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TESIS DOCTORAL

METALOPROTEINASA-9 Y DEPRESIÓN: ESTUDIO EN UN MODELO ANIMAL Y EN MUESTRAS CEREBRALES HUMANAS *POST-MORTEM*, Y PAPEL EN EL MECANISMO DE ACCIÓN DE ANTIDEPRESIVOS DE ACCIÓN RÁPIDA

PhD THESIS

METALLOPROTEINASE-9 AND DEPRESSION: A STUDY IN AN ANIMAL MODEL AND HUMAN *POST-MORTEM* BRAIN, AND ITS ROLE IN THE MECHANISM OF ACTION OF FAST-ACTING ANTIDEPRESSANT DRUGS

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CERTIFICAN:

Que la Tesis Doctoral titulada “Metaloproteinasa-9 y depresión: estudio en un modelo animal y en muestras cerebrales humanas *post-mortem*, y papel en el mecanismo de acción de antidepresivos de acción rápida”, “Metalloproteinase-9 and depression: a study in an animal model and human *post-mortem* brain, and its role in the mechanism of action of fast-acting antidepressant drugs”, ha sido realizada por Júlia Senserrich Guerrero, bajo su dirección en el Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC) (UC-CSIC-SODERCAN), con el fin de optar al grado de Doctor.

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ABBREVIATION LIST

Δ^9-THC: Δ^9-Tetrahydrocannabinol

5-HT: 5-Hydroxytryptamine (serotonin)

AD: Antidepressant drugs

AEMPS: *Agencia Española de Medicamentos y Productos Sanitarios*

AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BDNF: Brain-derived neurotrophic factor

C: Control

CB: Cannabinoid

CBD: Cannabidiol

CORT: Corticosterone

COVID-19: Coronavirus disease 2019

CRF: Cardiorespiratory failure

Cx: Cortex

DA: 3,4-Dihydroxyphenethylamine (dopamine)

DALYs: Disability-adjusted life-years

DSM-V: Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition

EMA: European Medicines Agency

EPM: Elevated plus maze

FAAH: Fatty acid amide hydrolase

FBS: Fetal bovine serum

FDA: Food and Drug Administration

GABA: γ-Aminobutyric acid

GPR55: Orphan G protein-coupled receptor 55

Hp: Hippocampus

HPA: Hypothalamic-pituitary-adrenal

HPLC: High performance liquid chromatography

IL-1: Interleukin-1

i.p.: Intraperitoneal

LDB: Light-dark box

MAOI: monoamine oxidase inhibitor

MDD: Major depressive disorder

MMPs: Matrix metalloproteinases

MMP-9 KO: MMP-9 knockout

MMP-9 OE: MMP-9 overexpressing

mTOR: Mammalian target of rapamycin

NA: Noradrenaline

NMDA: N-methyl-D-aspartate

NSF: Novelty suppressed feeding test

NSRIs: Noradrenaline selective reuptake inhibitors

OFT: Open field test

PFCx: Prefrontal cortex

PMI: *Post-mortem* interval

PPARγ: Peroxisome proliferator-activated receptor gamma

PSD95: Postsynaptic density protein 95

S.E.M.: Standard error mean

SIH: Stress-induced hyperthermia

SIT: Social interaction test

SPF: Specific-Pathogen-Free

SNRIs: Serotonin/norepinephrine reuptake inhibitors

SPT: Sucrose preference test

SSRIs: Serotonin selective reuptake inhibitors

TIMPs: Tissue inhibitors of metalloproteinases

TNF-α: Tumour necrosis factor-α

TrkB: Tyrosine kinase B

$TRPV_1$: Transient receptor potential vanilloid ion channels 1

TST: Tail suspension test

VEH: Vehicle

w/v: Weight/volume

WB: Western blot

WHO: World Health Organization

WT: Wild type

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INTRODUCTION

1. Major Depressive Disorder

1.1 General features

Major Depressive Disorder (MDD) is a chronic neuropsychiatric disease with a higher prevalence among mental health disorders, just behind anxiety disorder, affecting 3.6% of the World population in 2019, which corresponds to 279.6 million people worldwide (GBD, 2022). MDD has become a leading cause of disability worldwide that results in a huge socioeconomic cost, as it mainly affects people in its productive stage (figure 1) (Sicras-Mainar et al., 2010; Greenberg et al., 2021; GBD, 2022).

