Neurobiological determinants of depressive-like symptoms in rodents

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CHAPTER 1

General Introduction

1.1 The burden of depression-related symptoms: state of the art

Depression is the leading cause of disability worldwide and is a major contributor to the overall global burden of diseases, with high levels of suicide incidence (www.who.int, World Health Organisation website). According to the World Health Organization, it is estimated that 10% to 15% of the general population will experience clinical depression during their lifetime (Tsuang, Taylor, & Faraone, 2004). Currently, more than 350 million of people of all ages suffer from depression (www.who.int). Indeed, depressive disorders often start at young age, affecting lifestyle and usually becoming recurrent. The prevalence of depression is approximately doubled in females compared to males, and several studies suggest that the heritability of the disorder is significantly higher in women (Mill & Petronis, 2007). Depression core symptoms include depressed mood, anhedonia (reduced ability to experience pleasure from natural rewards), irritability, difficulties in concentrating, social withdrawal (withdrawal from social contact that derives from indifference or lack of desire to have social contact) and abnormalities in appetite and sleep, the so called "neurovegetative symptoms" (Krishnan & Nestler, 2008).

Depression has shown to be comorbid with several neuropsychiatric diseases, such as schizophrenia, bipolar disorders, Alzheimer's diseases, anxiety disorders, autism spectrum disorders (ASD) and stress-related diseases. In particular, anxiety-related disorders, such as obsessive-compulsive disorders and social anxiety disorder, are highly comorbid with depression, with up to 90% of patients experiencing clinical depression at some point in their lifetime (Ressler & Mayberg, 2007). Moreover, depression often occurs during the prodromic phase of Alzheimer's disease, schizophrenia and bipolar disorders.

1.2 The impact of dietary factors on depressive-like symptoms

Lifestyle, particularly environmental and dietary factors, have a great influence on the pathogenesis of depression. In this regard, dietary Polyunsaturated Fatty Acids (PUFA) have received great attention during the last decades. PUFAs are a family of lipids that are 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. 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;

Morgese, Tucci, et al., 2017; 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 the brain development and to maintain correct central nervous system (CNS) functionality. Experimental evidence in animals has demonstrated that DHA deficiency during early brain development is deleterious and permanent (Lo Van et al., 2016; Lozada, Desai, Kevala, Lee, & Kim, 2017; Maekawa et al., 2017). During embryonal life and lactation, PUFA intake exclusively depends on maternal diet (Lafourcade et al., 2011). Indeed, it has been reported that maternal malnutrition plays a crucial role in development of psychiatric complications in later adulthood. In particular, evidence from human studies indicate that maternal metabolic state and diet influence dramatically the risk for behavioural disorders in progeny (Sullivan, Riper, Lockard, & Valleau, 2015). Unfortunately, it is quite appropriate to assume that this nutritional-poor diet will be later perpetuated, considering that represents part of a lifestyle acquired during early childhood.

Modern western diets are characterized by deficiency in content of n-3 PUFA, in particular low consumption of fish in favour of baked and junk food has determined a dramatic increase of the n-6/n- 3 PUFA ratio. Indeed, such ratio moved from 1, typical of early 20^{th} century, up to 15 in industrialized countries (Simopoulos, 2009, 2011). Epidemiological evidences have established a negative correlation between n-3 PUFA consumption and development of anxiety and depressive symptoms as well as physiological distress (U. E. Lang, Beglinger, Schweinfurth, Walter, & Borgwardt, 2015; Larrieu, Madore, Joffre, & Laye, 2012; Ross, 2009). These findings were supported by clinical studies, indicating that the lack of n-3 PUFA in diet is linked to an increased susceptibility to neuropsychiatric disorders (Beydoun et al., 2015; Lucas, Kirmayer, Dery, & Dewailly, 2010; Murakami, Miyake, Sasaki, Tanaka, & Arakawa, 2010; Panagiotakos et al., 2010), while beneficial results with n-3 PUFA supplementation alone or in adjunctive therapies have been described in treatment of depressive-like disorders (Grosso, Pajak, et al., 2014; P. Y. Lin & Su, 2007).

Moreover, Evans and colleagues demonstrated that n-6 PUFA and their biosynthetic enzymes are useful biomarkers for measurements of depressive disorders and burden of diseases, suggesting that they should also be taken into consideration when n-3 PUFA role is investigated (Evans et al., 2015). However, studies involving humans are often polluted by uncontrollable variables and biases and some contrasting results and inconsistencies in clinical evaluations have also been

denounced (Grosso, Galvano, et al., 2014; Grosso, Pajak, et al., 2014). In this context, the use of animal model can be very useful, especially when diet-influenced outcomes need to be measured in offspring, since dosing, age of first assumption, along with duration of assumption are strictly monitored and are quite reliable.

Along with dietary deficiency, chronic stress is another environmental risk factor for the development of depressive symptoms and dysregulation of hypothalamic–pituitary adrenal (HPA) axis in response to chronic or repeated stressful events is a reported important mechanism (McEwen, Eiland, Hunter, & Miller, 2012). In this regard, low cerebral DHA content, secondary to poor diet, has been associated to increased anxiety-like behaviour induced by chronic mild stress paradigm in animals (Harauma & Moriguchi, 2011). Indeed, it has been reported that a diet poor in n-3 PUFA increased aggressive behaviour in rodents, while high n-3 PUFA diet was able to reduce the stress response (Fedorova & Salem, 2006; Ikemoto et al., 2001), indicating that n-3 PUFA deficiency plays a central role in the chronic stress modulation.

In this context, in chapter 2 and 3 of the present thesis, effects of n-3 PUFA deficient and n-3 PUFA enriched diets on female rat offspring have been investigated, in terms of depressive-like behaviours and alterations of neurochemical parameters related to chronic stress, anxiety and depression.

1.3 From neurobiology to neuropsychiatric symptoms: searching for new biomarkers

However, current nosology for the diagnosis of neuropsychiatric disorders classify each disorder into non-overlapping diagnostic categories. This separation is not based on their underlying etiology, but on convention-based clustering of qualitative symptoms of the disorder (Kas et al., Neuroscience & Biobehavioral reviews, submitted). Although these diagnostic categories are sufficient to provide the basis for general clinical treatments, they do not describe the underlying neurobiology that gives rise to individual symptoms. The ability to precisely link these symptoms to the underlying neurobiology would not only facilitate the development of better treatments, it would also allow physicians to help patients with a better understanding of the complexities and management of their illnesses (Kas et al., Neuroscience & Biobehavioral reviews, submitted). To realize this ambition, a paradigm shift is needed to raise awareness and to build an understanding of how neuropsychiatric diagnoses can be based on quantitative biological parameters. In this

regard, the main limit in the construction of biologically valid diagnoses is the lack of objective biomarkers. Moreover, the uncertain relationship between diagnosis and underlying etiology has created difficulties for the development of appropriate disease models and targeted treatments. Currently, there has been a rethinking of these diagnostic boundaries in regard to their usefulness in treatment and classification of neuropsychiatric disorders (Kas et al., Neuroscience & Biobehavioral reviews, submitted). This is partly based on the notion that there is more pathogenetic overlap between psychiatric and neurodegenerative disorders than previously thought, and that they might better be described as domains of cross-disorder-related traits rather than be classified into separate categories (Insel & Cuthbert, 2015; Kas et al., 2011; Krishnan & Nestler, 2008). To reach this purpose, animal models can be really helpful to longitudinally study behavioural alterations resembling human symptoms in a translational way, and ultimately investigate underlying neurobiology in order to deeper understand the etiology.

1.4 Revisiting behavioural paradigms and rodent models for a translational approach

In this thesis, we focused on depressive-like symptoms that occur in several neuropsychiatric and neurodegenerative diseases, and, using different animal paradigms and models, we tried to disentangle the heterogeneous neurobiology behind these symptoms.

In particular, the most used test to assess depressive-like behaviour is the Forced Swimming Test (FST). The FST is a reliable test widely used to evaluate depressive-like state and screen antidepressants activity in rodents (Li, Jiang, Song, Quan, & Yu, 2017). This test is based on learned helplessness that results in depressive-like symptoms, such as immobility increase and swimming and struggling decrease.

Disrupted sociability is an important behavioural aspect that needs to be taken into account to fully delineate a translational picture of symptoms related to depression.

The currently available behavioural tests to assess sociability are the social interaction test and the three chamber or social preference test. In the social interaction test, interaction between two animals is evaluated, while, in the three chamber test, the animal can choose between one empty chamber and one chamber with a stimulus animal. Thus, in these tests only dyadic interactions can be analyzed. Hence, these behavioural tests are not able to investigate social dynamics in a translational way, due to the interactions with no more than two animals at the same time. For

this purpose, semi-natural habitats have been developed. In nature, rodents live in large groups with organized social structures and dominance hierarchies (So, Franks, Lim, & Curley, 2015). One of the most interesting systems for the behavioural analyses of social group dynamics is the Visible Burrow System (VBS), developed by the Blanchard group (D. C. Blanchard et al., 2012; D. C. Blanchard et al., 1995; R. J. Blanchard, Yudko, Dulloog, & Blanchard, 2001; Pobbe et al., 2010). The VBS is a semi-natural environment resembling rodent ecological appropriate environment. It consists of an open-arena that is connected to continuously dark tunnels with multiple nests in order to mimic the natural burrows. Research using the VBS has been primarily focused on aggression, dominance and hierarchies in rats (R. J. Blanchard, Dulloog, et al., 2001; R. J. Blanchard, Yudko, et al., 2001; Buwalda, Koolhaas, & de Boer, 2017). However, during the last decade, attention has shifted towards the use of mice, thus encouraging the study of transgenic and mutant mice lines, resembling humane neuropsychiatric phenotypes. Among these lines, an interesting mutant strain is the BTBR T+tf/J (BTBR) inbred strain. The BTBR mice show deficits in social interaction, impaired communication, and repetitive behaviours, thus resembling the autism-like phenotype in humans (McFarlane et al., 2008). The BTBR behavioural deficits have been investigated in the VBS and subsequently validated using the Three Chamber test, by Pobbe et al. (Pobbe et al., 2010). In their VBS colonies, composed of four males, BTBR mice showed an impairment in all social behavioural domains, such as approach, aggressive behaviour and allo-grooming (Pobbe et al., 2010). Therefore, the BTBR strain appears to be a useful model to study social behavioural dysfunctions in a translational perspective.

In this regard, our group implemented a modified version of the VBS, adding two additional chamber in the burrows in order to have more nests. Moreover, to reproduce behaviours that naturally occur in colonies, we used mixed-sex colonies, using 2 females and 6 males for each VBS experiment. In chapter 4, we used the VBS to identify and validate behavioural readouts to assess sociability and social withdrawal features in BTBR and C57BL/6J control strain. In particular, C57BL/6J mouse strain has normal sociability and has been used as control strain in numerous preclinical studies (Cai et al., 2017; Hsieh, Wen, Miyares, Lombroso, & Bordey, 2017).

Furthermore, transgenic Knock-Out (KO) mice models for candidate genes involved in social pathways are becoming a growing field of research. In this context, cadherin superfamily, originally characterized as calcium-dependent cell-adhesion molecules, is now known to be involved in many biological processes, including cell recognition, cell signaling during

embryogenesis and formation of neural circuits (Bruining et al., 2015; Morishita & Yagi, 2007). In particular, protocadherin family, the largest subgroup within the cadherin superfamily, are predominantly expressed in the nervous system. Interestingly, recent evidence suggested that *Protocadherin 9* (*Pcdh9*) might be involved in schizophrenia and ASD pathogenesis (Hirabayashi & Yagi, 2014). Moreover, a recent study reported that the gene encoding *Pcdh9* might be considered as a novel risk factor for Major Depressive Disorder (MDD) (Xiao, Zheng, et al., 2017). In this regard, in chapter 5 of this thesis, we investigated VBS colonies composed of 2 Homozygous (HOM) KO *Pcdh9*, 2 Heterozygous (HET) KO *Pcdh9* and 2 Wild Type (WT) *Pcdh9* males, together with 2 WT *Pcdh9* females, in order to evaluate sociability and social withdrawal features in relation to genotype differences.

1.5 Unravelling neurobiological alterations underlying depressive-like symptoms

Disentangle the etiology of depressive-like symptoms is a hard challenge. Indeed, available techniques to analyze the aberrant function of brain circuits is based on either *post-mortem* studies, which have numerous limitations, or neuroimaging techniques, which rely on detecting changes in neuronal activity by using indirect markers of activation (Krishnan). Although these approaches have provided important insights into candidate brain regions, simple increases or decreases in regional brain activity are probably insufficient to explain the complex array of symptoms related to depression (Krishnan & Nestler, 2008). Therefore, neuropsychiatric symptomatology raises from heterogeneous neurobiology (Cummings, 2015), as a result of pathophysiological and biochemical alterations within several brain regions (Schiavone, Tucci, et al., 2017). This hypothesis is supported by several levels of evidence, in which neuropsychiatric symptoms are associated with underlying neurotransmitter system imbalances, including NA, DA, 5-HT, glutamate and gamma-aminobutyric acid (GABA), but also HPA axis dysfunctions, neurotrophins impairments and, recently, soluble beta amyloid involvement (Panza et al., 2010; Sweet et al., 2004; Wegener et al., 2004).

The monoamine hypothesis of depression

Depression has been associated with impaired neurotransmission of serotonergic, noradrenergic and dopaminergic pathways. This concept is now over fifty years old and arose from the empirical observation that depressive symptoms were influenced by the pharmacological manipulation of the monoaminergic system (Lanni, Govoni, Lucchelli, & Boselli, 2009; Sanacora, 2010). For

instance, reserpine, an antihypertensive first introduced in 1954, was found to deplete presynaptic stores of serotonin and noradrenaline and induce depression in some individuals (Lopez-Munoz, Bhatara, Alamo, & Cuenca, 2004). Moreover, iproniazid and imipramine had potent antidepressant effects in humans and were later shown to enhance central serotonin and noradrenaline transmission (Krishnan & Nestler, 2008). Since the catecholamine hypothesis of depression was first described, most antidepressant drug development has targeted the enhancement of monoamine neurotransmissions. For decades tricyclic antidepressants, that inhibit the reuptake of norepinephrine and serotonin, were the principal treatment choice for physicians. Therefore, monoamine hypothesis has been accepted as the most common hypothesis of major depression for a long period because of its simplicity and understandability (Boku, Nakagawa, Toda, & Hishimoto, 2017). Indeed, several evidence links depression to deficiencies in the neurotransmission of the monoamines 5-HT, NA and DA (D'Aquila, Collu, Gessa, & Serra, 2000; Popik et al., 2006). In this context, it has been suggested that a triple re-uptake inhibitor, resulting in an additive effect of enhancing neurotransmission in all three monoamine systems, might lead to improved efficacy and quicker onset of the antidepressant response (Marks, Pae, & Patkar, 2008). Although receiving considerable support, the monoamine hypothesis is considered restricted by several researchers (Joyce, 2007), as it does not provide a comprehensive explanation for the mechanism of actions of antidepressants and fails to explain why less than 50% of patients achieve full remission despite the numerous drugs available (Trivedi et al., 2006). For this reason, identification of new effective and safe treatment for depression is still a significant task and drugs targeting monoamine neurotransmissions alone are not able to fully cure all the behavioural symptoms and the different aspects and subtypes of depression.

GABAergic and glutamatergic neurotransmissions in depressive-like symptoms affecting the social sphere

During last decades, a number of evidence suggested that altered function of the amino acid neurotransmitter systems, especially GABA and glutamate systems, might contribute significantly to the etiology of neuropsychiatric disorders (Sanacora, 2010).

In this regard, glutamate is the major mediator of excitatory synaptic transmission in the mammalian brain (Maletic et al., 2007). Abnormal function of the glutamergic system has been implicated in the pathophysiology of several neuropsychiatric disorders, such as Huntington's chorea, epilepsy, Alzheimer's disease, schizophrenia and anxiety disorders (Hashimoto, Malchow,

Falkai, & Schmitt, 2013; Siegel & Sanacora, 2012). Increasing evidence indicated that abnormal activity of the glutamatergic system observed in patients affected by mood disorders is likely to contribute to impairments in synaptic and neural plasticity found in these patients (Lanni et al., 2009). Moreover, preclinical studies demonstrated a negative correlation between glutamatergic tone and sociability, reporting an increase in social interactions following suppression of glutamate neurotransmission, while activation of prefrontal cortex led to reduced social interactions (Kendell, Krystal, & Sanacora, 2005), suggesting that attenuation of glutamatergic tone might ameliorate depressive-like symptoms affecting sociability, such as social withdrawal.

Conversely, GABA is the most widely distributed inhibitory neurotransmitter in the mammalian central nervous system (Celio, 1986). GABAergic tone is involved in the synaptic transmission of 5-HT, NA and DA, and has been shown to act as a modulator of several behavioural processes, such as sleep, appetite, aggression, sexual behaviour, pain, thermoregulation and mood. In this regard, reduced GABA concentrations have been observed in plasma and cerebrospinal fluid of depressed patients (Bhagwagar & Cowen, 2008). Accordingly, neuroimaging data has shown lowered levels of GABA in the occipital cortex of depressed subjects (Price, Lee, Garvey, & Gibson, 2010) and patients suffering from schizophrenia, depression, ASD and bipolar disorders appear to have lowered central and peripheral GABA levels when compared to healthy controls (Lewis, 2014; Romeo, Choucha, Fossati, & Rotge, 2017). In particular, this lowered functionality is visible during the prodromal stage of the diseases, concomitantly with behavioural dysfunctions, such as disrupted sociability (Minzenberg et al., 2010). In this view, recent studies showed that decreasing GABA neurotransmission in prefrontal cortex and amygdala led to decreased sociability (Paine, Swedlow, & Swetschinski, 2017). Thus, changes in GABA signaling might mediate sociability dysfunctions, such as social withdrawal, which is an important early symptom of several neuropsychiatric diseases.

In conclusion, drugs aim to potentiate GABAergic and attenuate glutamatergic neurotrasmissions might be helpful to treat depressive-like symptoms, particularly in relation to the social sphere.

Nerve Growth Factor in depressive-like symptoms

Nerve growth factor (NGF), a key neurotrophin for the development of the nervous system, was initially discovered by Cohen and Levi-Montalcini, who won the Nobel Prize for this amazing discovery (Cohen, Levi-Montalcini, & Hamburger, 1954). Since first being discovered in 1979, NGF has been studied in different areas, such as neurology, angiogenesis, immunology, urology, and

others (Y. W. Chen et al., 2015). In the searching for neurobiological substrate involved in depressive-like states, NGF also play a significant role. NGF is an important member of the neurotrophins groups and is produced mainly in the cortex, hippocampus and hypothalamus, but also in the peripheral nervous system and immune system (Martino et al., 2013; Xiong et al., 2011). Evidence from animal studies reported decreased levels of NGF in specific brain areas of different mouse models, such as anxiety-related models, stress-induced diseases, learned helplessness and threatening treatment. All those mouse models are believed to represent forms of depressive-like models (Y. W. Chen et al., 2015). Accordingly, clinical studies have detected reduced levels of NGF in patients with major depression when compared with healthy individual controls (Diniz et al., 2014; Xiong et al., 2011). In addition, treatment with certain antidepressants has increased NGF levels in both clinical and experimental studies (Hassanzadeh & Rahimpour, 2011; Wiener et al., 2015). Furthermore, a significant decrease in serum NGF has been observed in patients with mild cognitive impairment, suggesting that the availability of NGF might be reduced at the onset of several neurodegenerative process (Schaub, Anders, Golz, Gohringer, & Hellweg, 2002). Since the identification of peripheral biomarkers to help in the diagnosis or to monitor the progression of mental diseases is still a field open to future research, NGF might be further investigated as a putative biomarker related to neurodegenerative disorders.

In addition, NGF and 5-HT are close and reciprocally regulated signals, thus the changes in NGF levels, acting through modifications of the 5-HT system, might help to disentangle the neurobiological mechanisms that give rise to depressive-like symptoms (Colaianna et al., 2010; Garcia-Alloza et al., 2004; Tapia-Arancibia, Aliaga, Silhol, & Arancibia, 2008).

HPA axis parameters related to depressive-like states

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, HPA axis hyperactivation is a crucial response to chronic stress. HPA axis hyperactivation is featured by increased hypothalamic corticotropin-releasing factor (CRF) expression and consequently elevated plasmatic glucocorticoid concentrations (T. Chen, Li, & Chen, 2009; Wang et al., 2010; Zhang et al., 2017). In regard to depressive-like symptoms, it has long been hypothesized that cortisol secretion is an important neurobiological characteristic of depressive disorders (Lee & Rhee, 2017). Moreover, a number of studies supported the hypothesis of HPA axis involvement in depression, reporting an increase in hypothalamic CRF in depressed

patients (Raadsheer, Hoogendijk, Stam, Tilders, & Swaab, 1994; Raadsheer et al., 1995), or a decrease of CRF receptors in the frontal cortex (Nemeroff, Owens, Bissette, Andorn, & Stanley, 1988), or a decreased sensitivity to negative feedback (Halbreich, Asnis, Shindledecker, Zumoff, & Nathan, 1985; Pfohl, Sherman, Schlechte, & Winokur, 1985; Young et al., 2004).

However, HPA axis dysfunctions have been found only in a subset of depressed patients (Varghese & Brown, 2001), suggesting that not all the depressive disorders share the same pathogenic pathways. Interestingly, HPA axis hyperactivity is highly related to anxiety disorders (Herman & Tasker, 2016; Y. T. Lin et al., 2017). In this regard, recent studies indicated that optogenetic inhibition of parvalbumin CRF neurons reduces anxiety-like behaviour, while stimulation induces anxiety-like behaviour (Fuzesi, Daviu, Wamsteeker Cusulin, Bonin, & Bains, 2016; Herman & Tasker, 2016).

Hence, future medications, pointing towards the modulation of HPA axis parameters, should be considered for the treatment of depressive-like disorders comorbid with anxiety states.

Soluble Amyloid Beta (Aβ1-42) peptide in depressive-like states

During the last decade the soluble Aβ1-42 peptide has gained great attention in the study of depression insurgence, also considering that such neuropsychiatric disease is highly comorbid with Alzheimer's Disease (AD) and other neurodegenerative illnesses (Colaianna et al., 2010; Morgese, Schiavone, & Trabace, 2017; Pomara & Sidtis, 2007; Schiavone, Tucci, et al., 2017; Sun et al., 2008). More recently, depressive signs have been potentially linked, in part, to the presence of soluble Aβ in the brain. Aβ peptides are physiologically produced from the Aβ protein precursor through beta and gamma secretase cleavage (Zetterberg, Mattsson, Shaw, & Blennow, 2010). They possess different brain area-selective neuromodulatory actions (Morgese, Schiavone, et al., 2017; Morgese et al., 2014; Mura et al., 2010; Trabace et al., 2007). Although the relationship among soluble Aβ, brain neurochemistry and depression remains complex, several studies have demonstrated an increased risk for the development of AD in individuals with late-life depression, indicating a prodromal state of AD (Dal Forno et al., 2005; Steffens et al., 1997; Sun et al., 2008). Similarly, it has been reported that depressed individuals are nearly twice as likely to develop dementia, often in the form of AD, compared with non-depressed individuals (Jorm, 2001). Aβ might have an effect on mood not limited to AD patients, indeed depressive-like states might precede or accompany dementia (Starkstein, Mizrahi, & Power, 2008). In this regard, our group has previously shown that central Aβ 1-42 administration in male rats was able to evoke a

depressive-like phenotype (Colaianna et al., 2010), characterized by increased immobility frequency in the Forced Swimming test and reduced cortical 5-HT and neurotrophins, such as NGF and Brain-Derived Neurotrophic Factor (BDNF). Since behavioural and neurochemical alterations were observed at a time at which amyloid plaques were not visible in the rat brain (Trabace et al., 2007), we could hypothesize that cerebral injection of soluble Aβ induced long-lasting neuronal circuits disruption ultimately responsible of depressive-like symptomatology (Schiavone, Tucci, et al., 2017).

1.6 Thesis aims and outline

The knowledge of the pathophysiology of depressive-like symptoms has evolved substantially from Galen's speculations in antiquity about an excess of black bile ("melancholia") to current evidence that incorporate lifestyle factors, genetic, endocrine, neurochemical and metabolic mediators, and cellular, molecular and epigenetic alterations. In this regard, considering the polysyndromic nature of depression, a multifactorial approach to better explore the etiopathogenesis of different depressive-like symptoms is warranted (Krishnan & Nestler, 2008).

Hence, the overall aim of this thesis was to investigate neurobiological determinants related to depressive-like symptoms. More specifically, by using different animal models and behavioural paradigms resembling human depressive-like symptoms, we evaluated the underlying neurobiological pathways, including monoamine system impairments, alterations in amino acids neurotransmissions, neurotrophin changes and HPA axis dysfunctions.

In particular, in chapter 2 and 3, we assessed the effect of n-3 PUFA in adult female offspring fed from conception with a diet poor in n-3 PUFA, or rich in n-3 PUFA, or a control diet. From a behavioural point of view, we performed Forced Swimming test to assess depressive-like behaviour and Open Field test to evaluate locomotor activity and anxiety-like behaviour. Moreover, we analyzed monoamine contents, in particular NA, DA, 5-HT and 5-HT turnover, HPA axis parameters, in particular hypothalamic CRF and plasmatic corticosterone, cortical NGF levels, plasmatic soluble Aβ levels, and, the last but not the least, GABA and glutamate levels.

Furthermore, we evaluated the effects of n-3 PUFA supplementation on a model of Aβ-induced depressive-like phenotype in adult female rats, performing the behavioural tests and neurochemical analyses listed above.

Moreover, in chapter 4 and 5 of this thesis, we implemented a modified version of the Visible Burrow System to study group-housed social dynamics and ultimately identify and validate behavioural readouts to assess sociability and social withdrawal features in mutant BTBR strain, transgenic *Pcdh9* line and C57BL/6J control strain. In addition, we investigated the neurobiological alterations underlying social behaviours, particularly focusing on GABA and glutamate neurotransmission.

CHAPTER 2

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

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Manuscript ready for submission

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 $A\beta_{1-42}$ 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 Aβ injection is able to elicit depressive-like phenotype in male rats. In the present study, we reproduced for the first time the Aβ-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 Aβ administration. Our results confirmed the Aβ-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 Aβ-induced depressive-like profile in female rats. In conclusion, the Aβ-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.

2.1 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 (Gorman, 2006). In this regard, several studies reported that depression is more prevalent in women compared to men (Gorman, 2006; Kokras et al., 2015; Marcus et al., 2005). Although the 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 (Marrocco & McEwen, 2016).In particular, estrogens modulate several neural and behavioural functions, including mood, cognitive function, blood pressure regulation, motor coordination, pain, and opioid sensitivity (McEwen & Milner, 2017). 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 (Borrow & Cameron, 2014). These evidence suggest that the antidepressant therapy should be personalized, taking into account also gender differences (Sloan & Kornstein, 2003; Thiels, Linden, Grieger, & Leonard, 2005). In addition, a series of studies indicated that estrogens modulate the metabolic production of different endogenous and exogenous molecules (M. Barton et al., 2017; Laredo, Villalon Landeros, & Trainor, 2014; Migliaccio, Davis, Gibson, Gray, & Korach, 1992). 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) (Burdge & Wootton, 2002; Giltay, Gooren, Toorians, Katan, & Zock, 2004). DHA is a key n-3 polyunsaturated fatty acid (PUFA) involved in the Central Nervous System (CNS) development (Colangelo et al., 2017) and, thus, fundamental during pregnancy and early stage of childhood (Echeverria, Valenzuela, Catalina Hernandez-Rodas, & Valenzuela, 2017). 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 (Morgese, Tucci, et al., 2017). DHA, AA and their mediators modulate several processes, such as signal pathways, membrane fluidity, neurotransmission, neuroinflammation and cell survival (Echeverria et al., 2017). During embryonic life and lactation, PUFAs intake exclusively depends on maternal diet, as the metabolic conversion of essential precursors cannot be accomplished (Lafourcade et al., 2011). Indeed, in utero exposure to unbalanced diet can be an important risk factor for mental disorders in later adulthood. 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 (Simopoulos, 2011). 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 PUFAs deficiency in mood disorders, such as Major Depressive Disorder (MDD) (Grosso et al., 2016; McNamara & Welge, 2016). Accordingly, different epidemiological studies reported an inverse correlation between n-3 PUFA intake and depressive symptoms among United States women (Beydoun et al., 2013; Beydoun et al., 2015). In this regard, 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 (Morgese, Tucci, et al., 2017) in male rats. The $A\beta_{1-42}$ peptide, produced through proteolytic cleavage of the amyloid precursor protein (APP), has been demonstrated to have powerful neurotoxic effects (Pomara & Sidtis, 2007). 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 (Schiavone, Tucci, et al., 2017; Sun et al., 2008). In our previous studies, we injected soluble $A\beta_{1-42}$ in the ventricular area of male rats, provoking a depressive-like phenotype (Colaianna et al., 2010), accompanied by reduced cortical 5-HT and neurotrophins, such as Nerve Grow Factor (NGF) and Brain-Derived Neurotrophic Factor (BDNF).

