UCMS procedure described by Willner, Muscat, and Papp (30), and adapted to mice by our laboratory (31–34). This animal model of depression consists of chronic exposure to various mild social and environmental stressors, none of which is sufficient alone to induce long-lasting effects. The stressors used vary, and they were applied in a different sequence each week to avoid any habituation. We excluded nociceptive stressors and food/water deprivation for ethical reasons. The emphasis in this model is on the chronic and variable nature of the stressors.

The stressors used consisted of removal of bedding, wetting the bedding, several repeated changes of bedding, tilting cages by 45° for varying times, placing ~2 cm of water in the home cage (after removing the bedding), exposure to rat bedding for 15 min, social stress 1 (placing a mouse in a cage that had previously belonged to another mouse), social stress 2 (placing a mouse in another animal's cage and then returning it to its own cage, where it would find that the cage had been occupied by another mouse), restrained stress in small tubes for varying times, lights on during the dark phase, lights off during the light phase, a succession of light and dark periods for 30 min, and switching the light/dark cycle for varying durations.

Parameters measured

Two parameters were measured throughout the UCMS procedure, i.e., condition of the coat and body weight. The condition of the coat was evaluated each week by examining the

coat on different parts of the body (head, neck, dorsal area, ventral area, tail, front and hind paws, and genital area). For each area, a score of 0 was applied if the coat was in good condition, and a score of 1 if it was in very poor condition (disordered, piloerection). The total score was the sum of the score for each area; thus a high score indicated that the coat was in poor condition. This method has been validated in a number of recent studies (31, 32, 35). Body weight was also measured each week until the end of the UCMS procedure.

n-3 PUFA supplementation

n-3 PUFA (léroDNV, Laboratoire léro; France) or vehicle devoid of n-3 PUFA (Frial oil; Lesieur, France) was administered daily at 1:30 PM by force feeding at a volume of 0.15 ml throughout the UCMS procedure. léroDNV contained 70% n-3 PUFA (w/w). Mice in the supplemented groups were thus receiving a dose of approximately 80 mg/day n-3 PUFA [6.1 mg DHA and 9.2 mg eicosapentaenoic acid (EPA)], corresponding to the dose that has been previously shown to allow maximal DHA incorporation in brain membranes (36).

Four groups of mice were formed: a nonstressed group receiving the vehicle (NS-V, n = 13), a stressed group receiving the vehicle (S-V, n = 14), a nonstressed group receiving n-3 PUFA (NS-PUFA, n = 14), and a stressed group receiving n-3 PUFA (S-PUFA, n = 14).

Behavioral tests

NSF test. The NSF test is a modified version of that used by Santarelli et al. (32). The testing apparatus consisted of a wooden 30 cm × 30 cm × 20 cm box with an indirect red light. The floor was covered with 2 cm sawdust. Twelve hours before the test, food was removed from the cages. At the time of testing, a pellet of food (regular chow) was placed on a white paper platform positioned in the center of the box. An animal was placed in a corner of the maze. The latency to manifestly chew the pellet was recorded within a 3 min period. This test induced conflicting motivation between the drive to eat the food pellet and the fear of venturing into the center of the arena. This test was performed at 10 AM.

Resident-intruder test. The resident-intruder test consisted of the introduction of an unknown animal into the home cage of test mice to measure their aggressiveness. The intruder was a naïve male C57BL/6J@Rj mouse, known for its high passivity and lack of aggression. When done in nonstressed mice, the animals were isolated 24 h before the test to become familiar with their novel environment. The bedding of the isolated stressed mice was changed 24 h before the test to standardize conditions between nonstressed and stressed mice. The test started when the intruder was placed in the home cage of the resident animal and lasted for 5 min. Two parameters were measured: the latency of the first attack and the frequency of attacks on the intruder. This test was performed at 3 PM.

Fatty acid analysis of phospholipid classes

Mice (n = 6 for each group) were euthanized by decapitation at the end of the behavioral tests. Brains were quickly removed, and the frontal cortex, hippocampus, and striatum were dissected out on ice, weighed, and frozen in liquid nitrogen. Total lipids were extracted by a modification of the method of Folch, Lees, and Sloane Stanley (37). Phosphatidylethanolamine (PE) was separated from total lipids on an aminopropyl-bonded silica gel cartridge (BAKERBOND spe™ Amino; Baker, USA) by the method of Alessandri and Goustard-Langelier (38). The fatty acids were methylated with BF3, and the fatty acid methyl esters were analyzed by gas liquid chromatography (Carlo Erba) (39) and identified by comparison with commercial standards of equivalent chain lengths. The results were expressed as mg fatty acids/100 mg total fatty acids (TFAs; wt %).

Monoamine analysis

Mice (n = 6–8 for each group) were euthanized by decapitation at the end of the behavioral tests. Brains were quickly removed, and the frontal cortex, hippocampus, and striatum were dissected out on ice and weighed. Each cerebral region was homogenized in 1 ml of a buffer containing 12 mM $HClO_4$, 0.1 mM EDTA, 0.5 mM $Na_2S_2O_5$, 3 mM octanesulfonic acid, and 3 mM heptanesulfonic acid with an Ultraturrax T25 at 4°C. After centrifugation at 30,000 *g* for 20 min at 4°C, 100 μl of the supernatant was stored at −80°C until use. Contents of norepinephrine (NE), DA, dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) were measured in each supernatant by HPLC, with electrochemical detection on a Concorde apparatus (Waters; St. Quentin-Yvelines, France). Samples were injected using a Rheodyne 7725i injector valve with a 20 μl injection loop. The mobile phase, consisting of 7% acetonitrile, 3% methanol, 90% 20 mM citric acid, 10 mM monobasic phosphate sodium, 3.25 mM octanesulfonic acid, 3 mM heptanesulfonic acid, 0.1 mM EDTA, 2 mM KCl, 6 ml/l o-phosphoric acid, and 2 ml/l diethylamine, pH 3, was pumped at 0.3 ml/min using a Gold 118 system (Beckman; Fullerton, CA). Separation was performed with a 3 μm C18, 3.2 × 100 mm reversed phase column (LC-22C, BAS; West Lafayette, IN). A glassy carbon working electrode set at 610 mV with reference to an in situ Ag/AgCl reference electrode was used to detect compounds. Signals were recorded and quantified with a Beckman Gold 118 integrator. Amounts of NE, DA, DOPAC, HVA, 5-HT, and 5-HIAA were calculated by comparing peak levels from the supernatant samples with those of external standards. Results are expressed as nmol/mg tissue.