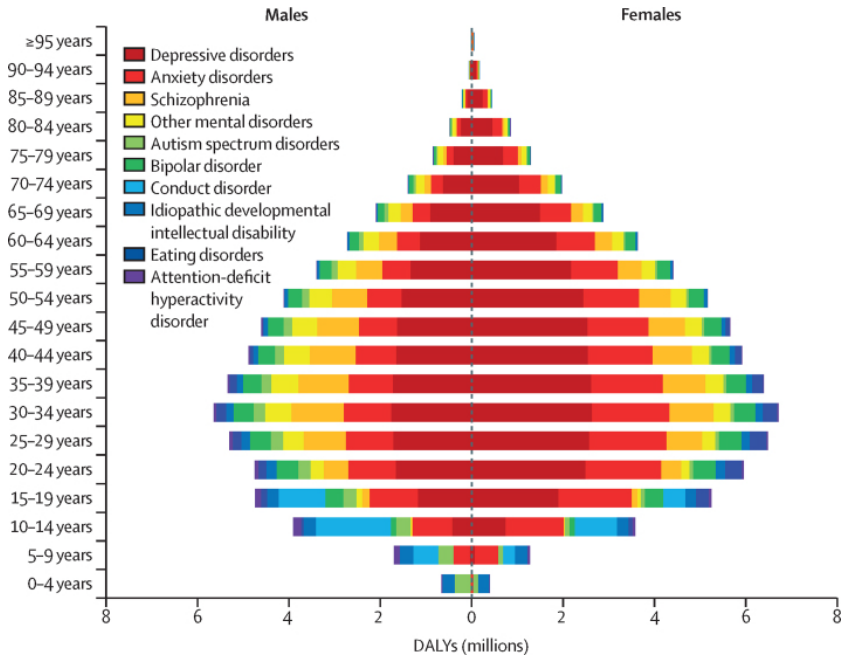

Figure 1. Global DALYs by mental disorder, sex and age, 2019. DALYs: disability-adjusted life-years. Obtained from GBD (2022).

Recently, the World Health Organization (WHO) reported that the COVID-19 pandemic has induced an exponential increase in depression and anxiety cases by a 25% (WHO, 2022). It is well known that the prevalence of depression is higher in women than in

men, affecting 2 women for every man diagnosed (Holden, 2005; Marcus et al., 2005; Salk et al., 2017). Moreover, the COVID-19 pandemic has a greater impact on the mental health of women and young people (WHO, 2022), which will probably affect the sex ratio, increasing the predominance of women suffering from depression.

Currently, the diagnosis of MDD is uniquely clinic and it is based on the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (American Psychiatric Association, 2013). According to the DSM-V, a patient with MDD must present at least 5 of the symptoms listed below, for at least 2 weeks. In addition, the symptomatology shall include (1) depressed mood or (2) anhedonia, and involve a disturbance of the normal, social, and occupational functionality of the patient. The diagnostic criteria of DSM-V for Major Depressive Disorder are briefly the following (American Psychiatric Association, 2013):

1. Depressed mood (such as feeling sad, empty, or hopeless).
2. Loss of interest or pleasure (anhedonia).
3. Increase/decrease in weight or appetite.
4. Insomnia or hypersomnia.
5. Psychomotor retardation or agitation (observable by others).
6. Loss of energy or fatigue.
7. Excessive or inappropriate worthlessness or guilt.
8. Impaired concentration or indecisiveness.
9. Thoughts of death or suicidal ideation/attempt.

In addition, patients often present other symptoms such as cognitive impairment and reduced libido (*see review* Perini et al., 2019).

Moreover, MDD presents comorbidity with other diseases, such as cancer, diabetes, and coronary heart diseases (Hu et al., 2020; Mondal et al., 2020; Moroianu et al., 2022), and other psychological illnesses, such as anxiety (Rush et al., 2005; Thaipisuttikul et al.,

2014; Kim and Schwartz, 2020). Some psychopathologies, like bipolar disorder and schizophrenia, also present depressive symptoms, which may lead to incorrect diagnoses (*see review* Hirschfeld, 2014; Rahim and Rashid, 2017).

1.2 Etiopathogenesis of depression

The etiopathology of MDD is still unknown. MDD is a complex heterogeneous disease in which multiple psychosocial, genetics and biological factors interact. However, several hypotheses have been proposed to explain the molecular and neurochemical mechanisms that may lead to depression. Understanding the mechanisms underlying the pathophysiology of MDD is highly important for the development of novel therapeutic approaches. A summary of the main hypotheses is presented below.

1.2.1 Monoaminergic hypothesis

The monoaminergic hypothesis was the first to be postulated and during the lasts decades, it has been the most important, proposing that a deficit of monoamines — serotonin (5-HT), dopamine (DA), and noradrenaline (NA) — and the lower activity of those neurotransmitter systems underly depression (Schildkraut, 1965; Coppen et al., 1967). This hypothesis was formulated after observing that the antihypertensive drug reserpine, which depletes catecholamine vesicles, induced depressive symptoms (Lemieux et al., 1956). In addition, the administration of iproniazid, a monoamine oxidase inhibitor (MAOI), used to treat tuberculosis, and imipramine, a drug that blocks the reuptake of amines, exerted an antidepressant effect mainly by inhibiting the degradation of monoamines, and the reuptake of serotonin and noradrenaline, respectively (*see review* Hillhouse and Porter, 2015). The monoaminergic hypothesis has been the basis for the development of new antidepressant drugs. Serotonin selective reuptake inhibitors (SSRIs), noradrenaline selective reuptake inhibitors (NSRIs), and dual serotonin/norepinephrine reuptake inhibitors (SNRIs) are more

selective and display fewer side effects. SSRIs have become the first-line antidepressants for MDD treatment (Gautam et al., 2017; Shelton, 2019).

