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Effects of n-3 PUFA enriched and n-3 PUFA deficient diets in naïve and A β -treated female rats

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ABSTRACT

Depression is one of the most common psychiatric diseases and the prevalence of depressive symptoms in women is almost twice compared to men, although the reasons of this gender difference are not fully understood yet. Recently, soluble A β _{1–42} peptide has been receiving great importance in the development of depression, also since depression is highly comorbid with Alzheimer's disease and other neurodegenerative illnesses. Accordingly, we have previously shown that central A β injection is able to elicit depressive-like phenotype in male rats. In the present study, we reproduced for the first time the A β -induced depressive-like model in female rats, evaluating behavioural and neurochemical outcomes. Moreover, we studied the effect of lifelong exposure to either n-3 PUFA enriched or n-3 PUFA deficient diet, in female rats, both intact and after central A β administration. Our results confirmed the A β -induced depressive-like profile also in female rats. Moreover, chronic exposure to n-3 PUFA deficient diet led to highly negative alterations in behavioural and neurochemical parameters, while lifelong exposure to n-3 PUFA enriched diet was able to restore the A β -induced depressive-like profile in female rats. In conclusion, the A β -induced depressive-like profile was reversed by n-3 PUFA supplementation, indicating a possible therapeutic role of n-3 PUFA in the treatment of the burden of depressive disorders.

1. Introduction

Depression is one of the most common psychiatric diseases and the prevalence of depressive symptoms has reached epidemic proportions during the last few decades [32]. In this regard, several studies reported that depression is more prevalent in women compared to men [32,40,50]. Although reasons of this gender difference are not fully understood yet, women show different response to sex hormones, that might ultimately influence behaviour and brain functions [51]. In particular, estrogens modulate several neural and behavioural functions, including mood, cognitive function, blood pressure regulation, motor coordination, pain, and opioid sensitivity [52]. In addition, it has been shown that estrogens also affect neurotrophic functions and monoamine neurotransmission in several brain areas, thus they might ultimately be involved in the pathogenesis of depressive-like disorders [9]. Such evidence suggests that the antidepressant therapy should be personalized, taking into account also gender differences [82,90]. In

addition, a series of studies indicated that estrogens modulate the metabolic production of different endogenous and exogenous molecules [6,43,58]. Among these molecules, it has been reported that estrogens stimulate the conversion of essential fatty acids into their longer chain metabolites, such as α -linolenic acid conversion into docosahexanoic acid (DHA) [11,31]. DHA is a key n-3 polyunsaturated fatty acid (PUFA) involved in the Central Nervous System (CNS) development [16] and, thus, it is fundamental during pregnancy and early stage of childhood [24]. DHA and arachidonic acid (AA, 20:4n-6) are biologically important PUFAs, and can be supplied either directly from diet or by metabolic conversion of their essential precursors α -linolenic acid (18:3n-3) and linoleic acid (18:2n-6), respectively [64]. DHA, AA and their mediators modulate several processes, such as signal pathways, membrane fluidity, neurotransmission, neuroinflammation and cell survival [24]. During embryonic life and lactation, PUFAs intake exclusively depends on maternal diet, as the metabolic conversion of essential precursors cannot be accomplished [42]. Indeed, *in utero*

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exposure to unbalanced diet can be an important risk factor for mental disorders in later adulthood [42]. Modern western diets are characterized by low fish consumption and more junk food, resulting in n-3 PUFA deficiency and abnormal n-6 PUFA increase, respectively [81]. This unbalanced n-6/n-3 ratio is considered to be detrimental for the CNS functioning. Indeed, recent research suggests an etiological role for n-3 PUFA deficiency in mood disorders, such as Major Depressive Disorder (MDD) [34,55]. Accordingly, different epidemiological studies reported an inverse correlation between n-3 PUFA intake and depressive symptoms among women in United States [7,8]. We have previously shown that lifelong deficiency of n-3 PUFA leads to a depressive-like phenotype associated with reduced serotonin (5-HT) levels and increased soluble amyloid beta ($A\beta$)_{1–42} concentrations [64] in male rats. The $A\beta$ _{1–42} peptide and its oligomeric forms have been demonstrated to have powerful neurotoxic effects [73,79]. Recently, soluble $A\beta$ _{1–42} peptide has been received great importance in the development of depression, also since depression is highly comorbid with Alzheimer's disease and other neurodegenerative illnesses [72,73,89]. In this regard, we have previously shown that the injection of a solution of soluble $A\beta$ _{1–42} in the ventricular area of male rats can evoke a depressive-like phenotype associated with reduced cortical 5-HT and neurotrophins, such as Nerve Growth Factor (NGF) and Brain-Derived Neurotrophic Factor (BDNF) [15,78].