Statistical analyses

All data are expressed as mean ± SEM. For monoamine assays and fatty acid analyses, means were compared by two-way ANOVA (PUFA supplementation × stress factors), followed by the posthoc Bonferroni test in case of significance. For behavioral studies and body weight follow-up, results were compared using nonparametric ANOVA from Kruskal-Wallis, followed by the posthoc Mann-Whitney U test in case of significance. Differences with $P < 0.05$ were considered significant. Statistical analyses were performed using Statistica 7.0 software (StatSoft®, Inc.; Tulsa, OK).

RESULTS

State of the coat and body weight

Coat state. Kruskal-Wallis test revealed significant differences in the state of the coat after 1 week of UCMS ($P < 0.001$) until the end of UCMS ($P < 0.001$) (**Fig. 1A**). The coat state of both stressed groups (groups S-V and S-PUFA) continued to deteriorate, and there was a significant difference compared with nonstressed mice after 1 week UCMS ($P < 0.001$) until the end of UCMS ($P < 0.001$). No effect of n-3 PUFA treatment was observed in stressed mice (groups S-V and S-PUFA), from week 1 until week 5. Deterioration was greater during weeks 5 and 7 in the

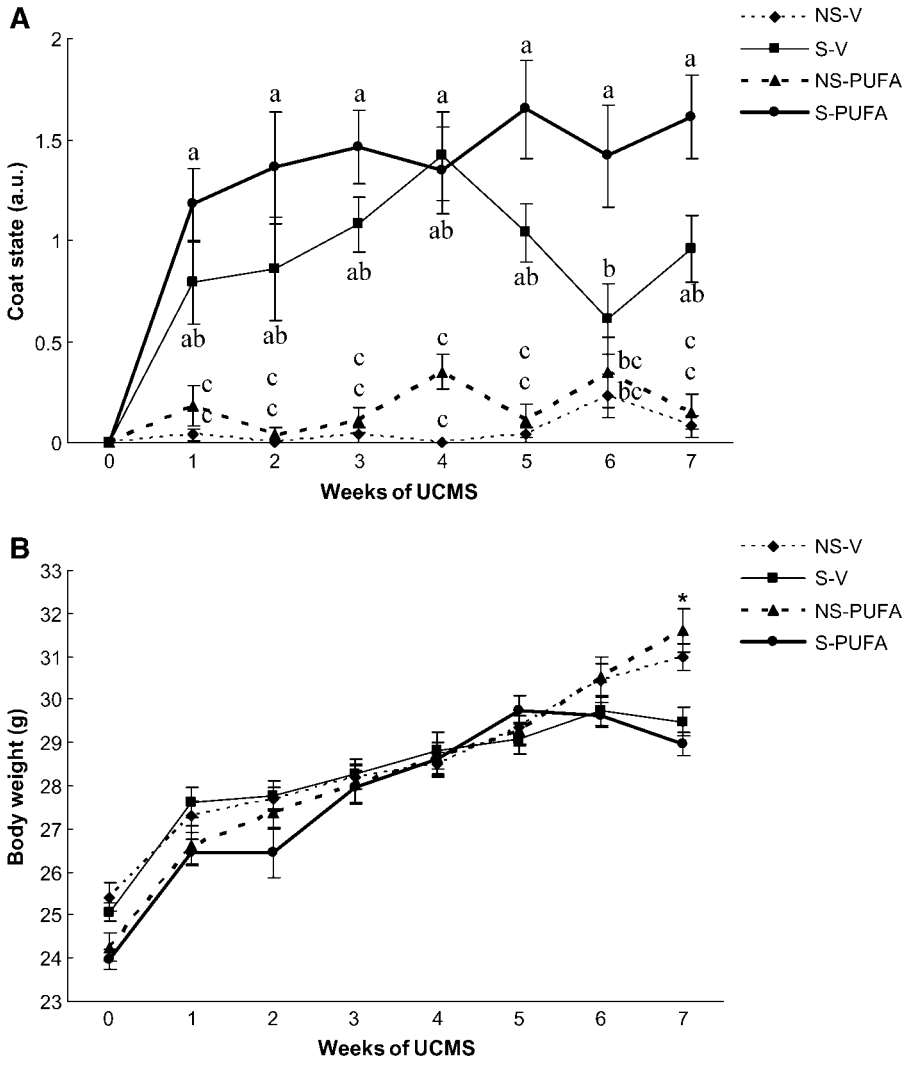

Fig. 1. Effects of unpredictable chronic mild stress (UCMS) on the state of the coat (A) and body weight (B). A: The condition of the coat was evaluated each week on eight different body areas and scored from 0 (good condition) to 1 (very poor condition). The total score represents the sum of each area. a–c, significantly different between groups ($P < 0.05$; ANOVA); B: *Significantly different between nonstressed groups and stressed groups ($P < 0.05$; ANOVA). Values are means ± SEM (n = 12 for each group). NS-V, nonstressed receiving vehicle; S-V, stressed receiving vehicle; NS-PUFA, nonstressed receiving n-3 PUFA; S-PUFA, stressed receiving n-3 PUFA.

stressed supplemented mice (group S-PUFA) than in the stressed unsupplemented mice (group S-V, $P = 0.0501$ and $P = 0.0568$, respectively), but the difference only reached statistical significance during week 6 ($P < 0.03$).

Body weight. No difference in body weight appeared among the four groups throughout the UCMS regimen (Fig. 1B), except during week 7, when the body weights of both nonstressed groups (NS-V and NS-PUFA) were significantly higher than those of the stressed groups (S-V and S-PUFA; $P < 0.03$ and $P < 0.001$, respectively).

Behavioral analyses

NSF test. The Kruskal-Wallis test showed significant differences between the four groups in latency to chew the pellet ($P < 0.001$) (**Fig. 2A**). n-3 PUFA supplementation resulted in increased latency to chew the pellet ($P < 0.02$ for S-V vs. S-PUFA and $P < 0.005$ for NS-V vs. NS-PUFA). UCMS had no effect in either test.