Although the majority of animal studies on depression use males in order to avoid the variability that hormonal cycle could induce (Altemus, 2006), the US National Institute of Health is strongly encouraging preclinical research on females (Kokras et al., 2015). For this reason, considering also the higher incidence of depressive disorders in women, the development of preclinical models of depressive-like profile in females is becoming necessary (D'Souza & Sadananda, 2017).

In the present study, we reproduced for the first time the Aβ-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 Aβ central administration.

2.2 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, Lieben, & Blokland, 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.

Aβ administration

The $A\beta_{1-42}$ peptide was purchased from Tocris (Bristol, UK) and was dissolved in sterile double-distilled water (vehicle) at a concentration of 4 µM as previously describe (Colaianna et al., 2010). All solutions were freshly prepared. 7-weeks-old rats were anesthetized with 3.6 ml/kg Equithesin intraperitoneally (i.p.; composition: 1.2 g sodium pentobarbital; 5.3 g chloral hydrate; 2.7 g MgSO4; 49.5 ml propylene glycol; 12.5 ml ethanol and 58 ml distilled water) and secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The skin was shaved, disinfected and cut with a sterile scalpel to expose the skull and a hole was drilled to insert the infusion needle (30-gauge stainless steel tubing; Cooper's Needles, Birmingham, UK). Coordinates for icv infusions were based on the atlas of Paxinos and Watson (1998): AP = - 0.5, ML = + 1.2 and DV = - 3.2 from bregma, with the incisor bar set at -3.3 mm. Soluble Aβ (5 µl) was delivered through a 25 µl Hamilton microsyringe at 2 µl/min infusion rate over a period of 2.5 min, with an additional 5 min allowed to elapse prior to removal of the infusion needle. Control rats were infused with vehicle only, because reverse $A\beta_{42-1}$, used in preliminary experiments, had no effect on the measured neurochemical parameters and was indistinguishable from vehicle alone (unpublished observations). The injection placement of needle track was verified at the time of dissection. All experimental procedures were performed 7 days after icv administration (SHAM or Aβ-treated groups).

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 (Porsolt, Bertin, & Jalfre, 1977). 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±1°C at 30 cm of height (Cryan, Valentino, & Lucki, 2005).

During the preconditioning period, animals were videotaped for 15 min. Then, rats were removed and dried before to be returned to their home cages. Twenty-four h later, each rat was positioned in the water-filled cylinder for 5 min. This session was video-recorded and subsequently scored by an observer blind to the treatment groups. During the test sessions, the frequency that rats spent performing the following behaviors were measured: struggling (time spent in tentative of escaping), swimming (time spent moving around the cylinder) and immobility (time spent

remaining afloat making only the necessary movements to keep its head above the water). Data were expressed as frequency on 5 sec counts.

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

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 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, Thermo Scientific Dionex, San Donato Milanese, Italy).

ELISA quantifications

PFC samples were analyzed for NGF quantifications by using ELISA kits provided by Cloud-Clone Corporation (Houston, Texas, USA). 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 soluble $A\beta_{1\text{-}42}$ 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. Behavioural and neurobiological data were analyzed by using one or two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* analyses, as required. P value was set at 0.05.

2.3 Results

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 and n-3 PUFA enriched diet on depressive-like behaviour, we performed the forced swimming test (FST). Our results show that n-3 PUFA deficient diet significantly increased the immobility frequency compared to control diet (Figure 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 (Figure 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 (Figure 1C, One-way ANOVA followed by Bonferroni's multiple comparison test, $F=4.929$, $P<0.05$ n-3 deficient versus CTRL).

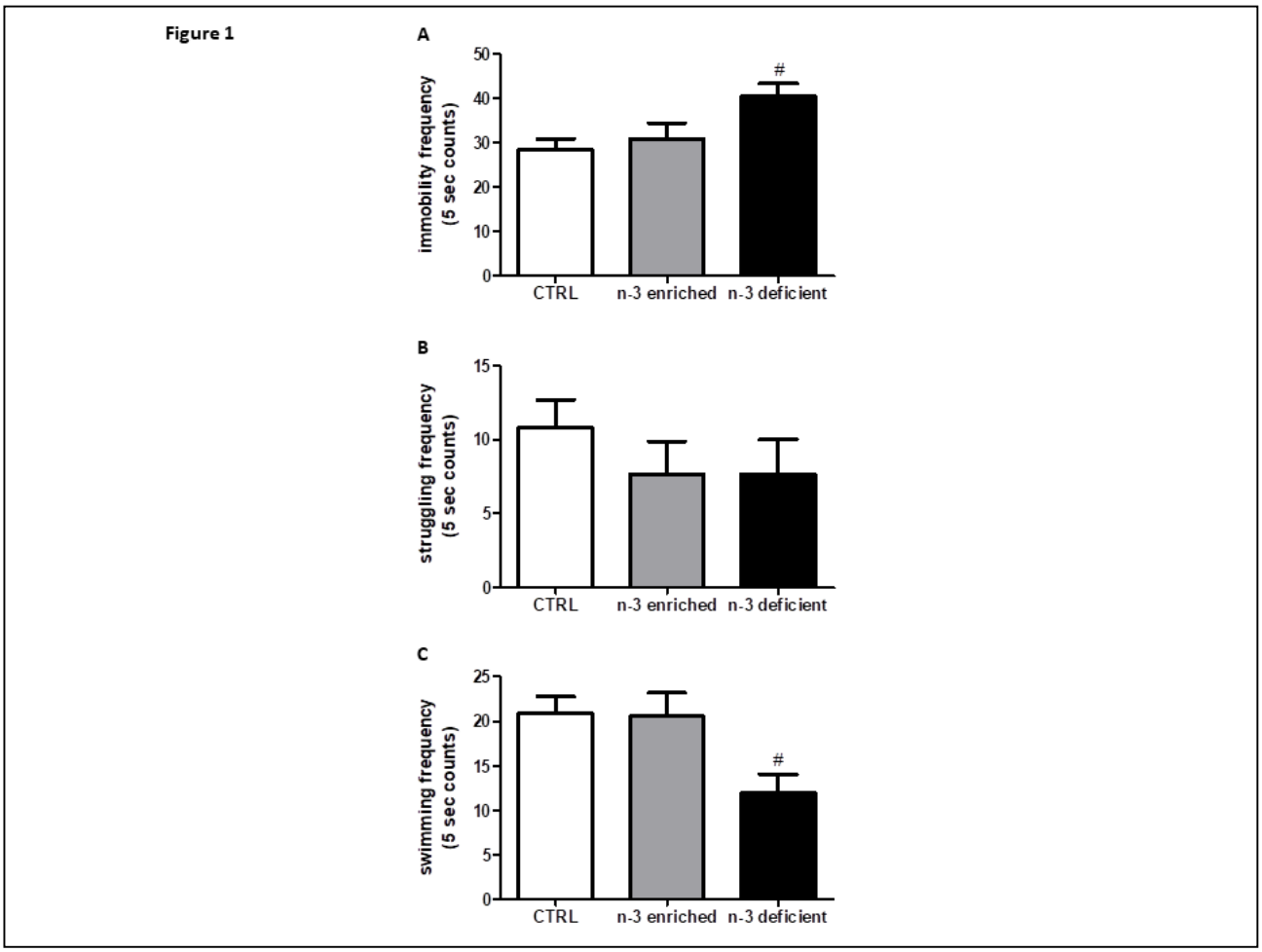

Figure 1 Effects of control, n-3 PUFA enriched and n-3 PUFA deficient diets on FST. Frequency measure of immobility (**A**), struggling (**B**), and swimming (**C**) behaviours in female rats fed from conception until 5 weeks post-weaning with control diet (*white bar*), n-3 PUFA enriched diet (*grey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

Effects of n-3 PUFA deficient diet on plasmatic Aβ levels

We quantified plasmatic soluble $A\beta_{1-42}$ peptide in offspring of rats fed with n-3 PUFA enriched and 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 Aβ levels compared to controls (Figure 2, one-way ANOVA followed by Bonferroni's multiple comparison test, $F=9.164$, $P<0.01$ n-3 deficient versus CTRL).

Figure 2

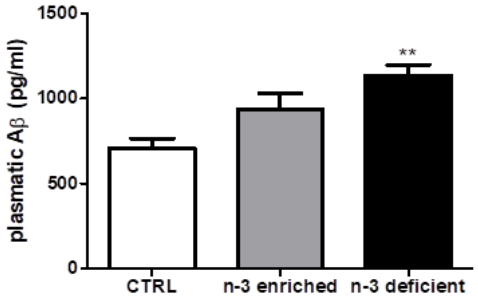

Figure 2 Effects of control diet (*white bar*), n-3 PUFA enriched diet (*grey bar*), and n-3 PUFA deficient diet (*black bar*).on plasmatic soluble Aβ levels. Data are expressed as mean ± SEM (n=6-7 per group). One-way ANOVA followed by Bonferroni's multiple comparison test $^{**}P < 0.01$ vs. CTRL

Effects of n-3 PUFA enriched diet on Aβ-induced depressive-like behaviour using FST

Our group has previously demonstrated that Aβ soluble peptide is able to evoke a depressive-like state (Colaianna et al.), thus we tested whether lifelong exposure to n-3 PUFA enriched diet would prevent such Aβ-induced alterations in female offspring rats. As shown in Figure 3A and 3C, n-3 PUFA enriched diet prevented the depressive effect of Aβ. Indeed, immobility frequency was significantly increased and swimming frequency was significantly reduced in Aβ treated rats compared to SHAM operated only in control animals (Figure 3A, Two-way ANOVA followed by Bonferroni's multiple comparison test; $F_{(1,32)}$=6.258 P<0.01, Aβ versus SHAM rats; Figure 3C, Two-way ANOVA followed by Bonferroni's multiple comparison test; $F_{(1,32)}$=13.57, P<0.01, Aβ versus SHAM rats), while no differences were evidenced in struggling frequency among groups (Figure 3B, Two-way ANOVA followed by Bonferroni's multiple comparison test; n. s.).

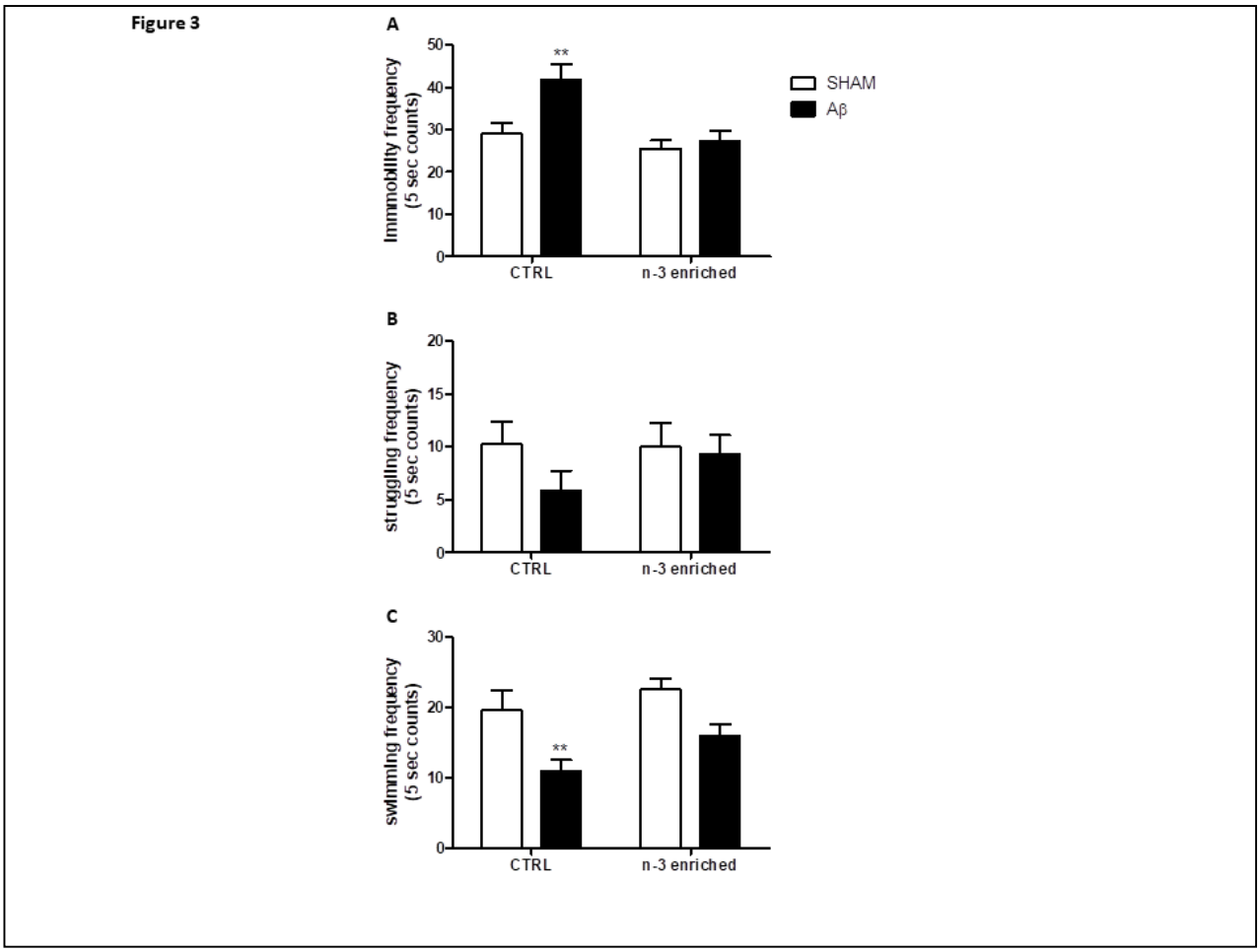

Figure 3 Effects of control and n-3 PUFA enriched diet on Aβ-induced depressive-like behaviour. Frequency measure of immobility (**A**), struggling (**B**), and swimming (**C**) behaviours in female rats SHAM-operated (*white bar*) and Aβ-operated (*black bar*). Data are expressed as mean ± SEM (n=9-12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

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

In order to better investigate behavioural results, we performed also neurochemical analyses. In particular, we quantified serotonin (5-HT) content and 5-HT turnover (5-HIAA/5-HT ratio) in PFC. We found that cortical 5-HT concentrations were significantly lower in animals pre- and post-natal fed with n-3 PUFA deficient diet compared to controls (Figure 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 (Figure 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 (Figure 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 (Figure 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 (Figure 4D, two-way ANOVA followed by Bonferroni's multiple comparison test, n.s.).

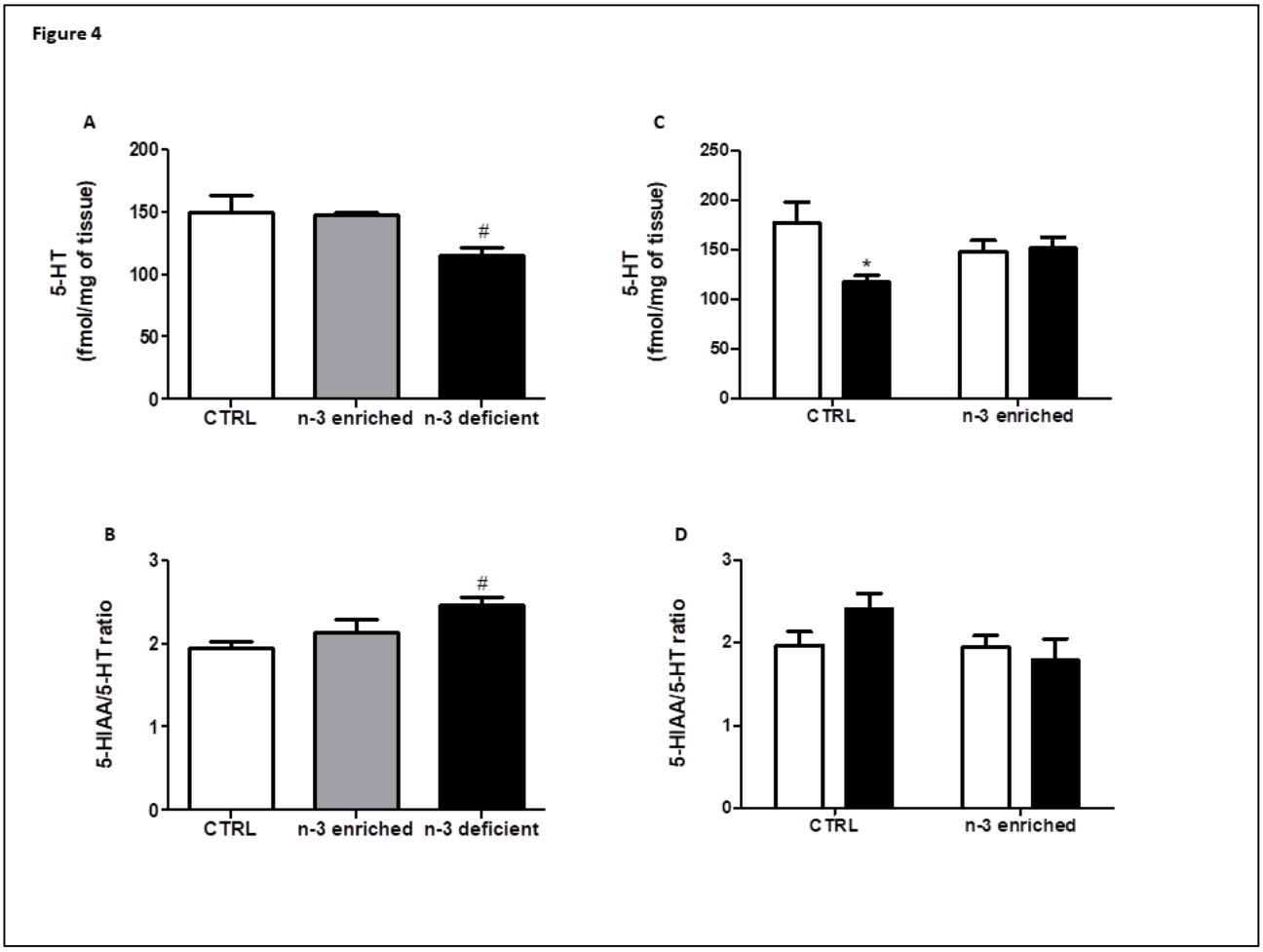

Figure 4 Effects of control (*white bar*), n-3 PUFA enriched (grey *bar*) and n-3 PUFA deficient (*dark bar*) diets on cortical 5-HT levels (**A**) and 5-HIAA/5-HT ratio (**B**) in naïve animal. Data are expressed as mean ± SEM. One-way ANOVA followed by Bonferroni's multiple comparison test, #P<0.05 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 Aβ-operated (*dark bar*) females. Data are expressed as mean ± SEM (n=5-7 per group). Two-way ANOVA followed by Bonferroni's multiple comparison test, *P<0.05 vs. SHAM-operated.

Effects of n-3 PUFA deficient and n-3 PUFA enriched diets on dopamine levels in PFC

We quantified cortical dopamine in female offspring fed with n-3 PUFA enriched; n-3 PUFA deficient or control diets and no significant differences were found (Figure 5A, One-way ANOVA followed by Bonferroni's multiple comparison test, n.s.). We also analyzed dopamine 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 dopamine content of Aβ-treated animals compared to SHAM operated only in n-3 PUFA fed animals, suggesting a specific interaction with dopaminergic system only in presence of Aβ (Figure 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).

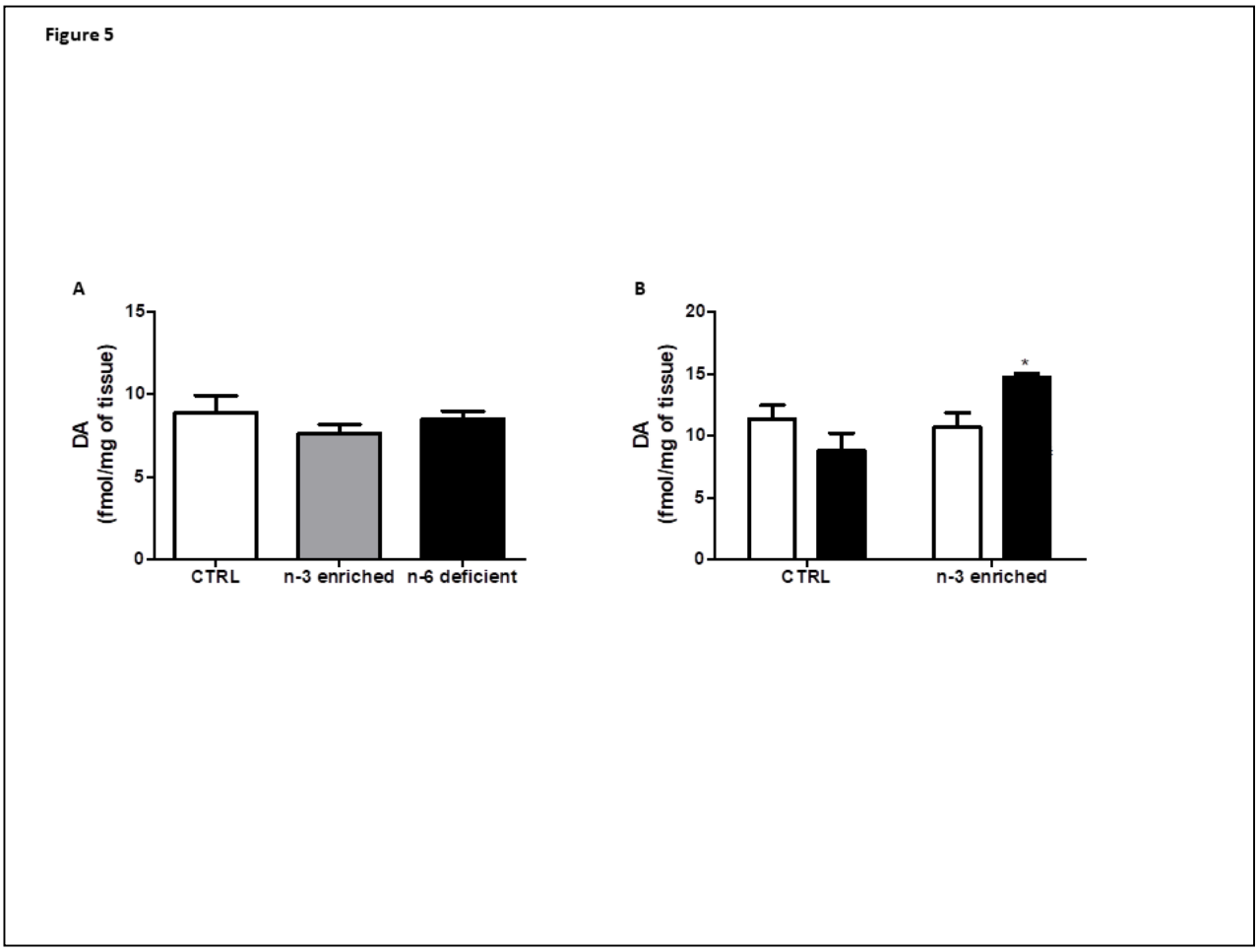

Figure 5 Effects of control (*white bar*), n-3 PUFA enriched (grey *bar*) and n-3 PUFA deficient (*dark bar*) diets on cortical DA levels in naïve (**A**), SHAM- (*white bar*) and Aβ-operated (*dark bar*) females (**B**). Data are expressed as mean ± SEM (n=6 per group). One- and Two-way ANOVA followed by Bonferroni's multiple comparison test *P<0.05 vs. SHAM-operated.

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

To endorse our results on behavioral analyses, we measured NGF protein levels in PFC of our experimental groups. 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 (Figure 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 after Aβ administration in n-3 PUFA fed animals compared to controls, still confirming a protective role of this diet towards Aβ-induced impairments (Figure 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).

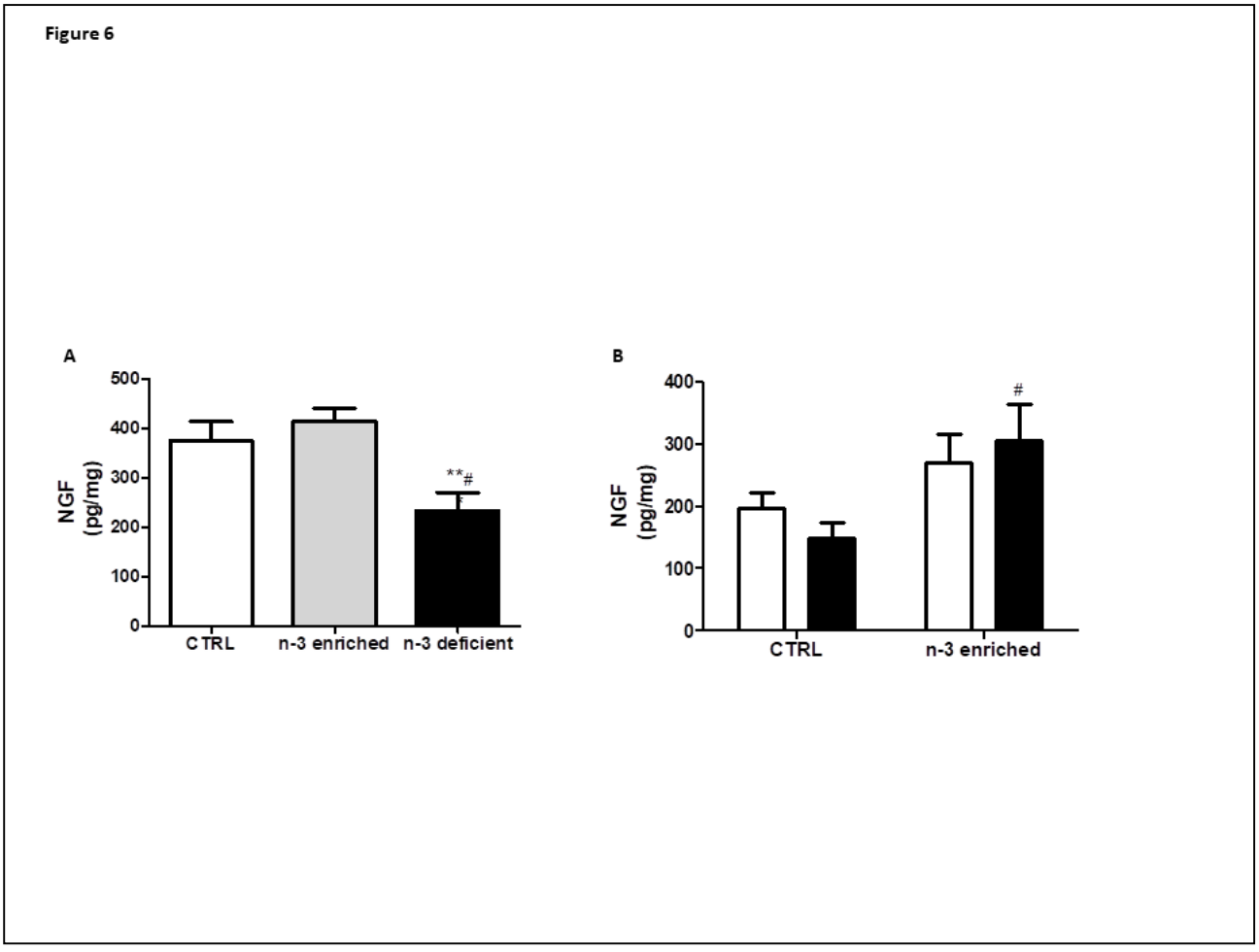

Figure 6 Effects of control (*white bar*), n-3 PUFA enriched (grey *bar*) and n-3 PUFA deficient (*dark bar*) diets on cortical NGF levels in naïve (**A**), SHAM- (*white bar*) and Aβ-operated (*dark bar*) females (**B**). Data are expressed as mean ± SEM

(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. Aβ-operated CTRL diet.

2.4 Discussion

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

From a behavioural point of view, 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 (Li et al., 2017). This test is based on learned helplessness that results in depressive-like symptoms, such as immobility increase and swimming and struggling decrease. These results are in line with our previous study, in which we reported a significant increase in immobility and decrease in swimming and struggling frequency in male rats fed with a diet poor in n-3 PUFA, confirming the positive effect of n-3 PUFA supplementation (Morgese, Tucci, et al., 2017). In order to rule out whether the increased immobility frequency and the decreased swimming frequency could be due to locomotor impairments, we performed OF test, whose results indicated no differences in vertical or horizontal activity in all experimental groups. Thus, the impairment retrieved in the FST could not be attributed to alteration in locomotion, but it might be related instead to neurobiological alterations induced by low n-3 PUFA consumption.