In order to avoid the variability that female hormonal cycle could induce [3], the majority of animal studies on depression use males. However, the US National Institute of Health is strongly encouraging preclinical research on females [40]. Hence, considering also the higher incidence of depressive disorders in women, the development of pre-clinical models of depressive-like profile in females is becoming necessary [18].

In the present study, we evaluated the effect of lifelong exposure to either n-3 PUFA enriched or n-3 PUFA deficient diet in female rats exposed to $A\beta$ _{1–42} administration.

2. Methods

2.1. Animals

Adult (250–300 g) Wistar rats (Harlan, S. Pietro al Natisone, Udine) were used in this study. They were housed at constant room temperature ($22 \pm 1^\circ\text{C}$) and relative humidity ($55 \pm 5\%$) under a 12 h light/dark cycle (lights on at the 7 A.M.) with *ad libitum* access to food and water.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines of the Italian Ministry of Health (D.L. 26/2014), the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2004), the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. All procedures involving animals were approved by the Italian Ministry of Health (protocol number: B2EF8.17) and were conducted in accordance to ARRIVE guidelines. Animal welfare was daily monitored through the entire period of experimental procedures. No signs of distress were evidenced, anyway all efforts were made to minimize the number of animals used and their suffering.

2.2. Diets

One male and two female rats were housed together for mating. Animals were exposed to specific diets mimicking lifelong n-3 PUFA deficiency or supplementation, as previously described [2,42,64]. After mating, dams were randomly assigned to the group fed with a diet (laboratorio dottori Piccioni srl, Gessate, Milan Italy) containing 6% total fat in the form of only rapeseed oil (n-3 enriched, rich in α linolenic acid 18:3n-3) or peanut oil (n-3 deficient, rich in linoleic acid 18:2n-6) throughout gestation and lactation. As control group, dams

were fed with a diet containing 6% total fat in the form of 3% of peanut oil plus 3% of rapeseed oil, called control diet. After weaning, offspring continued to be subjected to the same diet throughout life. All experiments were carried out in female eight-week-old rats. Estrus cycle synchronization was determined by vaginal smear and confirmed by serum estradiol concentration. However, considering that pro-estrous/estrus events under normal lighting schedules (as in the present study) tend to occur during the late afternoon to early hours of the morning [96] procedures involving alive animals were performed in the morning (09.00–12.00 h).

2.3. $A\beta$ administration

The $A\beta$ _{1–42} peptide was purchased from Tocris (Bristol, UK) and was freshly prepared in sterile double-distilled water (vehicle) at a concentration of 4 μM as previously described [15]. Seven-weeks-old rats were anaesthetized with 3.6 ml/kg Equithesin intraperitoneally (i.p.; composition: 1.2 g sodium pentobarbital; 5.3 g chloral hydrate; 2.7 g MgSO_4 ; 49.5 ml propylene glycol; 12.5 ml ethanol and 58 ml distilled water) (Sigma Aldrich, Milan Italy) and secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Coordinates from bregma for intracerebroventricular (icv) infusions were based on the atlas of Paxinos and Watson [69]: AP = -0.5 , ML = $+1.2$ and DV = -3.2 , (incisor bar at -3.3 mm). Soluble $A\beta$ (5 μl) was delivered through a 25 μl Hamilton microsyringe at 2 $\mu\text{l}/\text{min}$ infusion rate over a period of 2.5 min. Infusion needle was left in place for additional 5 min in order to avoid elapsing during removal. Control rats received vehicle only, because outcomes observed from preliminary used reverse $A\beta$ _{42–1} were indistinguishable from vehicle alone (unpublished observations). The needle track placement of was verified at the time of dissection. All experimental procedures were carried out 7 days after icv administration (SHAM or $A\beta$ -treated groups).

2.4. Forced swimming test

The forced swimming test (FST) is a reliable task for discriminating depressive state in animals and is widely used for predicting antidepressant properties of drugs [74]. On the first of the two test days, animals were placed individually in inescapable Perspex cylinders (diameter 23 cm; height 70 cm) filled with water at constant temperature of $25 \pm 1^\circ\text{C}$ at 30 cm of height [17].

During the preconditioning period, animals were videotaped for 15 min, then were returned to their home cages after drying. Twenty-four h later, each rat was positioned in the water-filled cylinder for 5 min and video-recorded. Frequencies of the following behaviors were scored by a blind observer: struggling (tentative of escaping), swimming (moving around the cylinder) and immobility (remaining afloat making only the necessary movements to keep its head above the water). Data were expressed as frequency on 5 s counts.

2.5. Post-mortem tissue analysis

Brains were immediately removed from euthanized rats, and cooled on ice for dissection of prefrontal cortex (PFC) and hypothalamic areas, according to the atlas of Paxinos and Watson [69]. Tissues were frozen and stored at -80°C until analyses were carried out.

