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Potentiation of omega-3 fatty acid antidepressant-like effects with low non-antidepressant doses of fluoxetine and mirtazapine

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ABSTRACT

Despite the advances in psychopharmacology, the treatment of depressive disorders is still not satisfactory. Side effects and resistance to antidepressant drugs are the greatest complications during treatment. Based on recent evidence, omega-3 fatty acids may influence vulnerability and outcome in depressive disorders. The aim of this study was to further characterize the omega-3 antidepressant-like effect in rats in terms of its behavioral features in the depression model forced swimming test either alone or in combination with antidepressants fluoxetine or mirtazapine. Ultimately, we prompted to determine the lowest dose at which omega-3 fatty acids and antidepressant drugs may still represent a pharmacological advantage when employed in combined treatments. Chronic diet supplementation with omega-3 fatty acids produced concentration-dependent antidepressant-like effects in the forced swimming test displaying a behavioral profile similar to fluoxetine but different from mirtazapine. Fluoxetine or mirtazapine at antidepressant doses (10 and 20 mg/kg/day, respectively) rendered additive effects in combination with omega-3 fatty acid supplementation (720 mg/kg/day). Beneficial effects of combined treatment were also observed at sub-effective doses (1 mg/kg/day) of fluoxetine or mirtazapine, since in combination with omega-3 fatty acids (720 mg/kg/day), antidepressants potentiated omega-3 antidepressant-like effects. The antidepressant-like effects occurred in the absence of changes in brain phospholipid classes. The therapeutic approach of combining omega-3 fatty acids with low ineffective doses of antidepressants might represent benefits in the treatment of depression, especially in patients with depression resistant to conventional treatments and even may contribute to patient compliance by decreasing the magnitude of some antidepressant dose-dependent side effects.

1. Introduction

The large increase in the ratio omega-6 to omega-3 family of dietary polyunsaturated fatty acids consumed in the modern diet has been proposed to increase the prevalence of depressive disorders and advanced as a rationale for ways to treat them (i.e., polyunsaturated fatty acid supplementation) (Hibbeln and Salem, 1995; Kris-Etherton et al., 2000; Simopoulos, 2003; Kang, 2003, Yehuda et al., 2003; Pella et al., 2003; Dubnov and Berry, 2003; Zampelas et al., 2003; Hamazaki and Okuyama, 2003). Among omega-3 polyunsaturated fatty acids, eiscosapentanoic acid (EPA) and docosahexaenoic acid (DHA) are essential for normal neuronal function.

Despite of the availability of many antidepressant drugs approved for the treatment of major depressive disorders, nearly 50% of patients are treatment-resistant (Papakostas, 2005, 2009). In addition, Lin et al. (1995) have reported a high rate of premature treatment discontinuation mostly due to drug side effects. Other therapeutical concerns are also faced in the treatment of bipolar disorders and perinatal and childhood depression, especially due to the queries regarding drug efficacy and safety.

Several natural compounds, including omega-3 fatty acids, have been suggested as potential augmenters of antidepressant drug effects especially in treatment-resistant depression (Fava, 2001; Nemets et al., 2002; Logan, 2003; Jazayeri et al., 2008). However, randomized controlled clinical trials have been slow to accrue. Most but not all trials with omega-3 indicate that these fatty acids are effective as an adjunctive treatment for unipolar depression and may also be

Abbreviations: BDNF, brain-derived neurotrophic factor; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NA, noradrenaline; ω3, omega-3 fatty acids; 5-HT, serotonin; SSRI, serotonin selective reuptake inhibitor.

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beneficial in other mood disorders (Owen et al., 2008). Besides, it has been recently reported the first clinical trial demonstrating omega-3 to be effective monotherapy in childhood depression (Nemets and Nemets, 2006). There is no doubt that further research is needed in this field to clarify the efficacy of omega-3 as monotherapy for the treatment of unipolar, bipolar, perinatal and childhood depression as well as the optimal dose and type of omega-3 supplement for each case.

Besides epidemiological and clinical studies, experimental models of depression in rodents are useful approaches to study the omega-3 fatty acid antidepressant-like effects in monotherapy (Carlezon et al., 2005; Lakhwani et al., 2007; Huang et al., 2008; Venna et al., 2009) and in combination with other psychotropic drugs such as antidepressants. Indeed, omega-3 fatty acid enriched diet and antidepressant treatments have proven to be beneficial in combined treatments in rats. For instance, fluoxetine and imipramine display additive effects when combined with omega-3 rich diets (Lakhwani et al., 2007; Venna et al., 2009).

In the present study we intended to further characterize the omega-3 antidepressant-like effect in rats in terms of its behavioral features in the depression model forced swimming test either alone or in combination with antidepressants fluoxetine or mirtazapine. At variance to previous reports, we studied the effect of chronic agent administration after behavioral despair has been induced in the animals. Ultimately, we prompted to determine the lowest dose at which combining omega-3 fatty acids and antidepressant drugs may still represent a pharmacological advantage to reverse the behavioral deficit when employed alone or in combined treatments.

2. Materials and methods

2.1. Animals

Experiments were carried out on male Wistar rats weighing 200–386 g. Rats were maintained on a 12 h light (0800–2000 h)–12 h dark cycle with free access to food and water except during testing. The rats were divided into four groups, housed in individual polyethylene cages of three or four rats (55×38×30 cm) and fed with standard pellet diet for controls (c) or diet supplemented with omega-3 fatty acids (ω3). Animals were employed only once in each test. All studies described were conducted in accordance with the Guide for Care and Use of Laboratory Animals provided by the National Institutes of Health, USA.

2.2. Drugs and treatment

Fluoxetine-HCl was kindly provided by Laboratorio Gador and mirtazapine by Laboratorio Raffo, Buenos Aires, Argentina**.** In all cases, drugs were administered intraperitoneally (i.p.) in a volume equivalent to 1 cc/kg and were prepared freshly each morning. Fluoxetine was dissolved in distilled water and mirtazapine was solubilized in isotonic saline solution (0.9% NaCl) plus three or four drops of glacial acetic. Glacial acetic acid solution in such a small quantity induced no effect per se in the forced swimming test or in locomotor activity (Rénéric et al., 2002). All of the control rats received injections of saline solution (0.9% NaCl). The doses of fluoxetine (10 mg/kg) and mirtazapine (20 mg/kg) were chosen as they produced the most robust effect in the forced swimming test in previous studies and under similar experimental conditions (Rénéric and Lucki, 1998; Egawa et al., 1995). Fluoxetine (1 mg/kg) or mirtazapine (1 mg/kg) doses that lack the antidepressant-like effect were chosen according to previous studies (Nowakowska and Chodera, 1999; Contreras, 2001). The antidepressant drugs or saline were given once a day for 16 days, with the final injection given 24 h before the test session, as previous studies (Detke et al., 1997) (Fig. 1). Four groups of rats were studied. Two groups were fed with rat casein 20% supplemented with fish oil and two were kept on normal rat casein 20% (Tables 1 and 2).