To confirm this hypothesis and find potential biomarkers of depression, several studies have measured the levels of monoamines and their metabolites in peripheral and central samples of MDD patients, leading to inconclusive results (*see reviews* Ricci and Wellman, 1990; Asberg, 1997). Later, researchers postulated that in addition to a disruption of the monoaminergic neurotransmitters, the sensitivity of their target receptors and the consequential intracellular signalling pathways may be also affected (Brigitta, 2002). In fact, it is suggested that there is a hypersensitization of the serotonergic and noradrenergic autoreceptors, as well as some heteroreceptors (Arango et al., 1990; 1995; Meana et al., 1992; Callado et al., 1998; Parsey et al., 2010; Valdizán et al., 2010). This is supported by the fact that the chronic administration of some antidepressant drugs induces autoreceptor desensitization, as in 5-HT_{1A} (Blier and de Montigny, 1994; Hervás et al., 2001; Hensler, 2002; Castro et al., 2003) and α_2-adrenergic receptors (Invernizzi et al., 2001; Mateo et al., 2001; *see review* Cottingham and Wang, 2012) in animal studies. This receptor desensitization has been also reported in human studies (García-Sevilla et al., 1990; Yatham et al., 1999; Gray et al., 2013; Rivero et al., 2014). It has been postulated that the time required for the desensitization of the autoreceptors is 2-4 weeks, as the time required to observe their therapeutic effect (Albert and François, 2010; Artigas, 2013; Duman et al., 2016; Commons and Linnros, 2019). However, not all antidepressant drugs down-regulate these monoaminergic receptors (Jeon and Kim, 2016).

Despite the evidence supporting the monoaminergic hypothesis, it presents some limitations. One-third of the patients are resistant to the classic antidepressant drugs (Rush et al., 2006; Al-Harbi, 2012; Ontiveros-Sánchez de la Barquera, 2017; Desmidt et al., 2022; Diep et al., 2022) and less than 50% of treated patients show a complete remission (Rush et al., 2006; Jeffrey et al., 2021).

1.2.2 Glutamatergic hypothesis

The glutamatergic hypothesis of depression proposes that a stress-induced overactivation of the glutamatergic system, and the maladaptive changes in structures that modulate the cognitive-emotional behaviour, leads to the appearance of depressive symptoms (*see reviews* Sanacora et al., 2012; Thompson et al., 2015). This hypothesis was postulated after observing the antidepressant effect of several N-methyl-D-aspartate (NMDA) receptor antagonists in some animal models (Trullas and Skolnick, 1990).

Indeed, preclinical studies have reported that acute stress increases extracellular glutamate levels in the prefrontal cortex (PFCx) (Treccani et al., 2014; Drouet et al., 2015), which in turn induces excitotoxicity (*see review* Popoli et al., 2011), reinforcing the role of a dysregulated glutamatergic system in the pathophysiology of depression. Glial cells are the main responsible for glutamate reuptake, and alterations in this cell type may lead to lower reuptake of extracellular glutamate and high glutamate levels in the synapse. In this line, a decreased density of glial cells has been reported in the cortex (Ongür et al., 1998; Cotter et al., 2001) and hippocampus (Cobb et al., 2016) of MDD patients. The NMDA receptors are overactivated, leading to excitotoxicity (*see review* Vanhoutte and Bading, 2003). The excessive release of glutamate would induce negative feedback by the activation of presynaptic autoreceptors, leading to a reduction of glutamate in the synaptic cleft (*see review* Kugaya and Sanacora, 2005). In this sense, a chronic stress animal model presents lower cortical glutamate levels (Son et al., 2018). However, there are discrepancies in the glutamate levels found in different brain areas of MDD patients (Auer et al., 2000; Yildiz-Yesiloglu and Ankerst, 2006; Hashimoto et al., 2007).

In line with this hypothesis, ketamine, a non-competitive NMDA receptor antagonist, displays a fast-acting and maintained antidepressant effect in MDD patients, even in treatment-resistant patients (Berman et al., 2000; Zarate et al., 2006). The Food and

Drug Administration (FDA) and the European Medicines Agency (EMA) approved esketamine, an enantiomer of ketamine, as a nasal spray for treating treatment-resistant MDD patients in 2019, becoming the first fast-acting antidepressant approved. Due to the effectiveness of ketamine, different research lines are focused on finding other molecules that modulate the glutamatergic system (*see reviews* Gerhard et al., 2016; Murrough et al., 2017). In fact, last year (2022), the FDA approved Auvelity™, the first oral fast-acting antidepressant. Auvelity™ is composed of dextromethorphan —a non-competitive NMDA receptor antagonist—, and bupropion —a dopamine and noradrenaline reuptake inhibitor— both already used in the clinic (*see review* Keam, 2022).

1.2.3 Neurotrophic/neuroplastic hypothesis

This hypothesis postulates that depression may be related to high cortical and hippocampal neuronal death, together with decreased synaptic plasticity, induced by stress (*see review* Duman et al., 1997). Exposure to chronic stress induces the activation of the hypothalamic-pituitary-adrenal (HPA) axis, promoting the release of glucocorticoids (*see review* Herman et al., 2016). It has been suggested that high glucocorticoids and glutamate levels, would decrease the expression of neurotrophic factors in the limbic system, specifically, the brain-derived neurotrophic factor (BDNF), resulting in neural atrophy, shortening of the dendrites and the decrease of dendritic spine density (*see reviews* Duman and Li, 2012; Herman et al., 2016).