Resident-intruder test. The Kruskal-Wallis test showed significant differences between the groups in latency to attack the intruder ($P < 0.005$) (Fig. 2B). The UCMS procedure resulted in a significant effect, inasmuch as it reduced the latency of agonistic behaviors ($P < 0.05$ for NS-V vs. S-V). Similar effects were observed for frequency of attack (data not shown). n-3 PUFA supplementation had no effect in this test.

Fatty acid profile of brain membranes

The major fatty acids in the PE of frontal cortex, hippocampus, and striatum are shown in **Tables 1**, **2**, and **3**,

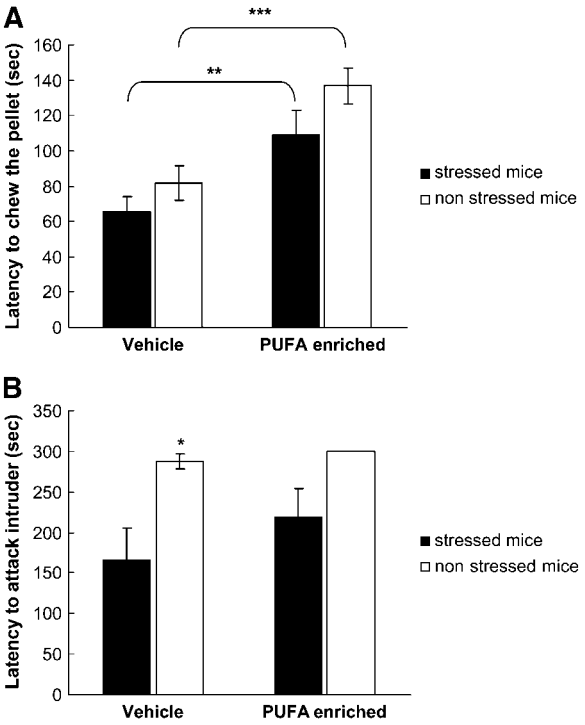

Fig. 2. Effects of UCMS and n-3 PUFA supplementation in the novelty suppression of feeding test (A) and in the resident-intruder test (B). A: ** $P < 0.02$; *** $P < 0.005$; B: * $P < 0.05$ (ANOVA). Values are means ± SEM (n = 12 for each group).

respectively. In the four groups of mice, saturated fatty acid (SFA) and PUFA accounted for the highest levels of fatty acids. The total SFA and total n-6 + n-3 PUFAs thus accounted for nearly 30% and 45% of the TFAs in each cerebral region, respectively.

Effect of UCMS in nonsupplemented mice

The UCMS procedure resulted in a slight but significant reduction in the 22:5n-6 content in the three cerebral areas studied (20% in the frontal cortex and hippocampus, 28% in the striatum), leading to a significantly reduced 22:5n-6/22:6n-3 ratio in the hippocampus and striatum ($P < 0.05$).

Effects of n-3 PUFA supplementation in nonstressed mice

Daily n-3 PUFA supplementation did not affect the SFA or the MUFA contents of brain membranes, but significantly increased the 22:6n-3 (DHA) levels in the frontal cortex (+9% in NS-PUFA compared with NS-V, $P < 0.05$) and hippocampus (+17%, $P < 0.05$). However, no incorporation of 20:5n-3 (EPA) was detected, despite the high content in the supplement. This resulted in significantly higher levels of total n-3 PUFA ($P < 0.05$). The n-3 PUFA supplementation induced a reduction of total n-6 PUFA levels in the three cerebral regions, i.e., 20% in the frontal cortex, 15% in the hippocampus, and 12% in the striatum, mainly owing to the decrease in 20:4n-6 and 22:5n-6 (decreased by almost 60% in the three regions, $P < 0.05$). These changes resulted in a significant reduction of the 22:5n-6/22:6n-3 ratio ($P < 0.05$), without affecting the total n-6 + n-3 PUFAs.

Interaction between stress and supplementation

In mice subjected to UCMS, n-3 PUFA supplementation resulted in only a 5–6% increase in 22:6n-3 (DHA) levels in the hippocampus and in frontal cortex ($P < 0.05$), whereas no change was observed in the striatum. Moreover, low levels of incorporation of 20:5n-3 (EPA) appeared in the three cerebral structures. The reduction in 22:5n-6 was much less in stressed than in nonstressed mice; levels were decreased by 50% in the frontal cortex, 35% in the hippocampus, and unchanged in the striatum. It should be noted that the reduction in levels of 20:4n-6 (arachidonic acid) was greater in the hippocampus and striatum of stressed mice (S-PUFA group) than in corresponding structures in nonstressed mice (NS-PUFA group).

Monoamine levels in brain tissues

Effects of UCMS. A number of reductions in tissue levels of monoamines were observed in stressed animals (group S-V) compared with controls (group NS-V) in the three brain regions studied. In the frontal cortex (**Table 4**), significantly reduced levels of NE were observed (51%, $P < 0.05$). In the hippocampus (**Table 5**), a 44%, although nonsignificantly reduced, level of NE was observed, whereas amounts of 5-HT and 5-HIAA were decreased 2-fold ($P <$

TABLE 1. Main fatty acid contents of PE in the frontal cortex

	Fatty Acid[a]		*mg/100 mg fatty acids*							
Group	SFA	MUFA	20:4n-6	22:5n-6	n-6 PUFA[b]	20:5n-3	22:6n-3	n-3 PUFA[c]	n-6+n-3 PUFA	22:5n-6/22:6n-3
NS-V	26.9 ± 0.6	10.2 ± 0.6	9.5 ± 0.3[a]	0.7 ± 0.0[a]	14.9 ± 0.4[a]	nd	29.3 ± 0.8[a]	29.6 ± 0.8[a]	44.6 ± 1.0	0.02 ± 0.00[a]
S-V	26.6 ± 0.7	11.0 ± 0.6	9.4 ± 0.3[a]	0.5 ± 0.0[b]	14.8 ± 0.4[a]	nd	29.3 ± 0.6[a]	29.5 ± 0.5[a]	44.3 ± 0.5	0.02 ± 0.00[a]
NS-PUFA	26.3 ± 0.7	11.2 ± 0.9	7.8 ± 0.2[b]	0.3 ± 0.0[c]	11.9 ± 0.4[b]	nd	31.7 ± 0.6[b]	32.4 ± 0.6[b]	44.3 ± 0.8	0.01 ± 0.00[b]
S-PUFA	26.5 ± 1.0	11.0 ± 0.3	7.7 ± 0.5[b]	0.3 ± 0.0[c]	12.0 ± 0.6[b]	0.1 ± 0.1	30.8 ± 1.7[b]	31.6 ± 1.7[b]	43.5 ± 2.3	0.01 ± 0.00[b]

MUFA, monounsaturated fatty acid; NS-PUFA, nonstressed mice receiving n-3 fatty acids; NS-V, nonstressed mice receiving vehicle; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; S-PUFA, stressed mice receiving n-3 fatty acids; S-V, stressed mice receiving vehicle; nd, not determined. Values are means ± SEM.