2.6. High-performance liquid chromatography (HPLC) quantifications

5-HT, 5-hydroxyindolacetic acid (5-HIAA) and dopamine (DA) concentrations were determined by HPLC coupled with an electrochemical detector (Ultimate ECD, Dionex Scientific, Milan, Italy). Separation was accomplished by a LC18 reverse phase column (Kinetex, 150 mm $\times$ 4.2 mm, ODS 5 μm ; Phenomenex, Castel Maggiore-Bologna, Italy) and detection was performed through a thin-layer amperometric cell (Dionex, ThermoScientifics, Milan, Italy) with a 5 mm diameter

glassy carbon electrode with a working potential set at 0.400 V (vs. Pd) as previously described [60]. The mobile phase consisted of a solution of 75 mM NaH_2PO_4 , 1.7 mM octane sulfonic acid, 0.3 mM EDTA, acetonitrile 10%, in distilled water, buffered at pH 3.0. Chemicals and reagents were purchased from Sigma Aldrich, Milan Italy. The flow rate was set at 0.7 ml/min by using an isocratic pump (Shimadzu LC-10 AD, Kyoto, Japan). Data acquisition and integration was performed through Chromeleon software (version 6.80, Thermo Scientific Dionex, San Donato Milanese, Italy).

2.7. ELISA quantifications

Cortical Nerve Growth Factor (NGF), hypothalamic corticotrophin releasing factor (CRF) and plasma corticosterone and soluble $\text{A}\beta_{1-42}$ quantifications were carried out by using ELISA kits provided by Cloud-Clone Corporation (Houston, Texas, USA). Assays were performed according to manufacturer's instructions. Briefly, tissues were diluted (10% tissue weight/total volume) with ice-cold medium containing phosphate-buffered saline (PBS) and protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy). After homogenization and centrifugation ($10,000 \times g$ at 4°C for 20 min), supernatant was collected and assays were performed according to the manufacturer's instructions. To normalize data and negate differences due to sample collection, protein concentration was determined by using the Pierce BCA assay kit (Thermo Fisher Scientific, San Donato Milanese, Italy). Analyses were carried out in duplicate to avoid intra-assay variations.

2.8. Statistical analyses

Results were expressed as mean $\pm$ S.E.M. Behavioural and neurobiological data were analyzed by using one or two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* analyses, as required by using GraphPad 5.0 (GraphPad Software, San Diego, CA). P value was set at 0.05.

3. Results

3.1. Effects of n-3 PUFA deficient diet on depressive-like behaviour using FST

To investigate the influence of lifelong exposure to n-3 PUFA deficient or n-3 PUFA enriched diet on depressive-like behaviour, we performed the FST. Our results show that n-3 PUFA deficient diet significantly increased the immobility frequency compared to control diet (Fig. 1A, One-way ANOVA followed by Bonferroni's multiple comparison test, $F = 4.351$, $P < 0.05$ n-3 deficient versus CTRL). Moreover, there were no significant differences in struggling frequency (Fig. 1B, One-way ANOVA followed by Bonferroni's multiple comparison test, n.s.), while swimming was significantly decreased in n-3 PUFA deficient diet-exposed animals (Fig. 1C, One-way ANOVA followed by Bonferroni's multiple comparison test, $F = 4.929$, $P < 0.05$ n-3 deficient versus CTRL).

3.2. Effects of n-3 PUFA deficient diet on plasmatic $\text{A}\beta$ levels

We quantified plasmatic soluble $\text{A}\beta_{1-42}$ peptide in offspring of rats fed with n-3 PUFA enriched or n-3 PUFA deficient diets. We found that animals exposed throughout their life to n-3 PUFA deficient diet had a significant increase in plasmatic $\text{A}\beta$ levels compared to controls (Fig. 2, one-way ANOVA followed by Bonferroni's multiple comparison test, $F = 9.164$, $P < 0.01$ n-3 deficient versus CTRL).