In addition, we quantified plasmatic concentrations of Aβ in female animals receiving a diet either rich or poor in n-3 PUFA. Our results showed that plasmatic Aβ 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 Aβ levels compared to controls, while high n-3 PUFA diet significantly decreased such levels (Morgese et al., 2016). Recently, the Aβ peptide, particularly in its soluble forms, is gaining more and more attention in the study of depressive disorders (Colaianna et al., 2010; Pomara & Sidtis, 2007; Schiavone, Tucci, et al., 2017; Sun et al., 2008). In this regard, our group has previously demonstrated that central Aβ 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 (Colaianna et al., 2010). Regarding Aβ and n-3 PUFA interaction, recent studies suggest a crucial role played

by n-3 PUFA in the production/clearance of the Aβ peptide (Hopperton, Trepanier, Giuliano, & Bazinet, 2016; Lim et al., 2005). Indeed, it has been shown that n-3 PUFA, by increasing membrane fluidity, promote the Aβ interaction with membrane lipid bilayers, influencing the peptide aggregation process (Emendato et al., 2016). Thus, we can speculate that in our model the decrease availability of n-3 PUFA in plasmalemma, secondary to deficiency in n-3-PUFA consumption, may lead to less interaction of Aβ species to the membrane, ultimately resulting in higher soluble Aβ levels.

To better understand the link between Aβ and PUFA and to investigate possible gender differences, we administered the soluble Aβ peptide in female offspring fed with n-3 PUFA enriched or control diet. 7 days after Aβ icv, we performed the FST and we found that in control animals immobility frequency was significantly increased and swimming frequency was significantly decreased in Aβ-treated females compared to SHAM operated animals, confirming the efficacy of the Aβ-depressive-like model also in females. Conversely, in n-3 PUFA fed animals, there were no differences between Aβ injected and SHAM operated animals, indicating a protective role of n-3 PUFA diet on the depressive-like phenotype induced by soluble Aβ 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 was significantly decreased in n-3 PUFA deficient females and 5-HIAA/5-HT ratio was significantly increased, confirming the deleterious effects of a diet poor in n-3 PUFA. Furthermore, cortical 5-HT was significantly reduced in Aβ-treated animals compared to SHAM operated animals, both fed with control diet, consolidating the Aβ-induced depressive-like profile. As widely known, 5-HT 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 (Salaminios et al., 2017). Moreover, in an interesting clinical study, Barton and Colleagues found an elevated brain 5-HT turnover in unmedicated patients with depression (D. A. Barton et al., 2008) and several studies also reported a decrease in brain 5-HT turnover after classical or natural antidepressant treatments (Ahmed & Azmat, 2017; S. H. Lin et al., 2015). Interestingly, we found that n-3 PUFA enriched diet was able to restore 5-HT levels in Aβ 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 (Patrick & Ames, 2015). In particular, DHA increases cell membrane fluidity in postsynaptic

neurons, thus, in low DHA conditions, the membrane becomes less fluid and the binding of serotonin to its receptor decreases significantly, due to the lower accessibility of serotonin receptors (Jones, Arai, & Rapoport, 1997; Paila, Ganguly, & Chattopadhyay, 2010). This effect is not limited to the serotonin receptors, but also affects the dopamine receptors and other neurotransmitter receptors (Paila et al., 2010). Furthermore, n-3 PUFA might influence serotonin neurotransmission acting through the inflammatory pathways. Interestingly, 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 (McNamara, Able, Rider, Tso, & Jandacek, 2010).

As regard other monoaminergic neurotransmissions, several evidence pointed out to an important role of dopaminergic system in the pathogenesis of depression (Finan & Smith, 2013; Hori & Kunugi, 2013; Tye et al., 2013). 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 Aβ injection, DA was significantly increased in animals exposed to n-3 PUFA enriched diet compared to SHAM operated animals.

Recent preclinical studies have indicated the involvement of dopaminergic receptors, either D1, D2 or D3, in the antidepressant effects (Pytka et al., 2016). In addition, it was shown that lesion of dopamine neurons in ventral tegmental area lead to dopamine depletion in the nucleus accumbens, producing depressive-like phenotype in the animals (Furlanetti, Coenen, & Dobrossy, 2016).

In this regard, 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 (Sublette et al., 2014). Moreover, Zimmer and Colleagues demonstrated that in n-3 PUFA deficient rats, dopamine vesicles are specifically decreased in frontal cortex, inducing modification in dopamine metabolism (Zimmer et al., 2002). 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 Aβ 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 (Iulita et al., 2017; Triaca & Calissano, 2016). 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 (Song, Zhang, & Manku, 2009). Interestingly, it has been reported that dopamine agonists, such as bromocriptine, bergolide, cabergolide, may promote the synthesis and secretion of NGF (Ohta et al., 2010; Ohta et al., 2003). These data are in line with our results, in which Aβ treated females exposed to n-3 PUFA enriched diet showed an increase in cortical dopamine and also in cortical NGF. These results suggest a beneficial effect of n-3 PUFA supplementation in Aβ- induced depressive-like symptoms, acting also through dopamine 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 (Gertsik, Poland, Bresee, & Rapaport, 2012; Jazayeri et al., 2008; J. J. Liu et al., 2013).

Although our group is a pioneer on the depressive-like model induced by soluble Aβ icv injection (Colaianna et al., 2010; Morgese et al., 2015; Morgese, Schiavone, et al., 2017; Morgese et al., 2014; Morgese, Tucci, et al., 2017; Schiavone, Tucci, et al., 2017; Trabace, 2014; Trabace et al., 2007; Tucci et al., 2014), this is the first study using female rats. In particular, female trends confirm male results regarding Aβ-induced depressive-like profile. Literature about estrogens involvement in the pathogenesis of depression is controversial. In this regard, 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 (Fink, Sumner, McQueen, Wilson, & Rosie, 1998; Kudielka & Kirschbaum, 2005; Soares, Castro, Reis-Henriques, Monteiro, & Santos, 2012). Nevertheless different studies reported the influence of social factors, such as marital and employment status, as a trigger in the development of depression in women (Bulloch, Williams, Lavorato, & Patten, 2009; Diaz, Guendelman, &

Kuppermann, 2014). From our point of view, females did not show differences compared to males, revealing the same Aβ-induced phenotype.

Furthermore, n-3 PUFA supplementation showed differences in naïve females compared to Aβ treated animals, indicating a positive effect only in presence of Aβ-induced dysfunctions. These results endorse the hypothesis of a possible therapeutic use of n-3 PUFA supplementation, acting in synergy with antidepressants or even alone. In support to our findings, several studies supported the benefits of n-3 PUFA supplementation in the treatment of *post partum* depressive symptoms (Chong et al., 2015; Sparling, Henschke, Nesbitt, & Gabrysch, 2017), while a recent study reported no efficacy of daily n-3 PUFA supplementation in the prevention of maternal depressive symptoms (Vaz, Farias, Adegboye, Nardi, & Kac, 2017).

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 (Beydoun et al., 2013; Beydoun et al., 2015; Grosso et al., 2016).

Although 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 their 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 (Grosso, Galvano, et al., 2014). Furthermore, 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 (Wiener et al., 2015).

To the best of our knowledge, this is the first study that evaluates the effect of Aβ icv injection in female rats, reporting depressive-like behaviour and neurochemical impairments. In conclusion, the Aβ-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.

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 neurotrasmissions (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 (Zelena, 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 video-tracking 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

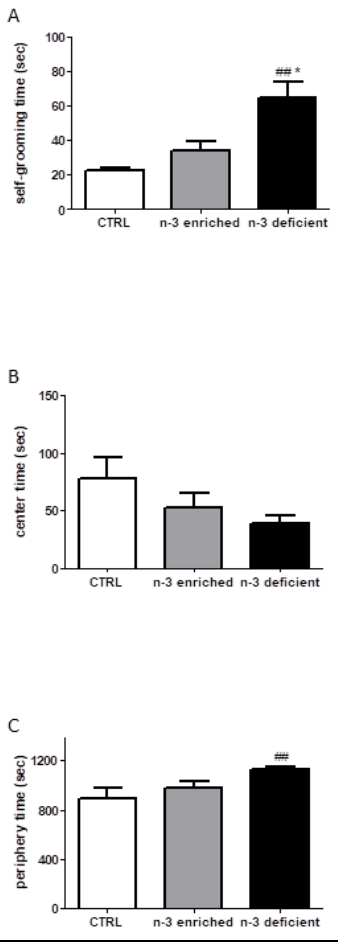

Figure 1 Effects of control, n-3 PUFA enriched and n-3 PUFA deficient diets on anxiety-like behaviours in the OF test. Time spent preforming self-grooming (**A**), time spent at the center of the arena (**B**), and time spent at the periphery of the arena (**C**) in female rats fed from conception until 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

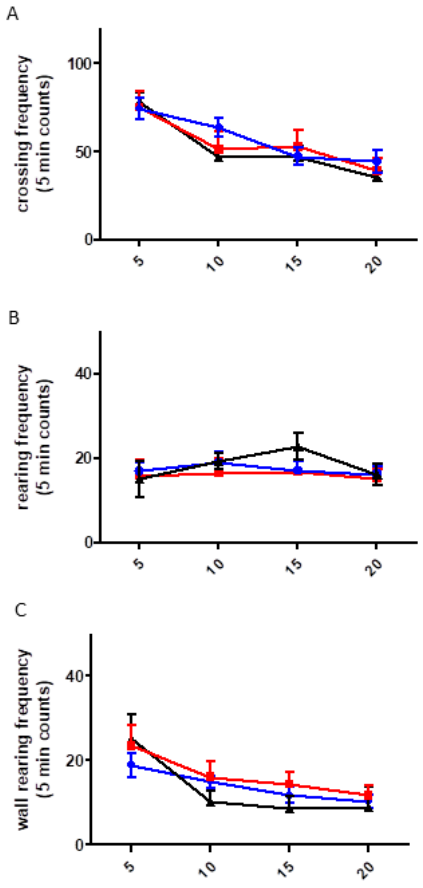

Figure 2 Effects of control, n-3 PUFA enriched and n-3 PUFA deficient diets on locomotor activity in the OF test. Frequency measure of crossing (**A**), rearing (**B**), and wall rearing (**C**) behaviours in female rats fed from conception until 5 weeks post-weaning with control diet *(blue line)*,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

(*black line*). Data are expressed as mean ± SEM. Two-way ANOVA RM followed by Bonferroni's multiple comparison test, n.s.

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

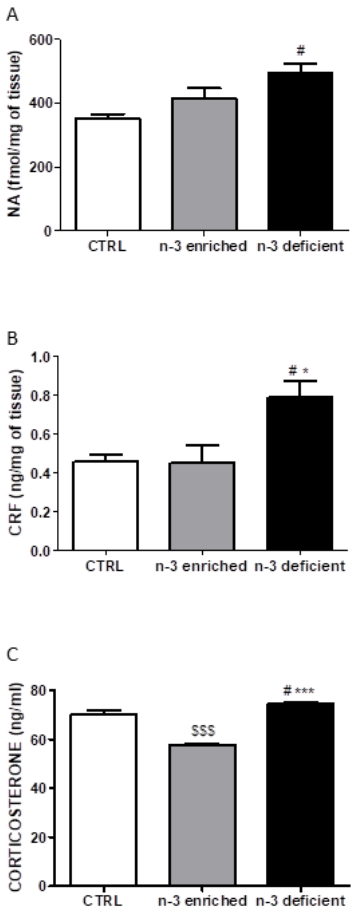

Figure 3 Effects of control, n-3 PUFA enriched and n-3 PUFA deficient diets on HPA axis parameters. Levels of hypothalamic NA (**A**), hypothalamic CRF (**B**), and plasmatic corticosterone (**C**) in female rats fed from conception until 5 weeks post-weaning with control diet (*white bar*), n-3 PUFA 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

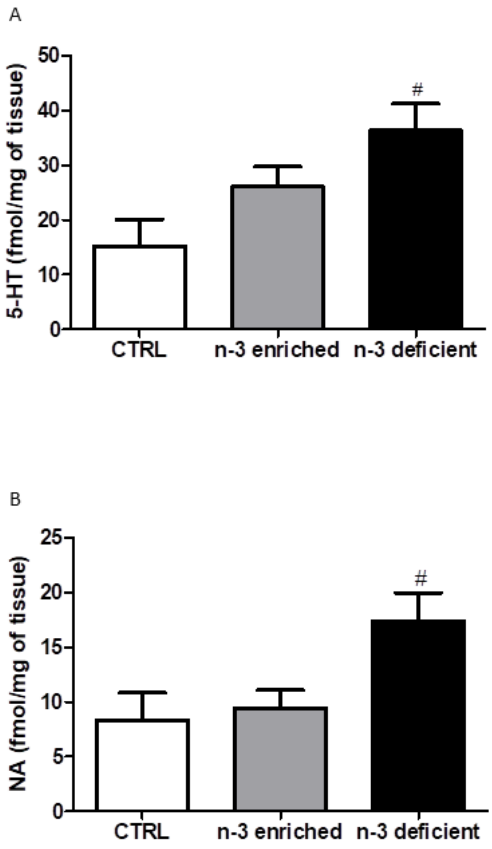

Figure 4 Effects of control (*white bar*), n-3 PUFA enriched (grey *bar*) and n-3 PUFA deficient (*dark bar*) diets on amygdaloidal 5-HT (**A**) and NA (**B**) levels. Data are expressed as mean ± SEM (n=4-6 per group). One-way ANOVA followed by Bonferroni's multiple comparison test, #P<0.05 vs. CTRL.

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) 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

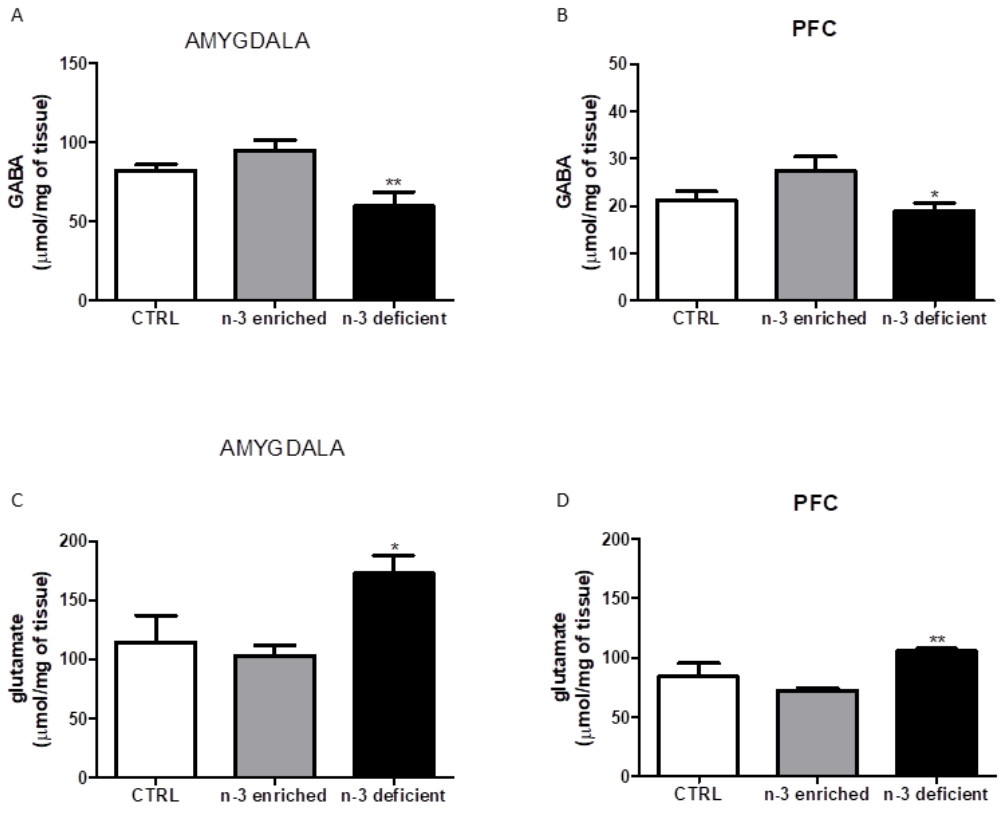

Figure 5 Effects of control (*white bar*), n-3 PUFA enriched (grey *bar*) and n-3 PUFA deficient (*dark bar*) diets on amygdaloidal GABA (**A**) and cortical GABA (**B**) levels, and on amygdaloidal glutamate (**C**) and cortical glutamate (**D**) levels. Data are expressed as mean ± SEM (n=5 per group). 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, Mhillaj, 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 adrenocorticotropin 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.

CHAPTER 4

The Visible Burrow System: a behavioural paradigm to assess sociability and social withdrawal in BTBR and C57BL/6J mice strains

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Abstract

Disrupted sociability and consequent social withdrawal are (early) symptoms of a wide variety of neuropsychiatric diseases, such as schizophrenia, autism spectrum disorders, depressive disorders and Alzheimer's disease. The paucity of objective measures to translationally assess social withdrawal characteristics has been an important limitation to study this behavioural alteration, both in human and rodents. The aim of the present study was to investigate sociability and social withdrawal in rodents using a behavioural paradigm, the Visible Burrow System (VBS). The VBS mimics a natural environment, with male and female rodents housed together in an enclosure where an open arena is connected to a continuously dark burrow system that includes 4 boxes connected by corridors. In this study, mixed-sex colonies of C57BL/6J and of BTBR mice have been investigated (n=8 mice per colony). Results showed marked differences between the two strains, in terms of sociability as well as social withdrawal behaviours. In particular, BTBR mice performed less social behaviours and have a preference for non-social behaviours compared to C57BL/6J mice. The lack of sociability in BTBR was further accompanied by reduced GABA and increased glutamate concentrations in PFC and amygdala. In conclusion, our study validated the use of the VBS as a behavioural paradigm to investigate sociability and social withdrawal features and their underlying neurobiology, to further develop new therapeutic treatments for behavioural dysfunctions that may be relevant across neuropsychiatric diseases.

4.1 Introduction

Several neuropsychiatric diseases share the same behavioural dysfunctions, such as anxiety, delusion, apathy and impaired social functioning (Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, DSM-5). Among these behavioural alterations, social withdrawal, defined as "withdrawal from social contact that derives from indifference or lack of desire to have social contact", appears to be an early manifestation of a wide variety of neuropsychiatric diseases, such as schizophrenia, major depressive disorders (MDD), Alzheimer's disease and autism spectrum disorders (ASD) (Green, 2016; Wilson & Koenig, 2014). Indeed, a deep analysis of social withdrawal behaviours and their underlying neurobiology has become necessary in order to find new therapeutic strategies to treat this important neuropsychiatric symptom. In this regard, mice can provide a good opportunity to study social behaviours, as they are highly social species and show distinct and robust social behaviours (Ricceri, Moles, & Crawley, 2007). However, to investigate sociability and social withdrawal dynamics in a translational way, novel approaches that take into account natural behavioural variation into account. In this context, the Visible Burrow System (VBS) has been developed to reproduce group-housed rodent behaviours in semi-natural settings (Arakawa, Blanchard, & Blanchard, 2007; Herman & Tamashiro, 2017; Melhorn, Elfers, Scott, & Sakai, 2017). The VBS mimics a natural environment, where male and female animals are housed together in an enclosure where an open arena, with an imposed diurnal photoperiod, is connected to a continuously dark burrow system, consisting of tunnels and small chambers as the underground burrows and nests of colonies into the wild (D. C. Blanchard et al., 2012; D. C. Blanchard et al., 1995; Buwalda et al., 2017; Pobbe et al., 2010). Although it has been used mainly to study dominance and hierarchy, this social housing model appears to be a useful tool to analyze social group behaviour dynamics that naturally occur in a mixed-sex colony (McEwen, McKittrick, Tamashiro, & Sakai, 2015). To validate the suitability of the VBS to study sociability and social withdrawal behaviours, mouse models with behavioural phenotypes affecting the social sphere need to be used. In particular, BTBR T+tf/J (BTBR) inbred mouse strain shows robust behavioural phenotypes with analogies to the core symptoms of ASD, such as deficits in social interaction, impaired communication, and repetitive behaviours (McFarlane et al., 2008; Molenhuis, de Visser, Bruining, & Kas, 2014; Pobbe et al., 2010). The BTBR strain shows lower social approach and abnormalities in reciprocal social interaction in comparison to the control C57BL/6J mice strain

when tested in standard behavioural assays, such as social interaction and social preference tests (Moy et al., 2007; Yang et al., 2007).

Furthermore, recent studies are focusing on the neural circuits underlying social behavioural alterations. A number of evidence suggest a key role played by corticolimbic circuitry, including the medial prefrontal cortex (PFC) and basolateral amygdala. Indeed, it has been reported that activation of PFC and amygdala leads to a reduced social preference in the three chamber preference test and reduced social interaction in the social interaction test (Sanders & Shekhar, 1995), while NMDA and AMPA receptors blockade, with consequent glutamatergic neurotransmission suppression, ultimately leads to an increase in social interaction in the social interaction test (Sajdyk & Shekhar, 1997). Accordingly, in an elegant study, Paine et colleagues showed that a decrease in GABA functioning in either medial PFC or basolateral amygdala, due to a bilateral injection of a GABA A antagonist, decreased social preference in the three chamber preference test and social interaction in the social interaction test (Paine et al., 2017).

Interestingly, the GABAergic system has also been investigated in clinical research focused on schizophrenia, depression and bipolar disorders (Lewis, 2014; Romeo et al., 2017). Patients suffering from these diseases appear to have lowered central and peripheral GABA levels when compared to healthy controls (Lewis, 2014; Romeo et al., 2017). Moreover, this lowered functionality is visible during the prodromal stage of the diseases (Minzenberg et al., 2010), and might ultimately represent a biomarker of symptomatic states in these patients (Romeo et al., 2017).

In the present study, the VBS has been validated as a behavioural paradigm to study sociability and social withdrawal behaviours in mice colonies. Hence, we studied BTBR and C57BL/6J mixed-sex colonies housed in VBS continuously for 5 days, evaluating all the kind of social and non-social behaviours. To further investigate the mechanisms underlying sociability and social withdrawal, we quantified GABA and glutamate in PFC and amygdala of each mouse in our colonies.

4.2 Materials and Methods

Animals

Adult C57BL/6J and BTBR male and female mice aged 14-22 weeks were used in this study. C57BL/6J mice were offspring of breeding pairs obtained from Janvier Labs (Le Genest-Saint-Isle, France) and BTBR mice were offspring of breeding pairs obtained from Jackson Laboratory (Bar Harbor, Maine, U. S.). Animals were

bred in the animal facilities of the University of Groningen. Animals were housed in standard polypropylene cages, 34 cm x 18 cm x 14 cm, in a group of two mice in a temperature-controlled room (temperature 21 ± 2 °C). All subjects were maintained on a 12-h light/dark cycle, with access to water and standard chow *ad libitum* in their home cages. All procedures were conducted in accordance with protocols approved by the University of Groningen.

Apparatus

The VBS' were built in-house at the University of Groningen, based on the design by Blanchard et al. (1995). Extra chambers (nests) were added to better study the social dynamics. The system consisted of two parts: an open arena (50cm x 50cm) with two stations where animals had access to food and water *ad libitum*, and a burrow (50cm x 25cm) with 4 chambers and a corridor. The open arena was subjected to a 12:12 L/D cycle (ZT0 at 08:00, see figure 3) and was open to the outside. The burrow of the VBS was closed using a polycarbonate lid that functioned as an infrared-pass filter. Thus the burrow was in complete darkness at all time, resembling the natural environment. Within the burrow 2 big chambers (7,5cm x 12,5cm) and 2 small chambers (7,5cm x 7,5cm) were placed with a tunnel connecting them to each other and to the open arena (see Figure 1). Behaviour in the VBS was recorded using a Bassler Cam GigE monochrome infrared sensitive camera (acA1300-60gm). Thus, due to its infrared sensitivity, the camera not only recorded behaviour in the open arena, but also could capture behaviour in the burrow through the polycarbonate lid.

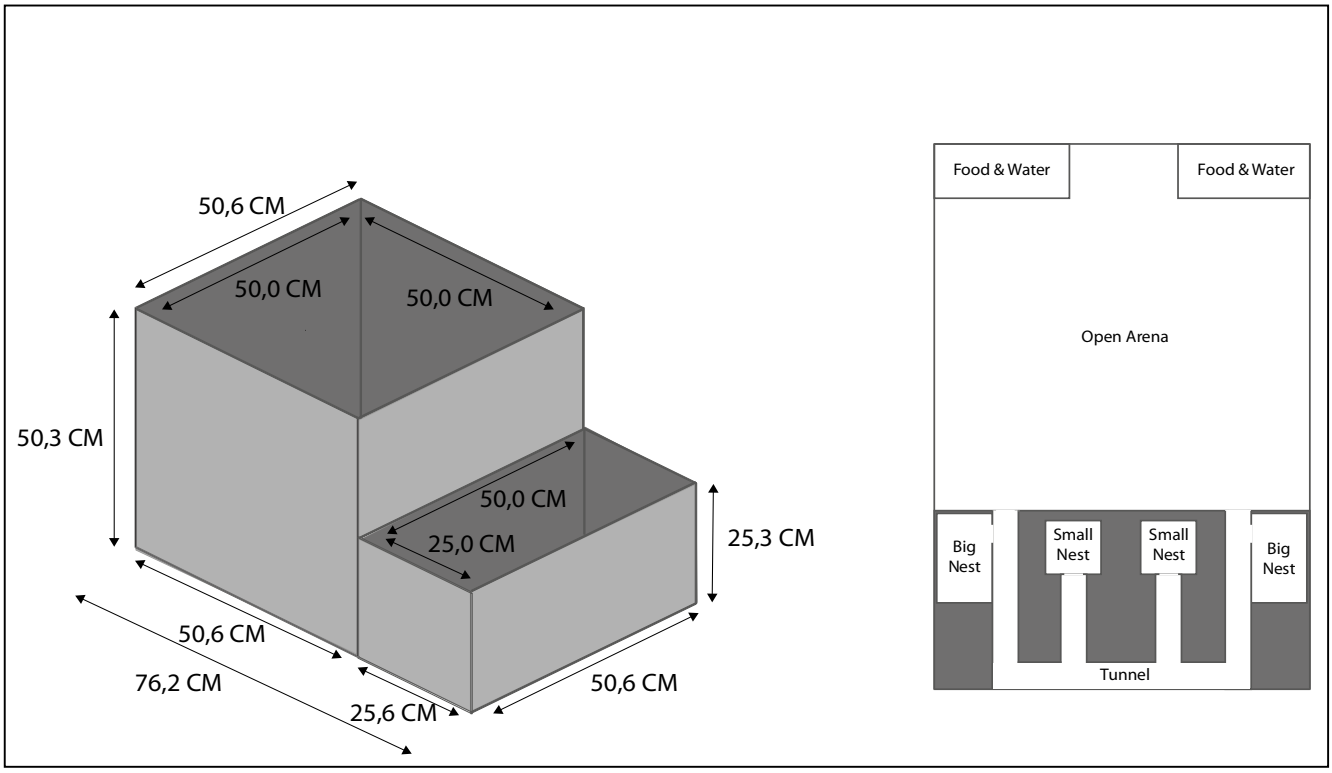

Figure 1 The modified Visible Burrow System (VBS)

Experimental procedures

Animals were placed in the experimental room two weeks before the start of the experiments. Each colony consisted of 6 male mice and 2 female mice of the same strain. Every colony contained no more than 2 littermates. Females were used to mimic the natural group-housed conditions in rodents. Females were previous sterilized by legating the oviducts and leaving the ovaries intact in order to maintain the estrous cycle. Estrous cycle was monitored every day before the start of the experiments. Two days before the start of the experiment males were marked with a commercial crème-based hair dye (Garnier Olia B++ Super blonde) to facilitate individual recognition of the animals. Animals were housed in the system for 8 days. During the experiment, the animals were recorded continuously. The animals were weighted at the beginning and at the end of the experiment in order to leave them undisturbed in the system.

Behavioural ethogram

Social and non-social behaviours scored are described in Table 1.

Table 1

Behavioural ethogram describing all the different behaviour scored.

