Neurobiological determinants of depressive-like symptoms in rodents
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CHAPTER 3

Detrimental effects of lifelong n-3 PUFA deficiency on stress- and anxiety-related parameters in female rat offspring

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Abstract

Chronic stress is generally known to exacerbate the development of a wide variety of neuropsychiatric diseases, such as depression and anxiety disorders. The prevalence of these stress-related psychiatric disorders is about twice as high in women compared to men. Diets, genetics and lifestyle contribute to the onset and progression of mental illnesses. As regarding dietary factors, Polyunsaturated Fatty Acids (PUFA) have received great attention during the last decades, particularly due to the trend towards a poor n-3 PUFA intake of modern Western diets. In this regard, we have previously demonstrated that female offsprings receiving from conception a diet poor in n-3 PUFA showed a depressive-like behaviour, accompanied by decreased cortical serotonin and nerve growth factor. In the present study we investigated behavioural and neurochemical consequences of lifelong n-3 PUFA deficiency and n-3 PUFA enrichment on stress- and anxiety-related parameters in female offspring. Our results showed that female rats exposed to n-3 PUFA deficient diet spent more time performing self-grooming and staying in the periphery of the arena in the open field test, both indexes of anxiety-like behaviour. Moreover, we found a hyperactivation of the HPA axis pathway in n-3 PUFA deficient female rats, accompanied by a significant increase in serotonin and noradrenaline content in amygdala. In addition, we found a significant decrease of GABA and increase in glutamate in both amygdala and prefrontal cortex of female rats fed with n-3 PUFA deficient diet compared to females fed with n-3 PUFA enriched diet. In conclusion, modern Western diets, lacking in n-3 PUFA, might elicit significant neurochemical alterations that can ultimately lead to stress-related disorders, such as depression and anxiety.
3.1 Introduction

Stress-related psychiatric disorders, such as depressive diseases, are about twice as high in women compared to men (Donner & Lowry, 2013; Kendler, Thornton, & Gardner, 2000; Weissman et al., 1993). Likewise, the US National Institute of Mental Health reports that the lifetime prevalence of an anxiety disorder is 60% higher in women than in men (Donner & Lowry, 2013; Kessler, Chiu, Demler, Merikangas, & Walters, 2005; Leach, Christensen, Mackinnon, Windsor, & Butterworth, 2008; McLean & Anderson, 2009) and that the onset, severity, clinical course, and treatment response of anxiety disorders differ significantly in women (Pinna, Costa, & Guidotti, 2009).

Although this gender difference is well defined in literature, the biological bases underlying such dissimilarity are not fully yet unraveled (Bangasser et al., 2010). The increase in prevalence of these neuropsychiatric disorders made mandatory the search of novel strategic approaches destined at their prevention.