Preclinical studies found atrophy of neurons, glial loss, and decreased neurogenesis, especially in the hippocampus and prefrontal cortex of animal models of depression exposed to chronic stress (Radley et al., 2004; Banasr et al., 2007; Czéh et al., 2007). Clinical studies also describe a decrease in the number of glial cells (*see* glutamatergic hypothesis), loss of synapses and neuronal density in the hippocampus and prefrontal cortex (Kang et al., 2012; Holmes et al., 2019), which may lead to the reduction of the

volume of these structures reported in depressed patients (Neumeister et al., 2005; Kronmüller et al., 2009; Zhuo et al., 2017; Kandilarova et al., 2019; Zheng et al., 2021). In addition, a reversion of the hippocampal and fronto-cortical atrophy has been observed in MDD patients that respond to antidepressant treatment (Sheline et al., 2003; MacQueen et al., 2008; Smith et al., 2013), in parallel to an increased expression of trophic factors (*see review* Castrén and Antila, 2017). Chronic treatment with classical antidepressant drugs is needed to achieve neurogenesis, indicating that hippocampal neurogenesis may also contribute to their therapeutic effect (Malberg et al., 2000).

The neurotrophic factors are biomolecules involved in the growth and survival of neurons, and in neuronal plasticity (*see review* Castrén et al., 2007). One of the most relevant factors is the **brain-derived neurotrophic factor (BDNF)** and its receptor tyrosine kinase B (TrkB), as they have been related to the pathophysiology of depression and the antidepressant drug action (*see review* Castrén and Monteggia, 2021). In fact, MDD patients present low peripheral BDNF levels (Molendijk et al., 2011; Yoshida et al., 2012; Qi et al., 2015), that have been correlated with the severity of the disease (Shimizu et al., 2003; Zhou et al., 2013). Moreover, BDNF levels and its receptor are decreased in several *post-mortem* brain areas of MDD patients (Dwivedi et al., 2003; Karege et al., 2005; Dunham et al., 2009; Guilloux et al., 2012; Tripp et al., 2012; Ray et al., 2014; *see reviews* Duman et al., 2016; Mosiołek, et al., 2021). On the other hand, antidepressant treatments reverse the decreased BDNF expression observed in MDD patients (Karege et al., 2005; Duman et al., 2016). These findings support the potential role of BDNF as a biomarker for the assessment of diagnosis and treatment effectiveness (*see review* Rana et al., 2021).

BDNF/TrkB pathway induces the activation of the **mammalian target of rapamycin (mTOR)** (figure 2). The mTOR pathway is involved in the regulation of different proteins translation, such as postsynaptic density protein 95 (PSD95), synapsin I, and BDNF itself, playing an important role in the synaptic plasticity and neurogenesis (*see review*

Cholewinski et al., 2021). Some discrepancies are reported regarding the activation of the mTOR pathway and mTOR protein levels in the prefrontal cortex of MDD patients (Jernigan et al., 2011; Salort et al., 2020). However, preclinical studies seem to obtain more consistent results. Thus, decreased mTOR pathway activation has been described in different rodent models of depression, that was normalized by antidepressant drug treatment (Zhong et al., 2014; Liu et al., 2015; Pazini et al., 2016; Xu et al., 2018; de Almeida et al., 2020; Xu et al., 2020; Yao et al., 2020). In addition, the pharmacological inhibition of mTOR abolished the antidepressant-like effect (Liu et al., 2015; Pazini et al., 2016; Xu et al., 2018; de Almeida et al., 2020; Xu et al., 2020), while its genetic deletion (Zhong et al., 2014), and the silencing of mTOR in the infralimbic cortex induces depressive-like behaviour in mice (Garro-Martínez et al., 2021), revealing a potential role of mTOR in the pathogenesis of depression and its treatment.

PSD95 is a scaffold protein located in the postsynaptic membrane of excitatory synapses. It binds to the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors, acting as an anchor to the cell membrane. This organization contributes to synaptic plasticity processes, affecting the long-term potentiation or depression (*see review* Stachowicz, 2022). In clinical studies, lower PSD95 protein levels have been observed in the PFCx of MDD patients (Feyissa et al., 2009; Rafalo-Ulinska et al., 2016). In addition, a reduction in PSD95 levels has been reported in *post-mortem* brain samples of people that committed suicide (Sowa-Kućma et al., 2013; Dean et al., 2016), which may suggest an association with the severity of the disease. Antidepressant drugs revert the decreased PSD95 levels in primary hippocampal cell cultures (Seo et al., 2014) and murine models of depression (Reinés et al., 2008; Leem et al., 2020). However, it must be considered that altered levels of PSD95 may enhance the number of NMDA receptors in the postsynaptic membrane and therefore boost NMDA-induced excitotoxicity (Sattler et al., 1999; Aarts et al., 2002; Fan et al., 2009) (figure 2).