3.3. Effects of n-3 PUFA enriched diet on $\text{A}\beta$ -induced depressive-like behaviour using FST

Our group has previously demonstrated that $\text{A}\beta$ soluble peptide is

Fig. 1. Effects of control, n-3 PUFA enriched and n-3 PUFA deficient diets on FST. Frequency measure of immobility (A), struggling (B), and swimming (C) behaviours in female rats fed from conception until 5 weeks post-weaning with control diet (white bar), n-3 PUFA enriched diet (grey bar), and n-3 PUFA deficient diet (black bar). Data are expressed as mean $\pm$ SEM ($n = 12$ –13 per group). One-way ANOVA followed by Bonferroni's multiple comparison test, [#] $P < 0.05$ vs. CTRL.

able to evoke a depressive-like state [15], thus we tested whether lifelong exposure to n-3 PUFA enriched diet would prevent such $\text{A}\beta$ -induced alterations in female offspring rats. As shown in Fig. 3A and C, n-3 PUFA enriched diet prevented the depressive effect of $\text{A}\beta$. Indeed, immobility frequency was significantly increased and swimming frequency was significantly reduced in $\text{A}\beta$ treated rats compared to SHAM operated only in control animals (Fig. 3A, Two-way ANOVA followed by Bonferroni's multiple comparison test; $F_{(1,32)} = 6.258$, $P < 0.01$, $\text{A}\beta$ versus SHAM rats; Fig. 3C, Two-way ANOVA followed by Bonferroni's multiple comparison test; $F_{(1,32)} = 13.57$, $P < 0.01$, $\text{A}\beta$ versus SHAM

Fig. 2. Effects of control diet (white bar), n-3 PUFA enriched diet (grey bar), and n-3 PUFA deficient diet (black bar) on plasmatic soluble Aβ levels. Data are expressed as mean $\pm$ SEM (n = 6–7 per group). One-way ANOVA followed by Bonferroni's multiple comparison test **P < 0.01 vs. CTRL.

Fig. 3. Effects of control and n-3 PUFA enriched diet on Aβ-induced depressive-like behaviour. Frequency measure of immobility (A), struggling (B), and swimming (C) behaviours in female rats SHAM-operated (white bar) and Aβ-operated (black bar). Data are expressed as mean $\pm$ SEM (n = 9–12 per group). Two-way ANOVA followed by Bonferroni's multiple comparison test **P < 0.01, vs. SHAM rats.

rats), while no differences were evidenced in struggling frequency among groups (Fig. 3B, Two-way ANOVA followed by Bonferroni's multiple comparison test; n. s.).

3.4. Effects of n-3 PUFA deficient and n-3 PUFA enriched diets on serotonin levels and turnover in PFC

Results showed that cortical 5-HT concentrations were significantly lower in animals pre- and post-natal fed with n-3 PUFA deficient diet compared to controls (Fig. 4A, one-way ANOVA followed by Bonferroni's multiple comparison test, $F = 3.546$, $P < 0.05$ n-3 deficient versus CTRL). Moreover, 5-HT turnover was significantly increased in n-3 PUFA deficient rats compared to controls animals (Fig. 4B, one-way ANOVA followed by Bonferroni's multiple comparison test, $F = 6.086$, $P < 0.05$ n-3 PUFA versus n-6/n-3 CTRL). We also quantified 5-HT content in PFC of female rats exposed during their entire life to n-3 PUFA enriched or control diet, 7 days after Aβ icv injection. In particular, Aβ injection significantly reduced 5-HT content in control rats (Fig. 4C, two-way ANOVA followed by Bonferroni's multiple comparison test, $F_{(1,17)} = 3.431$, $P < 0.05$ Aβ-treated vs SHAM operated rats), while in n-3 PUFA fed animals no differences were retrieved between groups, indicating a protective effect of this diet towards Aβ-induced impairment (Fig. 4C, two-way ANOVA followed by Bonferroni's multiple comparison test, n.s., Aβ-treated vs SHAM operated rats). In regard to 5-HT turnover, no differences were found among experimental groups (Fig. 4D, two-way ANOVA followed by Bonferroni's multiple comparison test, n.s.).

3.5. Effects of n-3 PUFA deficient and n-3 PUFA enriched diets on DA levels in PFC

We quantified cortical DA in female offspring fed with n-3 PUFA enriched; n-3 PUFA deficient or control diets and no significant differences were found (Fig. 5A, One-way ANOVA followed by Bonferroni's multiple comparison test, n.s.). We also analyzed DA content in PFC of female rats exposed during their entire life to n-3 PUFA enriched or control diets, 7 days after Aβ icv injection; we found a significant increase in DA content of Aβ-treated animals compared to SHAM operated only in n-3 PUFA fed animals (Fig. 5B, two-way ANOVA followed by Bonferroni's multiple comparison test, $F_{(1,20)} = 5.873$, $P < 0.05$, Aβ-treated vs SHAM operated rats). In regard to DA turnover, no differences were found among experimental groups (data not shown).

3.6. Effects of n-3 PUFA deficient and n-3 PUFA enriched diets on cortical NGF protein content

We found that NGF was significantly reduced in n-3 PUFA deficient animals compared to animal exposed to n-3 PUFA enriched and control diets (Fig. 6A, One-way ANOVA followed by Bonferroni's multiple comparison test, $F = 7.514$, $P < 0.001$ n-3 deficient versus n-3 enriched, $P < 0.05$ n-3 deficient vs CTRL diet).