Genetics and environmental factors have been shown to crucially endow to the onset and progression of mental illnesses (Alam, Abdolmaleky, & Zhou, 2017; Barrenger, Draine, Angell, & Herman, 2017; Papadimitriou, 2017; Xiao, Chang, & Li, 2017). In particular, among environmental factors, dietary factors gained great attention in the last decade. In this regard, the right consumption of Polyunsaturated Fatty Acids (PUFA) with diet has been in the spotlight. PUFAs are a family of lipids identified by the position of the last double bond in their structure. Among them, n-3 and n-6 PUFAs are biologically important molecules that mediate several processes, such as signal pathways, membrane fluidity, neurotransmission, neuroinflammation and cell survival (Echeverria et al., 2017). N-3 and n-6 PUFA 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 (Morgese & Trabace, 2016; Zuliani et al., 2009). N-3 PUFA include alpha linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), while n-6 PUFA include linoleic acid (LA) and arachidonic acid (AA). N-3 PUFA, in particular DHA, are crucial for brain development and for proper central nervous system (CNS) functionality (Echeverria et al., 2017; Maekawa et al., 2017). Experimental evidence in animals has demonstrated that DHA deficiency during early brain development is deleterious with permanent consequences (Lafourcade et al., 2011; Lo Van et al., 2016; Lozada et al., 2017; Maekawa et al., 2017). Accordingly, we have previously demonstrated that female and male offspring receiving for their entire life a diet poor in n-3 PUFA showed a depressive-like behaviour in the forced swimming test,
accompanied by decreased cortical serotonin (5-HT) and nerve grow factor (NGF) content (Morgese, Tucci, et al., 2017). In addition, we have reported that, in male rats, lifelong n-3 deficiency lead to increased vulnerability to stress (Morgese et al., 2016). Indeed, chronic stress is generally known to exacerbate the development of a wide variety of neuropsychiatric diseases, such as depression, fear and anxiety disorders (Z. P. Liu et al., 2014). In this regard, Hypothalamic-Pituitary-Adrenal (HPA) axis hyperactivation is a well-known hormonal response to chronic stress. Activation of the HPA axis, starting with hypothalamic increase in corticotropin-releasing-Factor (CRF), acting through Adeno-Cortico-Tropic Hormone (ACTH) and ultimately leading to an increase in plasmatic corticosterone (in animals) or cortisol (in humans), is modulated by several brain signaling systems (Stephens & Wand, 2012). Among these systems, monoamines and amino acids neurotransmissions need to be taken into account. In this context, a number of evidence has indicated alterations in both noradrenaline and HPA axis parameters in anxiety and affective disorders (Dunn & Berridge, 1990; Heinrichs & Koob, 2004; Owens & Nemeroff, 1991; Smagin, Heinrichs, & Dunn, 2001). In particular, it has been widely reported that noradrenaline can excite CRF-containing cells in the hypothalamic paraventricular nucleus to activate the HPA axis (Dunn & Swiergiel, 2008). Moreover, the amygdala plays a critical role in emotional disorders (Hakamata et al., 2017) and it has been shown that the amygdala regulates the HPA axis response, probably involving serotonin and noradrenaline neurotransmissions (Weidenfeld, Newman, Itzik, Gur, & Feldman, 2002). However, also GABA and glutamate neurotransmissions play an important role in the stress-induced HPA axis hyperactivation. Interestingly, it has been reported that glutamate, a well-known excitatory neurotransmitter, can activate the HPA axis inducing ACTH elevation (Zelenà, Mergl, & Makara, 2005), while depression of the GABAergic tone has been shown to contribute the HPA axis activation (Cullinan, Ziegler, & Herman, 2008; Herman, Mueller, & Figueiredo, 2004; Mikkelsen, Bundzikova, Larsen, Hansen, & Kiss, 2008). On the other hand, it has been proposed that CRF signaling is positively regulated by estrogens and that, after certain stressors, the hypothalamic CRF expression is greater in female, both in humans and rodents (Bangasser et al., 2010). Thus, in the present study we deeply investigated behavioural and neurochemical consequences of lifelong n-3 PUFA deficiency and n-3 PUFA enrichment on stress- and anxiety-related parameters in female offsprings. In particular, we performed Open Field test and analyzed HPA axis parameters, catecholamines and amino acids involved in stress- and anxiety-related mechanisms.
3.2 Materials and Methods

Animals

Adult (250-300g) Wistar rats (Harlan, S. Pietro al Natisone, Udine) were used in this study. They were housed at constant room temperature (22±1°C) and relative humidity (55±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 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.

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 (Aid et al., 2003; Lafourcade et al., 2011; Morgese et al., 2016). In particular, after mating dams were randomly assigned to the group fed with either a diet containing 6% total fat in the form of only rapeseed oil (n-3 enriched, rich in -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.

Effects on experiments carried out may be influenced by the time of their estrous cycle (Jans et al., 2007). Pro-estrous/estrus events tend to be dictated by lighting times, but under normal lighting schedules (as in the present study) tend to occur during the late afternoon to early hours of the morning (Witcher & Freeman, 1985). Hence, all animal procedures were performed in the morning (usually 09:00–12:00 h) to reduce estrous cycle effects. Serum estradiol concentrations were measured to take account of possible differences in the estrous cycle.
Open Field Spontaneous Locomotor Activity
The open field apparatus consisted of a circular arena, 75 cm diameter, made of dark plastic under dim lighting, as previously described by Monteggia et al. (Monteggia et al., 2007). The experimental sessions were videotaped by a camera fixed above the arena. Animals were acclimatized to the test room for 1 h before each test. Motor activity was measured by placing the rat into the center of the arena before a 20-min session. The scoring was performed using a videotracking motion analysis system (ANY-MAZE, San Diego Instrument, San Diego, CA). To assess general locomotor activity, the following behavioral parameters (expressed as frequency on 5 min counts) were scored: number of square limit crossings with both forepaws, rearing (standing with the body inclined vertically, forequarters raised), and wall rearing (standing on the hind limbs and touching the walls of the apparatus with the forelimbs). To investigate anxiety-related behaviour, we measured time spent performing general grooming activity consisting of the following: face grooming (strokes along the snout), head washing (semicircular movements over the top of the head and behind the ears), and body grooming (body fur licking) (Choleris, Thomas, Kavaliers, & Prato, 2001). Time spent in center and periphery was quantified as measure of anxiety-like behaviour.