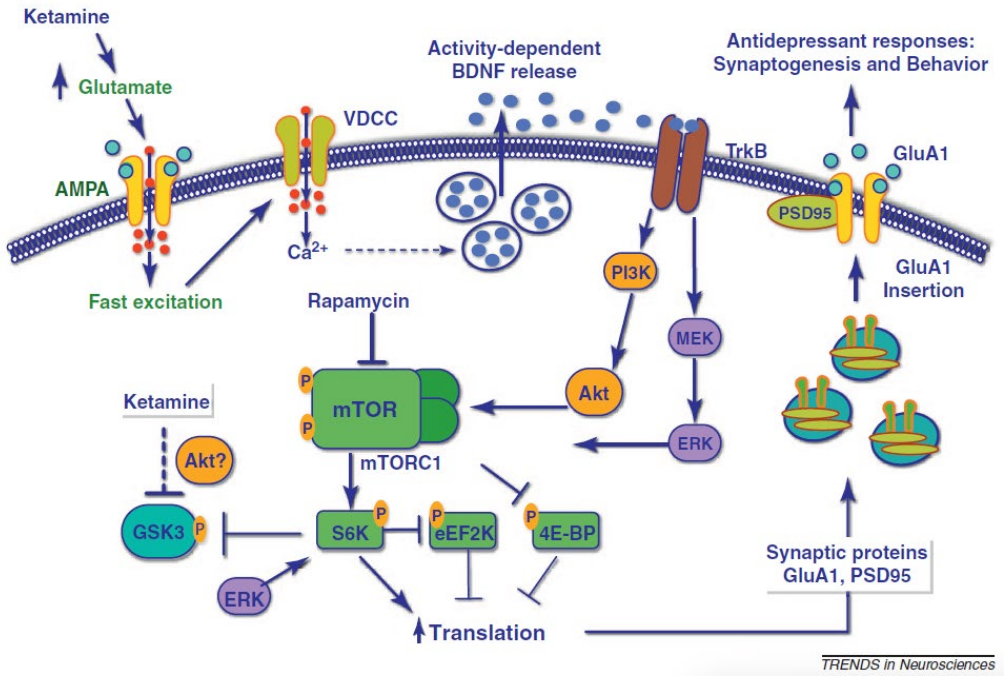

Figure 2. Graphical representation of neuronal mTORC1 pathway involved in depression pathophysiology and its treatment. mTOR signaling pathway implicated in the fast antidepressant effect of ketamine. Ketamine increases extracellular glutamate, inducing a fast depolarization that leads to the activation of voltage-dependent Ca^{2+} channels (VDCC) and the release of BDNF. This trophic factor binds to TrkB and stimulates downstream signaling pathways, which increases S6K, and the local translation of synaptic proteins such as PSD95 and the GluA1 subunit of the AMPA receptor, which is subsequently inserted into the membrane, increasing the synaptic plasticity. mTOR pathway is inhibited by the selective inhibitor rapamycin. 4E-BP: 4E binding protein; AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BDNF: brain-derived neurotrophic factor; eEF2K: eukaryotic elongation factor-2 kinase; ERK: extracellular signal-regulated kinase; MEK: MAP/ERK kinase; mTOR: mammalian target of rapamycin; PI3K: phosphatidyl inositol-3 kinase; PSD95: postsynaptic density protein 95; S6K: S6 kinase; TrkB: tyrosine kinase B receptor. Obtained from Duman and Voleti (2012).

Synapsin I is localized in the presynaptic terminals, primarily in GABAergic neurons. It binds to synaptic vesicles, participating in the maintenance of the stability, trafficking, and regulation of the release of neurotransmitters in the synaptic cleft (Khvotchev and Sun, 2009). Therefore, it plays an important role in neurogenesis (Barbieri et al., 2018)

and synaptic plasticity (Rocchi et al., 2019). Contradictory results have been reported in synapsin I mRNA expression in the PFCx of MDD patients (Kang et al., 2012; Cruceanu et al., 2013; Schmidt et al., 2015), while a lack of reports assesses its protein levels. Preclinical studies found decreased hippocampal synapsin I levels in different animal models of depression, that were normalized by antidepressant drug treatment (Liu et al., 2015; Xu et al., 2018). However, in cortical areas, different studies have reported contradictory results (Liu et al., 2015; Gao et al., 2019).

Brain areas such as the hippocampus and prefrontal cortex present decreased volume and neuronal plasticity. On the contrary, other brain areas, such as the amygdala, present more discrepant findings (Frodl et al., 2002; Hamilton et al., 2008; Rubinow et al., 2016; Lorenzetti et al., 2020).

1.2.4 Neuroinflammatory hypothesis

The neuroinflammatory hypothesis of depression advocates the role of inflammation in this disease. It was observed that pro-inflammatory cytokines are associated with a reduction in the monoaminergic availability, and with the activation of microglia inducing an increase in glutamate levels (*see reviews* Maes, 1999; Miller et al., 2010; Miller and Raison, 2016).

Indeed, an elevated presence of pro-inflammatory cytokines, even in humans or animals, induces “sickness behaviour” and mimics depressive symptomatology (*see review* Raison et al., 2006; Engler et al., 2017). Clinical studies in patients diagnosed with MDD found increased pro-inflammatory cytokines levels in the blood (Maes et al., 1995; Zou et al., 2018), cerebrospinal fluid (Levine et al., 1999; Martinez et al., 2012; Wang and Miller, 2018) and in *post-mortem* brain samples (Enache et al., 2019). In addition, peripheral levels of interleukin-1 (IL-1) and the tumour necrosis factor-α (TNF-α) positively correlate with the severity of depression and suicidal ideation (Martinez et

al., 2012; Zou et al., 2018). Interestingly, some anti-inflammatory agents displayed an antidepressant effect alone or as adjuvant treatment in MDD patients (Köhler-Forsberg et al., 2019; Bai et al., 2020).