BEHAVIOURS	DESCRIPTION
Social Exploration	Sniffing another animal, following another animal, playing with another animal
Approach	Moving towards another animal
Aggression	When the subject is biting, chasing, fighting other animals
Avoidance	submissive and avoidance behaviour. Submissive reactions to aggressive behaviour (i.e. not fighting back/surrendering). Also moving/running away from aggressive encounters and social contact/approaches
Huddling	Resting/huddling while in contact with conspecifics. When the subject resumes activity for more than 5 seconds, behaviour is not considered part of resting/huddling
Sexual activity	Mounting Female
Passive/Receiving social contact	Receiving social contact is scored when an animal does not react to social exploration of another animal (i.e. passive social interaction)
Allogrooming	When an animal is grooming another animal
Autogrooming	When an animal is grooming itself
Feeding/Drinking	Feeding/drinking from the feeding station
Environmental Exploration	Animal explores the surrounding environment, behaviour is not aimed towards another animal. (e.g. digging, locomotion, sniffing the walls)
Alone Inactivity	Resting whilst not being in bodily contact with another animal. When the subject resumes activity for more than 5 seconds, behaviour is not considered part of resting

Behavioural analyses

Behaviour was analyzed using The Observed 13 XT (Noldus Information Technology, Wageningen, The Netherlands). Each colony was observed for 10 minutes of six hours divided over the day. The six hours were previously tested and then selected for their representation of the full day and to cover most of the activity phase of the animals. The first 10 minutes of these hours were tested for their accuracy in representing the full hour. A total of 3 C57BL/6J and 2 BTBR colonies were scored manually for 5 days. Frequency and duration in seconds of every behaviour were scored for every 10 minutes of each of the chosen six hours and data were showed as frequency per day and time spent per day. The data of the 5 days scored were summed and showed in the overall behaviour in order to analyze strain differences.

Post mortem analyses

After 5 days of VBS colony housing, male mice were immediately euthanized by cervical dislocation and brains were collected, frozen in isopentane and stored at -80^{o}C.

For the standard-housed measurements, 4 adult C57BL/6J and 4 adult BTBR male mice, housed in standard cages, two per cage, were euthanized, brains were collected, frozen in isopentane and stored at -80^{o}C.

HPLC quantifications

GABA and glutamate concentrations in prefrontal cortex and amygdala 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, 4.60 nm; excitation length, 3.40 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.

Statistical analyses

Frequency and duration of each behaviour were tested for normality and then analyzed per day using Two-way ANOVA for repeated measures followed by a Bonferroni's post-hoc test. Differences in the overall behaviour and neurochemical data were tested for normality and then analyzed using unpaired *t*-test. Correlation between social behaviours and GABA tissue levels were analyzed by using Pearson correlation. Results were expressed as mean ± S.E.M. Statistical analyses were performed using Graph Pad 5.0 (GraphPad Software, San Diego, CA) for Windows. Differences were considered statistically significant when P value was less than 0.05.

4.3 Results

In order to validate the VBS as suitable tool to study social group behaviour dynamics that naturally occur in a mixed-sex colony, we analyzed BTBR and C57BL/6J colonies.

BTBR mice showed reduced social behaviours in VBS colony housing

We scored social exploration and huddling as social behaviours, for both frequency and duration. In particular, we found that the time spent performing social exploration during day 2 was significantly decreased in BTBR compared to control (Fig. 2A, Two-way ANOVA RM followed by Bonferroni, $F_{(1,28)}$=10.98, p<0.01 BTBR vs. C57BL/6J). In addition, the overall duration of social

exploration was significantly decreased in BTBR compared to controls (Fig. 2B, Unpaired *t*-test, p<0.01 BTBR vs. C57BL/6J). Moreover, the frequency of social exploration during day 2, 3 and 5 was significantly decreased in BTBR compared to control strain (Fig. 2C, Two-way ANOVA RM followed by Bonferroni, $F_{(1,28)}$=20.72, p<0.001, p<0.05 BTBR vs. C57BL/6J), and also the total frequency of social exploration was significantly decreased (Fig. 2D, Unpaired *t*-test, p<0.001 BTBR vs. C57BL/6J). On the other hand, there were no differences in time spent performing huddling in BTBR compared to controls (Fig. 2E, Two-way ANOVA RM followed by Bonferroni's, n. s. BTBR vs. C57BL/6J, Fig. 2F Unpaired *t*-test, n. s. BTBR vs. C57BL/6J), while frequency during day 1 was significantly decreased in BTBR compared to controls (Fig. 2G, Two-way ANOVA RM followed by Bonferroni, $F_{(1,28)}$=12.47, p<0.001 BTBR vs. C57BL/6J). Ultimately the total frequency of huddling was significantly reduced in BTBR compared to controls (Fig. 2H, Unpaired *t*-test, p<0.01 BTBR vs. C57BL/6J).

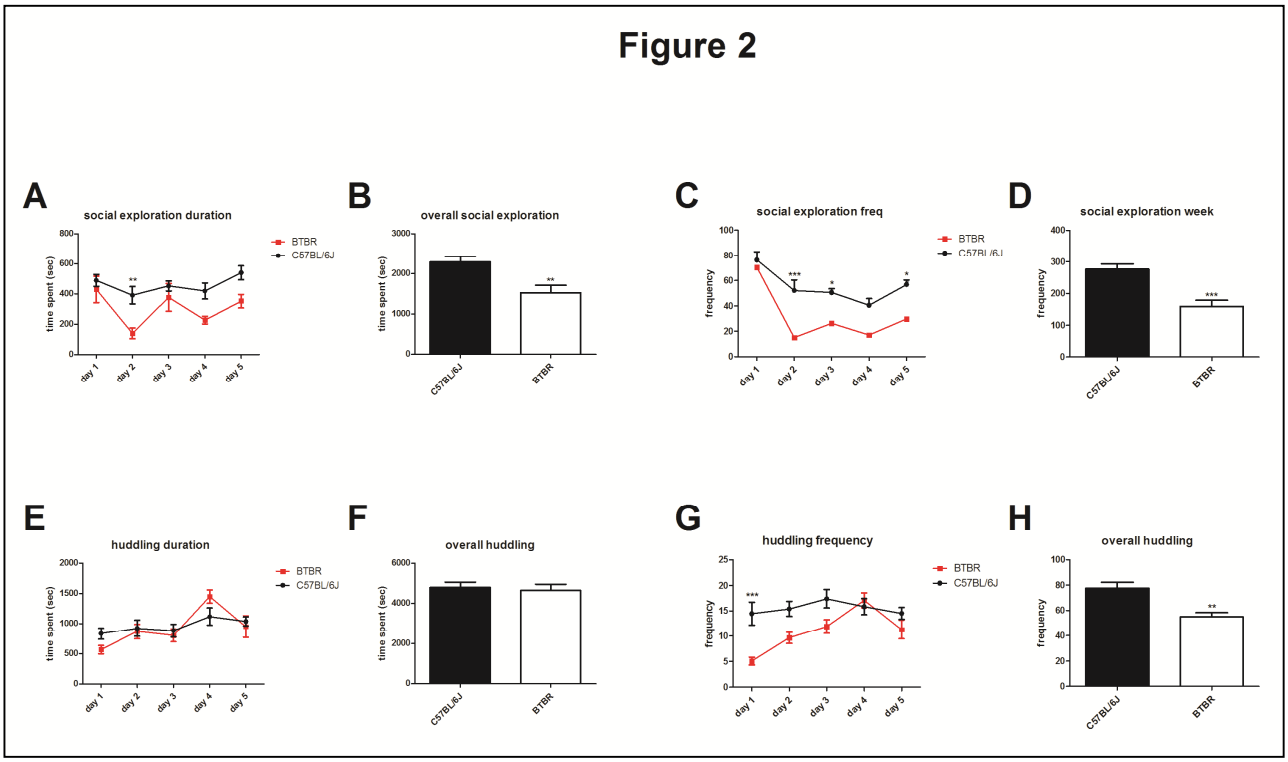

Figure 2 Duration and frequency of social behaviours in BTBR and C57BL/6J mice. Time spent performing social exploration per day (**A**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, **p<0.01 vs. C57BL/6J. Overall time spent performing social exploration (**B**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, **p<0.01 vs. C57BL/6J. Frequency of social exploration per day (**C**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, *p<0.05, **p<0.01 vs. C57BL/6J. Overall

frequency of social exploration (**D**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, ***p<0.001 vs. C57BL/6J. Time spent performing huddling per day (**E**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing huddling (**F**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, **p<0.01 vs. C57BL/6J. Frequency of huddling per day (**G**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, ***p<0.001 vs. C57BL/6J. Overall frequency of huddling (**H**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, **p<0.01 vs. C57BL/6J. Data are expressed as mean ± SEM (n=12 for BTBR group, n=18 for C57BL/6J group).

BTBR mice showed increased non-social behaviours in VBS colony housing

We scored alone inactivity, environmental exploration, avoidance and passive/receiving social contact behaviours as non-social behaviours. Our results showed that there were no differences in time spent performing alone inactivity per day between the two strains (Fig. 3A, Two-way ANOVA RM followed by Bonferroni's, n. s. BTBR vs. C57BL/6J), while the overall duration of alone inactivity was significantly increased in BTBR compared to control strain (Fig. 3B, Unpaired *t*-test, p<0.05 BTBR vs. C57BL/6J). Regarding frequency, there were no differences in both daily and overall alone inactivity between the two strains (Fig. 3C, Two-way ANOVA RM followed by Bonferroni's, n. s. BTBR vs. C57BL/6J, Fig. 3D Unpaired *t*-test, n. s. BTBR vs. C57BL/6J). In addition, the time spent performing environmental exploration was not significantly different between the two strains (Fig. 3E, Two-way ANOVA RM followed by Bonferroni's, n. s. BTBR vs. C57BL/6J, Fig. 3F, Unpaired *t*-test, n. s. BTBR vs. C57BL/6J). Moreover, the frequency of environmental exploration was significantly increased in BTBR mice during day 1 (Fig. 3G, Two-way ANOVA RM followed by Bonferroni, $F_{(1,28)}$=0.4863, p<0.001 BTBR vs. C57BL/6J), although no differences were detected in the overall frequency of environmental exploration between the two strains (Fig. 3H, Unpaired *t*-test, n.s. BTBR vs. C57BL/6J). Furthermore, we found that BTBR spent significantly more time performing avoidance behaviour during day 1 (Fig. 3I, Two-way ANOVA RM followed by Bonferroni, $F_{(1,28)}$=0.01953, p<0.001 BTBR vs. C57BL/6J), while no differences were found in the overall avoidance duration (Fig. 3J, Unpaired *t*-test, n. s. BTBR vs. C57BL/6J). These results were confirmed with the avoidance frequency that was significantly increased in BTBR mice compared to controls only during day 1 (Fig. 3K, Two-way ANOVA RM followed by Bonferroni, $F_{(1,28)}$=1.429, p<0.001 BTBR vs. C57BL/6J), and not in the overall avoidance frequency (Fig. 3L, Unpaired *t*-test, n. s. BTBR vs. C57BL/6J). Ultimately, time spent performing passive/receiving social contact behaviour did not differ between the two strains (Fig. 3M, Two-way ANOVA RM followed by Bonferroni's, n. s. BTBR vs. C57BL/6J, Fig. 3N, Unpaired *t*-test, n. s. BTBR vs. C57BL/6J). Also daily frequency of

passive/receiving social contact behaviour was not significantly different between BTBR and control animals (Fig. 3O, Two-way ANOVA RM followed by Bonferroni's, n. s. BTBR vs. C57BL/6J), while BTBR showed significantly more passive/receiving social contact frequency compared to controls (Fig. 3P, Unpaired *t*-test, p<0.05 BTBR vs. C57BL/6J).

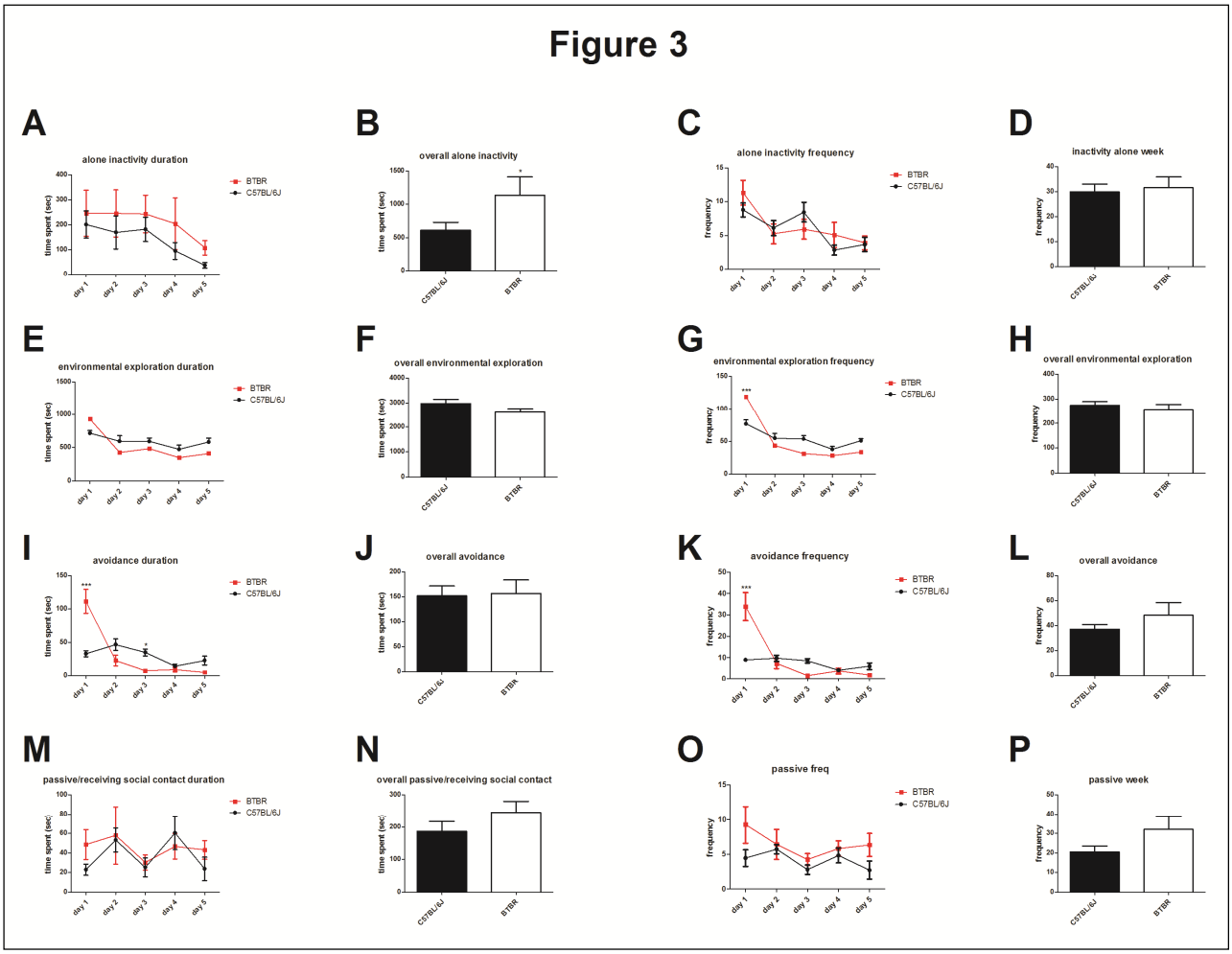

Figure 3 Duration and frequency of non-social behaviours in BTBR and C57BL/6J mice. Time spent performing alone inactivity per day (**A**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing alone inactivity (**B**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, *p<0.05 vs. C57BL/6J. Frequency of alone inactivity per day (**C**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of alone inactivity (**D**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, n.s. Time spent performing environmental exploration per day (**E**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing environmental exploration (**F**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, n.s. Frequency of environmental exploration per day (**G**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, ***p<0.001 vs. C57BL/6J. Overall frequency of environmental exploration (**H**) in

BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, n.s. Time spent performing avoidance per day (**I**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, ***p<0.001 vs. C57BL/6J. Overall time spent performing avoidance (**J**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, n.s. Frequency of avoidance per day (**K**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, ***p<0.001 vs. C57BL/6J. Overall frequency of avoidance (**L**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, n.s. Time spent performing passive/receiving social contact per day (**M**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing passive/receiving social contact (**N**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, n.s. Frequency of passive/receiving social contact per day (**O**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of passive/receiving social contact (**P**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, *p<0.05 vs. C57BL/6J. Data are expressed as mean ± SEM (n=12 for BTBR group, n=18 for C57BL/6J group).

BTBR mice showed novelty-induced aggressive behaviour in VBS colony housing

We scored daily aggressive behaviour for both frequency and duration. We found that BTBR showed significantly more time spent performing aggression during day 1 (Fig. 4A, Two-way ANOVA RM followed by Bonferroni's, $F_{(1,28)}$=2.782, p<0.001 BTBR vs. C57BL/6J), while there were no differences in the overall aggressive behaviour duration between the two strains (Fig. 4B, Unpaired *t*-test, n. s. BTBR vs. C57BL/6J). In addition, frequency of aggression was increased in BTBR during day 1 (Fig.4C, Two-way ANOVA RM followed by Bonferroni's, $F_{(1,28)}$=2.907, p<0.001 BTBR vs. C57BL/6J), while there were no differences detected for the total aggression frequency (Fig. 4D, Unpaired *t*-test, n. s. BTBR vs. C57BL/6J). Since aggressive behaviour could be influenced from sexual activity, we scored also sexual activity for both frequency and duration and we did not find any differences per day or in the total sexual activity between BTBR and control mice for both duration (Fig. 4E, Two-way ANOVA RM followed by Bonferroni's, n. s. BTBR vs. C57BL/6J, Fig. 4F, Unpaired *t*-test, n. s. BTBR vs. C57BL/6J) and frequency (Fig. 4G, Two-way ANOVA RM followed by Bonferroni's, n. s. Fig. 4H, n. s. BTBR vs. C57BL/6J, Unpaired *t*-test, n. s. BTBR vs. C57BL/6J).

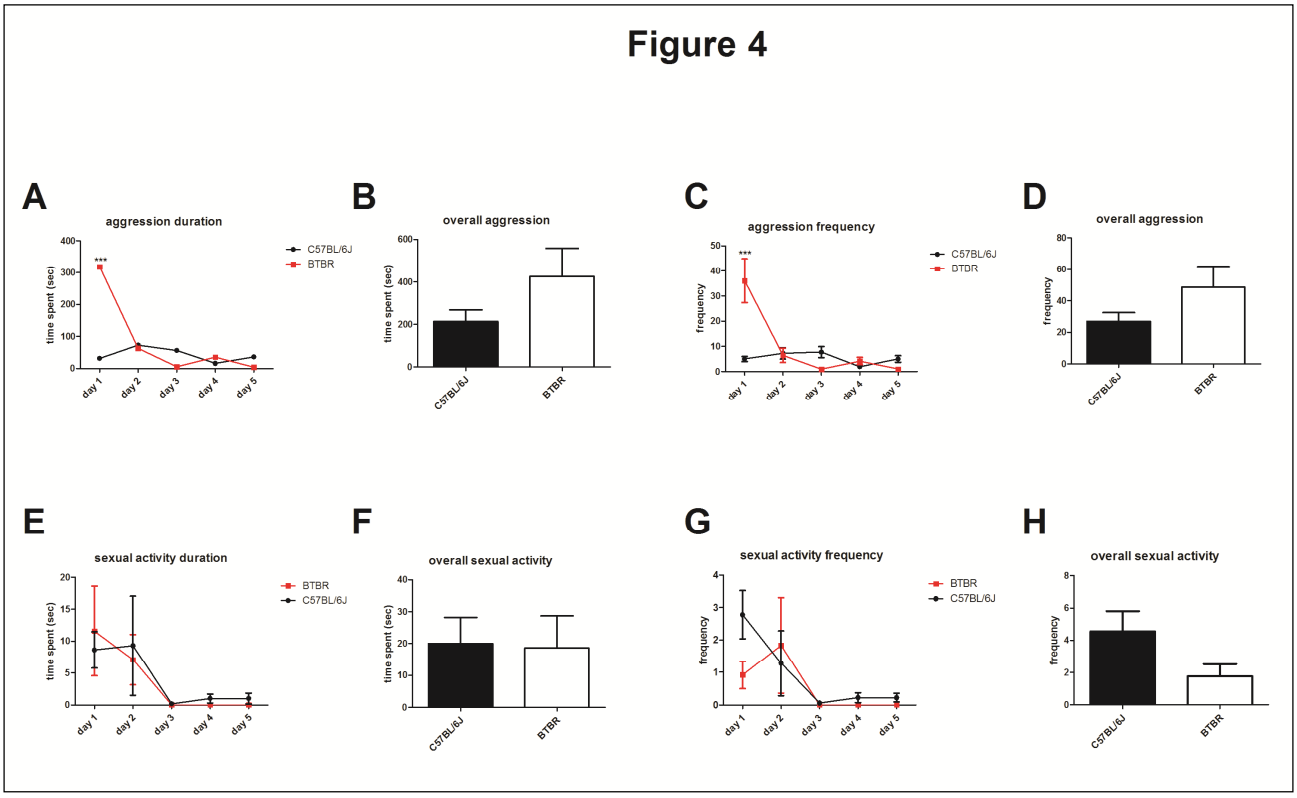

Figure 4 Duration and frequency of aggression and sexual activity in BTBR and C57BL/6J mice. Time spent performing aggression per day (**A**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, ***p<0.001 vs. C57BL/6J. Overall time spent performing aggression (**B**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, n.s. Frequency of aggression per day (**C**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, ***p<0.001 vs. C57BL/6J. Overall frequency of aggression (**D**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, n.s. Time spent performing sexual activity per day (**E**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing sexual activity (**F**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, n.s. Frequency of sexual activity per day (**G**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of sexual activity (**H**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, n.s. Data are expressed as mean ± SEM (n=12 for BTBR group, n=18 for C57BL/6J group).

BTBR mice showed increased grooming behaviour in VBS colony housing

We scored grooming behaviour, which includes both allogrooming and autogrooming. We found that BTBR spent significantly more time performing grooming compared to controls during day 3 and 5 (Fig.5A, Two-way ANOVA RM followed by Bonferroni's, F=3.687, p<0.05, p<0.01 BTBR vs. C57BL/6J) and in the overall grooming (Fig. 5B, Unpaired *t*-test, p<0.05 BTBR vs. C57BL/6J). Regarding frequency, BTBR showed significant less grooming frequency during day 1 compared to control mice (Fig. 5C, Two-way ANOVA RM followed by Bonferroni's, F=2.043, p<0.05 BTBR vs.

C57BL/6J), while the overall grooming frequency was not significantly different between the two strains (Fig. 5D, Unpaired *t*-test, n. s. BTBR vs. C57BL/6J).

To investigate whether the alteration in social activities in BTBR strain were due to alterations in general activity, we scored also the total activity of BTBR and C57BL/6J colonies, pooling all the active behaviours (social exploration, alone exploration, avoidance, passive, aggressive behaviour, sexual activity and grooming). Our results showed that there were no differences in time spent performing total activity in BTBR compared to controls during the daily scoring (Fig.5E, Two-way ANOVA RM followed by Bonferroni's, n. s. BTBR vs. C57BL/6J) and in the overall duration of total activity (Fig. 5F, Unpaired *t*-test, n. s. BTBR vs. C57BL/6J). Furthermore, we found an increase in total activity frequency during day 1 and a decrease during day 3 in BTBR mice compared to controls (Fig. 5G, Two-way ANOVA RM followed by Bonferroni's, $F_{(1,28)}=5.306$, $p<0.05$ BTBR vs. C57BL/6J), while there were no differences in the overall frequency of total activity between the two strains (Fig. 5H, Unpaired *t*-test, n. s. BTBR vs. C57BL/6J).

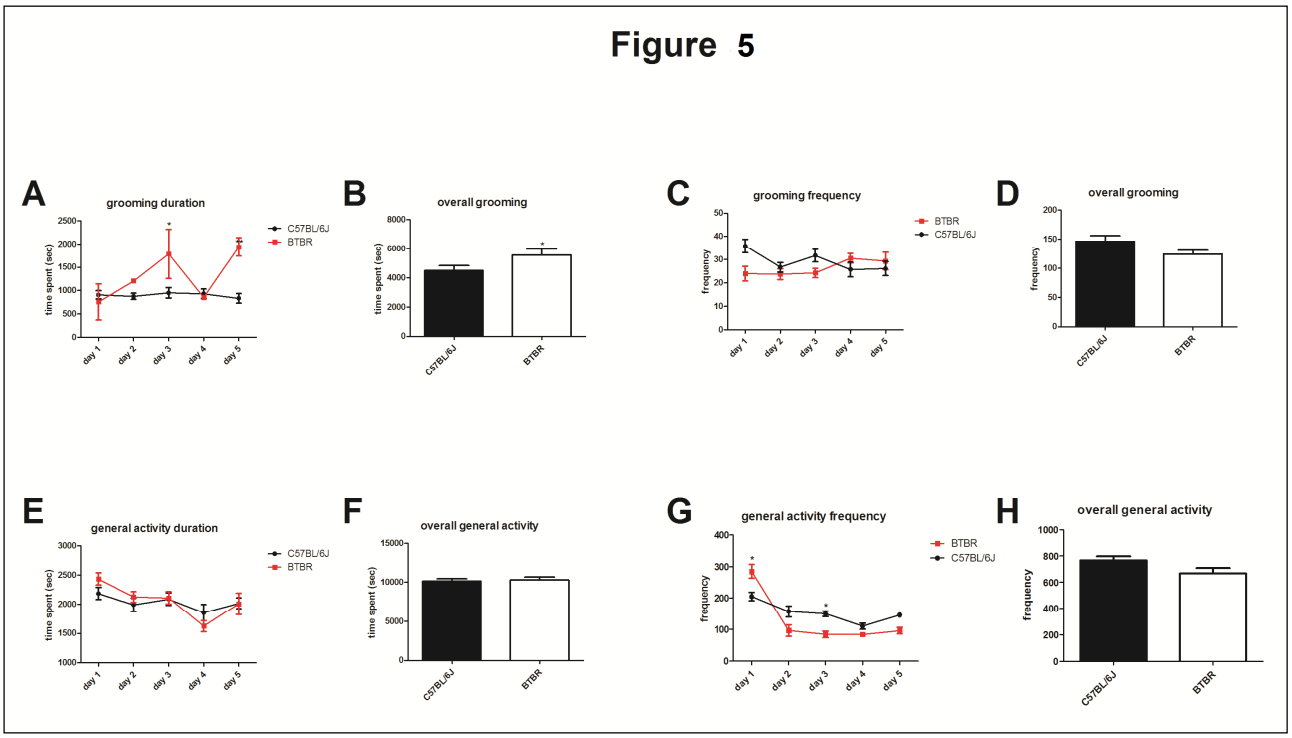

Figure 5 Duration and frequency of grooming and general activity in BTBR and C57BL/6J mice. Time spent performing grooming per day (**A**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, *p<0.05, ***p<0.001 vs. C57BL/6J. Overall time spent performing grooming (**B**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, *p<0.05 vs. C57BL/6J. Frequency of grooming per day (**C**) in BTBR (*red line*) and

C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, *p<0.05 vs. C57BL/6J. Overall frequency of grooming (**D**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, n.s. Time spent performing general activity per day (**E**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing general activity (**F**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, n.s. Frequency of general activity per day (**G**) in BTBR (*red line*) and C57BL/6J (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, *p<0.05 vs. C57BL/6J. Overall frequency of general activity (**H**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, n.s. Data are expressed as mean ± SEM (n=12 for BTBR group, n=18 for C57BL/6J group).

BTBR mice showed decreased GABA and increased glutamate concentrations in PFC and amygdala in VBS colony housing

To corroborate behavioural results with neurochemical analyses, we quantified GABA and glutamate levels in PFC and amygdala at the end of the VBS experiments. Our results showed a decrease in GABA levels in BTBR compared to control colonies, in both PFC and amygdala (Fig. 6A-B, Unpaired *t*-test, p<0.05, BTBR vs. C57BL/6J). Moreover, we found a significant increase in cortical and amygdaloidal glutamate levels in BTBR compared to C57BL/6J colonies (Fig. 6C, Unpaired *t*-test, p<0.001, BTBR vs. C57BL/6J, Fig. 6D, Unpaired *t*-test, p<0.01, BTBR vs. C57BL/6J).