Post-mortem tissue analysis
Rats were euthanized and brains were immediately removed and cooled on ice for dissection of target region, namely PFC, according to the atlas of Paxinos and Watson (1998). Tissues were frozen and stored at -80°C until analysis was performed.

HPLC quantifications
Serotonin (5-HT) and noradrenaline (NA) concentrations in amygdala and NA concentrations in hypothalamus were determined by HPLC coupled with an electrochemical detector (Ultimate ECD, Dionex Scientific, Milan, Italy). Separation was performed by a LC18 reverse phase column (Kinetex, 150 mm×4.2 mm, ODS 5 μm; Phenomenex, Castel Maggiore- Bologna, Italy). The detection was accomplished by a thin-layer amperometric cell (Dionex, ThermoScientifics, Milan, Italy) with a 5 mm diameter glassy carbon electrode at a working potential of 0.400 V vs. Pd. The mobile phase used was 75 mM NaH2PO4, 1.7 mM octane sulfonic acid, 0.3 mM EDTA, acetonitrile 10%, in distilled water, buffered at pH 3.0. The flow rate was maintained by an isocratic pump (Shimadzu LC-10 AD, Kyoto, Japan) at 0.7 ml/min. Data were acquired and integrated using Chromeleon software (version 6.80, Dionex, San Donato Milanese, Italy).
GABA and glutamate concentrations in prefrontal cortex were determined by HPLC using ODS-3 column (150 × 4.6 mm, 3 µm; INERTSIL) with fluorescence detection after derivatization with ophthalaldehyde/mercaptopropionic acid (emission length, 460 nm; excitation length, 340 nm). The mobile phase gradient consisted of 50 mM sodium acetate buffer, pH 6.95, with methanol increasing linearly from 2 to 30% (v/v) over 40 min. The flow rate was maintained by a pump (JASCO, Tokyo, Japan) at 0.5 ml/min. Results were analyzed by Borwin software (version 1.50; Jasco) and substrate concentration was expressed as µM.

**ELISA quantifications**

Hypothalamus samples were analyzed for CRF quantifications by using ELISA kits provided by Tebu-Bio (Magenta, Milan, Italy). Assays were performed according to the 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). Samples were homogenized and centrifuged at 10,000 x g at 4°C for 20 min. The 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 BCA assay kit. Each sample analysis was carried out in duplicate to avoid intra-assay variations.

Plasma samples were analyzed for corticosterone by using an ELISA kit provided by Cloud-Clone Corporation (Houston, Texas, USA). Assays were performed according to the manufacturer’s instructions. Each sample analysis was carried out in duplicate to avoid intra-assay variations.

**Statistical analyses**

Results were expressed as mean ± S.E.M. Statistical analyses were performed using Graph Pad 5.0 (GraphPad Software, San Diego, CA) for Windows. Behavioral and neurochemical data were tested for normality and then analyzed by using two-way analysis of variance (ANOVA) for repeated measures or one-way ANOVA followed by Bonferroni’s or Tukey’s multiple comparisons test, as required. Differences were considered statistically significant when P value was less than 0.05.

### 3.3 Results

**Effects of n-3 PUFA deficient diet on anxiety-related behaviour using the Open Field Test**

To investigate the effects of lifelong n-3 PUFA deficiency and lifelong n-3 PUFA supplementation on anxiety-related behaviour in adult female rats, we performed the Open Field Test (OF). Time
spent performing self-grooming is commonly considered as an index of anxiety-like state; in addition, anxiety-like behavior is usually positively correlated with time spent in periphery and inversely correlated with time spent in the center of the arena (ref). Our results showed that n-3 PUFA deficient diet significantly increased self-grooming compared to n-3 PUFA enriched and balanced diet (Figure 1A, One-way ANOVA followed by Tukey’s multiple comparison test, F=7.839, P<0.01 n-3 deficient versus CTRL, P<0.05 n-3 deficient vs. n-3 enriched). Moreover, in regard to time spent in the center, there were no differences among experimental groups (Figure 1B, One-way ANOVA followed by Tukey’s multiple comparison test, n.s.), while in n-3 PUFA deficient animals, the time spent in periphery was significantly increased compared to control animals (Figure 1C, One-way ANOVA followed by Tukey’s multiple comparison test, F=5.245, P< 0.01, n-3 deficient vs. CTRL).