[a] For each fatty acid, values with different superscripts ([a–c]) were significantly different between groups (two-way ANOVA; $P < 0.05$); n = 6 for all groups.

[b] n-6 PUFA = sum of 18:2n-6, 18:3n-6, 20:3n-6, 22:4n-6, and 22:5n-6.

[c] n-3 PUFA = sum of 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

TABLE 2. Main fatty acid contents of PE in the hippocampus

Group	Fatty Acid[a]		*mg/100 mg fatty acids*							
	SFA	MUFA	20:4n-6	22:5n-6	n-6 PUFA[b]	20:5n-3	22:6n-3	n-3 PUFA[c]	n-6+n-3 PUFA	22:5n-6/22:6n-3
NS-V	25.6 ± 1.4^{a,b}	11.0 ± 1.0	12.2 ± 1.0^{a}	0.7 ± 0.1^{a}	19.1 ± 1.4^{a}	nd	24.1 ± 1.7^{a}	24.4 ± 1.7^{a}	43.5 ± 3.0	0.03 ± 0.00^{a}
S-V	25.1 ± 0.6^{a,b}	10.2 ± 0.4	12.4 ± 0.3^{a}	0.5 ± 0.0^{b}	19.5 ± 0.3^{a}	nd	25.0 ± 0.7^{a}	25.3 ± 0.7^{a}	44.8 ± 0.5	0.02 ± 0.00^{b}
NS-PUFA	26.3 ± 1.2^{a}	10.9 ± 0.9	10.8 ± 0.6^{b}	0.3 ± 0.0^{c}	16.2 ± 0.6^{b}	nd	28.3 ± 1.4^{b}	29.1 ± 1.4^{b}	45.3 ± 1.8	0.01 ± 0.00^{c}
S-PUFA	24.9 ± 0.6^{b}	10.7 ± 0.4	10.2 ± 0.2^{b}	0.3 ± 0.0^{c}	16.1 ± 0.9^{b}	0.1 ± 0.1	26.4 ± 0.8^{c}	27.3 ± 0.9^{c}	43.4 ± 1.5	0.01 ± 0.00^{c}

Values are means ± SEM.

[a] For each fatty acid, values with different superscripts ($^{a–c}$) were significantly different between groups (two-way ANOVA; $P < 0.05$); n = 6 for all groups.

[b] n-6 PUFA = sum of 18:2n-6, 18:3n-6, 20:3n-6, 22:4n-6, and 22:5n-6.

[c] n-3 PUFA = sum of 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

0.05). A significant 60% decrease in NE and 45% decrease in HVA levels were measured in the striatum (**Table 6**).

Effects of n-3 PUFA supplementation. Supplementation with n-3 PUFA resulted in significant increases in the tissue levels of several monoamines in stressed mice, in both frontal cortex and hippocampus. Increased amounts of 5-HT were observed in S-PUFA compared with S-V groups in the frontal cortex (120%, $P < 0.05$, Table 4), and increased amounts of DOPAC were observed in nonstressed animals (97%, $P < 0.05$). In the hippocampus, n-3 PUFA supplementation also increased the levels of 5-HT and 5-HIAA in the stress condition (+93% and +60%, respectively, $P < 0.05$; Table 5). No effect of the supplementation was seen in the striatum (Table 6).

DISCUSSION

The purpose of the present study was to evaluate the effects of UCMS on several aspects of behavior and brain phospholipid fatty acid profiles and monoamine levels in mice receiving either a control diet or a diet supplemented with n-3 long-chain PUFAs throughout the stress procedure. The supplementation comprised daily administration of a mixture of DHA and EPA by force feeding, allowing precise control of the dose. It had been previously shown that n-3 PUFA, particularly DHA, can have an antistress effect in conditioned fear-induced freezing (20). Moreover, positive effects of EPA have been demonstrated on symptoms of depression in humans, whereas low n-3 PUFA plasma status has been reported to be associated with an increased risk of depression (as reviewed in Ref. 40).

We have previously shown in animal models that chronic diet deficiency in ALA was able to act on the release of monoamines and acetylcholine in brain regions involved in stress-related behavior (mainly the hippocampus and frontal cortex) (15, 16, 18, 39). 5-HT and acetylcholine release was greater in the basal state in the hippocampus but was reduced under neuronal activation in deficient rats. In addition, it appeared that serotonergic and muscarinic receptor binding might also be affected by the n-3 PUFA content of the diet (11, 41, 42).

On the basis of these findings, we hypothesized that n-3 PUFA supplementation could improve resistance to stress through action on monoaminergic neurotransmission. We used a model of UCMS in the mouse that had been shown to induce a depression-like state, which becomes apparent through deterioration in coat state (31–35). As expected, repeated exposure to mild stressors in the present study resulted in deterioration in the state of the coat that could be explained by a decrease in the mouse's grooming behavior related to conservation of resources in favor of coping behaviors toward the stress situation (31). The UCMS effect was also observed in the decrease in body weight gain during the last week. A clear effect of the UCMS procedure was also observed in the resident-intruder test, in which the decrease in latency to attack the intruder confirmed the increased level of aggressiveness in stressed animals (33). We observed that n-3 PUFA supplementation did not reduce coat deterioration in this model, or the aggressiveness in the resident-intruder test in stressed mice. Further, the coat state of stressed-