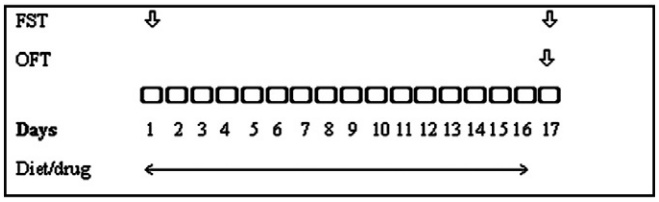

Fig. 1. Experimental design. Forced swimming and/or open field tests were performed in different groups of rats on day 1 and 17 after 16 day treatment with saline solution or antidepressants while receiving a control or omega-3 supplemented diet. FST: Forced swimming test, OFT: Open field test, Diet: control or omega-3 fatty acids, Drugs: fluoxetine or mirtazapine.

Omega-3 fatty acids were administered as a dietary supplement in food enriched with salmon oil for 16 days. This approach was selected because of difficulties in delivering high concentrations of this oil to rats by injection, and because short-term administration of omega-3 fatty acids failed to show evidence of antidepressant-like effects in mice using the forced swimming test (Shaldubina et al., 2002). The omega-3 group received normal rat casein 20% plus omega-3 fatty acids prepared by adding 4% fish oil containing 30% w/w omega-3 fatty acids, of which 17% is eicosapentaenoic acid (EPA) and 13% is docosahexaenoic acid (DHA). Then, this group received 12 mg omega-3 fatty acid per 1 g of diet. Each rat ate approximately 16–19 g of food each day depending on the assigned diet; i.e. for rats in the omega-3 condition, this amount of food contained a total of 0.24 g omega-3 fatty acids. As such, and considering the average rat weight in this group, animals in the omega-3 group were receiving 0.72 g/kg/day of omega-3 fatty acids (0.41 g EPA and 0.29 g DHA) at the time of testing. Omega-3 plus fluoxetine group received the same dose that was calculated considering the corresponding average weight and food intake in this group. Omega-3 fatty acids were supplemented each morning. Control animals were fed with standard diet (casein 20%). Other experiments were performed employing omega-3 doses ranged from 0.15 to 1 g/kg/day. Diets were equivalent in overall fat, protein, carbohydrate and caloric content.

2.3. Forced swimming test

This assay identifies treatments in rodents that possess antidepressant effects in humans (Porsolt et al., 1977; Detke et al., 1995). The procedure used was very similar to that described by Porsolt et al. (1978), except that the water was 30 cm deep and the pretest were

Table 1
Diet composition.

	Control	ω3 diet
	g/kg diet	
Calcium caseinate	200	200
Corn oil	50	50
Choline chlorhydrate	1.5	1.5
Vitamin mixture[a]	10	10
Mineral mixture[b]	35	35
Maltodextrin	696.9	696.9
Salmon Oil	–	11.93

[a] Composition of vitamin supplement triturated in sucrose (g/kg of diet): D-calcium panthotenate, 1.60 ; nicotinic acid, 3.00; D-biotin, 0.02; menadione, 0.029 ; thiamine HCl, 0.60; riboflavin, 0.60 ; folic acid, 0,20 ; dl-alpha-tocopherol acetate (500 u/gr), 15.00; retinyl palmitate, (400 ul/gr), 0.228; pyridoxine HCl, 0.70; cyanocobalamin 0,1 % (triturated in mannitol 1:1000), 2.50; cholecalciferol, (250000 U/gr), 0.40; sucrose, 975.123.

[b] Composition (g/kg of diet) as follows: K_2HPO_4, 322.5; $CaCO_3$, 357; NaCl, 74 ; MgO, 0.8; $MgSO_4 \cdot 7H_2O$, 146.9 ; $ZnSO_4 \cdot 5\ H_2O$, 0.63; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.008; KI, 0.0078; $Na_2SeO_3 \cdot 5H_2O$, 0.1025; iron and ammonium citrate, 6.06; ZnCl, 1.79; sucrose 91.

Table 2
Fatty acids (FA) composition of regular and experimental diets.[a]

Fatty acids	Experimental diet	
	Control	Fish oil
Myristic	0.11	7.6
Palmitoleic	0.16	9.74
C 16: 0 palmitic	6.75	16.25
C 18:0 stearic	3.04	4.10
C 18:1 ω9 oleico	29.2	13.05
C18:2 ω6 linoleic	58.8	1.67
C 18:3 ω3 alpha-linolenic	0.11	0.69
C20:4 ω6 arachidonic	0.24	1.15
C20:5 ω3 eicosapentaenoic	NC	17.24
C22:6 ω3 docosahexaenoic	NC	12.21

[a] Values are FA composition as a percentage of total dietary FA, determined by gas chromatography according to Fernandez et al. (2007).

performed 16 days before the test session. Porsolt (1981) noted that effects of pre-test sessions remained effective for as long as 2 weeks which was in agreement with our observations. Swimming sessions were conducted by placing rats in individual Plexiglas cylinders (46 cm tall × 20 cm in diameter) that had previously been filled with water (23−25 °C) up to 30 cm from bottom. All swimming session were carried out between 1200 and 1800 h.

At the end of both swimming sessions, rats were removed from the cylinders, dried with towels, placed in heated cages for 15 min, led them rest and recovery, and then returned to their home cages. The cylinders were emptied and cleaned between rats. Two sessions were conducted: an initial 15-min pre-test on day 1 followed by a 5-min test on day 17. Drugs were administered from day 1 to 16.

Each animal was assigned randomly to a treatment, and was only employed for one pre-test/test session.

2.4. Behavioral scoring

For behavioral sampling, rats were rated at 5 s intervals throughout the duration of the forced swimming session. At each 5 s interval, the predominant behavior was assigned to one of 3 categories: (1) immobility: floating in the water without struggling, and making only those movements necessary to keep the head above the water; (2) swimming: making active swimming motions, more than necessary to merely keep the head above water (i.e., moving around in the cylinder); and (3) climbing: making active movements with forepaws in and out of the water, usually directed against the walls. Scores for each behavior were expressed as total behavioral counts per 5-min session.

