Neurobiological determinants of depressive-like symptoms in rodents
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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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 serotonergic 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.