TABLE 3. Main fatty acid contents of PE in the striatum

Group	Fatty Acid[a]		*mg/100 mg fatty acids*							
	SFA	MUFA	20:4n-6	22:5n-6	n-6 PUFA[b]	20:5n-3	22:6n-3	n-3 PUFA[c]	n-6+n-3 PUFA	22:5n-6/22:6n-3
NS-V	24.3 ± 1.0	13.1 ± 2.0^{a}	11.6 ± 0.8^{a}	0.6 ± 0.1^{a}	18.0 ± 1.0^{a}	nd	23.2 ± 1.6	23.6 ± 1.6	41.5 ± 2.5^{a}	0.03 ± 0.00^{a}
S-V	23.1 ± 1.2	14.0 ± 0.8^{a}	11.2 ± 0.3^{a}	0.4 ± 0.1^{b}	17.2 ± 0.7^{a}	nd	23.5 ± 1.0	23.8 ± 1.1	41.0 ± 0.7^{a,b}	0.02 ± 0.00^{b}
NS-PUFA	23.6 ± 1.3	15.7 ± 2.0^{a}	11.4 ± 1.1^{a}	0.3 ± 0.0^{b}	15.8 ± 1.4^{b}	nd	25.2 ± 1.9	25.9 ± 1.8	41.6 ± 2.6^{a}	0.01 ± 0.00^{b}
S-PUFA	24.1 ± 1.9	13.7 ± 1.2^{b}	9.1 ± 0.2^{b}	0.4 ± 0.1^{b}	14.3 ± 0.5^{c}	0.1 ± 0.1	23.1 ± 2.4	24.1 ± 2.7	38.4 ± 2.3^{b}	0.02 ± 0.01^{c}

Values are means ± SEM.

[a] For each fatty acid, values with different superscripts ($^{a–c}$) were significantly different between groups (two-way ANOVA; $P < 0.05$); n = 6 for all groups.

[b] n-6 PUFA = sum of 18:2n-6, 18:3n-6, 20:3n-6, 22:4n-6, and 22:5n-6.

[c] n-3 PUFA = sum of 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

TABLE 4. Monoamine levels in the frontal cortex

Group	Monoamine[a]	*nmol/mg tissue*				
	NE	DA	DOPAC	HVA	5-HT	5-HIAA
NS-V[b]	1.95 ± 0.29[a]	2.45 ± 0.79	0.68 ± 0.15[a]	1.75 ± 0.76	1.05 ± 0.13[a,b]	1.18 ± 0.45
S-V[c]	0.95 ± 0.21[b]	1.71 ± 0.77	0.63 ± 0.22[a]	0.89 ± 0.68	0.64 ± 0.27[a]	0.88 ± 0.31
NS-PUFA[b]	2.18 ± 0.34[a]	2.63 ± 0.67	1.34 ± 0.36[b]	1.33 ± 0.50	1.28 ± 0.78[a,b]	0.89 ± 0.12
S-PUFA[c]	1.28 ± 0.30[b]	2.81 ± 1.39	0.88 ± 0.28[a]	1.28 ± 0.4	1.41 ± 0.25[b]	1.13 ± 0.28

Values are means ± SEM.
[a] For each monoamine, values with different superscripts ([a–c])were significantly different between groups (two-way ANOVA; $P < 0.05$).
[b] n = 6.
[c] n = 7.

supplemented mice was sometimes worse than that of their matched controls (stressed-vehicle mice). The sole effect of supplementation was seen in the NSF test, which involves food motivation and fear in a novel environment: in this situation, n-3 PUFA supplementation resulted in greater latency to chew the pellet in the novel environment, and this effect was unexpectedly independent of the UCMS procedure. In fact, we observed that in both stressed and nonstressed groups, the mice subjected to n-3 PUFA supplementation were more reluctant to accept the force-feeding procedure than were mice receiving the vehicle. This may be a consequence of the odor and/or taste of the PUFA-enriched mixture (fish and sea food odors), to which the mice seemed to have an aversion, resulting in reduction in their motivation to take food in the NSF test.

We measured the tissue levels of monoamines in several brain areas in the four experimental groups. In mice that did not receive n-3 PUFA supplementation, the UCMS induced a significant 50% to 60% decrease in the levels of NE in the frontal cortex and striatum, and a 44% (nonsignificant) decrease in the hippocampus. The tissue levels of 5-HT were also 40% to 65% decreased in the three brain regions studied, although this reduction was only statistically significant in the hippocampus. The levels of the 5-HT metabolite 5-HIAA were also significantly reduced in the hippocampus of stressed animals. The levels of DA and its metabolites, DOPAC and HVA, had a tendency to be slightly reduced in the regions where it was detectable (i.e., the frontal cortex and striatum), but no statistically significant difference was detected, except for the levels of HVA in the striatum. It has previously been shown that exposure to stressful stimuli increases the extracellular release of 5-HT in the hippocampus (43) and frontal cortex (44). It has been suggested that this type of consequence is a neuroadaptative process in response to stress, aimed at attenuating the adverse effects on behavior, and that a failure of this process may be involved in the occurrence of depression (45). In rat models of chronic mild stress, different results have been reported with regard to the function of monoaminergic systems, such as an increase in dopaminergic activity in the prefrontal cortex (46) or a decrease in the tissue levels of 5-HT (47). However, discrepant data have also been reported (48). In all conditions, these neurochemical modifications were improved by the administration of antidepressive drugs such as imipramine (46) or fluoxetine (47). With regard to the neurochemical findings, the reductions in tissue levels of 5-HT and NE detected at the end of the UCMS procedure may be related to signs of depression-like behavior observed in these animals.