Figure 1

Effects of control, n-3 PUFA enriched and n-3 PUFA deficient diets on anxiety-like behaviours in the OF test. Time spent preforming self-grooming (A), time spent at the center of the arena (B), and time spent at the periphery of the arena (C) 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 ± SEM (n=6-10 per group). One-way ANOVA followed by Bonferroni’s multiple comparison test *P<0.05 vs. n-3 enriched, ###P<0.01, ####P<0.001 vs. CTRL.

Effects of n-3 PUFA deficient or n-3 PUFA enriched diet on locomotor activity
In order to evaluate whether different diet exposure could impair locomotor activity, rearing, wall-rearing and crossing frequency were scored during the OF test. We did not find any dysfunctions associated to diets exposure, in either vertical or horizontal activity, as revealed by crossing, rearing, and wall rearing frequency measurements (Figure 2A–C, Two-way ANOVA for repeated measures followed by Bonferroni’s multiple comparison test, n.s.).

**Figure 2** Effects of control, n-3 PUFA enriched and n-3 PUFA deficient diets on locomotor activity in the OF test. Frequency measure of crossing (A), rearing (B), and wall rearing (C) behaviours in female rats fed from conception until 5 weeks post-weaning with control diet (blue line), n-3 PUFA enriched diet (red line), and n-3 PUFA deficient diet
Effects of n-3 PUFA deficient diet on HPA axis parameters

To corroborate behavioural with neurochemical data, we analyzed HPA axis parameters, quantifying NA and CRF in hypothalamus and corticosterone in plasma samples. We found that n-3 PUFA deficient diet leads to HPA axis dysfunctions. In particular, NA was significantly increased in n-3 PUFA deficient females compared to controls (Figure 3A, One-way ANOVA followed by Tukey’s multiple comparison test, F=8.232, P< 0.05, n-3 deficient vs. CTRL). Furthermore, hypothalamic CRF was significantly increased in n-3 PUFA deficient animals compared to n-3 PUFA enriched and controls (Figure 3B, One-way ANOVA followed by Tukey’s multiple comparison test, F=5.898, P< 0.05, n-3 deficient vs. n-3 enriched and CTRL). Finally, plasmatic corticosterone was significantly increased in animals fed with n-3 PUFA deficient diet compared to both n-3 PUFA enriched and control diets, while n-3 PUFA enriched diet significantly decreased corticosterone levels compared to control diet. (Figure 3C, One-way ANOVA followed by Tukey’s multiple comparison test, F=77.08, P<0.001, n-3 deficient vs. n-3 enriched; P< 0.05, n-3 deficient vs. CTRL; P<0.001 n-3 enriched vs. CTRL).
Figure 3  Effects of control, n-3 PUFA enriched and n-3 PUFA deficient diets on HPA axis parameters. Levels of hypothalamic NA (A), hypothalamic CRF (B), and plasmatic corticosterone (C) 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 ± SEM (n=4-6 per group). One-way ANOVA followed by Bonferroni’s multiple comparison test *P<0.05, **P<0.001 vs. n-3 enriched; #P<0.05 vs. CTRL; §§§P<0.001 vs. CTRL.