The behavioral sampling method differentiates classes of antidepressant drugs: for example, noradrenaline-reauptake inhibitors (NRIs) decrease immobility and increase climbing without affecting swimming, whereas SSRIs decrease immobility and increase swimming without affecting climbing (Heisler et al., 1997).

2.5. Open field test

This test was carried out to determine whether different treatments that were effective in the forced swimming test had non-specific effects on locomotor activity in rats previously exposed to forced swimming. Until day 16, these studies were conducted exactly as the forced swimming test studies: all rats underwent the first day of the forced swimming test but instead of re-testing in the forced swimming test on day 17, animals were subjected to an open field session. All animals were placed gently in the centre of the open field arena, owed to explore freely and its locomotion was measured by the number of squares entered with all four paws (counts), during a period of five minutes. The apparatus for the open field test consisted of a black, square open field (60 cm by 60 cm) with the floor divided in squares (15 × 15 cm) by means of white lines. Testing was performed between 1400 and 1700 h, illuminated with a 75 W electric bulb, hung 75 cm above it, in a quiet room. During all the experiments the laboratory room was dark. After each animal was removed, the open field was carefully cleaned with a damp cloth. The behavior was scored by an observer who was unaware of the experimental procedures previously performed on the animals and the results were expressed as mean ± S.E.M.

2.6. Phospholipid analysis

Upon termination of the experiment, the animals were sacrificed by CO_2 inhalation and decapitation. The freshly dissected hemispheres were used for lipid analysis. The cerebral cortex was then removed, followed by the hippocampus. All regions were frozen over dry ice and tissue samples were stored at −80 °C until analysis. Lipids were extracted by the method of Bligh and Dyer (1959). Phospholipids were separated by two-solvent one-dimensional thin-layer chromatography (TLC) in Silica gel G plates (0.25 mm thick). The first solvent system used was a mixture of chloroform/methanol/acetic acid/water (120:30:30:3 v/v/v/v) and the second one was chloroform/methanol/acetic acid/water (120:46:19:3 v/v/v/v). Lipids were detected with I_2 vapors. Spots of TLC plates corresponding to each phospholipid species were scraped off and phospholipids were quantified by the method of Fiske and Subbarrow (1925).

2.7. Statistical analysis

For each experiment, one- or two-way analysis of variance (ANOVA) with antidepressant treatment and diet as factors were performed. Subsequent post-hoc analysis was done using Dunnet's or Tukey's comparison tests. $P<0.05$ was considered as statistically significant.

3. Results

3.1. The omega-3 fatty acid antidepressant-like effect involves a decrease in immobility and an increase in swimming

To further evaluate the dose range of omega-3 fatty acids antidepressant-like effects and determine its pharmacological nature, the behavioral profile of omega-3 fatty acids in the forced swimming test was measured. Dietary supplementation with omega-3 fatty acids showed dose-dependent effects in the forced swimming test compared with saline controls (Fig. 2). Omega-3 (0.30, 0.72 and 1.00 g/kg/day) produced a dose-dependent decrease in immobility [$F(4, 30) = 4{,}46, P<0.05$] and a corresponding dose-dependent increase in swimming [$F(4, 30) = 6{,}79, P<0.001$] without significantly affecting the frequency of climbing [$F(4, 30) = 1{,}24$, P = NS] (Fig. 2A).

The effects of different doses of omega-3 fatty acids (0.15, 0.30, 0.72 and 1.0 g/kg/day) on locomotor activity are shown in Fig. 2B. Omega-3 fatty acids failed to modify the locomotor activity at doses ranging from 0.15 to 0.72 g/kg/day. However, locomotor activity was increased at dose of 1.0 g/kg of omega-3 [$F(4, 32) = 4.74$, $P<0.005$].

3.2. Combined treatments with omega-3 fatty acids and fluoxetine or mirtazapine at antidepressant doses render an additive effect

Behavioral effects produced in the forced swimming test by administration of fluoxetine (10 mg/kg, ip) or/and dietary supplementation with omega-3 fatty acids (0.72 g/kg/day) are shown in Fig. 3. Consistent with previous observations (Rénéric et al., 2002) fluoxetine reduced immobility (32% reduction) [$F(1, 37) = 20.91$, $P<0.0001$] and increased swimming (100%) [$F(1,37) = 24.30$, $P<0.0001$], without modifying the levels of climbing behavior [$F(1, 37) = 0.01$, P = NS], a pattern of behaviors consistent with a serotonergic mechanism of action (Rénéric

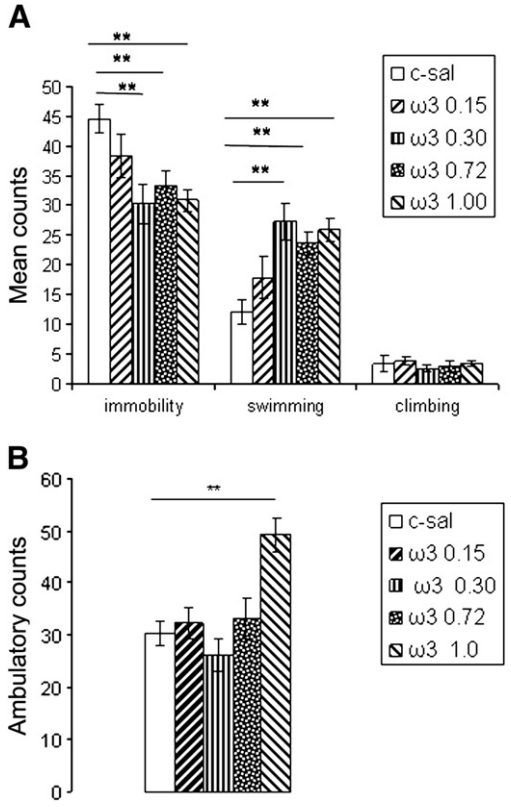

Fig. 2. Effects of dietary supplementation with omega-3 fatty acids on behaviors in the forced swimming test (FST) and open field test (OFT). (A) Omega-3 (ω3) fatty acids (0.15, 0.30, 0.72 and 1.0 g/kg/day) dose-dependently decreased immobility and increased swimming without affecting climbing in rats after 16 days. (B) Identical doses of omega-3 fatty acids used in the forced swimming test were tested in the open field test. Increased activity in this test was only seen with the highest omega-3 fatty acid dose (1 g/kg/day). Values are means ± S.E.M., $n = 5–10$ rats per group, ** $P<0.01$. Data were analyzed with one-way ANOVA, followed by Dunnett's test for comparison to control group.

et al., 2002). As shown in Fig. 2, omega-3 decreased immobility (23% reduction) [$F(1, 37) = 9.83$; $P<0.001$], and increased swimming (75%) [$F(1, 37) = 13.93$; $P<0.001$], without affecting climbing [$F(1, 37) = 0.90$, P = NS], a behavioral profile similar to that seen with fluoxetine. The combined fluoxetine/omega-3 fatty acid treatment also reduced immobility (51% reduction) ($P<0.01$) and increased swimming (165%) ($P<0.01$). The effect of the combination of drug and diet was significantly higher than the effects obtained after the individual administration of fluoxetine or omega-3 ($P<0.01$ vs both fluoxetine and omega-3) rendering an additive effect (Fig. 3A).