1.2.5 Interconnection between depression hypotheses

It should be noticed that the different hypotheses of depression are not mutually exclusive. In fact, some genetic and environmental factors categorized as risk factors for depression, such as psychosocial stressors, poor diet, sedentary lifestyle, and obesity, activate the HPA axis and the inflammatory response (*see reviews* Beurel et al., 2020; Mikulska et al., 2021). On the one hand, hyperactivation of the immune system promotes the desensitization of glucocorticoid receptors, disrupting the regulation of the corticotropin-releasing factor and cortisol release. On the other hand, the hyperactivation of the HPA axis through the corticotropin-releasing factor induces the production of pro-inflammatory cytokines (*see reviews* Raison et al., 2006; Jesulola et al., 2018), leading to physiopathological alterations that are related with depression. The HPA hyperactivation and the increase in pro-inflammatory cytokines may dysregulate the glutamatergic system, inducing excitotoxicity and decreasing the bioavailability of monoaminergic neurotransmitters, the synthesis of neurotrophic factors, brain neurogenesis and therefore contributing to the atrophy of neurons and glia (*see review* Ferrari and Villa, 2017) (figure 3).

The integration of the hypotheses of depression highlights the high complexity and heterogeneity of the etiopathogenesis of this disease. However, more research is needed for a deeper understanding of the neurobiology of depression that would contribute to the development of more effective and rapid-acting antidepressant treatments.

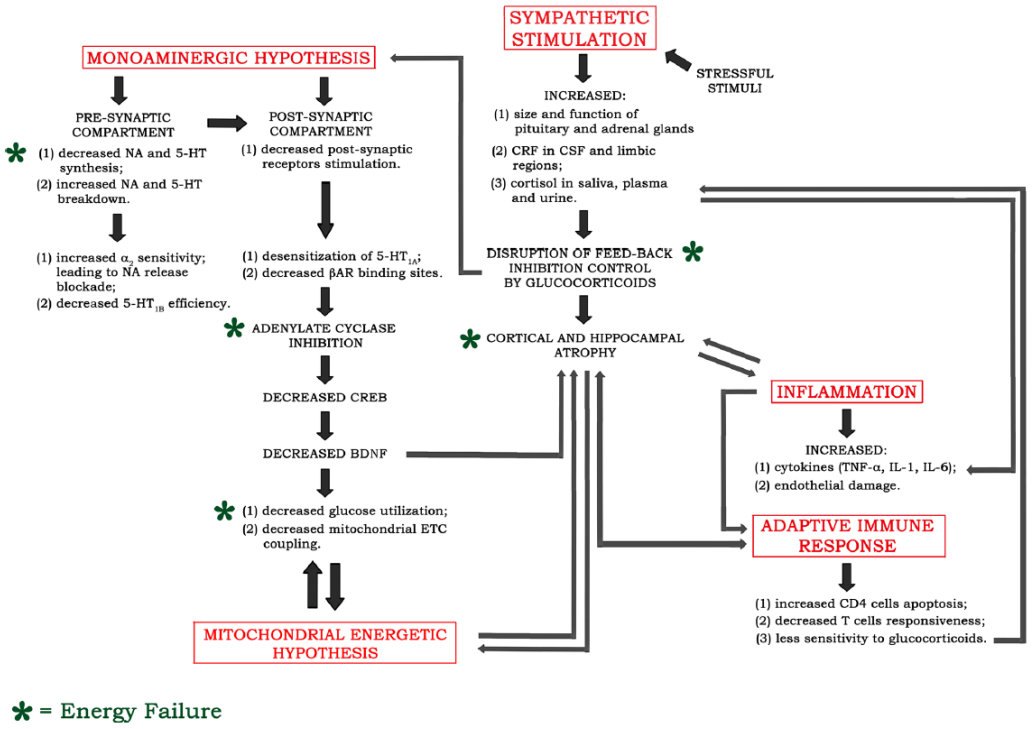

Figure 3. Schematic representation of the interconnection between depression hypotheses. βAR: β-adrenergic receptor; 5-HT: 5-hydroxytriptamine; BDNF: brain-derived neurotrophic factor; CD4: cluster of differentiation 4; CREB: cAMP response element-binding protein; CRF: corticotropin-releasing factor; CSF: cerebrospinal fluid; ETC: electron transport chain; IL: interleukin; NA: noradrenaline; TNF-α: tumour necrosis factor-α. Obtained from Ferrari and Villa (2017).

1.3 Neuronal circuits for depression and anxiety

It is still not well known the neural circuits alterations that may be involved in the depressive symptoms of major depressive disorder. A better understanding of these circuits may contribute to more effective therapeutic interventions (*see review* Spellman and Liston, 2020). Here we present a brief overview of the findings in the two brain areas most relevant to this thesis.

Alterations in the **prefrontal cortex** contribute to the development of anhedonic behaviour, negative processing biases (anxiety), learned helplessness (despair), and

executive functions (spatial working memory). MDD patients present a reduced volume of the prefrontal cortex which has been associated with the severity of the disease and the clinical response to the treatment (Marrus et al., 2015; Pirnia et al., 2016; Ward et al., 2019). However, studies assessing the neural activation of different PFCx regions in MDD patients, during the performance of tasks related to certain clinical manifestations showed opposite patterns of activation depending on the phenotype assessed (*see review* Pizzagalli and Roberts, 2022). For example, MDD patients display a hyperactivation of the dorsolateral PFCx in reward-related processes (Forbes et al., 2009), while in affective and cognitive tasks, this area is hypoactivated (Lawrence et al., 2004). Therefore, it is suggested that these alterations may be the consequence of the dysregulated connectivity of other brain areas with PFCx regions and the regulation of the excitatory/inhibitory synapses (*see review* Pizzagalli and Roberts, 2022). In this regard, most of the treatments used in depressive patients that do not respond to classical antidepressant drugs, such as ketamine, electroconvulsive therapy, and deep brain stimulation, normalize the neuronal activity in the PFCx, regulating specific neuronal populations (*see review* Hare and Duman, 2020).