Effects of n-3 PUFA deficient diet on monoamine neurotransmission in amygdala

To further explore the impairments in stress response due to lifelong n-3 PUFA deficient diet, we quantified NA and 5-HT levels in the amygdala. Interestingly, we found a significantly increase in female rats fed with n-3 PUFA deficient diet in both 5-HT and NA levels, suggesting an enhancement in the amygdaloidal neurotransmission induced by stress and anxiety (Figure 4A, One-way ANOVA followed by Tukey’s multiple comparison test, F=5.403, P<0.05 n-3 deficient diet vs. CTRL; Figure 4B, One-way ANOVA followed by Tukey’s multiple comparison test, F=4.789, P<0.05 n-3 deficient diet vs. CTRL).
Figure 4 Effects of control (white bar), n-3 PUFA enriched (grey bar) and n-3 PUFA deficient (dark bar) diets on amygdaloidal 5-HT (A) and NA (B) levels. Data are expressed as mean ± SEM (n=4-6 per group). One-way ANOVA followed by Bonferroni’s multiple comparison test, #P<0.05 vs. CTRL.

Effect of n-3 PUFA deficient diet on GABA and glutamate neurotransmission in amygdala and PFC

Ultimately, we analyzed GABA and glutamate content in amygdala and PFC of female rats fed with n-3 PUFA enriched and n-3 PUFA deficient diets. Our results showed a significant decrease in amygdaloidal GABA levels in n-3 PUFA deficient compared to n-3 PUFA enriched diet (Figure 5A, One-way ANOVA followed by Tukey’s multiple comparison test, F=7.001, P<0.01, n-3 deficient vs. n-3 enriched). Such a decrease was paralleled by a significant reduction in GABA levels in PFC (Figure 5B, One-way ANOVA followed by Tukey’s multiple comparison test, F=4.040, P<0.05, n-3 deficient vs. n-3 enriched). Moreover, n-3 PUFA deficient females showed a significant increase in glutamate content compared to females fed with n-3 PUFA enriched diet in both amygdala (Figure 5C, One-way ANOVA followed by Tukey’s multiple comparison test, F=6.209, P<0.05, n-3 deficient vs. n-3 enriched).
vs. n-3 enriched) and PFC areas (Figure 5D, One-way ANOVA followed by Tukey’s multiple comparison test, F=6.073, P<0.01, n-3 deficient vs. n-3 enriched).

**Figure 5**

Effects of control (white bar), n-3 PUFA enriched (grey bar) and n-3 PUFA deficient (dark bar) diets on amygdaloidal GABA (A) and cortical GABA (B) levels, and on amygdaloidal glutamate (C) and cortical glutamate (D) levels. Data are expressed as mean ± SEM (n=5 per group). One-way ANOVA followed by Bonferroni’s multiple comparison test, *P<0.05, **P<0.01 vs. n-3 enriched.

### 3.4 Discussion

In this study, the lifelong effects of diets enriched and deficient in n-3 PUFA on anxiety-like state and stress-induced dysfunctions in female rats have been evaluated, using behavioural and neurochemical tools. From a behavioural point of view, we performed the OF test. Besides the scoring of vertical and horizontal activity, to evaluate locomotor activity, the OF test is a valid tool for the evaluation of anxiety-like behaviours. In particular, when rodents are exposed to a new
environment, they are naturally inclined to move from the center towards the peripheral zone of the open field and closer to the limiting walls. This behaviour is considered as an index of timidity (Walsh & Cummins, 1976), and it is assumed to be an indicator of animal fear/anxiety state (Varela, Acanda de la Rocha, Diaz, & Lopez-Gimenez, 2017). Conversely, the animals that spent more time in the center of the arena are considered less fearful or anxious than those ones that prefer the peripheral area (Stanford, 2007). In addition, increased time spent performing self-grooming is also considered as an index of anxiety, since self-grooming in animals is an innate programmed behaviour that is controlled by a complex neural circuitry, and abnormal self-grooming behaviours are observed in many animal models of different anxiety disorders (Kalueff et al., 2016). In our experimental conditions, we found that n-3 PUFA deficient fed rats spent more time performing self-grooming and staying in the periphery of the arena, both indexes of anxiety-like behaviour. These findings cannot be related to impaired locomotion considering that no differences in vertical or horizontal activity among experimental groups were found.

Emotional disorders include both anxiety and depression and these two pathologies are highly comorbid. In this regard, we have previously shown that n-3 PUFA deficient diet has detrimental effects in both female and male rats, eliciting depressive-like alterations, such as increased immobility and decreased swimming frequency in FST, accompanied by reduced cortical 5-HT and increase in plasmatic soluble Aβ peptide (Morgese, Tucci, et al., 2017). Although lower levels of n-3 PUFA have been extensively correlated with major depressive disorder, less is known about PUFA status and anxiety disorders (J. J. Liu et al., 2013).