Administration of the antidepressant mirtazapine, which enhances both 5-HT and NA neurotransmissions, produced a behavioral pattern distinctive from that produced by fluoxetine. Mirtazapine (20 mg/kg, ip) significantly decreased immobility (34% reduction) [$F(1, 26) = 12, 91$, $P<0.005$] with a corresponding increase in both swimming (33%) [$F(1, 26) = 4.54$; $P<0.005$] and climbing (140%) [$F(1, 26) = 80,20$, $P<0.005$] (Fig. 3B). Dietary enrichment with omega-3 fatty acids (0.72 g/kg/day) decreased immobility (24% reduction) [$F(1, 26) = 6.38$; $P<0.05$] and increased swimming (66%) [$F(1, 26) = 11.60$, $P<0.005$] compared to the control group. The combined mirtazapine/omega-3 fatty acid treatment reduced immobility (56%) ($P<0.01$) and increased swimming (126%) and climbing (80%) ($P<0.01$). The effect of the combination of drug and diet was significantly stronger when compared to the effects obtained after the individual administration of mirtazapine or omega-3 ($P<0.05$ vs. both mirtazapine and omega-3) showing an additive effect (Fig. 3B).

The effects of fluoxetine (10 mg/kg) and/or omega-3 fatty acids (0.72 g/kg/day) on spontaneous locomotor activity in rats are shown in Fig. 3C. None of these treatments with antidepressant-like effects in the forced swimming test affected activity levels when rats were tested in an open field chamber instead of the forced swimming cylinders during the re-test (means ± S.E.M.) (Fig. 3C).

The effects of dietary supplementation with omega-3 fatty acids (0.72 g/kg/day) and/or mirtazapine (20 mg/kg) on spontaneous locomotor activity in rats are shown in Fig. 3D. Mirtazapine and omega-3 fatty acids, alone or in combination, did not interfere with locomotor responses ($P>0.05$).

3.3. Low non-antidepressant doses of fluoxetine and mirtazapine potentiate omega-3 fatty acid antidepressant-like effect

Effects of fluoxetine at a low non-antidepressant dose (1 mg/kg, i.p.) and/or omega-3 fatty acids (0.72 g/kg/day) on behaviors in the forced swimming test are shown in Fig. 4. A two-way ANOVA analyses revealed that, according to the initial results, omega-3 fatty acids induced a significant decrease in immobility (25% reduction) [$F(1, 15) = 30.75$, $P<0.005$] and increased swimming time (83%) [$F(1, 15) = 28.74$, $P<0.005$] (Fig. 4A), with no effect of the fluoxetine treatment [$F(1, 15) = 0.534$; $P>0.05$]. Co-administration of fluoxetine (1 mg/kg, i.p.) and omega-3 fatty acids significantly decreased the immobility time (45% reduction) ($P<0.01$) and increased swimming (158%) ($P<0.05$). A significant interaction among the diet and treatment ($P<0.05$) was also observed, indicating that fluoxetine potentiates omega-3 fatty acid antidepressant-like effect (Fig. 4A). It was also observed that fluoxetine doses lower than 1 mg/kg (0.1 and 0.5 mg/kg) failed to augment omega-3 antidepressant-like effects in combined treatments (data not shown).

An additional experiment was performed with a combination of ineffective doses of mirtazapine (1 mg/kg, i.p.) (Fig. 4B). This mirtazapine dose did not significantly change immobility time compared with the control group ($P>0.05$). In contrast, coadministration of mirtazapine (1 mg/kg, i.p.) and omega-3 fatty acids significantly decreased the immobility time (36% reduction) [$F(1, 20) = 43.61$, $P<0.005$] and increased swimming (127%) [$F(1, 20) = 39,1$; $P<0.005$] and climbing (75%) [$F(1, 20) = 11.1$, $P<0.005$] when compared to control animals; an interaction between treatment and diet was observed ($P<0.05$) indicating that mirtazapine potentiates omega-3 fatty acid antidepressant-like effect (Fig. 4B).

The effects of fluoxetine (1 mg/kg) and/or omega-3 fatty acids (0.72 g/kg/day) on spontaneous locomotor activity in rats are shown in Fig. 4C. None of the treatments with antidepressant-like effects in the forced swimming test affected activity levels when rats were tested in open field chamber instead of the forced swimming cylinders during the re-test (Fig. 4C).

The effects of dietary supplementation with omega-3 fatty acids (0.72 g/kg/day) and/or mirtazapine (1 mg/kg) on spontaneous locomotor activity in rats are shown in Fig. 4D. Despite of mirtazapine and omega-3 fatty acids alone did not interfere with locomotion responses, the combined treatment modified this behavioral parameter. However, this change cannot account for the improvement in the forced swimming test performance since the combined treatment induced a decrease rather an increase in locomotor performance ($P<0.05$).

3.4. Body weight gain analysis during chronic treatments with antidepressants and/or omega-3 fatty acids

Changes in body weight were evaluated with increasing doses of omega-3 fatty acids (0.15–1 g/kg/day). Diet supplementation did not induced changes in body weight gain at any dose of omega-3 fatty acids employed (Fig. 5A).