Interestingly, cortical NGF concentrations significantly increased in Aβ-treated n-3 PUFA fed animals compared to controls (Fig. 6B, Two-way ANOVA followed by Bonferroni's multiple comparison test, $F_{(1,16)} = 4.835$, $P < 0.05$ n-3 enriched vs CTRL diet).

3.7. Effects of n-3 PUFA deficient and n-3 PUFA enriched diets on hypothalamus pituitary adrenal (HPA) axis parameters

Results showed that hypothalamic CRF concentrations were significantly lower in animals pre- and post-natal fed with n-3 PUFA deficient diet compared to controls (Fig. 7A, one-way ANOVA followed by Bonferroni's multiple comparison test, $F = 5.898$, $P < 0.05$ n-3 deficient versus CTRL and n-3 enriched). Accordingly, corticosterone levels were increased in n-3 PUFA deficient rats and significantly decreased in

Fig. 4. Effects of control (white bar), n-3 PUFA enriched (grey bar) and n-3 PUFA deficient (black bar) diets on cortical 5-HT levels (A) and 5-HIAA/5-HT ratio (B) in naive animal. Data are expressed as mean $\pm$ SEM. One-way ANOVA followed by Bonferroni's multiple comparison test, [#] $P < 0.05$ vs. CTRL. Effects of control and n-3 PUFA enriched diet on cortical 5-HT levels (C) and 5-HIAA/5-HT ratio (D) in SHAM- (white bar) and A β -operated (black bar) females. Data are expressed as mean $\pm$ SEM ($n = 5-7$ per group). Two-way ANOVA followed by Bonferroni's multiple comparison test, ^{*} $P < 0.05$ vs. SHAM-operated.

n-3 enriched rats (Fig. 7B, one-way ANOVA followed by Bonferroni's multiple comparison test, $F = 77.08$, $P < 0.05$ n-3 deficient versus CTRL and $P < 0.001$ n-3 enriched vs CTRL and n-3 deficient). We also quantified CRF and corticosterone of female rats exposed during their entire life to n-3 PUFA enriched or control diet, 7 days after A β icv injection. As previously shown [63], no differences were retrieved in CRF while A β injection significantly reduced corticosterone content in CTRL rats (Fig. 7C two-way ANOVA followed by Bonferroni's multiple comparison test, n.s. and Fig. 7D, two-way ANOVA followed by Bonferroni's multiple comparison test, $F_{(1,18)} = 4.379$ $P < 0.05$ A β -treated vs SHAM operated rats).

4. Discussion

In the present study, we showed that lifelong exposure to n-3 PUFA deficient diet leads to impairments in behavioural and neurochemical parameters, while exposure to n-3 PUFA enriched diet is able to restore the A β -induced depressive-like profile in female rats.

From a behavioural point of view, we previously reported a significant increase in immobility and decrease in swimming and struggling frequency in FST in male rats fed with a diet poor in n-3 PUFA [64]. Here, we found similar results in female animals, indicating that in our experimental condition of poor n-3 PUFA intake, no sex differences are apparent.

Indeed, our results showed an increase in immobility frequency and a decrease in swimming frequency in FST in female adult offspring fed during their entire life with n-3 PUFA deficient diet. FST is a reliable test widely used to assess depressive-like state and screen

antidepressants activity in rodents [45]. This test is based on learned helplessness that results in depressive-like symptoms, such as immobility increase and swimming and struggling decrease. As in our previous studies, no locomotor impairment has been found in females (data not shown).

We showed that plasmatic A β levels were significantly increased in female rats fed with poor n-3 PUFA diet compared to controls. In good agreement, our previous study in male rats showed that a diet poor in n-3 PUFA increased plasmatic A β levels compared to controls, while high n-3 PUFA diet significantly decreased such levels [64]. Recently, the A β peptide, particularly in its soluble forms, is gaining more and more attention in the study of depressive disorders [15,73,78,89]. In this regard, our group has previously demonstrated that central A β administration can evoke a depressive like-phenotype in rats characterized by increased immobility frequency in the FST and by reduced cortical 5-HT and neurotrophin levels [15,62]. Regarding A β and n-3 PUFA interaction, recent studies suggest a crucial role played by n-3 PUFA in the production/clearance of the A β peptide [35,46]. Interestingly, it has been reported that A β aggregates interact with neuronal membranes at different levels. In particular, A β peptide affects membrane integrity, leading to neuron death, and lipid membranes mediate the transition from α -helical amyloid monomers into β -sheet oligomers [87]. Inclusion of PUFA in the lipid membrane, subsequent to their assumption as dietary supplements, effectively reduces the lipid ordering [94]. Thus, n-3 PUFA supplementation, by increasing membrane fluidity, promote the A β interaction with membrane lipid bilayers, influencing the peptide aggregation process [25]. Hence, we can speculate that in our model the decrease availability of n-3 PUFA in

Fig. 5. Effects of control (white bar), n-3 PUFA enriched (grey bar) and n-3 PUFA deficient (black bar) diets on cortical DA levels in naive (A), SHAM- (white bar) and A β -operated (black bar) females (B). Data are expressed as mean $\pm$ SEM ($n = 6$ per group). One- and Two-way ANOVA followed by Bonferroni's multiple comparison test ^{*} $P < 0.05$ vs. SHAM-operated.