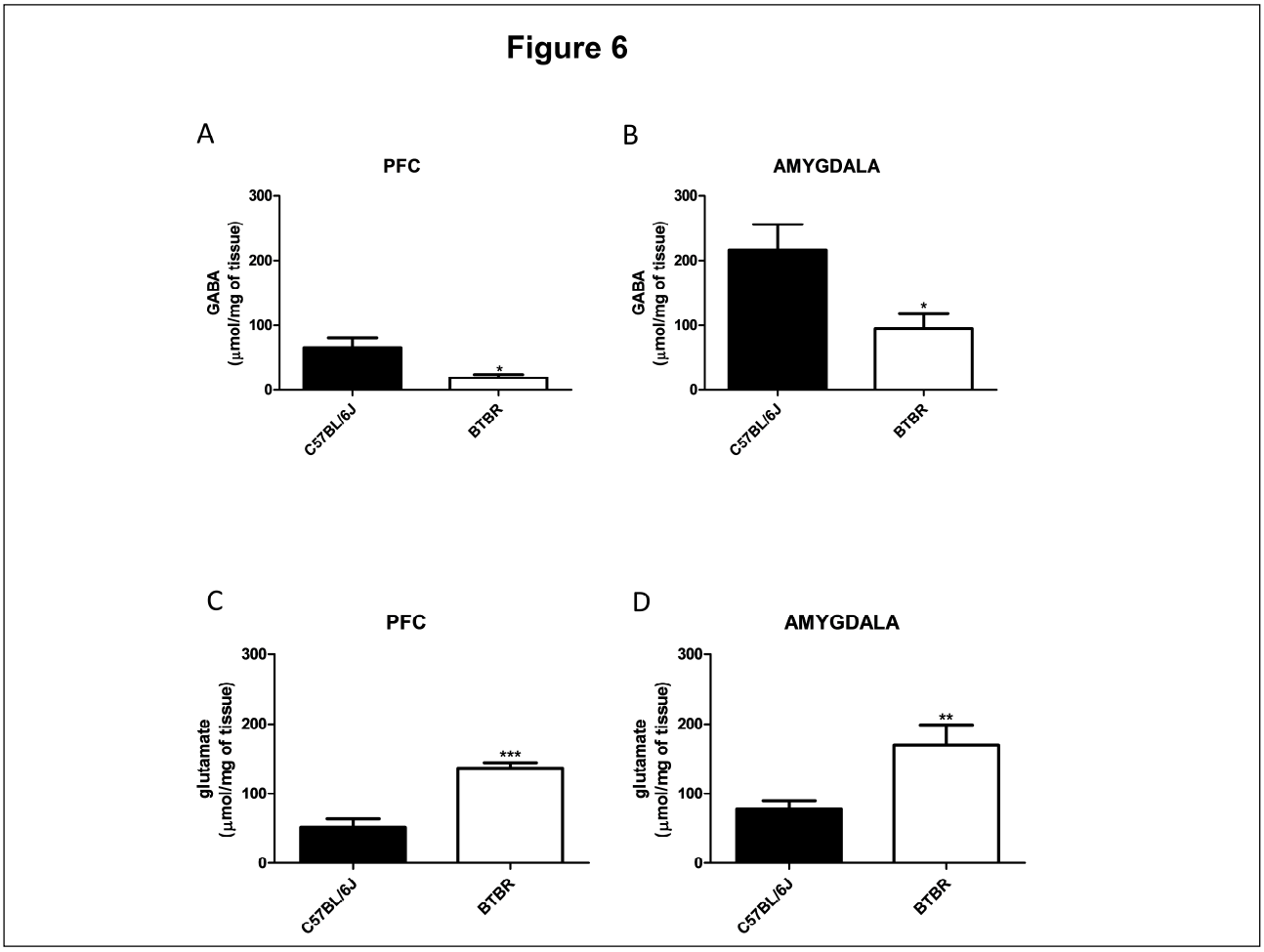

Figure 6 Cortical and amygdaloidal GABA and glutamate levels in BTBR and C57BL/6J mice. GABA levels in PFC (**A**) and amygdala (**B**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, *p<0.05 vs. C57BL/6J. Glutamate levels in PFC (**C**) and amygdala (**D**) in BTBR (*white bar*) and C57BL/6J (*black bar*) mice. Unpaired t-test, **p<0.01, ***p<0.001 vs. C57BL/6J. Data are expressed as mean ± SEM (n=12 for BTBR group, n=18 for C57BL/6J group).

Effect of VBS colony housing on GABA and glutamate concentrations in BTBR and C57BL/6J mice

In order to investigate the effect of the VBS colony housing on neurochemical outcomes, we quantified cortical and amygdaloidal GABA and glutamate levels in standard-housed animals and in VBS-housed animals in BTBR and C57BL/6J strains. We found an increase in GABA levels in both PFC and amygdala in VBS-housed C57BL/6J compared to the standard-housed C57BL/6J (Fig. 7A, Unpaired *t*-test, p<0.01, VBS-housed vs. standard-housed, Fig. 7B, Unpaired *t*-test, p<0.05, VBS-housed vs. standard-housed). Moreover, VBS-housed C57BL/6J showed a decrease in cortical and amygdaloidal glutamate levels compared to standard-housed C57BL/6J (Fig. 7C-D, Unpaired *t*-test, p<0.05, VBS-housed vs. standard-housed). As regarding BTBR strain, no differences were detected

in GABA and glutamate levels, in both PFC and amygdala, in VBS-housed compared to standard-housed mice (Fig. 7E-H, Unpaired *t*-test, n. s., VBS-housed vs. standard-housed).

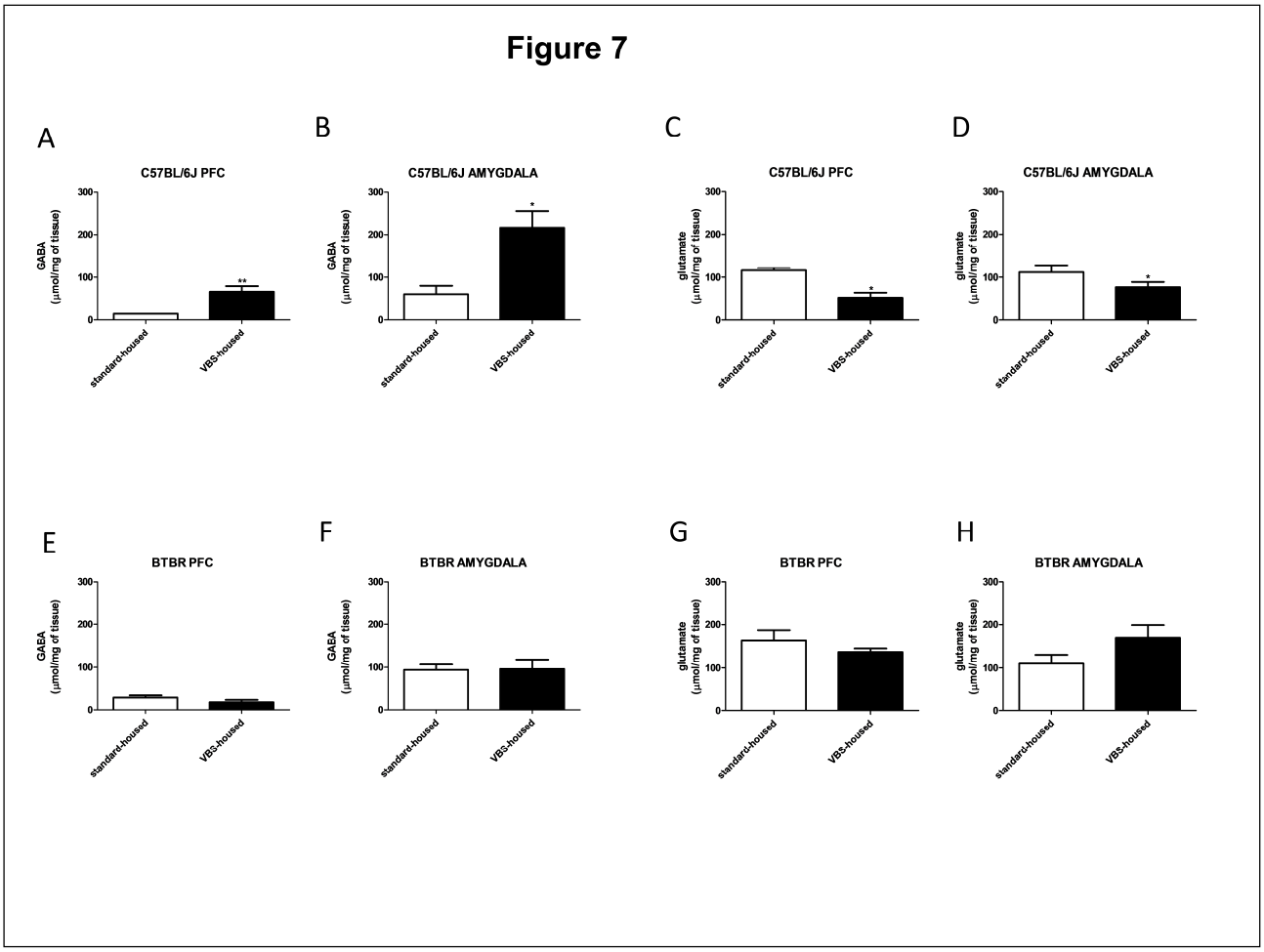

Figure 7 Effect of VBS colony housing on cortical and amygdaloidal GABA and glutamate levels in BTBR and C57BL/6J mice. GABA levels in PFC (**A**) and amygdala (**B**) in C57BL/6J mice housed in standard cages (*white bar*) and C57BL/6J VBS-housed (*black bar*) mice. Unpaired t-test, *p<0.05, **p<0.01 vs. C57BL/6J standard-housed. Glutamate levels in PFC (**C**) and amygdala (**D**) in C57BL/6J mice housed in standard cages (*white bar*) and C57BL/6J VBS-housed (*black bar*) mice. Unpaired t-test, *p<0.05 vs. C57BL/6J standard-housed. GABA levels in PFC (**D**) and amygdala (**E**) in BTBR mice housed in standard cages (*white bar*) and BTBR VBS-housed (*black bar*) mice. Unpaired t-test, n.s. Glutamate levels in PFC (**G**) and amygdala (**H**) in BTBR mice housed in standard cages (*white bar*) and BTBR VBS-housed (*black bar*) mice. Unpaired t-test, n.s. Data are expressed as mean ± SEM (n=8 for standard-housed C57BL/6J and BTBR groups, n=12 for VBS-housed C57BL/6J and BTBR groups).

Positive correlation between social exploration and amygdaloidal GABA in C57BL/6J but not BTBR mice colonies

In order to investigate the presence of correlations between social behaviours and GABA tissue levels, we performed Pearson correlation for each mouse for 2 C57BL/6J and 2 BTBR colonies. We did not found any correlation between social inactivity and cortical and amygdaloidal GABA levels for both strains (data not shown). Finally, we found a significant positive correlation between social exploration and GABA in amygdala in C57BL/6J mice (Fig. 8A, Pearson correlation, r^2=0,6093, p<0.01), while the correlation was not significant in BTBR mice (Fig. 8B, Pearson correlation, r^2=0,2509, n. s.).

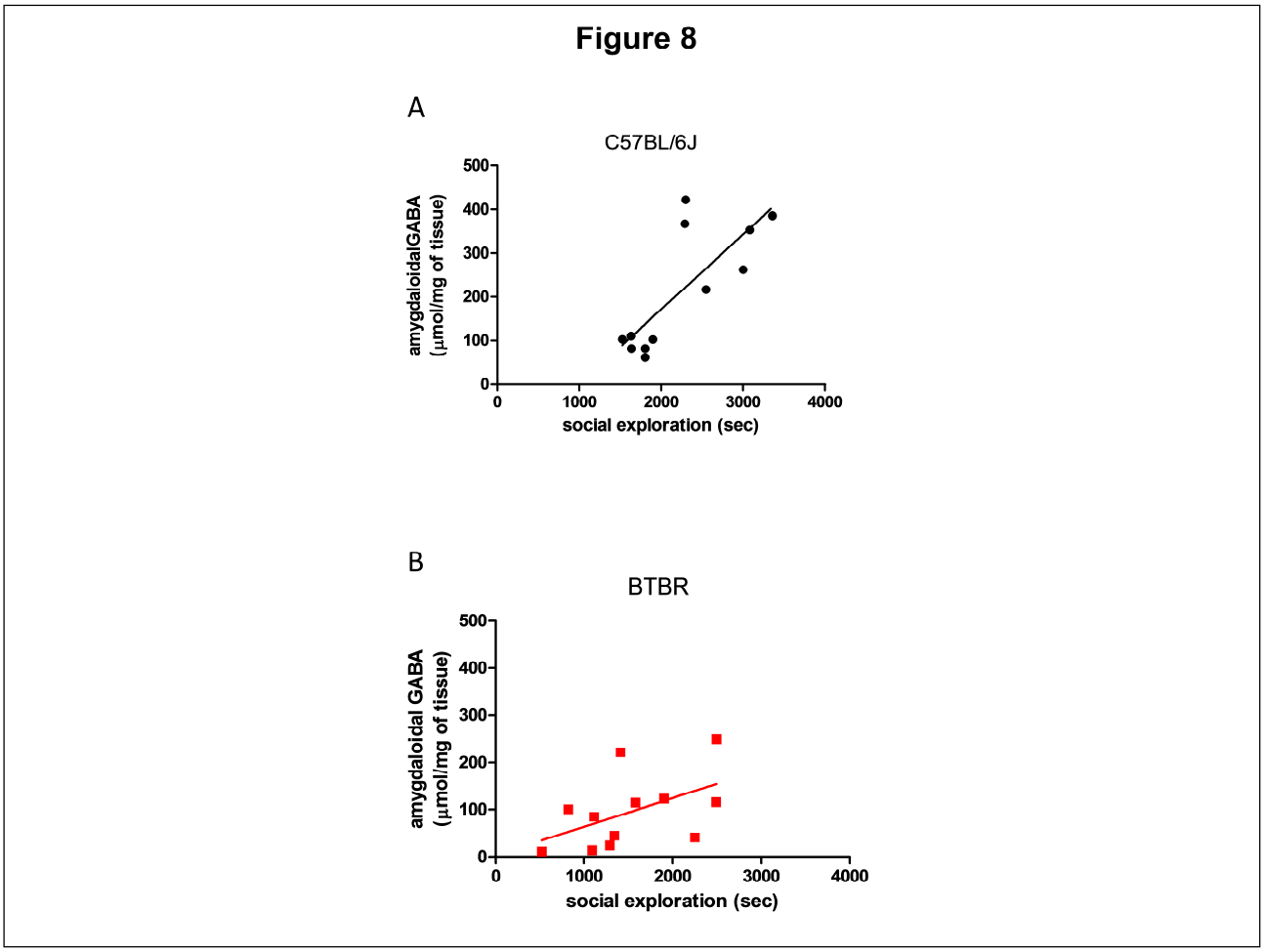

Figure 8 Correlation between social exploration and amygdaloidal GABA in C57BL/6J and BTBR colonies. Positive correlation between social exploration and amygdaloidal GABA in C57BL/6J mice housed in VBS colonies (**A**). Pearson correlation, r^2=0,6093, **p<0.01. Correlation between social exploration and amygdaloidal GABA in BTBR mice housed in VBS colonies (**B**). Pearson correlation, r^2=0,2509, n.s.

4.4 Discussion

In the present study, we investigated social dynamics and study social withdrawal features in BTBR and C57BL/6J colonies in the VBS paradigm. Our results showed that BTBR mice performed less social behaviours and have a preference for non-social behaviours compared to C57BL/6J mice. The lack of sociability in BTBR was further accompanied by reduced GABA and increased glutamate concentrations in PFC and amygdala.

In our study, we implemented a modified version of an earlier used VBS paradigm, namely by adding two additional chambers in the burrow, enabling animals to have more nests and thus mimic the natural environment as much as possible. Moreover, we used mixed-sex colonies to better reproduce the group-housed social dynamics that naturally occur in rodents (Buwalda et al., 2017). To the best of our knowledge, this is the first study using a scoring method that includes duration and frequency of the most important behaviours of group-housed animals. Indeed, our results showed the total burden of social and non-social behaviours, displaying a clear picture of BTBR and C57BL/6J behaviours in colony. In these regards, we found a decrease in time spent performing social exploration in BTBR mice compared to controls during day 2 and in the overall duration. The decrease in time spent performing social exploration in BTBR was accompanied by a decrease also in the frequency during day 2, 3, 5 and in the overall social exploration frequency. As widely known, BTBR mice are studied as an ASD model, because of their reduced sociability compared to the commonly used as control C57BL/6J strain (Pobbe et al., 2010; Ryan, Fraser-Thomas, & Weiss, 2017). Indeed, the most important features of ASD phenotype consist in social deficits and high levels of repetitive grooming (Pobbe et al., 2010; Silverman, Tolu, Barkan, & Crawley, 2010). However, the most used behavioural test to assess sociability is the three chamber test, in which social preference is tested towards only one stimulus animal (Moy et al., 2007). Hence, in our group-housed environment, we measured time spent and frequency of social behaviours for each component of the colony towards the other five males and two females, in order to untangle the social dynamics typical of the two studied strains. In this regard, we found a decrease of the overall huddling frequency in BTBR mice compared to controls, while no differences were detected in terms of duration. Our results are in line with previous studies from Blanchard group, in which they found a decrease in huddling frequency in BTBR compared to control mice (Pobbe et al., 2010). Although huddling is commonly considered a social inactive behaviour, it has also thermoregulatory functions. Factors such as social dominance, gender,

ambient temperature and thermogenic needs can all have an influence on the amount of huddling (see (Gilbert et al., 2010) for review).

As regarding non-social behaviours, BTBR showed a general preference compared to C57BL/6J. In particular, we found a significant increase in the overall duration of alone inactivity in BTBR mice, while no differences were detected in terms of frequency. Conversely, BTBR showed an increase in the frequency of environmental exploration during day 1, but no differences in time spent performing environmental exploration. In addition, avoidance behaviour was significantly increased in BTBR mice during day 1 for both frequency and duration. Ultimately, we found an increase of passive/receiving social contact behaviour in BTBR mice only in terms of overall frequency, but not overall duration.

Taken together, our results confirm that the BTBR strain display less sociability and a preference for non-social behaviours, also in a semi-natural mixed-sex housing condition.

Although our results are in line with previous literature regarding BTBR strain and decrease of sociability (Meyza et al., 2015), this is the first study showing also an effective increase in non-social behaviours. In particular, our results reported a trend towards social withdrawal behaviours in BTBR mice, opening to a deep investigation of the underlying neurobiology that gives rise to these behaviours.

Furthermore, we found an increase in aggressive behaviour in BTBR compared to C57BL/6J mice during day 1, in terms of both frequency and duration. Interestingly, after the first day, the aggressive behaviour in BTBR almost disappeared, suggesting a novelty-induced effect due to the new group housing condition. In this regard, very little is known about BTBR aggressiveness traits. Little aggressive behaviour was observed during social interaction test (Bolivar, Walters, & Phoenix, 2007) and resident-intruder paradigm (unpublished observations). Although aggression is not one of the core symptoms of ASD, ASD children display high levels of irritability, sometimes including aggressiveness towards others (Silverman et al., 2010) and caregiver surveys reported some episodes of aggression in ASD patients towards others ASD patients (Kanne & Mazurek, 2011; Pouw, Rieffe, Oosterveld, Huskens, & Stockmann, 2013). However, to fully evaluate aggressive behaviour features, hierarchy should to be taken into account. Dominance hierarchies are important aspects of animals living in social groups (Buwalda et al., 2017). Here, we wanted to investigate strain differences and validate the suitability of VBS as a paradigm to study social and

non-social behaviours. Future studies will be conducted to analyze individual animal behaviours and hierarchy formation within the colonies.

Since sexual activity is an important trigger for aggressive behaviour, we decided to use mixed-sex colonies and analyze their sexual activity. Our results showed that there were no strain differences in sexual activity duration and frequency. Accordingly with aggressive behaviour results, sexual activity was performed only during day 1 and 2 in BTBR colonies. Considering that females were monitored before the beginning of the experiment, avoiding to start the experiment during the sexual receptivity phase, these results further confirm the novelty-induced effect due to the new housing condition in BTBR mice.

As regarding grooming behaviour, we found an increase during day 3 and day 5 and in the overall duration of grooming in BTBR compared to C57BL/6J mice, while no differences were reported in terms of frequency. These data suggest that BTBR performed more grooming for a more prolonged time compared to C57BL/6J mice, indicating a less initiation of the behaviour. As widely reported, BTBR strain display high levels of repetitive behaviours, such as persistent self-grooming and murble-burying (Amodeo, Jones, Sweeney, & Ragozzino, 2012; Jones-Davis et al., 2013; McFarlane et al., 2008; Molenhuis et al., 2014; Moller & Raahave, 1974; Pobbe et al., 2010). In line with the previous literature, our increase in time spent performing grooming behaviour might be interpreted as repetitive behaviour that BTBR mice perform towards themselves (self-grooming) and towards others (allo-grooming). For this reason, we decided to pool together self- and allo-grooming, due to the their repetitive features and not to consider allo-grooming as a social behaviour, as differently reported in other VBS studies (Pobbe et al., 2010).

Finally, we also checked general activity, to assess whether social and non-social strain differences were due to alteration in activity and we did not find any differences between the two strains. In support of this findings, Silverman and colleagues demonstrated that BTBR have the same response of C57BL/6J in terms of activity and locomotion (Silverman, Babineau, Oliver, Karras, & Crawley, 2013).

From a neurochemical point of view, we found a significant decrease of GABA levels in PFC and amygdala in BTBR compared to C57BL/6J animals. The decrease in GABA was accompanied by an increase in glutamate levels, respectively in PFC and amygdala of BTBR mice. Recently, GABA involvement in sociability pathways is receiving great interest. In this regard, our results are consistent with those of Paine et colleagues, who demonstrated that a decrease in GABA functions

in PFC and basolateral amygdala lead to a decrease in the social interaction and in the social preference tests, without affecting general anxiety, reward or locomotion (Paine et al., 2017). However, different social factors contribute to sociability dysfunctions, such as social motivation, social anxiety and social cognition (Kennedy & Adolphs, 2012), hence future studies will be conducted to assess the involvement of these different social components in the sociability impairment.

Furthermore, it has been widely demonstrated that imbalances in the excitatory and inhibitory synaptic transmission might be responsible of severe neuropsychiatric-related symptoms (Gao & Penzes, 2015; Nelson & Valakh, 2015; Sorce et al., 2010; Yizhar et al., 2011). In an elegant study, Yizhar and colleagues found a reduction in social interactions and social preference when activating optogenetically cortical pyramidal neurons (Yizhar et al., 2011). Moreover, it has been reported that lesions in the medial PFC increased social behaviour in the social interaction test (Shah & Treit, 2003). In conclusion, the decrease in GABA and the corresponding increase in glutamate in PFC and amygdala might be responsible of the decrease in social behaviour and increase in social withdrawal characteristics in BTBR strain. Thus, enhancing GABA neurotransmission could be a possible therapeutic strategy to treat social withdrawal symptoms that primarily occur in many neuropsychiatric and neurodegenerative diseases.

In addition, we evaluated the effect of VBS colony housing condition on GABA and glutamate neurotransmission in the two studied strains. Interestingly, we found that GABA was increased and glutamate was decreased in both PFC and amygdala in C57BL/6J housed in VBS compared to C57BL/6J housed in standard cages. Otherwise, BTBR did not follow the same trend of C57BL/6J, indeed no differences were detected in GABA and glutamate levels between the VBS colonies and the standard cage housing condition. Thus, C57BL/6J mice showed a neurochemical response to the highly social housing conditions, not found in BTBR strain. The BTBR neurochemical non-response to VBS housing conditions might explain their tendency to perform social withdrawal behaviours in the colony. Our findings are consistent with a preclinical study from Crawley group, in which they showed that BTBR mice have poor abilities to modulate their responses to different social partners, resembling social cognition deficits in ASD patients (Yang et al., 2012).

To further corroborate neurochemical data with behavioural outcomes, we searched for correlations between social exploratory behaviour and GABA levels in amygdala for each individual mouse within every colony. We found a significant positive correlation between social exploratory

activity and amygdaloidal GABA in C57BL/6J, but not in BTBR colonies. These results endorse the hypothesis that GABA neurotransmission deeply affect sociability and that, in physiological condition such as in control C57BL/6J mice, GABAergic tone is able to modulate the response to different social environments.

Our study validated the use of the VBS as a behavioural paradigm to deeply analyze sociability and social withdrawal behaviours, investigating mixed-sex group-housed dynamics in rodents. In conclusion, the VBS can be used as a tool to study behavioural dysfunctions and their underlying neurobiology, ultimately helping to design effective treatments for behavioural symptoms observed across neuropsychiatric diseases.

CHAPTER 5

Studying social group behaviours in *Pcdh9* deficient mice using the Visible Burrow System

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Manuscript in preparation

Abstract

Genetic deletion of genes related to neuropsychiatric disorders allow us to study gene function in relation to disease features. In this regard, genetic ablation of *Pcdh9* gene has recently been associated to sensory processing deficits and impaired social interactions, that may be relevant for symptomatology across the neuropsychiatric spectrum. To study social behavioural phenotypes under semi-natural conditions, we have recently implemented the Visible Burrow System (VBS), a highly social habitat that may provide useful insights in group-housed social dynamics in a translational way. Recently, we have shown the suitability of the VBS to investigate social withdrawal features and their underlying neurobiology in a mouse model for ASD-like behavioural phenotypes. In this study we investigated for the first time mixed-genotype colonies in the VBS. In particular, we evaluated sociability and social withdrawal features in *Pcdh9* Wild Type (WT), Heterozygous (HET) and Homozygous (HOM) gene knockout (KO) mice, housed together in VBS. In light of the known sensory deficits in this genetic mouse model, we also investigated GABAergic and glutamatergic alterations in the somatosensory cortex of *Pcdh9* WT, KO HET and KO HOM mice. In this preliminary study, our results reported no differences in terms of social behaviours and non-social behaviours among the three genotypes. Moreover, no genotype differences were detected in GABA content in somatosensory cortex, for both standard housing condition and VBS colony housing. Interestingly, glutamate levels were significantly increased in *Pcdh9* KO HOM mice housed in standard cages, while this increase was not found in HOM *Pcdh9*-deficient mice housed in the VBS, suggesting a beneficial effect of this highly social environment on glutamate increase induced by *Pcdh9* genetic ablation. In conclusion the VBS, beyond its employment as a tool to assess sociability in a translational way, might be further used as a behavioural paradigm to test pharmacological treatments aiming at restoring social dysfunctions commonly occurring in several neuropsychiatric disorders, such as social withdrawal.

5.1 Introduction

Mental disorders are defined and classified in the Diagnostic and Statistical Manual of Mental Disorders (DSM–5), edited by the American Psychiatric Association (APA), in order to improve diagnoses, treatments and researches (www.apa.org, APA website). The burden of mental disorders is continuously growing with significant impacts on health in all countries of the world (www.apa.org, APA website). During the last decades, diagnosis in psychiatry only focused on subjective symptoms and observable signs (Goodkind et al., 2015). Although symptoms are an important starting point, genetics and neurobiology underlying these symptoms need to be deeply investigated. Interestingly, genetic and clinical analyses found similarities across a wide variety of diagnoses, suggesting that a common neurobiological substrate across mental illnesses might exist (Goodkind et al., 2015). In addition, genetic analyses have identified common polymorphisms associated with a large range of neuropsychiatric diseases and comorbidity among them is considerably higher than expected (Brugger & Howes, 2017). Moreover, several mental diseases, such as schizophrenia, Autism Spectrum Disorders (ASD), Alzheimer's disease, anxiety disorders and major depression diseases, share a number of common symptoms, especially regarding the social sphere (Goodkind et al., 2015).

In this context, disrupted sociability and consequent social withdrawal are recently receiving great attention as important symptoms that deeply affect the quality of life in neuropsychiatric patients. However, the pathophysiology underlying these symptoms still need to be fully elucidated and current treatments are not able to improve these relevant behavioural alterations (Wilson & Koenig, 2014).

Furthermore, rodents can be a helpful tool to study behavioural alterations related to neuropsychiatric diseases and their underlying neurobiology and genetics. In this regard, to assess behavioural alterations in a translational way, habitats mimicking the natural environment in which group-housed dynamics can be deeply analyzed might be highly useful. In our study, we implemented a modified version of the Visible Burrow System (VBS), a semi-natural habitat providing burrows and an open area for mixed-sex rodent colonies, developed by Blanchard group (D. C. Blanchard et al., 2012; D. C. Blanchard et al., 1995; R. J. Blanchard, Dulloog, et al., 2001).

Moreover, considering the important role of genetic factors in the development of mental diseases, genetic manipulation in rodents can help to obtain suitable models resembling human neuropsychiatric symptoms. In this context, cadherin superfamily, originally characterized as

calcium-dependent cell-adhesion molecules, is now known to be involved in many biological processes, including cell recognition, cell signaling during embryogenesis and formation of neural circuits (Morishita & Yagi, 2007). In particular, protocadherin family, the largest subgroup within the cadherin superfamily, are predominantly expressed in the nervous system. Interestingly, recent evidence suggested that *Protocadherin 9* (*Pcdh9*) is involved in sensory processing deficits and impaired social interaction (Bruining et al., 2015) that may be relevant in a wide variety of neuropsychiatric disorders, such as schizophrenia and ASD pathogenesis (Hirabayashi & Yagi, 2014; Morishita & Yagi, 2007). Moreover, Xiao and colleagues reported that the gene encoding *Pcdh9* might be considered as a novel risk factor for Major Depressive Disorder (MDD) (Xiao, Zheng, et al., 2017).