Body weight was evaluated in the four groups before fluoxetine (10 mg/kg) and/or omega-3 fatty acids (0.72 g/kg) treatment and after 16 days of treatment. Body weight gain during these treatments is shown in Fig. 5. Body weight was significantly lower in animals

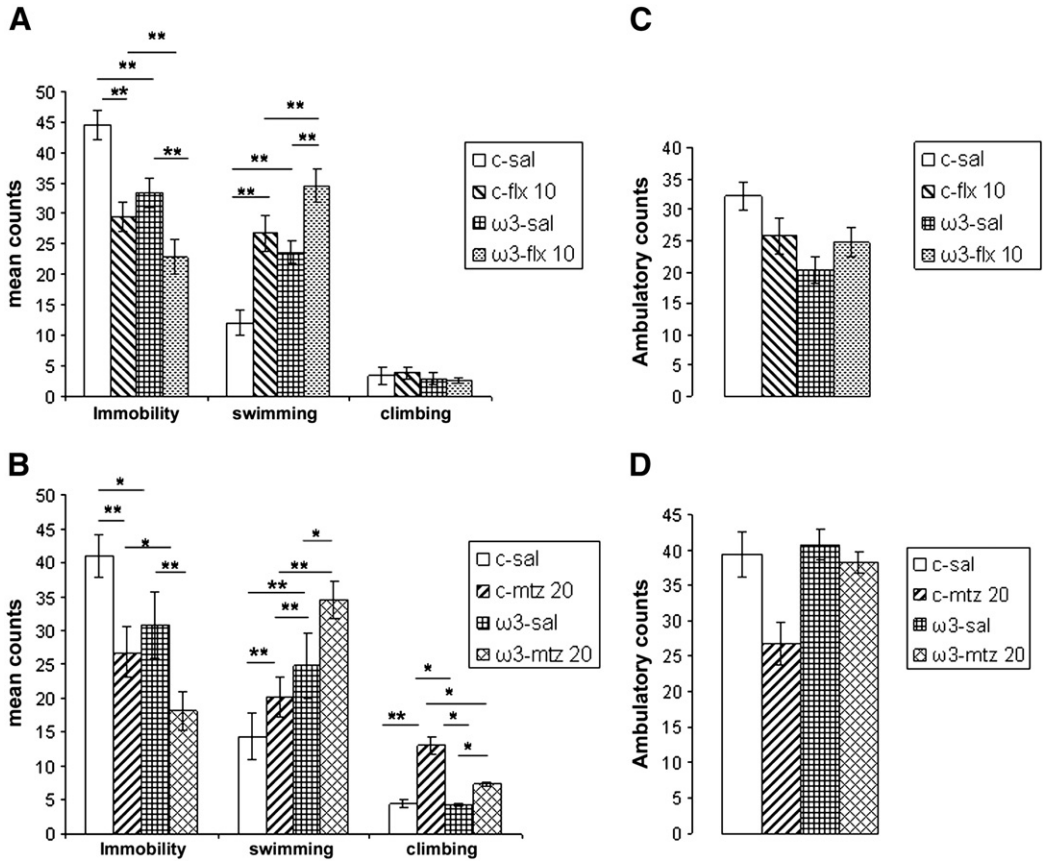

Fig. 3. Effects of the standard antidepressant drugs, omega-3 fatty acids and their combined treatments on behaviors in the forced swimming test (FST) and open field test (OFT). (A) Fluoxetine (10 mg/kg) and/or omega-3 fatty acids decreased immobility and increased swimming without affecting climbing. The combined treatment showed an additive effect. (B) Mirtazapine (20 mg/kg) and/or omega-3 fatty acids decreased immobility and increased swimming and climbing behaviors. The combined treatment showed an additive effect. (C) Fluoxetine (10 mg/kg) and/or omega-3 fatty acids (0.72 g/kg) failed to change the exploratory activity. (D) The omega-3 fatty acids and/or mirtazapine (20 mg/kg) treatments did not show any significant effect in the open field test. Bars represent the mean number of counts over the 5-min period of the test (±S.E.M). *P<0.05, ** P<0.01, $n = 7–12$ rats per group. c-sal, control diet- saline; c-flx 10, control diet-fluoxetine 10; c-mtz 20, control diet-mirtazapine 20; ω3-sal, omega-3 diet-saline; ω3-flx 10, omega-3 diet-fluoxetine 10; ω3-mtz 20, omega-3 diet-mirtazapine 20. Data were analyzed with two-way ANOVA, followed by Tukey's test for multiple comparisons.

treated with 10 mg/kg of fluoxetine alone or in combination with omega-3 fatty acids as compared to control group [$F(1, 45) = 54.24$, $P<0.0001$] and consistent with previous observations (Heisler et al., 1997) (Fig. 5B). Although in a lesser extent, similar results were obtained with the lowest dose of fluoxetine (1 mg/kg) (Fig. 5C).

Chronic treatment with mirtazapine (20 mg/kg) alone or in combination with omega-3 fatty acids (0.72 g/kg/day) significantly decreased body weight gain (Fig. 5D). Different results were obtained with the lowest doses of mirtazapine; despite of mirtazapine alone (1 mg/kg) produced no effect, the combined treatment with omega-3 fatty acids (0.72 g/kg/day) induced an increase in body weight gain [$F(1, 22) = 20.41$, $P<0.005$] (Fig. 5E).

3.5. Behavioral responses of antidepressants and/or omega-3 fatty acids in the absence of changes in brain phospholipid classes

The effects of fluoxetine (10 mg/kg) and/or omega-3 fatty acid (0.72 g/kg) were studied on phospholipids in the cerebral cortex (Fig. 6A) and hippocampus (Fig. 6B). We observed none of the treatments with antidepressant-like effects in the forced swimming test affected the content of diverse phospholipid classes either in cerebral cortex or hippocampus as seen by the similar contribution of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol and phosphatidylserine to the total phospholipid concentration in each experimental group.

4. Discussion

The purpose of the present work was to study the behavioral features of the omega-3 fatty acid antidepressant-like effect in the forced swimming test in rats alone or in combination with antidepressant drugs. Dose range, changes in locomotor activity in the open field test, weight gain and possible brain membrane modification in the phospholipid content were also examined. We particularly searched for different combined antidepressant plus omega-3 fatty acid treatments at low doses that might provide therapeutic benefits in terms of final antidepressant action and side effect profile.

Stress has been reported to be a predisposing factor for depression. In this study, we used the forced swimming test, a well-accepted model to test the antidepressant-like action of agents. The forced swimming test employs forced swimming stimuli as stressor to generate a behavior characterized by increased immobility time. The appearance of this behavior has been associated to changes in neurotransmitters and cell signalling pathways in the brain (Tardito et al., 2006).