Fig. 6. Effects of control (white bar), n-3 PUFA enriched (grey bar) and n-3 PUFA deficient (black bar) diets on cortical NGF levels in naïve (A), SHAM- (white bar) and A β -operated (black bar) females (B). Data are expressed as mean $\pm$ SEM (n = 5–6 per group). One- and Two-way ANOVA followed by Bonferroni's multiple comparison test [#]P < 0.05 vs. CTRL, ^{**}P < 0.01 vs. n-3 enriched, [#]P < 0.05 vs. A β -operated CTRL diet.

plasmalemma, secondary to deficiency in n-3-PUFA consumption, may lead to less interaction of A β species to the membrane, ultimately resulting in higher soluble A β levels.

To better understand the interaction between A β and PUFA and to investigate possible gender differences, we administered the soluble A β peptide in female offspring fed with n-3 PUFA enriched or control diet, reproducing the A β -depressive-like model also in females. Conversely, in n-3 PUFA fed animals, there were no differences between A β injected and SHAM operated animals, indicating a protective role of n-3 PUFA diet on the depressive-like phenotype induced by soluble A β injection. From a neurochemical point of view, we focused on 5-HT, 5-HT metabolism and DA in PFC. In this regard, we found that cortical 5-HT levels were significantly decreased in n-3 PUFA deficient females and 5-HIAA/5-HT ratio was significantly increased, confirming the detrimental effects of a diet poor in n-3 PUFA. Furthermore, cortical 5-HT content was significantly reduced in A β -treated animals compared to SHAM operated animals, both fed with control diet, endorsing the A β -induced depressive-like profile. As widely known, 5-HT levels and its metabolism impairment are strongly involved in the pathogenesis of depression and Selective Serotonin Reuptake Inhibitors (SSRI) are the most used pharmacological treatment for major depressive disorder [77]. Moreover, in an interesting clinical study, Barton and Colleagues found an elevated brain 5-HT turnover in unmedicated patients with depression [5] and several studies also reported a decrease in brain 5-HT turnover after classical or natural antidepressant treatments [1,47]. Interestingly, we found that n-3 PUFA enriched diet was able to restore

5-HT levels in A β treated animals. Among the several mechanisms that have been proposed to explain the influence of n-3 PUFA on the 5-HT synthesis, release and function in the brain, one of the most important might be the DHA modulation of 5-HT receptors accessibility [68]. In particular, DHA increases cell membrane fluidity in postsynaptic neurons, thus, in low DHA conditions, the membrane becomes less fluid and the binding of 5-HT to its receptors decreases significantly, due to the lower accessibility of 5-HT receptors [39,67]. This effect is not limited to the 5-HT receptors, but might also affect the DA receptors and other neurotransmitter receptors [67].

Furthermore, n-3 PUFA might influence 5-HT neurotransmission acting through the inflammatory pathways. In this regard, we have recently shown that acute A β injection used in our model leads to systemic inflammatory state in treated rats. Indeed, we reported significant increase in levels of circulating cytokines, such as interleukin 1 α and β , interleukin 12, tumor necrosis factor α and in pro-inflammatory enzymes such as cyclooxygenase II (COX II) [56] and an early activation of the inducible Nitric oxide synthase [60]. Moreover, increased microglial and astrocyte activation was present in our animal model [56]. In addition, sub-chronic treatment with a selective COX II inhibitor reversed the pro-depressive phenotype by reducing the immobility frequency in the FST and preventing the cortical serotonergic impairment found in our model [61]. Thus, we can speculate that in the present experimental conditions the amelioration in the depressive symptoms observed after n-3 PUFA rich diet in A β treated rats might be linked to the anti-inflammatory properties ascribed to such lipids.