In view of the sensory processing deficits that have been observed in *Pcdh9* deficient mice, glutamate is the excitatory neurotransmitter of the thalamocortical inputs to the primary visual, auditory and somatosensory cortices, thus it is crucial for the cortical hierarchic structure controlling the interactions with the environment (Tecchio et al., 2011). Indeed, thalamocortical relay neurons receive ascending and descending glutamatergic excitatory inputs and are subjected to GABAergic inhibitory input which shapes the sensory information conveyed to the cortex (Vahle-Hinz, Detsch, Siemers, & Kochs, 2007).

In this study we investigated for the first time mixed-genotype colonies in VBS. In particular, we evaluated sociability and social withdrawal features in *Pcdh9* Wild Type (WT), KO heterozygous (HET) and KO homozygous (HOM) mice, housed together in VBS. We also investigated GABAergic and glutamatergic alterations in the somatosensory cortex of *Pcdh9* WT, KO HET and KO HOM mice.

5.2 Materials and Methods

Animals

Adult *Pcdh9* WT, KO HET and KO HOM male and female mice aged 14-22 weeks were used in this study. Animals were bred in the animal facilities of the University of Groningen. Animals were housed in standard polypropylene cages, 34 cm x 18 cm x 14 cm, in a group of two mice in a temperature-controlled room (temperature 21 ± 2 °C). All subjects were maintained on a 12-h light/dark cycle, with access to water and standard chow *ad libitum* in their home cages. All

procedures were conducted in accordance with protocols approved by the University of Groningen.

Generation and Breeding of *Pcdh9*-Knockout Mice

Pcdh9-deficient mice were generated using a standard procedure (Bruining et al., 2015). Briefly, a targeting vector was designated to delete the second exon of the mouse *Pcdh9* gene, which encodes extracellular, transmembrane, and part of cytoplasmic domains. The targeting vector was constructed by using RPCI-23BAC library (Genetycs, Tsukuba). The 17.2-kb genomic DNA fragment was cloned into pBRSDT. The 5' homology arm was a HindIII/SacI-digested 8.7-kb fragment, and a floxed PGK-neo positive selection marker was placed at its downstream. The 3' homology arm was a SacI/NheI-digested 8.8-kb fragment, and a diphtheria toxin A fragment containing a poly-A signal was added as a negative selection marker. We obtained homologous recombinants using TT-2 embryonic stem cells. Mice possessing the neo cassette were produced using standard procedures for chimeric mouse production. Successful gene targeting was confirmed by Southern blot analysis. The isogenic mice were then used to generate time-mated HET breeding pairs to obtain WT and mutant mice from at least three different litters to mimic the background of the parental CSS14 and C57BL/6J controls. Chimeric mice were crossed to C57BL/6J females. Initially, F1 hybrids from HET matings were generated. Repeated backcrossing with C57BL/6J mice (>10 generations) was conducted to ensure an isogenic C57BL/6J background (Bruining et al., 2015). For the *Pcdh9* KO and WT mice all analyses were performed blind for *Pcdh9* genotype.

Genotyping

Total RNA was isolated from mouse ear clips according to the manufacturer's instructions using the TRIzol procedure (BoomLab, Meppel, The Netherlands). RNA concentrations and purity were measured with a NanoDrop® spectrophotometer (Thermo Scientific, Eindhoven, The Netherlands). For conversion into cDNA, the cDNA Synthesis Kit (BoomLab, Meppel, The Netherlands) was used and the reverse transcription reaction was performed in a thermocycler (PTC-200, MJ Research) with a 3-step program: 10 min at 25°C followed by 60 min at 42°C and a final 5 min step at 85°C. The machine was set to cool down automatically to 4°C after the end of the final step. cDNA samples were then used immediately for Real-Time PCR. Real-Time PCR was performed by using DreamTaq polymerase in accordance with the manufacturer's instructions (Thermo Scientific, Eindhoven, The Netherlands). For the target genes the primers used were *Pcdh9*R1 (5'-ACCAGTCTGTAGACAAGGCT -3'), *Pcdh9*R22 (5'- TACCCGGTAGAATTGACCTGCAG -3'), *Pcdh9*F1 (5'-

GTGGCTGTCTCCACATAAGA -3'). The mixture was prepared by adding the cDNA sample, followed by dNTP mix (Thermo Scientific, Eindhoven, The Netherlands) and 10X primers for the target genes. The samples were then placed in thermal cycler machine and run following thermal cycling conditions: step 1: 94°C for 2 min, step 2: 94°C for 30 sec, step 3: 60°C for 45 sec, step 4: 72°C for 1 min, step 5: from step 2, 32x times, step 6: 72°C for 7 min, step 7: 10°C until the end. At the end of the reaction, the PCR mixture was loaded with 6x loading dye and run together with a DNA Marker on 1.5% agarose gel for electrophoresis (approx. 30 minutes at 90V). The following profiles can be expected: 350bp: *Pcdh9* WT; 350bp and 500bp: *Pcdh9* KO HET; 500bp: *Pcdh9* KO HOM.

Apparatus

The VBS' were built in-house at the University of Groningen, based on the design by Blanchard et al. (D. C. Blanchard et al., 1995). Extra chambers (nests) were added to better study the social dynamics. The system consisted of two parts: an open arena (50cm x 50cm) with two stations where animals had access to food and water *ad libitum*, and a burrow (50cm x 25cm) with 4 chambers and a corridor. The open arena was subjected to a 12:12 L/D cycle (ZT0 at 08:00, see figure 3) and was open to the outside. The burrow of the VBS was closed using a polycarbonate lid that functioned as an infrared-pass filter. Thus the burrow was in complete darkness at all time, resembling the natural environment. Within the burrow 2 big chambers (7,5cm x 12,5cm) and 2 small chambers (7,5cm x 7,5cm) were placed with a tunnel connecting them to each other and to the open arena (see Figure 1). Behaviour in the VBS was recorded using a Bassler Cam GigE monochrome infrared sensitive camera (acA1300-60gm). Thus, due to its infrared sensitivity, the camera not only recorded behaviour in the open arena, but also could capture behaviour in the burrow through the polycarbonate lid.

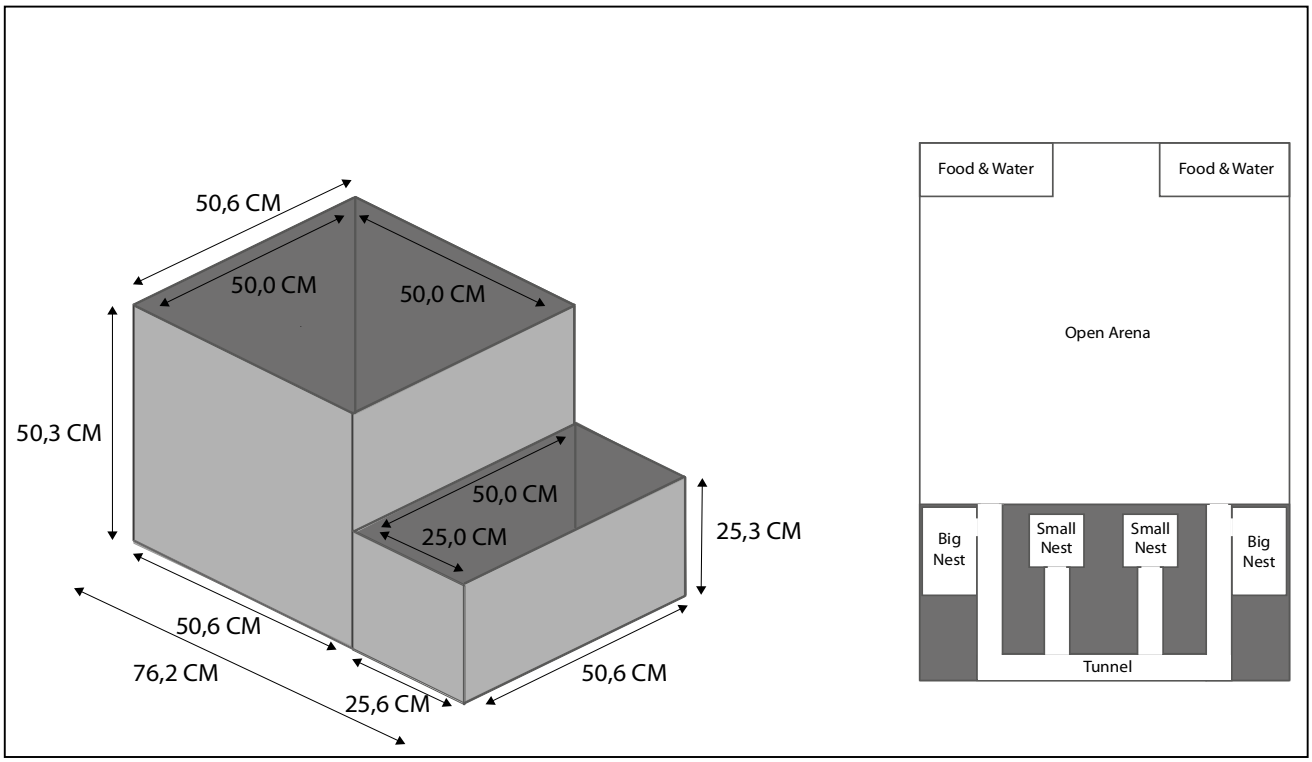

Figure 1 The modified Visible Burrow System

Experimental procedures

Animals were placed in the experimental room two weeks before the start of the experiments. Each colony consisted of 6 male mice, 2 *Pcdh9* WT, 2 *Pcdh9* KO HOM and 2 *Pcdh9* KO HET, and 2 *Pcdh9* WT female mice. Every colony contained no more than 2 littermates. Females were used to mimic the natural group-housed conditions in rodents. Females were previous sterilized by legating the oviducts and leaving the ovaries intact in order to maintain the estrous cycle. Estrous cycle was monitored every day before the start of the experiments. Two days before the start of the experiment males were marked with a commercial crème-based hair dye (Garnier Olia B++ Super blonde) to facilitate individual recognition of the animals. Animals were housed in the system for 8 days. During the experiment, the animals were recorded continuously. The animals were weighted at the beginning and at the end of the experiment in order to leave them undisturbed in the system. We performed and analyzed a total of 3 mixed-genotypes colonies.

Behavioural ethogram

Social and non-social behaviours scored are described in Table 1.

Table 1

Behavioural ethogram describing all the different behaviour scored.

BEHAVIOURS	DESCRIPTION
Social Exploration	Sniffing another animal, following another animal, playing with another animal
Approach	Moving towards another animal
Aggression	When the subject is biting, chasing, fighting other animals
Avoidance	submissive and avoidance behaviour. Submissive reactions to aggressive behaviour (i.e. not fighting back/surrendering). Also moving/running away from aggressive encounters and social contact/approaches
Huddling	Resting/huddling while in contact with conspecifics. When the subject resumes activity for more than 5 seconds, behaviour is not considered part of resting/huddling
Sexual activity	Mounting Female
Passive/Receiving social contact	Receiving social contact is scored when an animal does not react to social exploration of another animal (i.e. passive social interaction)
Allogrooming	When an animal is grooming another animal
Autogrooming	When an animal is grooming itself
Feeding/Drinking	Feeding/drinking from the feeding station
Environmental Exploration	Animal explores the surrounding environment, behaviour is not aimed towards another animal. (e.g. digging, locomotion, sniffing the walls)
Alone Inactivity	Resting whilst not being in bodily contact with another animal. When the subject resumes activity for more than 5 seconds, behaviour is not considered part of resting

Behavioural analyses

Behaviour was analyzed using The Observed 13 XT (Noldus Information Technology, Wageningen, The Netherlands). Each colony was observed for 10 minutes of six hours divided over the day. The six hours were previously tested and then selected for their representation of the full day and to cover most of the activity phase of the animals. The first 10 minutes of these hours were tested for their accuracy in representing the full hour. A total of 3 mixed-genotype *Pcdh9* colonies were scored manually for 5 days. Frequency and duration in seconds of every behaviour were scored for every 10 minutes of each of the chosen six hours and data were showed as frequency per day and

time spent per day. The data of the 5 days scored were summed and showed in the overall behaviour in order to analyze strain differences.

Post mortem analyses

After 8 days of VBS colony housing, mice were immediately euthanized by cervical dislocation and brains were collected, frozen in isopentane and stored at -80^{o}C.

For the standard-housed measurements, 4 adult *Pcdh9* KO HET, KO HOM and WT male mice, housed in standard cages, two per cage, were euthanized, brains were collected, frozen in isopentane and stored at -80^{o}C.

HPLC quantifications

GABA and glutamate concentrations in somatosensory 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, 4.60 nm; excitation length, 3.40 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.

Statistical analyses

Frequency and duration of each behaviour were tested for normality and then analyzed per day using Two-way ANOVA for repeated measures followed by a Bonferroni's post-hoc test. Differences in the overall behaviour and neurochemical data were tested for normality and then analyzed using One-way ANOVA followed by Bonferroni's post-hoc test. Results were expressed as mean ± S.E.M. Statistical analyses were performed using Graph Pad 5.0 (GraphPad Software, San Diego, CA) for Windows. Differences were considered statistically significant when P value was less than 0.05.

5.3 Results

In order to evaluate *Pcdh9* genotype differences, we analyzed mixed-sex colonies formed by 2 *Pcdh9* WT, 2 *Pcdh9* KO HET and 2 *Pcdh9* KO HOM males, with 2 *Pcdh9* WT females, housed together in VBS. Moreover, we measured GABA and glutamate content in the somatosensory cortex of mice housed in the VBS colonies and mice housed in standard cages, for the three different genotypes, respectively.

Social behaviours in mixed-genotype VBS colonies

We scored social exploration, huddling, aggression and sexual activity as social behaviours, for both frequency and duration. In particular, we found that duration and frequency of social exploration during the daily scoring and the overall were not significantly different among the three groups (Fig. 2A, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2B One-way ANOVA followed by Bonferroni, n.s.; Fig. 2C, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2D One-way ANOVA followed by Bonferroni, n.s.). In addition, our results did not show any difference in terms of huddling behaviour among the different genotypes, for both frequency and duration (Fig. 2E, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2F One-way ANOVA followed by Bonferroni, n.s.; Fig. 2G, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2H One-way ANOVA followed by Bonferroni, n.s.). Moreover, there were no differences in time spent performing aggression, and also in frequency of aggression, among *Pcdh9* KO HOM, KO HET and WT (Fig. 2I, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2J One-way ANOVA followed by Bonferroni, n.s.; Fig. 2K, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2L One-way ANOVA followed by Bonferroni, n.s.). Ultimately, we did not find significant differences in time spent and frequency of sexual activity (Fig. 2M, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2N One-way ANOVA followed by Bonferroni, n.s.; Fig. 2O, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2P One-way ANOVA followed by Bonferroni, n.s.).

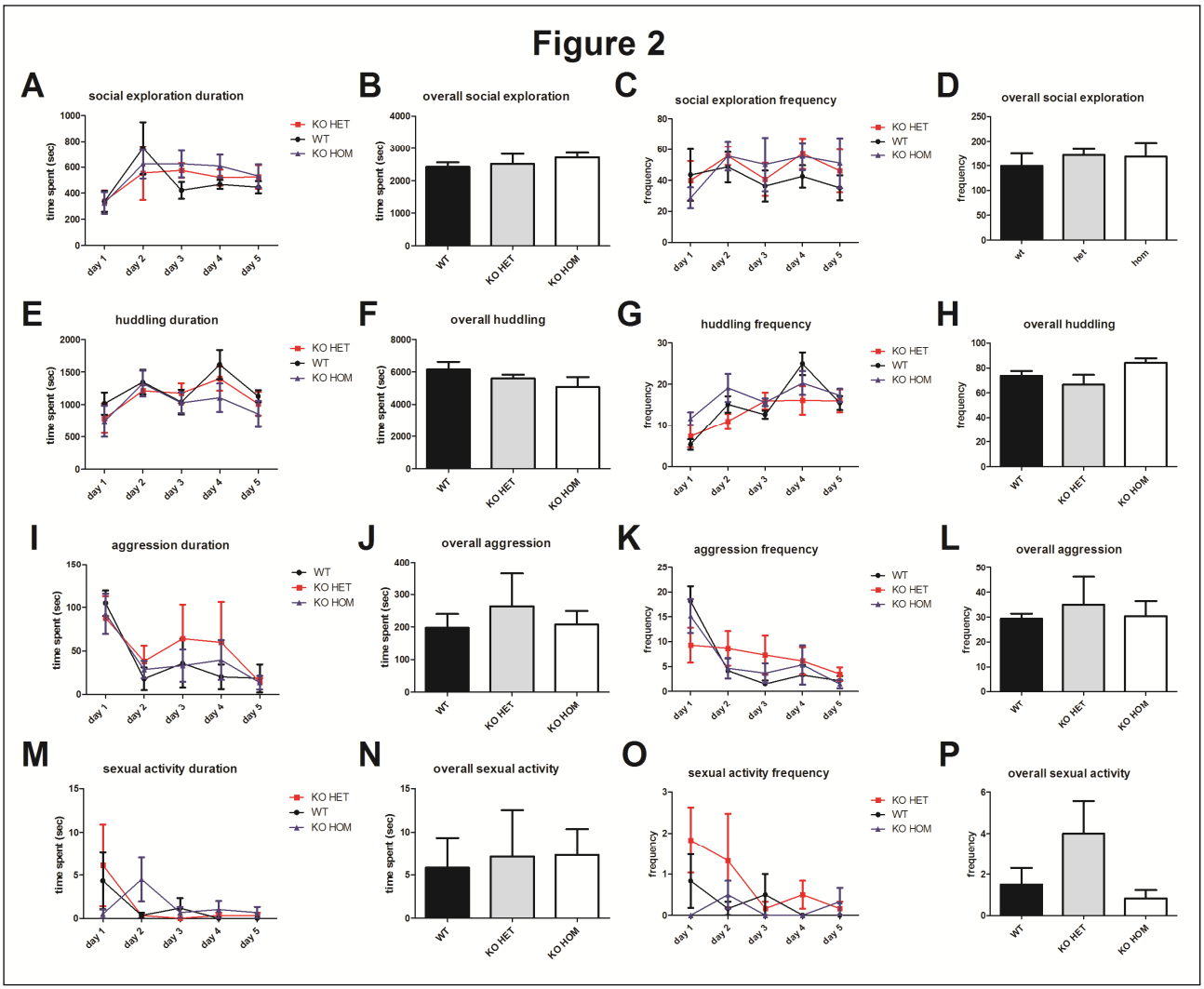

Figure 2 Duration and frequency of social behaviours in mixed-genotype *Pcdh9* colonies. Time spent performing social exploration per day (**A**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing social exploration (**B**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of social exploration per day (**C**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of social exploration (**D**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Time spent performing huddling per day (**E**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing huddling (**F**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of huddling per day (**G**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of huddling (**H**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Time spent performing aggression per day (**I**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s.

Overall time spent performing aggression (**J**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of aggression per day (**K**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of aggression (**L**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Time spent performing sexual activity per day (**M**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing sexual activity (**N**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of sexual activity per day (**O**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of sexual activity (**P**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Data are expressed as mean ± SEM (n=6 per group).

Non-social behaviours in mixed-genotype VBS colonies

We scored environmental exploration, alone inactivity, avoidance and passive/receiving social contact as non-social behaviours, for both frequency and duration. In particular, we found that duration and frequency of environmental exploration during the daily scoring and the overall were not significantly different among the three groups (Fig. 3A, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3B One-way ANOVA followed by Bonferroni, n.s.; Fig. 3C, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3D One-way ANOVA followed by Bonferroni, n.s.). In addition, our results did not show any difference in terms of alone inactivity among the different genotypes, for both frequency and duration (Fig. 3E, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3F One-way ANOVA followed by Bonferroni, n.s.; Fig. 3G, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3H One-way ANOVA followed by Bonferroni, n.s.). Moreover, there were no differences in time spent performing avoidance behaviour, and also in frequency of avoidance, among *Pcdh9* KO HOM, KO HET and WT (Fig. 3I, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3J One-way ANOVA followed by Bonferroni, n.s.; Fig. 3K, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3L One-way ANOVA followed by Bonferroni, n.s.). Ultimately, we did not find significant differences in time spent and frequency of passive/receiving social contact behaviour (Fig. 3M, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3N One-way ANOVA followed by Bonferroni, n.s.; Fig. 3O, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3P One-way ANOVA followed by Bonferroni, n.s.).

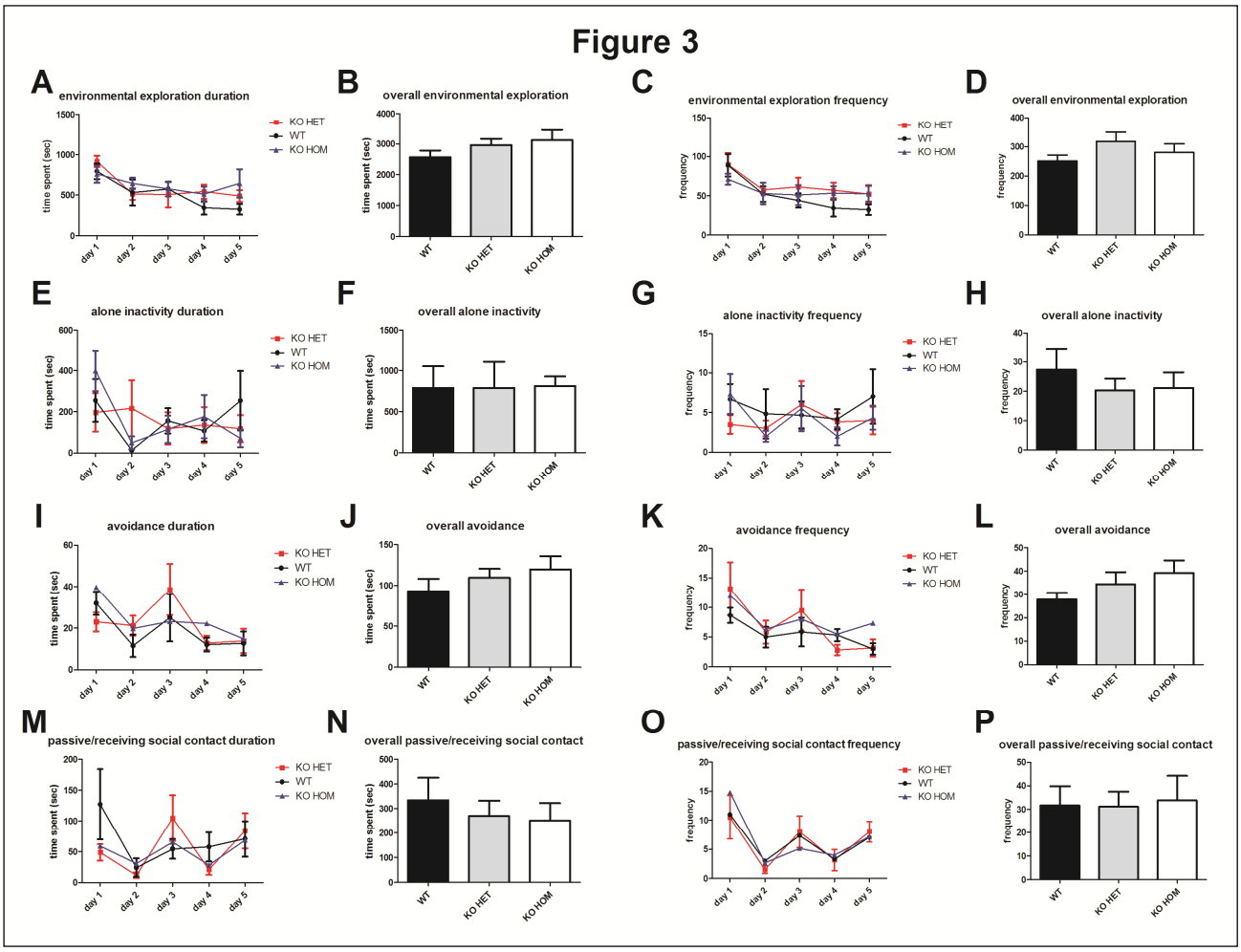

Figure 3 Duration and frequency of non-social behaviours in mixed-genotype *Pcdh9* colonies. Time spent performing environmental exploration per day (**A**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing environmental exploration (**B**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of environmental exploration per day (**C**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of environmental exploration (**D**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Time spent performing alone inactivity per day (**E**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing alone inactivity (**F**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of alone inactivity per day (**G**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of alone inactivity (**H**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Time spent performing avoidance per day (**I**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing avoidance (**J**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of

avoidance per day (**K**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of avoidance (**L**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Time spent performing passive/receiving social contact per day (**M**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing passive/receiving social contact (**N**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of passive/receiving social contact per day (**O**) in *Pcdh9* KO HET (*red line*), KO HOM (*blue line*) and *Pcdh9* WT (*black line*) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of passive/receiving social contact (**P**) in *Pcdh9* KO HOM (*white bar*), KO HET (*gray bar*) and WT (*black bar*) mice. One-Way ANOVA followed by Bonferroni, n.s. Data are expressed as mean ± SEM (n=6 per group).

Increased glutamate content in somatosensory cortex in standard-housing conditions, but not in VBS colony housing

We measured GABA and glutamate content in the somatosensory cortex of mixed-genotype VBS colonies and *Pcdh9* WT, KO HET and KO HOM mice housed in standard cages, in order to investigate genotype differences.

Our results showed that no genotype differences were detected in GABA content in standard-housed mice (Fig. 4A, One-way ANOVA followed by Bonferroni, n.s.), while there was a significant increase in glutamate levels in somatosensory cortex of Pcdh9 KO HOM compare to KO HET and WT mice (Fig. 4B, One-way ANOVA followed by Bonferroni, $F=15.72$, $P<0.01$ KO HOM vs. KO HET and WT). Moreover, we did not found any difference in GABA and glutamate contents in somatosensory cortex among the three different groups (Fig. 4C-D, One-way ANOVA followed by Bonferroni, n.s.).

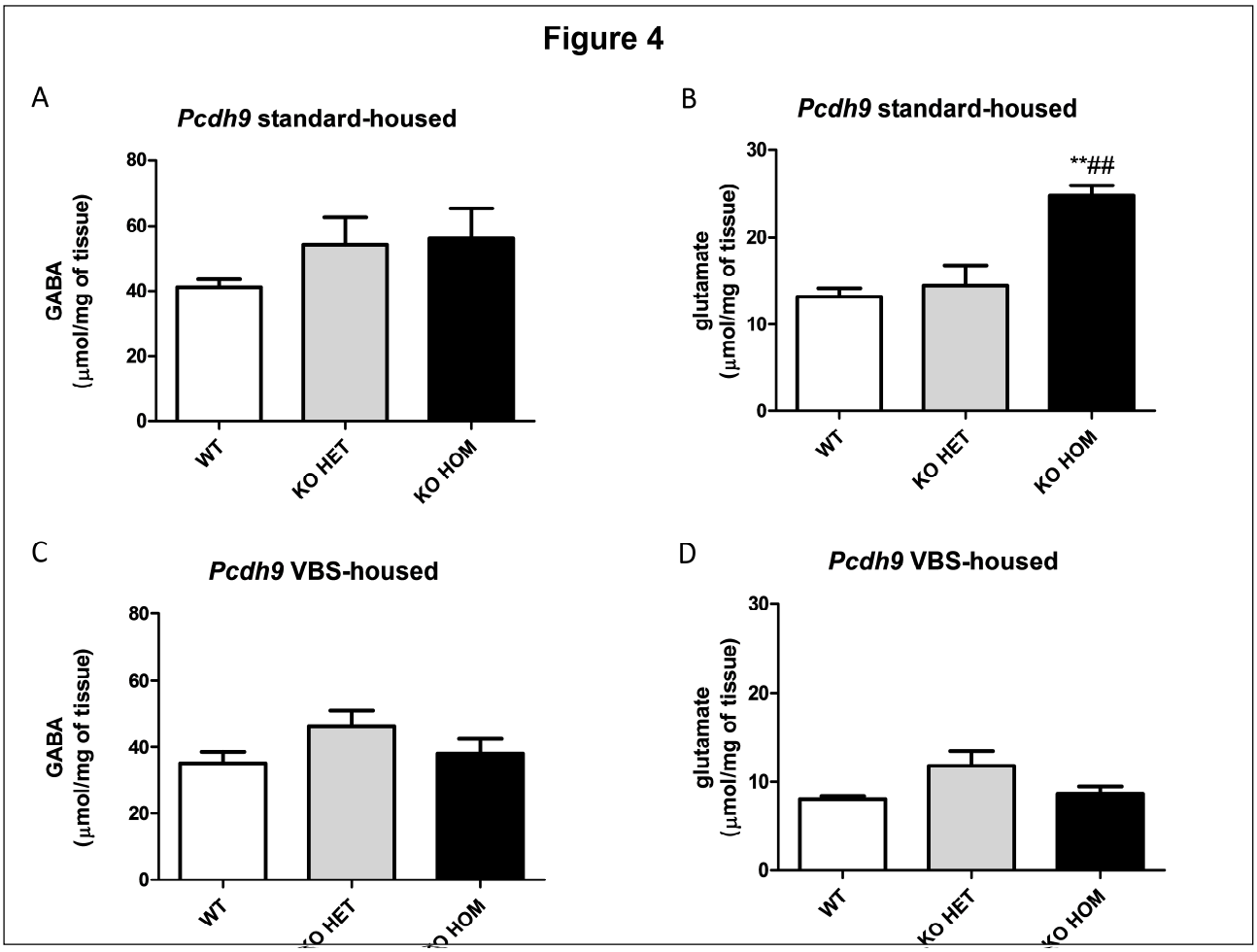

Figure 4 Effect of VBS colony housing on GABA and glutamate levels in somatosensory cortex of *Pcdh9* KO HOM, HET and WT mice. GABA (**A**) levels in somatosensory cortex of *Pcdh9* WT (*white bar*), KO HET (*gray bar*) and KO HOM (*black bar*) mice housed in standard cages. One-Way ANOVA followed by Bonferroni, n.s. Glutamate (**B**) levels in somatosensory cortex of *Pcdh9* WT (*white bar*), KO HET (*gray bar*) and KO HOM (*black bar*) mice housed in standard cages. One-Way ANOVA followed by Bonferroni, **p<0.01 vs. WT, ##p<0.01 vs. KO HET. GABA (**C**) and glutamate (**D**) levels in somatosensory cortex of *Pcdh9* WT (*white bar*), KO HET (*gray bar*) and KO HOM (*black bar*) mice housed in VBS colonies. One-Way ANOVA followed by Bonferroni, n.s.