In our forced swimming test design, a pre-test is done first, chronic drug treatment is then administered, and the final test is presented (Borsini et al., 1984; 1985; Delini-Stula et al., 1988; Mancinelli et al., 1987). Our protocol has at least two important advantages. First, administration of agents after the pre-test stressor allows the

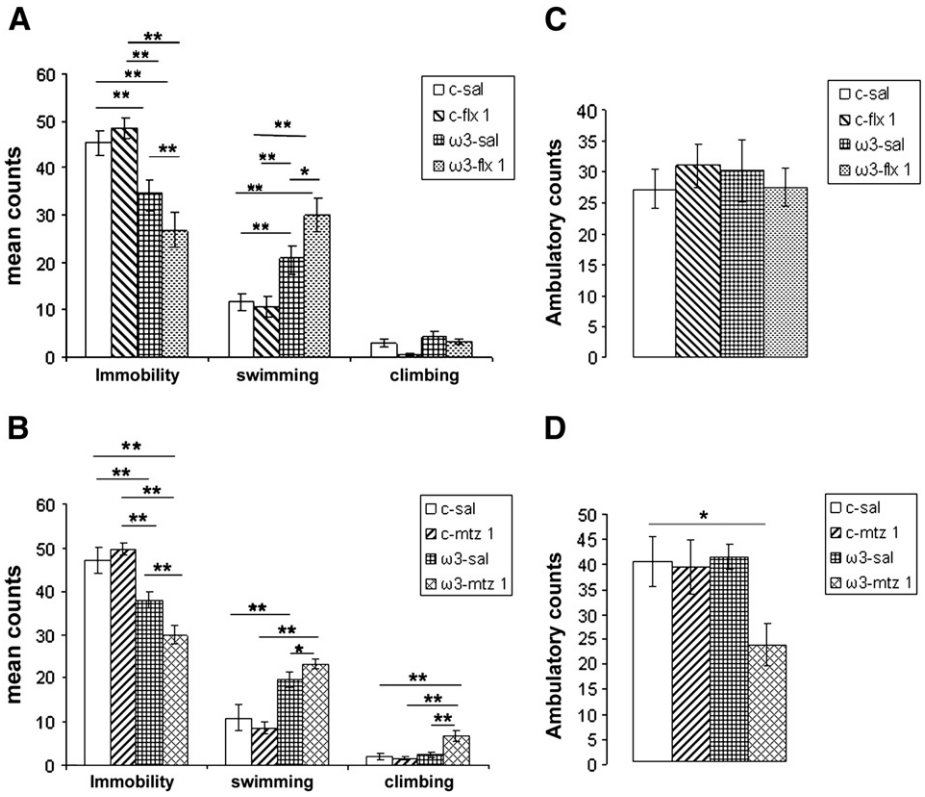

Fig. 4. Effects of sub-effective doses of standard antidepressant drugs, omega-3 fatty acids and their combined treatments on behaviors in the forced swimming test (FST) and open field test (OFT). (A) A low non-antidepressant dose of fluoxetine (1 mg/kg) failed to modify any behavioral parameter in the forced swimming test. In combined treatments, fluoxetine potentiated the omega-3 fatty acid antidepressant-like effects. (B) Mirtazapine (1 mg/kg) did not affect any behavioral parameters measured in the forced swimming test. In combined treatments, mirtazapine potentiated the omega-3 fatty acids antidepressant-like effects. (C) Fluoxetine (1 mg/kg), omega-3 fatty acids and their combined treatments did not affect the locomotor activity. (D) Among groups, only mirtazapine (1 mg/kg) plus omega-3 fatty acid treatment decreased the exploratory activity. Bars represent the mean number of counts over the 5-min period of the test (± S.E.M.). *$P<0.05$, ** $P<0.01$, $n=6$–12 rats per group. c-sal, control diet-saline; c-flx 1, control diet-fluoxetine 1; c-mtz 1, control diet-mirtazapine 1; ω3-sal, omega-3 diet-saline; ω3-flx 1, omega-3 diet-fluoxetine 1; ω3-mtz 1, omega-3 diet-mirtazapine 1. Data were analyzed with two-way ANOVA, followed by Tukey's test for multiple comparisons.

evaluation of drug action after the behavioral despair is induced in the animals (Reinés et al., 2004; 2008). Second, giving the study drug after the swimming pre-test session avoids taking as a performance improvement some anxiolytic effects described for antidepressants (Rickels et al., 1993; Schweizer and Rickels, 1993). This might be due to the fact that anxiolytics may ameliorate the stressor impact on the animal behavior (Borsini et al., 1989; De Pablo et al., 1989; Flugy et al., 1992).

Since previous studies in rats have studied omega-3 fatty acid antidepressant-like effects when chronically administered before stressor stimuli presentation (Carlezon et al., 2005; Lakhwani et al., 2007; Huang et al., 2008), we report here that omega-3 supplementation also exerts antidepressant-like actions in the forced swimming test after the behavioral despair is induced in the animals. Moreover, the decrease in immobility or the increase in swimming behavior induced in the forced swimming test by this treatment can be considered as specific since they are not attributable to changes in locomotor activity.

By comparing the behavioral features in the forced swimming test, it can be inferred that the omega-3 fatty acid antidepressant-like effect displays the same profile as shown by the antidepressant fluoxetine but not by mirtazapine. Serotonin (5-HT) selective reuptake inhibitors (SSRI), such as fluoxetine, enhance 5-HT neurotransmission while mirtazapine enhances both 5-HT and noradrenaline (NA) neurotransmissions. Either 5-HT or NA has been involved in the pathogenesis and recovery from depression (Blier et al., 1990; Caldecott-Hazard et al., 1991).

When analyzing omega-3 fatty acid antidepressant-like effects, diet composition, omega-3 dose and duration of the chronic treatment should be also considered for comparisons. For instance, acute and chronic EPA administration, contrary to that expected, failed to reduce the immobility time and to enhance the anti-immobility effects of imipramine administration (Shaldubina et al., 2002). Some studies have reported omega-3 antidepressant-like effects after 28–42 days of administration but not before day 10 (Carlezon et al., 2005; Lakhwani et al., 2007; Huang et al., 2008). Herein, we described the omega-3 antidepressant-like effects after 16 days of administration.

Our results demonstrate in agreement with previous reports (Lakhwani et al., 2007) that omega-3 fatty acid supplementation in combination with chronic antidepressant doses of fluoxetine has significantly higher antidepressant-like effects than individual treatment and shows an additive effect at therapeutic doses of antidepressants. From our findings, this conclusion can be extent to other antidepressant drugs with different mechanisms of action such as the dual 5-HT and NA reuptake inhibitor mirtazapine. It is worth noticing that the above mentioned results showed by Lakhwani et al. (2007) involve an antidepressant drug administration 24 h previous to the stressor stimulus presentation instead of the 16-day treatment we employed in this study. Venna et al. (2009) have also reported in mice an additive effect with imipramine in acute treatment after chronic administration of omega-3 fatty acids.