The **hippocampus** is deeply associated with cognition and the regulation of emotions and stress. This brain area is linked with memory impairment and suicidal ideation in depression (*see review* Zhang et al., 2022). Similar to PFCx, MDD patients have decreased hippocampal volume that seems to be linked with the duration of the disease (Roddy et al., 2019). Different studies reported an increased or decreased hippocampal activity in MDD patients (*see review* Levy et al., 2018), that has been associated with the different behaviour-related tasks evaluated. It has been described that MDD patients present altered connections between different hippocampal regions with other brain areas, such as the ventrolateral PFCx (Hao et al., 2020). Moreover, antidepressant treatment induces an increase in the hippocampal volume (Sheline et al., 2003; Boldrini et al., 2013; Fu et al., 2013), and reverts the hyperactivation of the hippocampus in response to emotional stimuli in MDD patients (Delaveau et al., 2011). All these findings

suggest the important role of fine-tuned prefrontal and hippocampal neural circuits to obtain an antidepressant effect.

1.4 Sexual dimorphism in Major Depressive Disorder

The molecular mechanisms underlying the sexual dimorphism observed in depression that is involved in the different prevalence, symptomatology, and treatment efficacy, are still unclear. For instance, women present more severe symptoms (*see review* Kokras and Dalla, 2014), although they appear to respond better to SSRIs drugs than men (*see review* Sramek et al., 2016). It should be considered that different hormonal stages, like puberty, pregnancy and menopause, could also influence the antidepressant response (*see review* Sramek et al., 2016).

Several factors have been associated with the phenotypical sexual dimorphism in MDD, such as sexual hormones, vulnerability to stressors, environment, and epigenetics. In this regard, a meta-analysis of the gene expression of three corticolimbic brain areas (dorsolateral PFCx, subgenual anterior cingulate cortex, and basolateral amygdala) in men and women diagnosed with MDD reported a different transcriptional profile between sexes. Strikingly, from those genes showing a differential expression in both sexes (73 out of 1588 transcripts), 21 genes changed in the same direction, while 52 did it in opposite directions (Seney et al., 2018). Moreover, a recent study reported an up-regulation of a specific long noncoding RNA, which they named FEDORA, in the PFCx and blood of depressed women, but not men. The levels of this RNA in blood were also associated with the pharmacological response to ketamine in women. Furthermore, the increased expression of FEDORA in the PFCx of mice induced anxious- and depressive-like behaviours only in females (Issler et al., 2022).

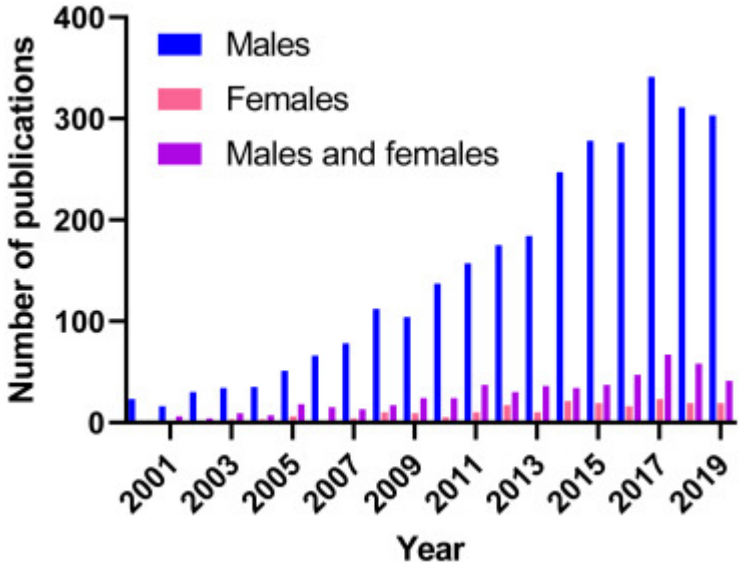

Figure 4. Graphical representation of the number of publications per gender in murine chronic stress models for depression, from 2000 to 2019. Obtained from Lopez and Bagot (2021).

Paradoxically, although the prevalence rate of MDD is twice in women than in men (Holden, 2005; Marcus et al., 2005; Salk et al., 2017), most of the preclinical studies are performed in male animal models of depression (*see* figure 4). Male animals are more popularly used as they present less variability than female animals, mainly attributed to the oestrus cycle (*see review* Lovick and Zangrossi, 2021). Furthermore, contrary to what has been reported in humans, male animals show more susceptibility to developing a depressive-like phenotype, while female seems to be more resilient. This discrepancy may be explained as most of the animal models have been validated only in males (*see reviews* Cryan and Mombereau, 2004; Lopez and Bagot, 2021). Some of the gender differences detected in several animal models of depression are associated with the behavioural phenotype, the treatment response, the serotonergic system, and neurotrophic factors levels (*see reviews* Borrow and Cameron, 2014; Kokras and Dalla, 2014). However, these differences may also differ depending on the strain and the model used.

In the last few years, the research using female animal models is increasing, but herein, we would like to manifest the urge to address preclinical research to define optimal female models of neuropsychiatric diseases, and their use for deeper knowledge of sex bias.