5.4 Discussion

In the present study, we investigated social dynamics in mixed-genotype and mixed-sex *Pcdh9* colonies in the VBS paradigm. Our preliminary analysis showed that there were no differences in terms of social behaviours and non-social behaviours among the three genotypes, for both duration and frequency. Additional analyses (e.g., including the implementation of location data and increasing the number of colonies tested) are needed to further strengthen these data.

Moreover, no genotype differences were detected in GABA content in somatosensory cortex, for both standard housing condition and VBS colony housing. Interestingly, glutamate levels were significantly increased in *Pcdh9* KO HOM mice housed in standard cages, while this increase was not found in HOM *Pcdh9*-deficient mice housed in the VBS.

We investigated social and non-social behaviours in mixed-sex and mixed-genotype colonies, using a modified VBS version, with four nests in a continuously dark burrow and a large open arena, mimicking the natural rodent housing condition. In addition, we housed together *Pcdh9* WT and KO HET and HOM animals, aiming to analyze genotype differences.

In particular, our results did not show genotype differences in social exploration, huddling, aggression and sexual activity. Thus, there were no differences in terms of social behaviours duration and frequency. Moreover, also non-social behaviours, in particular environmental exploration, alone inactivity, avoidance and passive/receiving social contact, did not show any significant genotype difference.

Interestingly, *Pcdh9* has been recently identified as an interesting candidate gene associated with ASD (Hirabayashi & Yagi, 2014; Morishita & Yagi, 2007). In this regard, Bruining and colleagues demonstrated that HOM *Pcdh9*-deficient mice show specific deficits in long-term social recognition, accompanied by additional impairments in sensorimotor development reflected by early touch-evoked biting, rotarod performance, and sensory gating deficits (Bruining et al., 2015). However, the recognition deficits in *Pcdh9* KO mice were not associated with alterations in perception, multi-trial discrimination learning, sociability, behavioral flexibility or fear memory (Bruining et al., 2015). In line with our results, also Bruining and colleagues did not found genotype differences in three-chamber test, the most used dyadic test to assess sociability in rodents.

However, we did not investigated sociability in a standard environment or social behaviours among *Pcdh9* colonies composed by the same genotype, thus future studies are required to better understand HOM *Pcdh9* KO social phenotype without the presence of social stimuli. Indeed, VBS colonies formed by mixed-genotype and mixed-sex mice are considered highly social environment, hence we hypothesized that strong social stimuli might be helpful to improve probable social deficits.

Social deficits are an important core symptoms of ASD and schizophrenia. The positive effect of the social stimuli in the mixed-genotype VBS colonies might reflect the importance of the behavioural therapy in patients. In accordance with our hypothesis, occupational therapists,

together with teachers, support autistic student participation in classrooms in order to stimulate sociability and increase socialization (Mills & Chapparo, 2017). Moreover, a recent study reported that trained parents, working together with their children, are able to improve their social skills (Dogan et al., 2017), suggesting the beneficial effects that social stimuli can have.

Furthermore, social deficits, in particular social withdrawal and anhedonia, are considered negative symptoms of schizophrenia. These symptoms, associated with cognitive (such as attention and memory deficits) and positive symptoms (such as delusions and hallucinations) significantly impair patients' personal and professional lives (Balhara & Verma, 2012). In particular, negative symptoms often lead to homelessness and are thought to be the primary driver for suicide in schizophrenics (Balhara & Verma, 2012).

In addition, a number of clinical evidence support the importance of employment among schizophrenic patients (Carmona, Gomez-Benito, Huedo-Medina, & Rojo, 2017; Llerena, Reddy, & Kern, 2017; Martini et al., 2017). In particular, work is considered one of the main forms of social organization and few individuals with mental illnesses find work opportunities (Martini et al., 2017). In an interesting clinical study, it has been reported that negative symptoms hinder job obtainment and work outcomes in people with schizophrenia, rather than cognitive and positive symptoms, thus an enhanced understanding of the domains of negative symptoms is vital in order to develop treatments that translate into better employment outcomes (Llerena et al., 2017). Antipsychotic pharmacotherapy has shown some success in alleviating positive symptoms, although treatment options for negative and cognitive symptoms remain very limited. Consequently, there is an urgent need to decipher the underlying causes of these symptoms and develop new pharmacological strategies.

In this regard, we have previously shown the suitability of VBS to investigate social withdrawal features and their underlying neurobiology in a mice model of ASD (Bove et al., submitted).

Thus, we quantified GABA and glutamate content in somatosensory cortex of *Pcdh9* WT, HOM KO and HET KO housed in VBS colonies and in standard cages. Our results showed that there were no differences in GABA content among the three genotypes in both VBS colonies and standard housing condition. Otherwise, glutamate was significantly increased only in HOM *Pcdh9*-deficient mice housed in standard cages, while no genotype differences were found in glutamate levels among VBS colonies. In this regard, it has been reported that altered glutamate transmission is a common feature of many neuropsychiatric conditions, including schizophrenia (O'Donovan,

Sullivan, & McCullumsmith, 2017). Moreover, a number of neuropsychiatric diseases associated to schizophrenia-like symptoms are characterized by alterations in sensory processing and perception (Gonzalez-Maeso et al., 2008). In addition, recent evidence reported that drugs interacting with metabotropic glutamate receptors show potential for the treatment of neuropsychiatric diseases (Aghajanian & Marek, 2000; Marek, 2004; Patil et al., 2007). Thus, glutamate neurotransmission in somatosensory cortex area might participate to the development of schizophrenia-like symptoms. Interestingly, a recent evidence reported that a plant-derived compound was able to reduce glutamate-evoked excitotoxicity in the somatosensory cortex acting through the inhibition of non-NMDA glutamate receptors (Borbely et al., 2016). Thus, attenuation of glutamatergic tone might represent a new treatment strategy in different brain diseases, such as schizophrenia, autism, bipolar disorders, major depression and mental retardation (Contractor, Mulle, & Swanson, 2011).

Intriguingly, the VBS, beyond its employment as a tool to assess sociability in a translational way, might also be used as a behavioural paradigm to further test pharmacological treatments aiming at restoring social dysfunctions commonly occurring in several neuropsychiatric disorders, such as social withdrawal.

To the best of our knowledge, this is the first study that evaluated mixed-genotype colonies in VBS. In particular, we showed no differences in sociability and social withdrawal features among *Pcdh9* WT, KO HOM and KO HET, indicating no disrupted sociability of *Pcdh9*-deficient mice when housed together with WT in the VBS. Moreover, the glutamate increase found in HOM *Pcdh9*-deficient mice housed in standard cages was not retrieved in VBS colony housing, suggesting a beneficial effect of this highly social environment on glutamate increase induced by *Pcdh9* genetic ablation.

CHAPTER 6

General discussion

In the present thesis, different animal models and behavioural alterations resembling human depressive-like symptoms shared across neuropsychiatric diseases have been investigated. In addition, we evaluated the neurobiological changes underlying these symptoms, with a focus on the analyses of monoamine and aminoacids neurotransmission, Hypothalamic-Pituitary-Adrenal (HPA) axis parameters and Nerve Grow Factor (NGF) levels. Our results showed that different neurobiological determinants are involved in the etiology of depressive-like symptoms, depending on several aspects, such as comorbidity, lifestyle, social, environmental and dietary factors.

As regarding dietary factors, the influence of Polyunsaturated Fatty Acids (PUFA) on depressive-like states has received great attention during the last decades. In this context, modern Western diets, characterized by low fish consumption in favour of more baked and junk food, have become particularly rich in n-6 PUFA and extremely poor in n-3 PUFA. To assess the consequences of this unbalanced n-6/n-3 PUFA ratio, in chapter 2 and 3 we investigated the effects of n-3 PUFA deficiency in adult female offspring. Since several studies reported that depression is more prevalent in women compared to men (Gorman, 2006; Kokras et al., 2015; Marcus et al., 2005) and that women are more likely to experience comorbid anxiety and depression in response to chronic stress, we focused our attention on female animals (Takahashi et al., 2017). Although the reasons of this gender difference are not fully understood yet, it has been reported that women show different responses to sex hormones, which might ultimately influence behaviour and brain functions (Marrocco & McEwen, 2016). In addition, 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) (Burdge & Wootton, 2002; Giltay et al., 2004). DHA is a key n-3 PUFA involved in the Central Nervous System (CNS) development, and experimental evidence in animals has demonstrated that DHA deficiency during early brain development is deleterious and permanent (Lo Van et al., 2016; Lozada et al., 2017; Maekawa et al., 2017). In our experiments, we used adult female rats fed from conception with three different diets, a diet poor in n-3 PUFA (n-6/n-3 ratio of 20:1), a diet enriched in n-3 PUFA (n-6/n-3 ratio of 1:2) and a control diet (n-6/n-3 ratio of 5:1). Our results reported that chronic exposure to n-3 PUFA deficient diet leads to highly detrimental consequences in behavioural and neurochemical parameters related to depressive- and anxiety-like symptoms. From a behavioural point of view, we performed Forced Swimming Test (FST) and Open Field test. In particular, in the FST, we found an increase in immobility and a decrease in swimming frequency in n-3 PUFA deficient females, suggesting that

n-3 PUFA deficiency is able to induce a depressive-like behaviour. Moreover, in the Open Field test, we showed an increase in time spent performing self-grooming and time spent in the periphery of the arena, both indexes of anxiety-like behaviour. Hence, our behavioural results showed that lifelong n-3 PUFA deficiency is able to elicit depressive- and anxiety-like symptoms in female rats. Therefore, we investigated neurochemical changes underlying these behavioural alterations. Firstly, we focused on monoamine systems commonly studied in depressive-like pathogenesis, measuring 5-HT and 5-HT metabolism, NA and DA. As regarding serotoninergic neurotransmission, we found a decrease in cortical 5-HT and an increase in 5-HT turnover in n-3 PUFA deficient females. Therefore, we hypothesized that n-3 PUFA deficiency influences serotonin neurotransmission acting through the inflammatory pathways. Accordingly, McNamara and colleagues showed that n-3 PUFA deficiency was positively correlated with pro-inflammatory cytokine production, ultimately leading to an increase in central 5-HT turnover, while n-3 PUFA supplementation prevented this negative effect (McNamara et al., 2010). As regarding NA and DA, no differences in prefrontal cortex were detected among the different diets. Interestingly, we found an increase in amygdaloidal NA and 5-HT content in n-3 PUFA deficient females, suggesting that n-3 PUFA deficiency affects this brain area deeply related to stress, emotional and anxiety-like disorders. Indeed, 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 accordance with our results, it has been demonstrated that stressors increase noradrenaline release in amygdala, leading to anxiety disorders induced by stress (Hakamata et al., 2017; Weidenfeld et al., 2002), and in addition, Johnson and colleagues showed that serotonin depletion in the basolateral amygdala led to a decrease in anxiety-like behaviour in social interaction and open field tests (Johnson et al., 2015). Hence, our results indicated that lifelong n-3 PUFA deficiency is able to evoke depressive and anxiety-like symptoms, affecting monoamine neurotransmissions, in particular NA, 5-HT and 5-HT turnover, not only in prefrontal cortex, but also in amygdala.

Moreover, chronic stress and adverse lifestyle have been indicated as triggering and worsening factors for neuropsychiatric diseases development. In particular, chronic stress is considered an important risk factor for the development of depressive-like states (Lee & Rhee, 2017). In this regard, 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 and depression in children and adolescents (Herman & Tasker, 2016; Kallen et al., 2007). 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. Accordingly, elevated cortisol levels and HPA axis dysregulation represent the most frequent alteration occurring in patients affected by major depressive disorder (Stetler & Miller, 2011).

Furthermore, the central role of soluble Aβ peptide in stress response is becoming evident, although the exact biological mechanism leading to Aβ accumulation after stress challenges has to be fully elucidated yet (Morgese, Schiavone, et al., 2017). In this regard, increased CRF and glucocorticoid levels have been associated with high soluble Aβ levels (Catania et al., 2009; Dong & Csernansky, 2009; Morgese, Schiavone, et al., 2017). In addition, we have previously shown that Aβ central injection elicits HPA axis dysfunctions in male rats (Morgese et al., 2014).

During the last decades, increasing evidence are pointing towards the emerging role of soluble Aβ in the pathophysiology of neurodegenerative illnesses, not only limited to Alzheimer's disease. In addition, data from preclinical research have associated different risk factors for depression with increased soluble Aβ production in the brain (Catania et al., 2009; Schiavone, Tucci, et al., 2017). In this regard, our group has previously shown that central Aβ1-42 administration in male rats was able to evoke a depressive-like phenotype (Colaianna et al., 2010), characterized by increased immobility frequency in the FST and reduced cortical 5-HT. Thus, in this thesis, we administered for the first time the soluble Aβ peptide in female rats, confirming the depressive-like phenotype we found in males also in female animals. In particular, we found an increase in immobility and a decrease in swimming frequency in FST in female rats fed with the control diet, accompanied by a decrease in cortical 5-HT. Once the animal model of ab-induced depressive-like symptoms in females has been validated, we assessed the effect of n-3 PUFA enriched diet on Aβ -treated female rats. Interestingly, our results showed that n-3 PUFA lifelong supplementation was able to restore the Aβ -induce deficits in depressive-like behaviour in FST and the reduction in cortical 5-HT, suggesting a protective role of n-3 PUFA towards the Aβ -induced depressive-like phenotype. Moreover, Aβ-treated females fed with n-3 PUFA enriched diet showed increased DA and NGF, endorsing the beneficial and potentially therapeutic effect of n-3 PUFA supplementation. In accordance with our results, observational studies evidenced that fish oil supplementation or increased n-3 PUFA blood levels are linked to reduced risk of cognitive decline and depression (P.

Y. Lin & Su, 2007; Vinot et al., 2011), and n-3 PUFA have been shown to ameliorate cognitive performances and neurodegenerative processes (Bo et al., 2017; Lauritzen et al., 2017; McNamara, Asch, Lindquist, & Krikorian, 2017). In addition, several studies supported the benefits of n-3 PUFA supplementation in the treatment of *post partum* depressive symptoms (Chong et al., 2015; Sparling et al., 2017; Vaz et al., 2017).

Interestingly, in our experiments n-3 PUFA supplementation showed differences in naïve females compared to Aβ treated animals, indicating a positive effect only in presence of Aβ-induced dysfunctions. These results endorse the hypothesis of a possible therapeutic use of n-3 PUFA supplementation, acting in synergy with antidepressants or even alone.

Furthermore, we found an increase in plasmatic Aβ in n-3 PUFA deficient female rats, suggesting that soluble AB peptide might be involved in the pathogenesis of depressive-like symptoms and that, ultimately, might be used as a putative plasmatic biomarker for a number of neuropsychiatric diseases exhibiting depressive-like symptoms.

Moreover, we found a decrease in cortical Nerve Grow Factor (NGF) levels in female rats fed with a diet poor in n-3 PUFA. Interestingly, clinical studies have detected reduced levels of NGF in patients with major depression when compared with healthy individual controls (Diniz et al., 2014; Xiong et al., 2011). In addition, treatment with certain antidepressants has increased NGF levels in both clinical and experimental studies (Hassanzadeh & Rahimpour, 2011; Wiener et al., 2015). Evidence from animal studies reported decreased levels of NGF in specific brain areas of different mouse models related to depression, such as anxiety disorders and stress-induced diseases (Y. W. Chen et al., 2015). In accordance with our results, Balogun et al. reported a decrease of NGF mRNA expression in C57BL/6J mice fed with low n-3 PUFA diet (Balogun & Cheema, 2014). Finally, NGF might contribute to the neurobiological mechanisms that give rise to depressive-like symptoms.

Taken together, our data suggest that monoamine impairments, accompanied by NGF alterations and HPA axis dysfunctions might be considered as important neurobiological determinants contributing to the pathogenesis of depressive-like symptoms induced by n-3 PUFA deficiency and soluble ab administration.

Furthermore, during last decades, a number of evidence suggested that altered function of the aminoacid neurotransmitter systems, especially gamma-aminobutyric acid (GABA) and glutamate systems, might contribute significantly to the etiopathogenesis of neuropsychiatric disorders (Sanacora, 2010). In this regard, we found a significant decrease in 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 accordance with our results, reduced GABA concentrations have been observed in plasma and cerebrospinal fluid of depressed patients (Bhagwagar & Cowen, 2008), and neuroimaging data has shown lowered levels of GABA in the occipital cortex of depressed subjects (Price et al., 2010). In addition, patients suffering from schizophrenia, depression, autism spectrum disorders (ASD) and bipolar disorders appear to have lowered central and peripheral GABA levels when compared to healthy controls (Lewis, 2014; Romeo et al., 2017). As regarding 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 et al., 2011; Hashimoto et al., 2007; Ogawa et al., 2017). In addition, there are several studies suggesting that glutamate is elevated in plasma of depressed patients (Kendell et al., 2005). Furthermore, increasing preclinical evidence suggests that glutamate plays an important role in the activation of the HPA axis, by inducing the adrenocorticotropin hormone (Zelena et al., 2005).

Therefore, our result indicated that GABA and glutamate tone are both involved in anxiety- and depressive-like symptoms induced by n-3 PUFA deficiency.

On the other hand, recent studies showed that decreasing GABA led to decreased sociability (Paine et al., 2017). Thus, in order to deeply investigate depression core symptoms in a translational way, the social sphere need to be taken into account. An important depressive-like symptom affecting the social sphere is social withdrawal. Social withdrawal, defined as lack of desire to have social contact, is an early symptom of a wide variety of neuropsychiatric diseases, including schizophrenia, ASD and major depression. In this regard, in chapter 4 and 5, we investigated behavioural alterations related to sociability and social withdrawal, using a behavioural paradigm called the Visible Burrow System (VBS). In order to fully evaluate social dysfunctions, a group-housed environment is needed, indeed the VBS is a semi-natural environment that mimic natural open spaces and burrows in order to analyze social dynamics that naturally occur in rodent colonies. Thus, we used the VBS to study C57BL/6J colonies as control strain and two different mutant lines, BTBR inbred strain and *Pcdh9*-deficient line. BTBR strain is a widely used strain for its similarities with human ASD deficits, such as repetitive behaviour, impaired communication and reduced social interactions (Cai et al., 2017; Yoshimura et al., 2017).

Pcdh9 gene KO mice have altered sensory information processing and social behaviour, phenotypes that are relevant across the neuropsychiatric spectrum (Hirabayashi & Yagi, 2014; Morishita & Yagi, 2007; Xiao, Zheng, et al., 2017). In particular, in chapter 4, we investigated social dynamics and studied social withdrawal features in BTBR and C57BL/6J colonies in the VBS paradigm. Our results showed that BTBR mice performed less social behaviours and have a preference for non-social behaviours compared to C57BL/6J mice in a group-housed mixed-sex environment. Although our results are in line with previous literature regarding BTBR strain and decrease of sociability (Meyza et al., 2015), this was the first study showing also an effective increase in non-social behaviours. In particular, our results reported a trend towards social withdrawal in BTBR mice, opening to a deep investigation of the underlying neurobiology that gives rise to this important symptom.

Hence, our study validated the suitability of VBS as a behavioural paradigm to assess sociability and social withdrawal features, investigating mixed-sex group-housed dynamics in rodents.

Interestingly, social withdrawal is also considered one of the core negative symptoms of schizophrenia (Seillier & Giuffrida, 2016, 2017). The negative symptoms of schizophrenia can be classified into primary negative symptoms, which are etiologically related to the pathophysiology of schizophrenia, and secondary negative symptoms, which result from other factors, such as positive symptoms, medication, depression and anxiety (Kirkpatrick, 2014). It has been demonstrated that the negative symptoms, such as blunted affect, alogia, social withdrawal, anhedonia and avolition, affect the patients' quality of life more than positive symptoms and are more difficult to treat (Foussias & Remington, 2010; Hanson, Healey, Wolf, & Kohler, 2010). Consequently, there is an urgent need to unravel the underlying causes of these symptoms and develop new pharmacological strategies.

In this regard, in chapter 5, we investigated VBS colonies composed of *Pcdh9* HOM and HET KO and WT, in order to evaluate social features in *Pcdh9*-deficient mice. Interestingly, in this preliminary analysis we found no differences in terms of social behaviours and non-social behaviours among the three genotypes, indicating no disrupted sociability of *Pcdh9*-deficient mice when housed together with WT in the VBS.

However, we did not investigated sociability in a standard environment or social behaviours among *Pcdh9* colonies constituted by the same genotype, thus future studies are required to better understand HOM *Pcdh9* KO social phenotype without the presence of social stimuli. Also,

the study was performed in a relatively low numbers of colonies, and will need expansion of this current data set. Indeed, VBS colonies formed by mixed-genotype and mixed-sex mice are considered highly social environment, and these strong social stimuli might be helpful improve putative social deficits.

In conclusion, the VBS can be used as a tool to study behavioural dysfunctions and might be further used as a behavioural paradigm to test pharmacological treatments aiming at restoring social dysfunctions commonly occurring in several neuropsychiatric disorders, such as social withdrawal.

However, VBS paradigm still has to be scored manually. This big disadvantage does not allow to track all the behaviour over the full period of time and thus the throughput is low. Further studies are currently being conducted to develop an automatic tracking system. The automatic system would also be helpful to investigate social networks, dominance and hierarchy within colonies.

Furthermore, in order to investigate neurobiology behind sociability and social withdrawal, we analyzed GABA and glutamate content in prefrontal cortex and amygdala of C57BL/6J and BTBR colonies and we found a significant decrease of GABA and a significant increase of glutamate in both areas of BTBR mice. These results are in line with recent evidence suggesting that attenuation of GABA tone might result in the disruption of sociability (Paine et al., 2017). However, different social factors contribute to sociability dysfunctions, such as social motivation, social anxiety and social cognition (Kennedy & Adolphs, 2012), hence future studies will be conducted to assess the involvement of these different social components in the sociability impairment. Moreover, it has been widely demonstrated that imbalances in the excitatory and inhibitory synaptic transmission might be responsible of severe neuropsychiatric-related symptoms (Gao & Penzes, 2015; Nelson & Valakh, 2015; Sorce et al., 2010; Yizhar et al., 2011). Accordingly with our results, a reduction in social interactions and social preference when activating optogenetically cortical pyramidal neurons has been showed (Yizhar et al., 2011). In addition, it has been reported that lesions in the medial prefrontal cortex increased social behaviour in the social interaction test (Shah & Treit, 2003). In conclusion, the decrease in GABA and the corresponding increase in glutamate in prefrontal cortex and amygdala might be responsible of the decrease in social behaviour and increase in social withdrawal characteristics in BTBR strain. Thus, enhancement of GABA neurotransmission and consequent attenuation of

glutamatergic tone might be a possible therapeutic strategy to treat social withdrawal symptoms that primarily occur in many neuropsychiatric and neurodegenerative diseases.

Furthermore, we measured GABA and glutamate levels in somatosensory cortex of *Pcdh9* colonies and we found that there were no differences in GABA content among the three genotypes in both VBS colony and standard housing condition. These results are in line with behavioural outcomes, in which no genotype differences in sociability were detected. Otherwise, glutamate was significantly increased only in HOM *Pcdh9*-deficient mice housed in standard cage, while no genotype differences were found in glutamate levels among VBS colonies.

In this regard, a number of neuropsychiatric diseases, such as psychosis associated to schizophrenia-like symptoms, are characterized by alterations in sensory processing and perception (Gonzalez-Maeso et al., 2008).

Interestingly, Bruining and colleagues, using quantitative-trait locus mapping, demonstrated that HOM *Pcdh9*-deficient mice showed long-term social recognition impairments, suggesting an important involvement of *Pcdh9* in the sensory cortex development and sensorimotor phenotypes (Bruining et al., 2015). In addition, recent evidence reported that drugs interacting with metabotropic glutamate receptors show potential for the treatment of mental diseases (Aghajanian & Marek, 2000; Marek, 2004; Patil et al., 2007). Thus, we hypothesized that glutamate neurotransmission in somatosensory cortex area participate to the development of the excitotoxicity reported in different neuropsychiatric symptoms.

In conclusion, the glutamate increase found in HOM *Pcdh9* KO housed in standard cages and not found in HOM *Pcdh9* KO housed in VBS colonies points towards a putative beneficial effect of this highly social environment on glutamate excitotoxicity induced by *Pcdh9* deletion.

Overall conclusions

In the present thesis, depressive-like symptoms shared among different neuropsychiatric disorders have been investigated, using animal models and behavioral paradigms. In addition, different neurobiological substrates underlying depressive-like symptoms have been analyzed.

Considering the heterogeneous nature of depressive-like symptoms, we developed a multifactorial approach based on different neurobiological determinants, interconnected with each other. Thus, the influence of social, environmental and dietary factors, together with comorbidities, need to be considered to ultimately target the correct neurobiological substrates that give rise to different

depressive-like symptoms shared across several brain diseases. This transdiagnostic perspective opens a new scenario towards the progression of precision medicine, ultimately aiming to develop new effective and safe treatments for individual depressive-like symptoms.

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ENGLISH SUMMARY

Depression is one of the most common psychiatric diseases; indeed the prevalence of depressive symptoms has reached epidemic proportions during the last few decades. Several studies reported that depression is more prevalent in women compared to men. Although the reasons for this gender pre-dominance in depression is not understood, women show different hormonal responses that might ultimately influence behaviours and brain functions.

The core symptoms of depression include depressed mood, anhedonia (reduced ability to experience pleasure from natural rewards), irritability, difficulties in concentrating, social withdrawal (withdrawal from social contact that derives from indifference or lack of desire to have social contact) and abnormalities in appetite and sleep, the so called “neurovegetative symptoms”.

Depression has shown to be comorbid with several neuropsychiatric diseases, such as schizophrenia, bipolar disorders, Alzheimer’s diseases, anxiety disorders, autism spectrum disorders (ASD) and stress-related diseases. Moreover, depression often occurs during the prodromic phase of Alzheimer’s disease, schizophrenia and bipolar disorders.

Diets, genetics and lifestyle contribute to the onset and progression of mental illnesses. 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, in chapter 2 and 3 of the present thesis, effects of n-3 PUFA deficient and n-3 PUFA enriched diets on female rat offspring have been investigated. Our results reported that chronic exposure to n-3 PUFA deficient diet leads to highly detrimental consequences in behavioural and neurochemical parameters related to depressive- and anxiety-like symptoms. In particular, we found an increase in immobility and a decrease in swimming frequency in Forced Swimming test in n-3 PUFA deficient females, and, in the Open Field test, we showed an increase in time spent performing self-grooming and time spent in the periphery of the arena. Hence, our behavioural results showed that lifelong n-3 PUFA deficiency is able to elicit depressive- and anxiety-like symptoms in female rats. Therefore, we investigated neurochemical changes underlying these behavioural alterations. We found a significant decrease in cortical serotonin and Nerve Growth Factor in n-3 PUFA deficient females, accompanied by an increase in serotonin turnover. Moreover, in chapter 3, we showed that n-3 PUFA deficient diet led to hyperactivation of the HPA axis, in particular increase in hypothalamic noradrenaline and corticotrophin-releasing factor and

also increase in plasmatic corticosterone, accompanied by an increase in amygdaloidal noradrenaline and serotonin and an increase in glutamate and a decrease of GABA in both prefrontal cortex and amygdala. Ultimately, we found an increase in plasmatic soluble beta amyloid $(A\beta)_{1-42}$ peptide in females exposed to n-3 PUFA deficient diet.