Recent evidence leads to hypothesize that n-3 PUFA may possess an anxiolytic effect, in addition to their antidepressant properties. Interestingly, Vinot and colleagues showed a reduction in anxiety in non-human primates following n-3 PUFA supplementation (Vinot et al., 2011). One mechanism involved in the beneficial effects of n-3 PUFA supplementation might be the PUFA regulation of immune responses to stress. In particular, it has been showed that n-3 PUFA supplementation reduces oxidative stress and pro-inflammatory cytokines, which are elevated in anxiety- and depressive-like states (Calder, 2006; Skouroliakou et al., 2010). In addition, a number of preclinical studies suggest that n-3 PUFA deficiency and additional stressors might converge in a pathologic synergism, resulting in the development and progression of anxiety disorders (Kiecolt-Glaser et al., 2007; J. J. Liu et al., 2013; Skouroliakou et al., 2010). In this context, a series of studies reported a strong correlation between anxiety disorders and chronic stress (Buffalari &
Grace, 2009; Hill & Patel, 2013; McEwen, 2007). In particular, the hyperactivity of HPA axis induced by chronic stress is highly related to depressive- and anxiety-like behaviours (Y. T. Lin et al., 2017). Interestingly, we found a deep alteration of the HPA axis pathway in n-3 PUFA deficient female rats, with a significant increase in hypothalamic NA and CRF and in plasmatic corticosterone. Indeed, it has been widely demonstrated that the HPA axis becomes active in response to stress and recent studies found that higher cortisol concentrations during stressful conditions are associated with high levels of anxiety in children and adolescents (Kallen, Ferdinand, & Tulen, 2007). Accordingly with our results, Larrieu et al. showed that n-3 PUFA supplementation prevents HPA axis hyperactivity and neuronal atrophy in PFC, inducing resilience to stress-induced emotional and neuronal impairments (Larrieu et al., 2014). In conclusion, n-3 PUFA enriched diet might be helpful for the treatment of stress-induced disorders and anxiety-like states. Here we also corroborated behavioral data with neurochemical quantification in brain areas crucially involved in stress response and anxiety-like disorders such as amygdala and PFC. In particular, we found a significant increase in 5-HT and NA content in amygdala of female rats treated with n-3 PUFA deficient diet. The amygdala has a pivotal role in emotional disorders and its functions are strongly modulated from stressful conditions and events (Hill et al., 2013). Hyperactivation of the amygdala following chronic stress is believed to be one of the primary mechanisms underlying the increased propensity for anxiety-like behaviours and pathological states (Hill et al., 2013). In particular, it has been demonstrated that stressors increase NA release in amygdala and an excessive increase could desensitize the α1-adrenergic receptors and contribute to the hyperexcitability of the amygdala, leading to anxiety disorders induced by stress (Hakamata et al., 2017; Weidenfeld et al., 2002). In addition, also amygdaloidal 5-HT content is involved in anxiety mechanisms. In a recent study, Johnson and colleagues pharmacologically depleted 5-HT in the basolateral amygdala nuclei complex and their results showed a decrease in anxiety-like behaviour in social interaction and open field tests (Johnson et al., 2015). Moreover, the amygdala is known to modulate the function of the HPA axis, but the mechanisms of this effect are still not clear (Weidenfeld et al., 2002). In this regard, Feldman and colleagues suggested that amygdaloidal 5-HT has an excitatory effect on the HPA axis (Feldman, Newman, Gur, & Weidenfeld, 1998), while Ma and colleagues reported that NA release in medial amygdala facilitates activation of HPA axis after acute stress (Ma & Morilak, 2005). Hence, the amygdaloidal
increase in noradrenaline and serotonin levels might contribute to the increase in anxiety indexes found in the OF test and to the HPA axis hyperactivation in n-3 PUFA deficient females. Furthermore, we found a significant decrease of GABA in both amygdala and PFC of female rats fed with n-3 PUFA deficient diet compared to females fed with n-3 PUFA enriched diet. On the other hand, amygdaloidal and cortical glutamate levels were significantly increased in n-3 PUFA deficient female rats compared to females fed with n-3 PUFA enriched diet. In this regard, dysfunctions of the central GABA system have been associated with anxiety disorders (Lydiard, 2003; Nemeroff, 2003; Nutt & Malizia, 2001) and it has been largely reported that an enhancement of GABAergic tone exerts anxiolytic effects (Kalueff, Kaluyeva, & Maillet, 2017; A. P. Lang & de Angelis, 2003; Nemeroff, 2003; Rosenthal, 2003; Stahl, 2004). In line with our results, Manzanares and colleagues sustained the hypothesis that the behavioural and neurophysiological consequences of chronic stress might be partially explained by the attenuation of GABAergic inhibition in the basolateral amygdala, ultimately leading to neuronal hyperexcitability (Rodriguez Manzanares, Isoardi, Carrer, & Molina, 2005). Interestingly, recent studies have suggested a possible involvement of DHA in the potentiation of GABA activity. In particular, it has been reported that DHA enhances the binding of diazepam to the GABA receptor in the cortical cell membrane (Takeuchi, Iwanaga, & Harada, 2003), while bicuculline, a GABA A antagonist, dramatically increased the negative effects of DHA deficiency (van Elst et al., 2014). In this regard, GABA A receptor functions might be influenced by PUFAs modulation of the membrane fluidity. Moreover, it has been suggested that n-6 PUFA increase, secondary to n-3 PUFA deficiency, might inhibit GABAergic neurotransmission and consequently cause neuronal excitability acting through phospholipase A2 or phospholipase C activation (Schwartz & Yu, 1992; van Elst et al., 2014). These results suggest that DHA might modulate neuronal excitability partially via a GABA-dependent mechanism (Sogaard et al., 2006) and that n-3 PUFA deficiency might ultimately lead to chronic stress and anxiety-related impairments acting also through GABAergic alterations.