The most interesting conclusion that can be obtain from our findings is that supplementation with omega-3 fatty acids and their combination with chronic low non-antidepressant doses of fluoxetine or mirtazapine has a significantly higher antidepressant-like effect than omega-3 fatty acids alone, suggesting that the omega-3 fatty acid antidepressant-like effect can be potentiated with inactive doses of antidepressants.

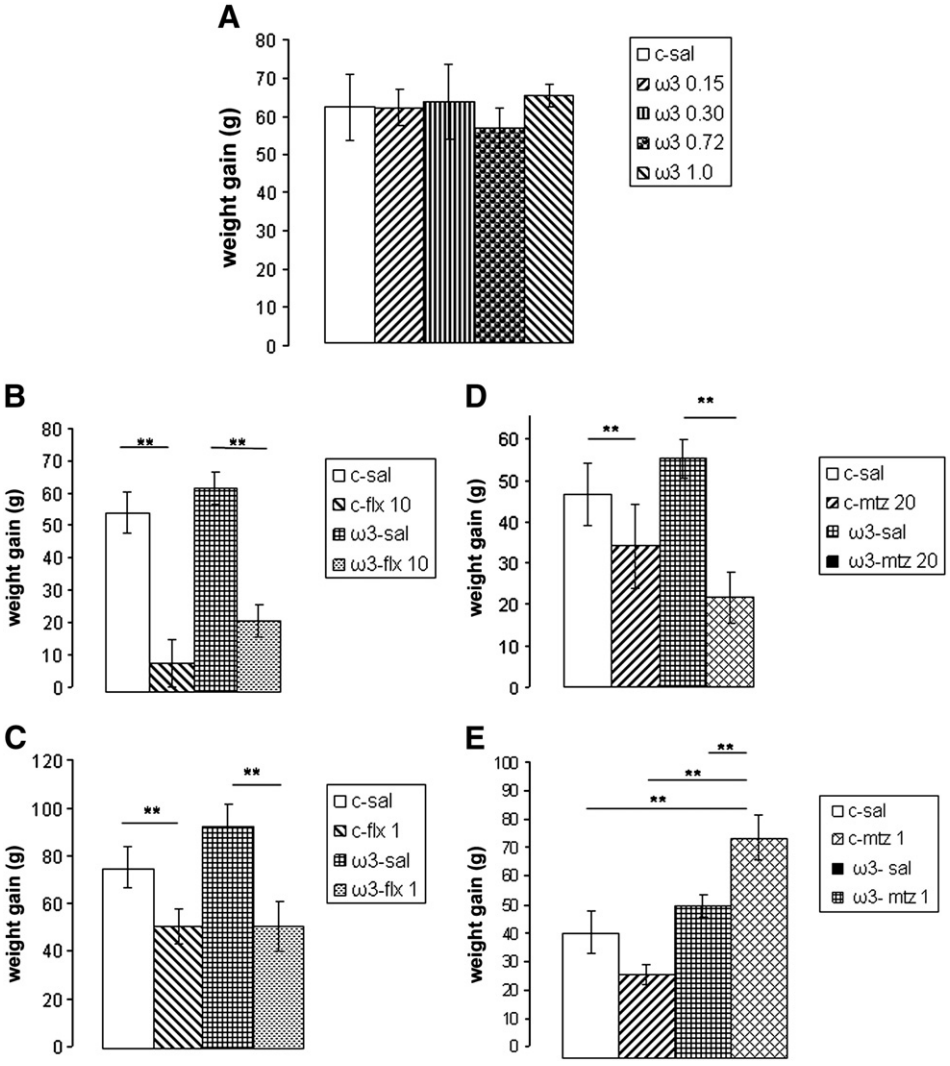

Fig. 5. Effects of the standard antidepressant drugs, omega-3 fatty acids and their combined treatments on rat weight gain. (A) Omega-3 (ω3) fatty acid diets did not affect the rat weight gain. (B) Rats given fluoxetine (10 mg/kg) alone or in combination with omega-3 fatty acid gained significantly less weight than control rats. (C) Fluoxetine (1 mg/kg) alone or in combination of omega-3 fatty acid gained significantly less weight than control rats. (D) Rats given mirtazapine (20 mg/kg) alone or in combination of omega-3 fatty acid gained significantly less weight than control rats. (E) Mirtazapine (1 mg/kg) did not affect the weight gain in rats maintained on the control diet. However, rats given mirtazapine (1 mg/kg) in combination with omega-3 fatty acids gained significantly more weight than control rats or rats maintained on the omega-3 fatty acid diet. Results are expressed as mean ± S.E.M, ** $P<0.01$, $n=5$–17 rats per group. Data were analyzed with one-way ANOVA (A) or two-way ANOVA (B–E), followed by Tukey's test for multiple comparisons.

To our knowledge this is the first report of the synergistic effect of combining omega-3 fatty acid supplementation with chronic antidepressant drug with different mechanisms of action at low non-antidepressant doses. Venna et al. (2009) have reported somewhat similar to that reported herein for fluoxetine and mirtazapine but for imipramine and omega-3 fatty acids both at ineffective doses. It should be pointed out that in our experimental conditions omega-3 fatty acid antidepressant-like effect potentiation with ineffective doses of fluoxetine and mirtazapine proved to be dose dependent. It should be also mentioned that an omega-3 fatty acid dose lacking antidepressant-like effect has been reported to potentiate the antidepressant-like effect of uridine (Carlezon et al., 2005).

Antidepressants are known to induce body weight changes after chronic administration. As previously described (Heisler et al., 1997), we observed that chronic fluoxetine administration dose-dependently reduces body weight. Regarding mirtazapine, clinical studies have shown increments in body weight as a consequence of adverse metabolic effects (Laimer et al. 2006). However, we observed decrements in body weight gain in rats with the highest mirtazapine dose employed (20 mg/kg, i.p.). In contrast, mirtazapine increases rat body weight gain only when administered at low doses (1 mg/kg, i.p.) along with omega-3 fatty acid supplementation. Contrary to antidepressant drugs, omega-3 fatty acids at doses from 0.10 to 1.00 mg/kg fail to induce any major change in body weight gain after chronic administration which could also represent an advantage when used in monotherapy. It should be noted that although omega-3 fatty acid supplementation does not counteract antidepressant induced-body weight changes, it would allow using lower antidepressant doses of fluoxetine associated to less effect on body weight.