Interestingly, soluble $A\beta_{1-42}$ peptide is 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 Aβ injection is able to elicit depressive-like phenotype in male rats. Thus, in chapter 2, we reproduced for the first time the Aβ-induced depressive-like model in female rats, evaluating behavioural and neurochemical outcomes. Our results confirmed the Aβ-induced depressive-like profile also in female rats. Moreover, the Aβ-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. Taken together, our data suggest that monoamine impairments, accompanied by Nerve Growth Factor alterations and HPA axis dysfunctions, might be considered important neurobiological determinants contributing to the pathogenesis of depressive-like symptoms induced by n-3 PUFA deficiency and soluble Aβ administration.

During the last decades, diagnosis in psychiatry only focused on subjective symptoms and observable signs. Although symptoms are an important starting point, genetics and neurobiology underlying these symptoms need to be deeply investigated. To achieve this purpose, animal models can be really helpful to longitudinally study behavioural alterations resembling human symptoms, and ultimately investigate the underlying neurobiology in order to unravel the etiopathogenesis. Therefore, in this thesis, we focused on depressive-like symptoms that occur in several neuropsychiatric and neurodegenerative diseases, and, using different animal paradigms and models, we tried to disentangle the neurobiological determinants behind these symptoms.

Intriguingly, in order to deeply investigate depression core symptoms in a translational way, the social sphere need to be taken into account. An important depressive-like symptom affecting the social sphere is social withdrawal. Social withdrawal, defined as lack of desire to have social contact, is an early symptom of a wide variety of neuropsychiatric diseases, including schizophrenia, Autism Spectrum Disorders (ASD) and major depression.

In chapter 4 and 5, we investigated behavioural alterations related to sociability and social withdrawal, using a behavioural paradigm called the Visible Burrow System (VBS). The VBS is a

semi-natural environment, with burrows and an open area, useful to study social dynamics that naturally occur in mixed-sex rodent colonies, firstly developed by Blanchard group. In particular we identified and validated behavioural readouts to assess sociability and social withdrawal features in C57BL/6J mice colonies, used as control strain, and two mutant lines, BTBR inbred strain and a *Pcdh9*-deficient line. The BTBR strain is a widely used strain for its similarities with human ASD deficits, such as repetitive behaviour, impaired communication and reduced social interactions, while *Pcdh9* gene Knockout (KO) mice are known to affect social behaviour in mice and may, through the core deficit in sensory processing be relevant to a wide variety of neuropsychiatric disorders, such as schizophrenia, major depression and ASD.

Our results showed that BTBR mice performed less social behaviours and have a preference for non-social behaviours compared to C57BL/6J mice in the VBS. Thus, our results reported a trend towards social withdrawal in BTBR mice, opening to a deep investigation of the underlying neurobiology that gives rise to this important symptom. Hence, our study validated the suitability of VBS as a behavioural paradigm to assess sociability and social withdrawal features. Conversely, we found no differences in terms of social behaviours and non-social behaviours among VBS colonies composed of *Pcdh9* Homozygous (HOM) and Heterozygous (HET) KO and Wild Type (WT) mice, indicating no disrupted sociability of *Pcdh9*-deficient mice when housed together with WT in the VBS. In this regard, future studies are required to better understand the HOM *Pcdh9* KO social phenotype without the presence of social stimuli. Indeed, VBS colonies formed by mixed-genotype and mixed-sex mice are considered highly social environment, and these strong social stimuli might be helpful to improve putative social deficits. In conclusion, the VBS can be used as a tool to study behavioural dysfunctions and might be further used as a behavioural paradigm to test pharmacological treatments aiming at restoring social dysfunctions that commonly occur in several neuropsychiatric disorders, such as social withdrawal.

Furthermore, in order to investigate neurobiology behind sociability and social withdrawal, in chapter 4, we analyzed GABA and glutamate content in prefrontal cortex and amygdala of C57BL/6J and BTBR colonies and we found a significant decrease of GABA and a significant increase of glutamate in both areas of BTBR mice.

Intriguingly, the decrease in GABA and the corresponding increase in glutamate in prefrontal cortex and amygdala might be responsible for the observed decrease in social behaviour and increase in social withdrawal characteristics in BTBR strain. Thus, enhancement of GABA

neurotransmission and consequent attenuation of glutamatergic tone might be a possible therapeutic strategy to treat social withdrawal symptoms that primarily occur in many neuropsychiatric and neurodegenerative diseases.

Moreover, in chapter 5, we measured GABA and glutamate levels in somatosensory cortex of *Pcdh9* colonies and we found that there were no differences in GABA content among the three genotypes in both VBS colonies and standard housing condition. Otherwise, glutamate was significantly increased only in HOM *Pcdh9*-deficient mice housed in standard cage, while no genotype differences were found in glutamate levels among VBS colonies. Hence, the glutamate increase found in HOM *Pcdh9* KO mice housed in standard cages and not found in HOM *Pcdh9* KO mice housed in VBS colonies points towards a putative beneficial effect of this highly social environment on glutamate increase induced by *Pcdh9* deletion.

In conclusion, in the present thesis, we investigated the heterogeneity underlying the neurobiology of depressive like-symptoms, that might be shared across different neuropsychiatric disorders. In this way a multifactorial perspective will be developed. Hence, in order to improve the current pharmacological approach and further develop new safe and effective treatments, the influence of social, environmental and dietary factors, together with comorbidities, need to be considered to ultimately target the correct neurobiological substrates that give rise to different depressive-like symptoms shared across various brain diseases.

NEDERLANDSE SAMENVATTING

Depressie is één van de vaakst voorkomende psychiatrische aandoeningen. In de afgelopen decennia is de prevalentie van depressieve symptomen toegenomen tot epidemische proporties. Daarnaast, geven verscheidene wetenschappelijke studies aan dat depressies vaker voorkomen bij vrouwen. Hoewel de reden voor dit verschil tussen de geslachten niet geheel duidelijk is, is wel bekend dat vrouwen anders reageren op geslachtshormonen die uiteindelijk hun invloed hebben op het gedrag en de hersenen.

De kernsymptomen van depressie bestaan uit een sombere stemming, verlies van interesse of plezier, prikkelbaarheid, moeite met concentreren, sociale terugtrekking (het terugtrekken van sociaal contact dat voorkomt uit onverschilligheid of een gebrek aan verlangen naar sociaal contact) en abnormaliteiten in eetlust en slaap, de zogenaamde “neurovegetatieve symptomen”.

Wetenschappelijk onderzoek heeft aangetoond dat depressie vaak een comorbide stoornis is bij verschillende neuropsychiatrische aandoeningen, zoals schizofrenie, bipolaire stoornissen, de ziekte van Alzheimer, angststoornissen, autisme spectrum stoornissen en stress-gerelateerde aandoeningen. Daarnaast uit depressie zich vaak in de prodromale fase van de ziekte van Alzheimer, schizofrenie en bipolaire stoornissen.

Het dieet, de genen en de levensstijl zijn allen factoren die bijdragen aan het ontstaan en de progressie van mentale stoornissen. Gelet op de factoren betreffende het dieet hebben meervoudig onverzadigde vetzuren de laatste decennia veel aandacht ontvangen, dit komt vooral doordat er in Westerse diëten weinig n-3 vetzuren (ook wel omega-3 vetzuren) worden genuttigd. In hoofdstuk 2 en 3 van dit proefschrift worden de effecten van diëten met een tekort aan n-3 vetzuren en rijk aan n-3 vetzuren op het nageslacht van vrouwelijke ratten bestudeerd. Onze resultaten geven aan dat chronische blootstelling aan diëten met een tekort aan n-3 vetzuren leidt tot zeer negatieve consequenties in zowel gedrags- als neurochemische parameters gerelateerd aan symptomen die vergelijkbaar zijn met depressie en angststoornissen. We vonden met name een verhoogde immobiliteit en een verlaagde zwem frequentie in de “Forced Swimming” test in n-3 vetzuur deficiënte vrouwtjes. Daarnaast poetsen deze vrouwtjes zichzelf meer en spendeerden ze meer tijd in de periferie in de “Open Field” test. Deze resultaten, met betrekking tot het gedrag, laten zien dat een levenslange deficiëntie van n-3 vetzuren kan zorgen voor symptomen vergelijkbaar met depressie en angststoornissen in vrouwelijke ratten. Dit heeft ertoe geleid dat we de neurochemische veranderingen die ten grondslag liggen aan deze gedragsveranderingen

zijn gaan bestuderen. We vonden een significante verlaging van corticale Serotonine en zenuwgroeifactor (beter bekend als nerve growth factor of NGF) in n-3 vetzuur deficiënte vrouwtjes, wat gepaard ging met een verhoging van de omzetting van Serotonine. Daarnaast lieten we in hoofdstuk 3 zien dat het n-3 vetzuur deficiënte dieet leidde tot hyperactivatie van de HPA-as, voornamelijk een verhoging van hypothalamisch noradrenaline en "corticotropin-releasing factor" en ook een verhoging in plasma corticosteron, wat samenging met verhoogd amygdaloidale noradrenaline en serotonine en een verhoging van glutamaat en verlaging van GABA in zowel de prefrontale cortex als de amygdala. Tenslotte vonden we een verhoging van plasma oplosbaar bèta amyloïde $(A\beta)_{1-42}$ peptide in vrouwtjes die blootgesteld waren aan het dieet met een tekort aan n-3 vetzuren.

Oplosbaar $A\beta_{1-42}$ peptide krijgt veel aandacht in de literatuur omdat het een rol speelt in de ontwikkeling van depressie, maar ook omdat depressie zeer vaak co morbide is met de ziekte van Alzheimer en andere neuropsychiatrische aandoeningen. In deze lijn hebben we in het verleden laten zien dat centrale Aβ injecties kunnen zorgen voor een depressie-achtig fenotype in mannelijke ratten.

In hoofdstuk 2 hebben we, voor het eerst, het Aβ-geïnduceerde depressie-achtige model in vrouwelijke ratten gereproduceerd, waarbij we hebben gelet op gedragsmatige en neurochemische uitkomsten. Onze resultaten bevestigen het Aβ-geïnduceerde depressie-achtige profiel ook in vrouwelijke ratten. Verder lieten we zien dat het Aβ-geïnduceerde depressie model kon worden tenietgedaan door supplementatie van n-3 vetzuren. Dit wijst op een mogelijk therapeutische rol voor n-3 vetzuren in de behandeling van depressieve stoornissen. Samen suggereert onze data dat mono-amine verminderingen, vergezeld door veranderingen in NGF en disfuncties in de HPA-as beschouwd kunnen worden als belangrijke neurobiologische factoren die bijdragen aan de pathogenese van depressie-achtige symptomen geïnduceerd door een tekort aan n-3 vetzuren en de toediening van oplosbaar Aβ.

In de afgelopen decennia richtte de diagnose in de psychiatrie zich alleen op subjectieve symptomen en observeerbare verschijnselen. Hoewel symptomen een belangrijk beginpunt zijn, is het nodig om de onderliggende genetica en neurobiologie grondig te bestuderen. Om dit doel te kunnen bereiken kunnen diermodellen van grote dienst zijn om longitudinaal gedragsveranderingen te bestuderen die vergelijkbaar zijn met humane symptomen en uiteindelijk de onderliggende neurobiologie bloot te leggen die ten grondslag ligt aan het ontstaan en het

verloop van het ziektebeeld. Om deze reden concentreren we ons, in dit proefschrift, op depressie-achtige symptomen die voorkomen bij verschillende neuropsychiatrische aandoeningen en neurodegeneratieve ziektebeelden. Door gebruik te maken van verschillende diermodellen en paradigma's proberen we de neurobiologische factoren achter deze symptomen te ontrafelen. Om de kernsymptomen van depressie te kunnen doorgronden op translationele wijze, is het belangrijk om het sociale domein in acht te nemen. Een belangrijk symptoom van depressie binnen het sociale domein is sociale terugtrekking. Sociale terugtrekking kan worden gedefinieerd als een gebrek aan verlangen naar sociaal contact. Het uit zich al in de vroege fase van verscheidene neuropsychiatrische aandoeningen, waaronder schizofrenie, ASS en depressie.

Hierop gelet bestudeerden we in hoofdstuk 4 en 5 de veranderingen in gedrag die gerelateerd zijn aan sociabiliteit en sociale terugtrekking, waarbij we gebruik maken van een paradigma genaamd het "Visible Burrow System" (VBS). Het VBS is een semi-natuurlijke omgeving, met een open arena en een gangensysteem, dat gebruikt kan worden om de sociale dynamiek te bestuderen die uit zichzelf wordt gevormd in kolonies van gemixte geslachten. Dit paradigma werd als eerste gebruikt en ontwikkeld door de Blanchard groep. In het bijzonder identificeerden en valideerden wij gedragsmatige factoren om sociabiliteit en sociale terugtrekking te bestuderen in C57BL/6J muizen kolonies (gebruikt als referentie) en in kolonies van twee mutant lijnen, de BTBR inteelt lijn en een lijn waarbij het gen *Protocadherin 9* (*Pcdh9*) mist. De BTBR lijn wordt door velen gebruikt om zijn gelijkenissen met de beperkingen die te zien zijn in het humane ASS, zoals repetitief gedrag, verslechterde communicatie en verminderde sociale interacties. Daarnaast is recent ontdekt dat *Pcdh9* is betrokken bij sensorische problemen zoals ook gezien in neuropsychiatrische aandoeningen zoals schizofrenie, depressie en ASS. Onze resultaten laten zien dat de BTBR muis minder sociaal gedrag laten zien en dat ze een voorkeur hebben voor niet-sociaal gedragingen in het VBS vergeleken met de C57BL/6J controle lijn. Hiermee laten onze resultaten een trend richting sociale terugtrekking zien in de BTBR lijn, wat de kans biedt om de onderliggende neurobiologie te onderzoeken die ten grondslag ligt aan dit centrale symptoom. Ons onderzoek bevestigt hiermee de geschiktheid van het VBS als een paradigma om factoren gerelateerd aan sociabiliteit en sociale terugtrekking te bestuderen. Daarentegen vonden we geen verschillen in sociaal en niet-sociaal gedrag in kolonies die bestonden uit muizen homozygoot en heterozygoot deficiënt voor *Pcdh9* en wild type muizen (met alle genen intact). Hieruit kunnen we opmaken dat *Pcdh9* deficiënte muizen geen verstoorde sociabiliteit laten zien in het VBS wanneer deze samen in

een kolonie leven met wild type muizen. Hierop gelet is het nodig om verder onderzoek te doen om een beter begrip te krijgen van het sociale fenotype van de homozygote *Pcdh9*-deficiënte muis zonder de aanwezigheid van sociale stimuli. Kolonies in het VBS die bestaan uit muizen van verschillende genotypes en geslachten kunnen namelijk worden gezien als een sociaal rijke omgeving en deze sterk sociale stimuli kunnen helpen bij het herstellen van de vermoedelijk sociale problemen. Hieruit kunnen we concluderen dat het VBS kan worden gebruikt als hulpmiddel om gedragsmatige problemen te bestuderen en dat het in de toekomst kan worden gebruikt als paradigma om farmaceutische interventies te testen gericht op het herstellen van sociale dysfuncties die veel voorkomend zijn in verscheidene neuropsychiatrische aandoeningen, zoals sociale terugtrekking.

Om de neurobiologie achter sociabiliteit en sociale terugtrekking te bestuderen hebben we in hoofdstuk 4 de aanwezigheid van de neurotransmitters GABA en glutamaat in de prefrontale cortex en de amygdala van C57BL/6J en BTBR muizen kolonies. In de BTBR lijn vonden we een significante verlaging van GABA en een significante verhoging van glutamaat in zowel de prefrontale cortex als de amygdala. De verlaging van GABA en de corresponderende verhoging van de aanwezigheid van glutamaat in beiden hersengebieden zouden verantwoordelijk kunnen zijn voor de geobserveerde vermindering van sociaal gedrag en de toenamen van factoren gerelateerd aan sociale terugtrekking. Dit wekt de suggestie dat een verbetering van de GABA neurotransmissie en de daaruit volgende vermindering van de glutamerge concentratie een mogelijke strategie zou kunnen zijn om de symptomen sociale terugtrekking te kunnen behandelen zoals die voorkomen in vele neuropsychiatrische aandoeningen en neurodegeneratieve ziektebeelden.

Daarnaast hebben we in hoofdstuk 5 GABA en glutamaat niveaus gemeten in de somatosensorische cortex van de *Pcdh9* kolonies. Hier vonden we dat er geen verschil is in GABA tussen de drie genotypes, niet in de VBS kolonies en ook niet in de standaard huisvesting condities. Daarentegen was glutamaat significant verhoogd in de homozygote deficiënte muizen voor *Pcdh9* in de normale omstandigheden. Terwijl geen verschil was gevonden in glutamaat concentraties in de VBS kolonies. Dit wekt het vermoeden op dat de sociaal rijke omgeving in de VBS kolonies een voordelig effect heeft op de glutamaat concentraties in de muizen die homozygoot deficiënt zijn voor *Pcdh9*.

Samenvattend, in dit proefschrift hebben we de heterogene onderliggende neurobiologie van depressie-achtige symptomen onderzocht, die voorkomen bij meerdere neuropsychiatrische ziektes. Hierdoor is een multifactorieel perspectief gecreëerd. Om vooruit te komen in het ontwikkelen van nieuwe effectieve en veilige behandelingen voor deze ziektes, moet de invloed van sociale, omgevings- en dieet factoren overwogen worden. Dit is nodig om uiteindelijk de juiste neurobiologische substraten te vinden die zorgen voor de depressieve symptomen voorkomend bij een variëteit aan neuropsychiatrische ziektebeelden.

SOMMARIO

La depressione è una delle malattie psichiatriche più frequenti, la cui prevalenza ha raggiunto proporzioni epidemiche negli ultimi decenni. A questo proposito, diversi studi riportano una maggior incidenza di depressione nelle donne rispetto agli uomini, sebbene le ragioni di questa differenza di genere non siano state ancora pienamente comprese. I principali sintomi della depressione includono umore depresso, anedonia (ridotta capacità di provare appagamento o interesse per attività comunemente ritenute piacevoli), irritabilità, difficoltà di concentrazione, ritiro sociale e anomalie dell'appetito e del sonno, i cosiddetti "sintomi neurovegetativi".

Numerosi disordini mentali come la schizofrenia, i disturbi bipolari, il Morbo di Alzheimer, i disturbi d'ansia, i disturbi dello spettro autistico (ASD) e le malattie correlate allo stress, possono manifestarsi come comorbidità della malattia depressiva. Inoltre, la depressione si presenta spesso durante la fase prodromica del Morbo di Alzheimer, della schizofrenia e dei disturbi bipolari.

Diete, fattori genetici e stile di vita contribuiscono all'insorgenza e alla progressione delle malattie mentali. Per quanto riguarda i fattori dietetici, gli acidi grassi polinsaturi (PUFAs) hanno ricevuto grande attenzione negli ultimi decenni, soprattutto a causa dell'aumentato consumo di junk food, tipico dei Paesi Occidentali, che ha portato ad una drammatica riduzione nell'assunzione di n-3 PUFA e ad uno smodato aumento del consumo di n-6 PUFA. A questo proposito, nei capitoli 2 e 3 di questa tesi, sono presentati i nostri dati riguardanti gli effetti di una dieta ricca di n-3 PUFA e di una dieta povera di n-3 PUFA su ratte femmine sottoposte, dal concepimento fino all'età adulta, alle sopracitate supplementazioni dietetiche. I nostri risultati hanno mostrato che l'esposizione cronica alla dieta povera di n-3 PUFA può portare ad alterazioni di parametri comportamentali e neurochimici, correlate a sintomi depressivo-ansiosi. In particolare, nelle ratte sottoposte alla dieta povera di n-3 PUFA, è stato riscontrato un aumento della frequenza dell'immobilità e una diminuzione della frequenza di attività nel test del nuoto forzato (FST). In un altro set di animali sottoposti allo stesso trattamento, l'Open Field test ha mostrato un aumento del tempo trascorso facendo self-grooming e di quello speso nella periferia dell'arena. Pertanto, i nostri risultati comportamentali suggeriscono che la protratta carenza di n-3 PUFA è in grado di indurre sintomi simil-depressivi e simil-ansiogeni nelle ratte femmine. Abbiamo successivamente studiato le possibili alterazioni neurochimiche alla base di queste alterazioni comportamentali, identificando una significativa diminuzione della serotonina (5-HT) corticale e del fattore di crescita neuronale

(NGF) nelle ratte esposte alla dieta povera di n-3 PUFA, accompagnati da un aumento del turnover della serotonina. Inoltre, nel capitolo 3, abbiamo mostrato che la dieta povera in n-3 PUFA determina un'iperattivazione dell'asse ipotalamo-ipofisi-surrene (HPA), mediante l'aumento della noradrenalina e del fattore di rilascio della corticotropina (CRF) in ipotalamo e l'incremento del corticosterone plasmatico (accompagnati da un aumento di noradrenalina e serotonina in amigdala), così come da un incremento del glutammato e una diminuzione dell' acido gamma-aminobutirrico (GABA) sia nella corteccia prefrontale che nell'amigdala. In ultima analisi, le ratte femmine esposte ad una dieta povera di n-3 PUFA hanno mostrato un aumento plasmatico del peptide beta amiloide solubile $(A\beta)_{1-42}$.

Il peptide solubile $A\beta_{1-42}$ sta recentemente assumendo grande importanza nella patogenesi della depressione, in quanto la depressione si presenta frequentemente come comorbidità sia della malattia di Alzheimer sia di altre malattie neurodegenerative. A questo proposito, il nostro gruppo ha precedentemente dimostrato che la somminiztrazione intracerebroventricolare (icv) di Aβ è in grado di evocare un fenotipo simil-depressivo in ratti adulti maschi. Nel capitolo 2 di questo lavoro di tesi, abbiamo riprodotto per la prima volta il modello simil-depressivo indotto dalla somministrazione di Aβ nelle ratte femmine, valutando parametri comportamentali e neurochimici. I nostri risultati hanno confermato il profilo depressivo indotto da Aβ anche nelle ratte femmine. Inoltre, il profilo simil-depressivo Aβ-indotto è stato revertito nelle ratte esposte ad una dieta ricca di n-3 PUFA, indicando un possibile effetto benefico della supplementazione di n-3 PUFA nel trattamento dei disturbi depressivi. In conclusione, i nostri dati suggeriscono che le alterazioni della trasmissione monoaminergica, accompagnate da modifiche del NGF e disfunzioni dell'asse HPA, possono essere considerate importanti determinanti neurobiologici che contribuiscono alla patogenesi dei sintomi simil-depressivi indotti dalla carenza di n-3 PUFA e dalla somministrazione del peptide Aβ solubile.

Negli ultimi decenni le diagnosi dei disturbi psichiatrici sono state basate essenzialmente su sintomi soggettivi e su segni osservabili. Sebbene i sintomi costituiscano un importante punto di partenza, i fattori genetici e neurobiologici sottesi a questi sintomi devono essere approfonditi. Pertanto, l'impiego di modelli animali può risultare molto utile al fine di studiare longitudinalmente le alterazioni comportamentali traslabili ai sintomi nell'uomo e, in ultima analisi, indagare sulla neurobiologia sottesa per identificarne l'eziopatogenesi.

Pertanto, in questo lavoro di tesi, sono stati investigati i meccanismi neurobiologici alla base di sintomi simil-depressivi comuni a molte malattie neuropsichiatriche e neurodegenerative, utilizzando diversi paradigmi e modelli animali.

Per poter analizzare in maniera approfondita e traslazionale i principali sintomi simil-depressivi, occorre tener conto anche della sfera sociale. Un importante sintomo simil-depressivo che colpisce la sfera sociale è il ritiro sociale. Il ritiro sociale, definito come la mancanza di desiderio di avere contatti sociali, è un sintomo precoce di un'ampia varietà di malattie neuropsichiatriche, tra cui la schizofrenia, l'ASD e la depressione maggiore.

A questo proposito, nel capitolo 4 e 5, abbiamo investigato le alterazioni comportamentali relative alla socialità e al ritiro sociale, utilizzando un nuovo paradigma comportamentale chiamato "Visible Burrow System" (VBS). Il VBS è un ambiente semi-naturale, costituito da un tunnel e numerose tane costantemente al buio e un'arena con un normale ciclo luce/buio (12h/12h), utile per analizzare le dinamiche sociali che si verificano naturalmente nelle colonie di roditori. In particolare, abbiamo individuato alterazioni comportamentali correlabili al ritiro sociale in colonie di topi C57BL/6J, utilizzati come ceppo di controllo, e due linee transgeniche, i topi BTBR e i topi *Pcdh9*-Knockout (KO). I topi BTBR sono ampiamente utilizzati per le loro somiglianze con i deficit tipici dell'ASD, poiché presentano comportamenti stereotipati, disfunzioni nella vocalizzazione e ridotte interazioni sociali, mentre i *Pcdh9*-KO sono stati recentemente studiati come modelli simil-schizofrenici, simil-depressivi e simil-autistici.

I nostri risultati hanno mostrato che i topi BTBR presentano deficit nei comportamenti sociali e hanno una preferenza per i comportamenti non sociali rispetto ai topi C57BL/6J nel VBS, con caratteristiche tipiche del ritiro sociale. Al contrario, non abbiamo trovato differenze di socializzazione nelle colonie composte da *Pcdh9* omozigoti e eterozigoti KO e Wild Type (WT), indicando l'assenza di deficit della sfera sociale nei topi *Pcdh9* KO stabulati insieme ad i WT nel VBS. A questo proposito, studi futuri saranno focalizzati su una maggiore caratterizzazione del fenotipo sociale dei *Pcdh9* KO, in assenza di stimoli sociali. Infatti, le colonie, formate da topi di genotipo misto e di sesso misto, sono considerate setting altamente sociali, utili a migliorare i deficit sociali eventualmente riscontrati. In conclusione, il nostro studio ha validato l'utilità del VBS come paradigma comportamentale per studiare le disfunzioni sociali e potrebbe essere ulteriormente impiegato per testare trattamenti farmacologici mirati al loro ripristino.

A questo punto, un'analisi della possibili alterazioni neurobiologiche responsabili del ritiro sociale si è resa necessaria. A tal fine, nel capitolo 4 di questo lavoro di tesi, abbiamo analizzato il contenuto di GABA e glutammato nella corteccia prefrontale e nell' amigdala di colonie di topi C57BL/6J e BTBR, identificando una significativa diminuzione del GABA e un significativo aumento del glutammato in entrambe le aree nei topi BTBR. Abbiamo così ipotizzato che la diminuzione di GABA e il corrispondente aumento del glutammato nella corteccia prefrontale e nell'amigdala potrebbero essere responsabili della ridotta socializzazione e dell'aumento del ritiro sociale nei topi BTBR. Pertanto, la stimolazione della neurotrasmissione GABAergica e l'attenuazione del tono glutammatergico potrebbero rappresentare possibili targets terapeutici per il trattamento del ritiro sociale, tipico di numerose malattie neuropsichiatriche e neurodegenerative.

Inoltre, nel capitolo 5 di questa tesi, abbiamo misurato i livelli di GABA e glutammato nella corteccia somato-sensoriale di colonie di topi *Pcdh9* e abbiamo evidenziato l'assenza di differenze nel contenuto di GABA tra i tre genotipi, sia nei topi raggruppati in colonie nel VBS, che nei topi stabulati nelle gabbie standard. Contrariamente, i topi *Pcdh9*-KO omozigoti, stabulati nelle gabbie standard, hanno riportato un aumento di glutammato rispetto ai topi KO eterozigoti ed ai WT, mentre nessuna differenza di genotipo è stata riscontrata nei livelli di glutammato delle le colonie del VBS. Di conseguenza, l'aumento del glutammato riportato dai *Pcdh9* KO omozigoti stabulati in gabbie standard e non riscontrato nei KO omozigoti raggruppati nelle colonie del VBS, suggerisce un effetto positivo di questo ambiente altamente sociale, in grado di revertire l'aumento di glutammato indotto dalla deficienza del gene *Pcdh9*.

In conclusione, il presente lavoro di tesi è stato focalizzato sullo studio di differenti pathways neurobiologici in grado di provocare sintomi simil-depressivi caratteristici di molti disturbi neuropsichiatrici, al fine di sviluppare un approccio multifattoriale.

Al fine di migliorare le attuali opzioni terapeutiche e sviluppare nuovi trattamenti sicuri ed efficaci, occorre necessariamente considerare l'influenza dei fattori sociali, ambientali e alimentari, così come il ruolo delle comorbidità nell'eziopatogenesi dei sintomi simil-depressivi, in maniera da individuare i corretti substrati neurobiologici responsabili della comparsa di tali sintomi.

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“Happiness is real only when shared.”

LIST OF PUBBLICATIONS

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