Fig. 7. Effects of control (white bar), n-3 PUFA enriched (grey bar) and n-3 PUFA deficient (black bar) diets on hypothalamic CRF (A) and plasma corticosterone (B) in naïve animal. Data are expressed as mean $\pm$ SEM. One-way ANOVA followed by Bonferroni's multiple comparison test, [#]P < 0.05 vs. CTRL and ^{*}P < 0.05 and ^{**}P < 0.01 vs. n-3 deficient. Effects of control and n-3 PUFA enriched diet on cortical 5-HT levels (C) and 5-HIAA/5-HT ratio (D) in SHAM- (white bar) and A β -operated (black bar) females. Data are expressed as mean $\pm$ SEM (n = 5–7 per group). Two-way ANOVA followed by Bonferroni's multiple comparison test, ^{*}P < 0.05 vs. SHAM-operated.

Indeed, it has been shown that enhanced n-3 PUFA consumption leads to reduced membrane levels of arachidonic acid following their incorporation into the phospholipids of inflammatory cell membranes at expenses of n-6 PUFA [12,13]. Accordingly, McNamara and Colleagues showed that n-3 PUFA deficiency was positively correlated with pro-inflammatory cytokine production, lead to an increase in central 5-HT turnover, while n-3 PUFA supplementation prevented this negative effect [53,54]. Furthermore, it has been shown that n-3 PUFA deficiency leads to the development of a pro-inflammatory condition in the central nervous system [20,49]. Clinical studies on healthy subjects reported an inverse correlation between blood n-3 PUFA levels and pro-inflammatory markers, such as cytokines and the acute phase protein C-reactive protein [26,57]. Furthermore, depressed patients exhibited elevated cytokines levels, significant blood DHA deficits and increased central 5-HT turnover [23,59]. Thus, these data would suggest that reduced n-3 PUFA levels might up-regulate the pro-inflammatory cascades and increase the 5-HT turnover, ultimately contributing to depression pathogenesis.

As regard other monoaminergic neurotransmissions, several evidence pointed out to an important role for dopaminergic system in the pathogenesis of depression [27,36,92]. In our model, we found no differences in cortical DA in naïve animals fed with n-3 PUFA enriched or n-3 PUFA deficient diet, but after A β injection, DA was significantly increased in animals exposed to n-3 PUFA enriched diet compared to SHAM operated animals.

Several studies indicated that certain antidepressants act also on dopaminergic system [19], targeting dopaminergic receptors [76], preventing dopamine re-uptake [70] or increasing extracellular DA levels [75]. Recently, it was reported that lesions of the ascending dopaminergic pathway at the level of the ventral tegmental area were able to reproduce depressive-like phenotype in the animals [29].

Conversely, very little is known about relationships between PUFA status and dopaminergic functioning in major depression. In a clinical study, DHA was inversely correlated with homovanillic acid, the main DA metabolite, in cerebrospinal fluid, indicating a possible link between n-3 PUFA status and dopaminergic tone in the brain [88]. Moreover, Zimmer and Colleagues demonstrated that in n-3 PUFA deficient rats, DA and D2 receptors amount was significantly decreased and also DA vesicles were specifically decreased in frontal cortex [97]. The mechanism leading to this modification might involve different pathways, such as vesicle turnover and membrane fluidity. Hence, even if drugs acting on dopaminergic system play a marginal role in the treatment of depression, this still remains a field open for future investigations.

In the searching for biological substrate involved in depressive state, neurotrophins also play a significant role. In this regard, we found a decrease of cortical NGF in female rats exposed to n-3 PUFA deficient diet, further confirming the putative pro-depressive effect of a diet poor in n-3 PUFA. Moreover, in A β treated animals, NGF was significantly increased in n-3 PUFA fed animals compared to controls, supporting the hypothesis of a possible therapeutic effect of n-3 PUFA in pathological conditions. Accordingly, it has been demonstrated that neurotrophic factors are relevant in neurodegenerative diseases, such as Parkinson's and Alzheimer's disease [37,91]. Since neurodegenerative symptoms also occur in depression, a neurotrophin hypothesis of depression has been raised. In particular, it has been shown that NGF decrease contributes to the etiology of depression [84]. Interestingly, it has been reported that DA agonists, such as bromocriptine, bergolide, cabergolide, may promote the synthesis and secretion of NGF [65,66]. These data are in line with our results, in which A β treated females exposed to n-3 PUFA enriched diet showed an increase in cortical DA and also in cortical NGF. These results suggest a beneficial effect of n-3 PUFA supplementation in A β -induced depressive-like symptoms, acting also through DA neurotransmission. Our hypothesis is supported by previous clinical studies in randomized placebo-controlled trials in which the antidepressant efficacy of n-3 PUFA supplementation is equivalent

to and also additive to the effects of classical antidepressants [30,38,48].

Although several epidemiological studies reported a prevalence of depressive symptoms in women, in our experimental paradigm female rats did not show differences compared to males, revealing the same A β -induced phenotype.