The main finding of the present study was that supplementation with n-3 PUFA seemed to have a reversing effect on the reduced 5-HT levels that were induced by the UCMS. As previously noted, tissue levels of 5-HT were 40% to 65% decreased in the three brain regions studied of unsupplemented stressed animals, although this reduction was only statistically significant in the hippocampus. Under n-3 PUFA supplementation, these levels were rather similar (frontal cortex and striatum) or increased (hippocampus) between stressed and nonstressed mice. Although this "reversal" was proven for 5-HT levels, it was less clear for NE, which was also reduced in S-PUFA versus NS-PUFA mice in the frontal cortex, but very similar between both groups in the hippocampus. For DA and metabolites, it was also difficult to observe clear effects of the n-3 supplementation on the consequences of stress. Thus, in the frontal cortex, we obtained a nonsignificant 30–40% reduction in the levels of DA and HVA in stressed animals, and similar levels

TABLE 5. Monoamine levels in the hippocampus

Group	Monoamine[a]	*nmol/mg tissue*				
	NE	DA	DOPAC	HVA	5-HT	5-HIAA
NS-V[b]	2.32 ± 0.31	nd	nd	nd	2.05 ± 1.32[a]	2.93 ± 0.83[a,c]
S-V[c]	1.29 ± 1.08	nd	nd	nd	1.10 ± 0.40[b]	1.40 ± 0.44[b]
NS-PUFA[b]	1.93 ± 0.69	nd	nd	nd	1.39 ± 0.40[a,b]	1.70 ± 0.29[b,c]
S-PUFA[c]	1.90 ± 0.93	nd	nd	nd	2.12 ± 0.63[a]	2.24 ± 0.61[c]

Values are means ± SEM.
[a] For each monoamine, values with different superscripts ([a–c]) were significantly different between groups (two-way ANOVA; $P < 0.05$).
[b] n = 6.
[c] n = 7.

TABLE 6. Monoamine levels in the striatum

Group	Monoamine[a]	*nmol/mg tissue*				
	NE	DA	DOPAC	HVA	5-HT	5-HIAA
NS-V[b]	2.03 ± 1.00[a]	35.82 ± 12.60	10.82 ± 4.70	9.92 ± 4.31[a]	4.00 ± 2.81	2.73 ± 1.37
S-V[c]	0.83 ± 0.66[b]	29.00 ± 6.90	6.43 ± 1.22	5.47 ± 0.95[b]	1.41 ± 1.13	1.64 ± 0.46
NS-PUFA[b]	nd	35.55 ± 9.18	8.27 ± 1.81	10.61 ± 4.43[a]	3.81 ± 0.86	1.70 ± 0.52
S-PUFA[c]	nd	35.43 ± 11.87	8.96 ± 1.93	5.86 ± 2.99[b]	3.15 ± 2.00	1.95 ± 0.80

Values are means ± SEM.
[a] For each monoamine, values with different superscripts ([a,b]) were significantly different between groups (two-way ANOVA; $P < 0.05$).
[b] n = 6.
[c] n = 7.

were observed between stressed and nonstressed animals under n-3 PUFA supplementation. In the striatum, the levels of HVA were significantly reduced in stressed compared with nonstressed animals, in unsupplemented as well as in supplemented groups. As mentioned above, these neurochemical effects of supplementation were not associated with effects on the physical or behavioral signs resulting from the stress procedure, indicating dissociation between the stress-induced modifications in monoaminergic parameters and the stressed-induced modifications in physical and behavioral parameters.

Supplementation with n-3 PUFA was also associated with several changes in the fatty acid composition of brain phospholipid membranes, inasmuch as it increased the incorporation of DHA in a range of variations specific to each cerebral region according to the stress condition. The increase in DHA in nonstressed mice was between 9% and 17%, the maximum observed in the hippocampus. In stressed conditions, there was no increase in DHA in the striatum, and it reached only 6% in the hippocampus and in the frontal cortex. However, n-3 PUFA supplementation resulted in low levels of incorporation of EPA in cerebral membranes in stressed mice but not in nonstressed animals. These findings suggest that the UCMS procedure might prevent the incorporation of DHA into phospholipid membranes. It has previously been shown that psychological stress increases lipid peroxidation activity in the mouse (49) and rat (50, 51) brain, in a cerebral region-specific manner. DHA has a highly oxidation-prone chemical structure, and this could contribute to the lower DHA levels incorporated into brain phospholipids of UCMS-subjected mice. However, the appearance of EPA also suggested higher retroconversion of DHA.

The UCMS imposed on nonsupplemented mice for 8 weeks resulted in a reduction in the 22:5n-6 levels in the PE of the frontal cortex, hippocampus, and striatum. Although this PUFA was present at very low concentrations in cerebral phospholipids, there may be stress-induced consequences on brain membrane properties. The differences in DHA content of cerebral structures observed in unsupplemented nonstressed mice were exacerbated by n-3 PUFA supplementation, illustrating the previously described differences in accretion (9, 39, 52). Incorporation of DHA in supplemented mice was higher in the frontal cortex than in the striatum and the hippocampus. As expected, n-3 PUFA supplementation induced a reduction in n-6 PUFA. In particular, we showed that 22:5n-6 levels, which were already low in brain membranes in unsupplemented animals, were substantially changed in supplemented nonstressed mice.

In conclusion, the present findings showed that UCMS resulted in depression-like behavior in mice associated with changes in brain phospholipid fatty acid composition and monoamine levels. Daily n-3 PUFA supplementation reversed the 5-HT stress-induced effects but without impact on the on the physical state or behavior of the animals. This suggests that n-3 long-chain PUFAs can improve resistance to stress through attenuation of the impact of the UCMS on specific aspects of cerebral function.