As regard glutamatergic neurotransmission, it has been widely demonstrated the role of glutamate excitotoxicity in the pathogenesis of different mental illnesses, including schizophrenia, bipolar disorders, Alzheimer’s disease, anxiety-related disorders and major depressive disease (Frisardi, Panza, & Farooqui, 2011; Hashimoto, Sawa, & Iyo, 2007; Ogawa et al., 2017; Schiavone, Mhllaj, et al., 2017). In this regard, our group has previously demonstrated a significant increase of cortical glutamate in a rat model of Aβ-induced depressive-like disorders (Tucci et al., 2014),
unpublished data). Moreover, a recent study showed a key role of glutamate pathways abnormalities within the cortico-striatal-thalamo-cortical circuitry and temporal lobes in obsessive-compulsive disorder pathogenesis, one of the most common anxiety disorder (Vlcek, Polak, Brunovsky, & Horacek, 2017). Furthermore, increasing preclinical evidence suggests that glutamate plays an important role in the activation of the HPA axis, by inducing the adrenocorticotropic hormone (Zelena et al., 2005). Indeed, activation of glutamatergic projections to the amygdala and to the nucleus tractus solitarius is implicated in the stress response (Mathew et al., 2001). Interestingly, it has been reported that DHA is a physiological inhibitor of glutamate uptake (Berry et al., 2005; Grintal et al., 2009). Thus, the reduced amount of DHA released at the synapses of n-3 PUFA deficient animals would lead to less inhibition of glutamate uptake, and ultimately to a reduced efficacy at some glutamatergic synapses involved in memory formation, providing an explanation for the cognitive deficits associated with n-3 PUFA deficiency (Grintal et al., 2009). These results are in line with our data, in which we reported an increase of glutamate in PFC and amygdala of n-3 PUFA deficient females, accompanied by an increase of HPA axis parameters, pointing towards an involvement of glutamatergic neurotransmission on the HPA axis hyperactivation. Hence, HPA axis modulators and glutamate antagonists might converge in the same pathways to treat chronic stress and anxiety-related disorders, and ultimately n-3 PUFA might be beneficial to protect glutamatergic neurotransmission from damage induced by stress, possibly preventing the development of stress-related disorders, such as depression or anxiety.

To the best of our knowledge, this is the first study that deeply investigated the negative consequences of a diet deficient in n-3 PUFA on stress- and anxiety-related neurochemical parameters in female rats. Our results, indicate that modern Western diets, characterized by such a low n-3 PUFA content, might elicit significant neurochemical alterations that can ultimately lead to stress-related disorders, such as depression and anxiety and particular attention should be paid in female population that is already more susceptible to these disorders.