Several neurophysiological mechanisms have been proposed to explain the relationship between omega-3 polyunsaturated fatty acids and depression. The two omega-3 fatty acids, EPA and DHA, appear to decrease the production of proinflammatory eicosanoids derived from arachidonic acid, such as interleukin-1 beta, interleukin-2, interleukin-6, interferon-gamma, and tumor necrosis factor alpha which has been associated to depression (Mamalakis et al., 2002; Logan, 2003; Boissonneault et al., 2000). It has been demonstrated that, by reducing the production of these proinflammatory cytokines, omega-3 fatty acids inhibit hypothalamic–pituitary–adrenal axis activity and decrease corticosterone levels (Logan, 2003; Song et al., 2003, 2004, 2007). This mechanism might contribute to reduce memory consolidation induced by exposure to aversive stimuli (Brinks et al., 2009). Omega-3 fatty acid

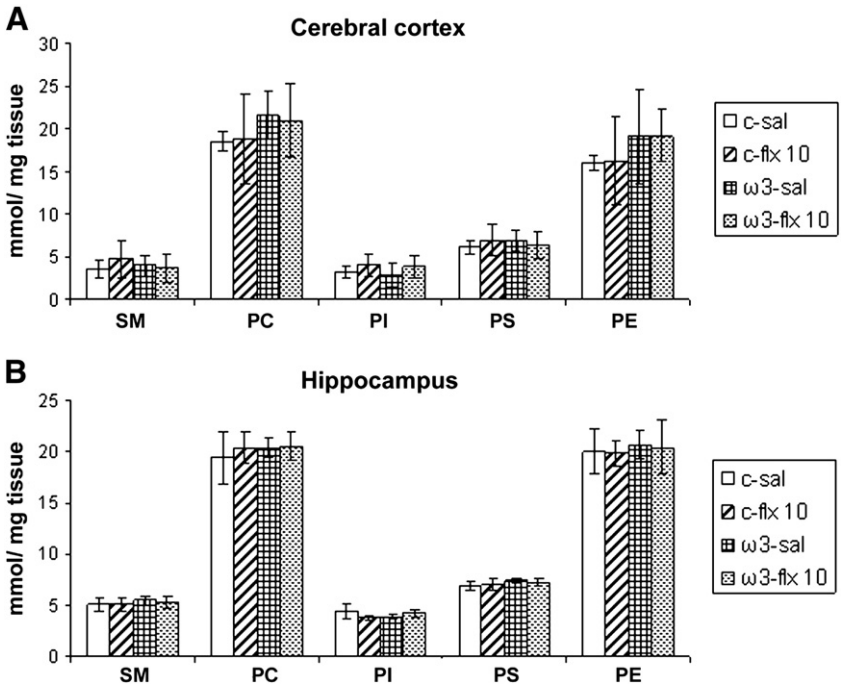

Fig. 6. Effects of Fluoxetine (10 mg/kg), omega-3 fatty acids and their combined treatments on the phospholipid content of cerebral cortex (A) and hippocampus (B). None of the treatments with antidepressant-like effects in the forced swimming test modified the fatty acid composition. Results are expressed as mean ± S.E.M for individual phospholipids. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PI, phosphatidylinositol; PS; phosphatidylserine. Data were analyzed with two-way ANOVA.

deficiency also decreases brain-derived neurotrophic factor (BDNF) (Ikemoto et al., 2000), a neurotrofin related to neuronal plasticity and neurogenesis that is found diminished in depressed patients and increased after antidepressant treatments (Saffet et al., 2005). Indeed, DHA-rich diets have been found to increase the levels of activated forms of hippocampal Akt and CaMKII, crucial steps by which BDNF exerts its action on synaptic plasticity and learning and memory, and with even greater elevation by a combination of diet and exercise (Wu et al., 2008). Similarly to that shown for antidepressant drugs, brain plasticity modulation has been proposed for omega-3 fatty acids as well (Venna et al., 2009). Another consistently proposed mechanism is related to the abundance of DHA in central nervous system membrane phospholipids, where it plays a vital role in maintaining membrane integrity and fluidity (Yehuda et al., 1998). In fact, DHA has been found to alter the membrane compartimentalization of several signalling proteins, thus modulating cellular function (Stillwell et al., 2005).

The analysis of brain phospholipid content after 16 day-supplementation with omega-3 fatty acids and/or antidepressant drug treatments suggests that either omega-3 antidepressant-like effect or the synergism observed in combination with antidepressants are not attributable to changes in the brain membrane contribution of each phospholipid class. These observations are in agreement with Porta et al. (2009) who previously reported some beneficial effects of omega-3 fatty acids on neurotransmission regardless of changes in brain membrane phospholipid composition. It has been also described that brain membrane fatty acid composition is not modified after short-term administration (about a week) of an omega-3 fatty acid-enriched diet (Girón et al., 1995), but is affected in the third rat generation when each of these is treated for two months with these fatty acids (Chalon et al., 1998). Another interesting hypothesis to be control is weather the omega-3 enriched diet might modify at some point antidepressant pharmacokinetic and so that contributing in some extent to the synergistic effect observed in combined treatments. In this regards, preliminary results from our laboratory show no differences in the fluoxetine plasma concentration between control and omega-3 animals on day 17 according the experimental design described in Fig. 1 (data not shown). Further experiments aimed to thoroughly analyze the pharmacokinetic parameters of fluoxetine and its metabolite norfluoxetine in animals fed with an omega-3 enriched diet could shed light on this matter.

To sum up, this study provides evidence to support the hypothesis that omega-3 fatty acids can be effective as an adjunctive treatment for those with treatment-resistant depression. Particularly, combined fluoxetine or mirtazapine and omega-3 fatty acid treatment, employing low inactive doses of the antidepressants, may help reduce the doses of antidepressant drugs and, in consequence, the magnitude of their side-effects. Furthermore, our work also support the robust antidepressant-like effect of omega-3 fatty acids when employed in monotherapy, as it has been reported in a recent clinical trial for the treatment of childhood depression (Nemets et al., 2006). Overall, our data could have important consequences in the medical practice and in the development of new antidepressant treatment strategies. Our findings may be particularly important in cases of depression resistant to conventional treatments, in cases where clinical trials with omega-3 as a treatment are still inconclusive or lacking, such as for the treatment of bipolar disorders and postnatal depression and in cases with queries regarding the efficacy and safety of antidepressant medication such as childhood depression.

5. Conclusions

This study shows the existence of significant synergistic effect of combining omega-3 fatty acid supplementation with chronic antidepressant drugs with different mechanisms of action at low ineffective doses. These findings might contribute as new therapeutic approaches and could mean higher response rates and lower magnitude of some side effects associated with antidepressant treatments.

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