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REFERENCES

1. Clandinin, M. T. 1999. Brain development and assessing the supply of polyunsaturated fatty acid. *Lipids.* **34:** 131–137.
2. Cunnane, S. C., V. Francescutti, J. T. Brenna, and M. Crawford. 2000. Breast-fed infants achieve a higher rate of brain and whole body docosahexaenoate accumulation than formula-fed infants not consuming dietary docosahexaenoate. *Lipids.* **35:** 105–111.
3. Fedorova, I., and N. Salem, Jr. 2006. Omega-3 fatty acids and rodent behavior. *Prostaglandins Leukot. Essent. Fatty Acids.* **75:** 271–289.
4. Francès, H., C. Monier, M. Clement, A. Lecorsier, M. Debray, and J. M. Bourre. 1996. Effect of dietary alpha-linolenic acid deficiency on habituation. *Life Sci.* **58:** 1805–1806.
5. Levant, B., J. D. Radel, and S. E. Carlson. 2004. Decreased brain docosahexaenoic acid during development alters dopamine-related behaviors in adult rats that are differentially affected by dietary remediation. *Behav. Brain Res.* **152:** 49–57.
6. Vancassel, S., C. Blondeau, M. S. Lallemand, M. Cador, A. Linard, M. Lavialle, and F. Dellu-Hagedorn. 2007. Hyperactivity in the rat is associated with spontaneous low level of n-3 polyunsaturated fatty acids in the frontal cortex. *Behav. Brain Res.* **180:** 119–126.
7. DeMar, J. C., K. Ma, J. M. Bell, M. Igarashi, D. Greenstein, and S. I. Rapoport. 2006. One generation of n-3 polyunsaturated fatty acid deprivation increases depression and aggression test scores in rats. *J. Lipid Res.* **47:** 172–180.
8. Lim, S. Y., and H. Suzuki. 2002. Dose-response effect of docosahexaenoic acid ethyl ester on maze behavior and brain fatty acid composition in adult mice. *Int. J. Vitam. Nutr. Res.* **72:** 77–84.
9. Carrié, I., M. Clément, D. de Javel, H. Francès, and J. M. Bourre. 2000. Phospholipid supplementation reverses behavioral and biochemical alterations induced by n-3 polyunsaturated fatty acid deficiency in mice. *J. Lipid Res.* **41:** 473–480.
10. Morigushi, T., and N. Salem. 2003. Recovery of brain docosahexaenoate leads to recovery of spatial task performance. *J. Neurochem.* **87:** 297–309.