In this regard, literature about estrogens involvement in the pathogenesis of depression is controversial. Indeed, there are several studies supporting the influence of hormonal fluctuations in the development of mental illnesses, in particular pointing toward the importance of sex hormones as neuro-endocrine modulators [28,41,83]. On the other hand, different studies reported the influence of social factors, such as marital and employment status, as a trigger in the development of depression in women [10,22].

Furthermore, n-3 PUFA supplementation showed differences in naïve females compared to A β treated animals, indicating a positive effect only in presence of A β -induced dysfunctions. These results endorse the hypothesis of a possible therapeutic use of n-3 PUFA supplementation in depressive-like symptoms. In support to our findings, several studies supported the benefits of n-3 PUFA supplementation in the treatment of *post partum* depressive symptoms [14,85], while a recent study reported no efficacy of daily n-3 PUFA supplementation in the prevention of maternal depressive symptoms [93].

Conversely, the deleterious effects of n-3 PUFA deficiency are widely shown and the inverse correlation between low n-3 PUFA intake and increase of depressive-like symptoms is extensively reported [7,8,34].

However, the underlying mechanisms of action are still unclear and different hypotheses have been raised, mainly based on up or down regulation of physiological n-3 PUFA pathways. Among these pathways, we focused on the modulation of n-3 PUFA supplementation or deficiency on monoamine neurotransmission, especially 5-HT, DA and 5-HT metabolism. In particular, the highly unsaturated nature of EPA and DHA results in the influence of membrane fluidity and signal transduction. Thus, n-3 PUFA supplementation provokes changes that may affect different neurotransmitter systems, particularly altering the regulation of dopaminergic and serotonergic neurotransmission, which are dysfunctional in depressed patients [33]. However, for sake of clarity it should be noted that we cannot completely ascertain that behavioural and neurochemical outcomes evidenced in our study are necessarily cause related, thus future investigations in the way may be warranted. Importantly, we also confirmed that lifelong exposure to a diet poor in n-3 PUFA leads to altered stress response [64]. Indeed, here we found increased hypothalamic CRF and plasma corticosterone after the exposure to such diet. These outcomes are in line with literature. Indeed, increased HPA axis activation has been reported after pro-inflammatory stimuli in n-3 deficient animals [21] and transgenic mice displaying higher n-3 PUFA resulted protected [20]. HPA dysregulation secondary to chronic stress is a risk factors for the development of depressive state. Accordingly, patients affected by major depressive disorder hold higher cortisol levels leading to HPA axis dysregulation [86]. Interestingly, n-3 PUFA enrichment of diet prevented the dysfunction of HPA induced by A β , confirming the putative protective role of these lipids.

On the other hand, DHA is important in brain development and plays a critical role in neuronal signaling pathways regulated by neurotrophins. In particular, high cortical accretion of DHA upregulates mRNA expressions of key neurotrophins, such as NGF. Consistent with the health benefits of n-3 PUFA intake, NGF increase has been shown to ameliorate the symptoms of neurodegenerative disorders, such as Alzheimer's depressive disorder [95]. In this sense, it should also taken into consideration that the levels of A β _{1–42} in cerebrospinal fluid (CSF) of depressed patients tend to change accordingly to the depression phase with lower levels associated with an improvement of symptoms [71]. In particular, CSF A β _{1–42} levels have been inversely correlated with cerebral levels considering that aggravation of depressive symptomatology corresponds to increased amyloid deposition [71].

However, such depressive-phase dependent change in A β _{1–42} level is not paralleled by A β _{1–40}. Thus, Pomara and colleagues suggested that reduction in CSF A β _{1–42} linked to improved symptoms can actually rely on the presence of oligomeric forms that can mask some A β _{1–42} epitopes and hence rendered the peptide undetectable. Oligomeric A β _{1–42} (A β O) forms have been shown to be very toxic and their toxicity is inversely related to the assembly size, being the smaller the most noxious [80]. Accordingly, we have previously shown that a single A β O injection rapidly activated glial cells and increased pro-inflammatory cytokine expression and lead to memory impairment with the involvement of toll like receptor 4 (TLR4) [4]. Furthermore, a depressive like profile associated with aberrant activation of the cerebral innate immunity, particularly TLR4, and decreased serotonergic tonus have been reported in animals acutely injected with A β O [44]. In this model, we used an acute injection of a solution mostly consisting of monomeric A β _{1–42} at the time of the injection [60], however we cannot completely exclude that 7 days after the icv administration small assembly of oligomeric forms can actually be occurred.

In conclusion, in our study we have evaluated the effect of A β icv injection in female rats, reporting depressive-like behaviour and neurochemical impairments. Our data support the hypothesis that the n-3 PUFA supplementation can represent a possible therapeutic approach for depressive state linked to A β burden.

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6. Conflict of interests

The authors declare no conflict of interests.

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