11. Chalon, S. 2006. Omega-3 fatty acids and monoamine neurotransmission. *Prostaglandins Leukot. Essent. Fatty Acids.* **75:** 259–269.
12. Kuperstein, F., E. Yakubov, P. Dinerman, S. Gil, R. Eylam, N. Salem, Jr., and E. Yavin. 2005. Overexpression of dopamine receptor genes and their products in the postnatal rat brain following maternal n-3 fatty acid dietary deficiency. *J. Neurochem.* **95:** 1550–1562.
13. Zimmer, L., S. Hembert, G. Durand, P. Breton, D. Guilloteau, J. C. Besnard, and S. Chalon. 1998. Chronic n-3 polyunsaturated fatty acid diet-deficiency acts on dopamine metabolism in the rat frontal cortex: a microdialysis study. *Neurosci. Lett.* **1240:** 177–181.
14. Zimmer, L., S. Delion-Vancassel, G. Durand, D. Guilloteau, S. Bodard, J. C. Besnard, and S. Chalon. 2000. Modification of dopamine neurotransmission in the nucleus accumbens of rats deficient in n-3 polyunsaturated fatty acids. *J. Lipid Res.* **41:** 32–40.
15. Zimmer, L., S. Delion-Vancassel, S. Cantagel, P. Breton, S. Delamanche, D. Guilloteau, G. Durand, and S. Chalon. 2002. The dopamine mesocorticolimbic pathway is altered by a chronic deficiency in n-3 polyunsaturated fatty acids in the rat. *Am. J. Clin. Nutr.* **75:** 662–667.
16. Chalon, S., S. Vancassel, L. Zimmer, D. Guilloteau, and G. Durand. 2001. PUFA and cerebral function: focus on monoaminergic neurotransmitters. *Lipids.* **36:** 937–944.
17. Reisbick, S., and M. Neuringer. 1997. Omega-3 fatty acid deficiency and behavior: a critical review and directions for future research. *In* Handbook of Essential Fatty Acid Biology: Biochemistry, Physiology, and Behavioral Neurobiology. S. Yehuda and D. I. Mostofsky, editors. Humana Press Inc., Totowa. 397–426.
18. Kodas, E., L. Galineau, S. Bodard, S. Vancassel, D. Guilloteau, J. C. Besnard, and S. Chalon. 2004. Serotoninergic neurotransmission is affected by n-3 polyunsaturated fatty acids in the rat. *J. Neurochem.* **89:** 695–702.
19. Kodas, E., S. Vancassel, B. Lejeune, D. Guilloteau, and S. Chalon. 2002. Reversibility of n-3 fatty acid deficiency-induced changes in dopaminergic neurotransmission in rats: critical role of developmental stage. *J. Lipid Res.* **43:** 1209–1219.
20. Takeuchi, T., M. Iwanaga, and E. Harada. 2003. Possible regulatory mechanism of DHA-induced anti-stress reaction in rats. *Brain Res.* **964:** 136–143.
21. Hamazaki, K., S. Sawazaki, T. Nagasawa, Y. Nagao, Y. Kanagawa, and K. Yazawa. 1999. Administration of docosahexaenoic acid influences behavior and plasma catecholamine levels at times of psychological stress. *Lipids.* **34:** S33–S37.
22. Hamazaki, K., M. Itomura, M. Huan, H. Nishizawa, S. Sawazaki, M. Tanouchi, S. Watanabe, T. Hamazaki, K. Terazawa, and K. Yazawa. 2005. Effect of omega-3 fatty acid-containing phospholipids on blood catecholamine concentrations in healthy volunteers: a randomized, placebo-controlled, double-blind trial. *Nutrition.* **21:** 705–710.
23. Hamazaki, T., M. Itomura, S. Sawazaki, and Y. Nagao. 2000. Anti-stress effects of DHA. *Biofactors.* **13:** 41–45.
24. Hibbeln, J. R. 2002. Seafood consumption, the DHA content of mothers' milk and prevalence rates of postpartum depression: a cross-national, ecological analysis. *J. Affect. Disord.* **69:** 15–29.
25. Otto, S. J., R. H. de Groot, and G. Hornstra. 2003. Increased risk of postpartum depressive symptoms is associated with slower normalization after pregnancy of the functional docosahexaenoic acid status. *Prostaglandins Leukot. Essent. Fatty Acids.* **69:** 237–243.
26. Hibbeln, J. R. 1998. Fish consumption and major depression. *Lancet.* **351:** 1213–1216.
27. Peet, M., B. Murphy, J. Shay, and D. F. Horrobin. 1998. Depletion of omega-3 fatty acid levels in red blood cell membranes of depressive patients. *Biol. Psychiatry.* **43:** 315–319.
28. Maes, M., A. Christophe, J. Delanghe, C. Altamura, H. Neels, and H. Y. Meltzer. 1999. Lowered omega 3 polyunsaturated fatty acids in serum phospholipids and cholesteryl esters of depressed patients. *Psychiatry Res.* **85:** 275–291.
29. Willner, P. 1997. Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology (Berl.).* **134:** 319–329.
30. Willner, P., R. Muscat, and M. Papp. 1992. An animal model of anhedonia. *Clin. Neuropharmacol.* **15 (Suppl.):** 550A–551A.
31. Ducottet, C., G. Griebel, and C. Belzung. 2003. Effects of the selective nonpeptide corticotropin-releasing factor receptor 1 antagonist antalarmin in the chronic mild stress model of depression in mice. *Prog. Neuropsychopharmacol. Biol. Psychiatry.* **27:** 625–631.
32. Santarelli, L., M. Saxe, C. Gross, A. Surget, F. Battaglia, S. Dulawa, N. Weisstaub, J. Lee, R. Duman, O. Arancio, et al. 2003. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science.* **301:** 805–809.
33. Mineur, Y. S., D. J. Prasol, C. Belzung, and W. E. Crusio. 2003. Agonistic behavior and unpredictable chronic mild stress in mice. *Behav. Genet.* **33:** 513–519.
34. Ducottet, C., and C. Belzung. 2004. Behaviour in the elevated plus-maze predicts coping after subchronic mild stress in mice. *Physiol. Behav.* **81:** 417–426.
35. Griebel, G., J. Simiand, C. Serradeil-Le Gal, J. Wagnon, M. Pascal, B. Scatton, J. P. Maffrand, and P. Soubrie. 2002. Anxiolytic- and antidepressant-like effects of the non-peptide vasopressin V1b receptor antagonist, SSR149415, suggest an innovative approach for the treatment of stress-related disorders. *Proc. Natl. Acad. Sci. USA.* **99:** 6370–6375.
36. Alessandri, J. M., C. Poumès-Ballihaut, B. Langelier, M. H. Perruchot, G. Raguenez, M. Lavialle, and P. Guesnet. 2003. Incorporation of docosahexaenoic acid into nerve membrane phospholipids: bridging the gap between animals and cultured cells. *Am. J. Clin. Nutr.* **78:** 702–710.
37. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* **226:** 497–506.
38. Alessandri, J. M., and B. Goustard-Langelier. 2001. Alterations in fatty acid composition of tissue phospholipids in the developing retinal dystrophic rat. *Lipids.* **36:** 1141–1152.
39. Aïd, S., S. Vancassel, C. Poumès-Ballihaut, S. Chalon, P. Guesnet, and M. Lavialle. 2003. Effect of a diet-induced (n-3) polyunsaturated fatty acid depletion on cholinergic parameters in the rat hippocampus. *J. Lipid Res.* **44:** 1545–1551.
40. Sinclair, A. J., D. Begg, M. Mathai, and R. S. Weisinger. 2007. Omega 3 fatty acids and brain: review of studies in depression. *Asia Pac. J. Clin. Nutr.* **16:** 391–397.
41. du Bois, T. M., W. Bell, C. Deng, and X. F. Huang. 2005. A high n-6 polyunsaturated fatty acid diet reduces muscarinic M2/M4 receptor binding in the rat brain. *J. Chem. Neuroanat.* **29:** 282–288.
42. du Bois, T. M., C. Deng, W. Bell, and X. F. Huang. 2006. Fatty acids differentially affect serotonin receptor and transporter binding in the rat brain. *Neuroscience.* **139:** 1397–1403.
43. Linthorst, A. C. E., R. G. Penalva, C. Flachskamm, F. Holsboer, and J. M. H. M. Reul. 2002. Forced swim stress activates rat hippocampal serotonergic neurotransmission involving a corticotrophin-releasing hormone receptor-dependent mechanism. *Eur. J. Neurosci.* **16:** 2441–2452.
44. Storey, J. D., D. A. F. Robertson, J. E. Beattie, I. C. Reid, S. N. Mitchell, and D. J. K. Balfour. 2006. Behavioural and neurochemical responses evoked by repeated exposure to an elevated open platform. *Behav. Brain Res.* **166:** 220–229.
45. Graeff, F. G., F. S. Guimaraes, T. G. C. S. De Andrade, and J. F. W. Deakin. 1996. Role of 5-HT in stress, anxiety, and depression. *Pharmacol. Biochem. Behav.* **54:** 129–141.
46. Bekris, S., K. Antoniou, S. Daskas, and Z. Papadopoulou-Daifoti. 2005. Behavioral and neurochemical effects induced by chronic mild stress applied to two different rat strains. *Behav. Brain Res.* **161:** 45–59.
47. Li, J-M., L-D. Kong, Y-M. Wang, C. H. K. Cheng, W-Y. Zhang, and W-Z. Tan. 2003. Behavioral and biochemical studies on chronic mild stress models in rats treated with a Chinese traditional prescription Banxia-houpu decoction. *Life Sci.* **74:** 55–73.
48. Di Chiara, G., P. Loddo, and G. Tanda. 1999. Reciprocal changes in prefrontal and limbic dopamine responsiveness to aversive and rewarding stimuli after chronic mild stress: implications for the psychobiology of depression. *Biol. Psychiatry.* **46:** 1624–1633.
49. Matsumoto, K., K. Yobimoto, N. T. T. Huong, M. Abdel-Fattah, T. Van Hien, and H. Watanabe. 1999. Psychological stress-induced enhancement of brain lipid peroxidation via nitric oxide systems and its modulation by anxiolytic and anxiogenic drugs in mice. *Brain Res.* **839:** 74–84.
50. Liu, J., W. Xiaoyan, M. K. Shigenaga, H. C. Yeo, A. Mori, and B. N. Ames. 1996. Immobilization stress causes oxidative damage to lipid, protein, and DNA in the brain of rats. *FASEB J.* **10:** 1532–1538.
51. Sahin, E., and S. Gumuslu. 2004. Alterations in brain antioxidant status, proteins oxidation and lipid peroxidation in response to different stress models. *Behav. Brain Res.* **155:** 214–248.
52. Favrelière, S., L. Barrier, G. Durand, S. Chalon, and C. Tallineau. 1998. Chronic dietary n-3 polyunsaturated fatty acids deficiency affects the fatty acid composition of plasmenylethanolamine and phosphatidylethanolamine differently in rat frontal cortex, striatum and cerebellum. *Lipids.* **33:** 401–407.