1.5 Animal models of depression

Animal models are essential tools in research for a better understanding of the mechanisms underlying the pathophysiology of different diseases and to find new therapeutic targets. However, still there is not an animal model of depression that perfectly mimics the human disease. Several models with high predictive validity have been established, each reproducing a different subset of physiological, endocrinological, and behavioural alterations typical of depression in humans. Unfortunately, not all the depressive manifestations observed in humans can be modelled in animals. Some symptoms are purely human, such as guilt feeling, suicidal ideation, or sad mood (*see reviews* Cryan et al., 2002; Cryan and Mombereau, 2004; Gururajan et al., 2019).

It has been proposed that the ideal animal model of depression should have, at least, the following basic features (figure 5) (Dedic et al., 2011):

- Construct or etiologic validity: the phenotype observed in the animal model is based on the same mechanisms underlying the human disease.
- Face validity: the animal model exhibits similar parameters (behavioural, biochemical, anatomical, and neuropathological) to the human.
- Predictive validity: a response to pharmacological treatments.

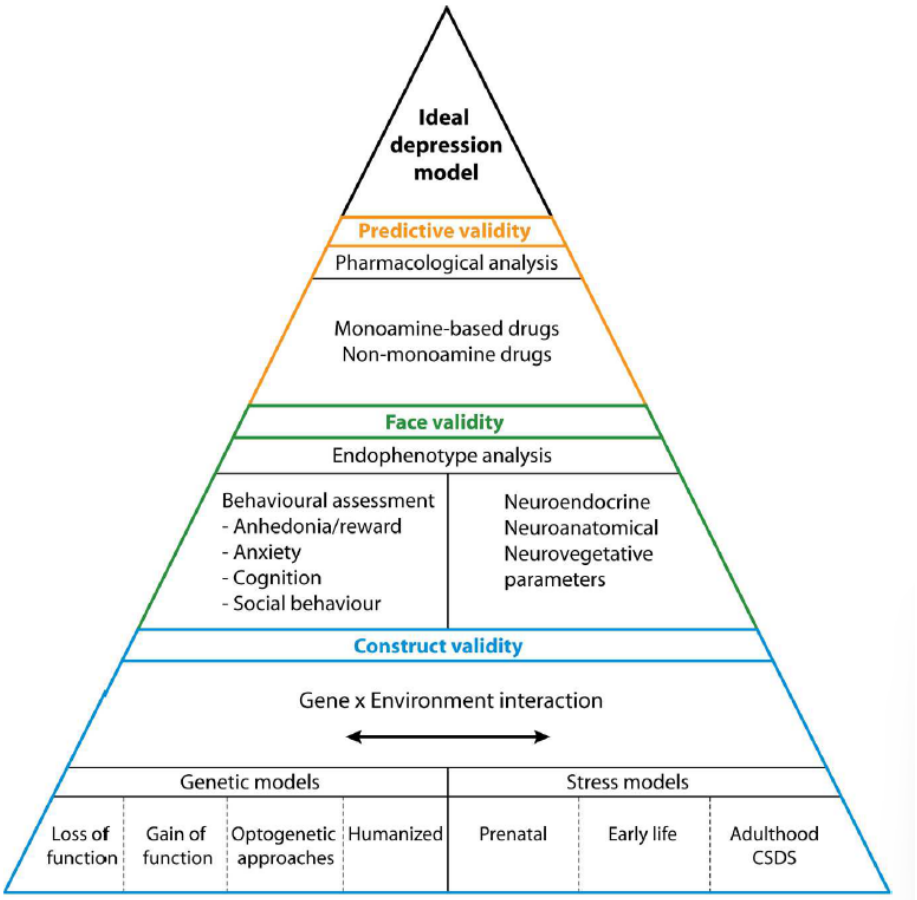

Figure 5. Features underlying an ideal animal model of depression. Obtained from Dedic et al. (2011).

The different animal models of depression could be classified depending on the procedure applied to obtain it. For instance: genetic models, stress models (chronic unpredictable mild stress, social stress, maternal deprivation, learned helplessness), pharmacological models (reserpine-induced model, corticosterone-induced model), surgical manipulation (olfactory bulbectomy), and inflammation (lipopolysaccharide injection), among others (Márquez-Herrero et al., 2019). In the present thesis, we have used the chronic corticosterone-induced mouse model of depression.

Chronic corticosterone mouse model

The chronic corticosterone animal model is endowed with a great degree of construct, face and predictive validity (Gourley and Taylor, 2009). This animal model in rodents mimics the hyperactivation of the HPA axis that could be observed after exposure to chronic stress, which increases the levels of cortisol or glucocorticoids in humans and animals, respectively (*see review* Smith and Vale, 2006). It presents behavioural alterations consistent in an anxious- and depressive-like phenotype. The chronic corticosterone model of depression displays behavioural despair, anhedonia, decreased grooming, and anxiety in several tests, among others (Gourley et al., 2008; David et al., 2009; Vidal et al., 2019; Garro-Martínez et al., 2020; Amigo et al., 2021; Breviario et al., 2023). In addition, this model exhibits molecular, neurochemical, and morphological disease-related changes, such as the dysregulation of the HPA axis function (Johnson et al., 2006), alterations of the serotonergic neurotransmission (Garro-Martínez et al., 2020), reduced hippocampal neurogenesis (Murray et al., 2008), dendritic atrophy and reduced BDNF levels in the hippocampus and PFCx (Cerqueira et al., 2005; Dwivedi et al., 2006; Yau et al., 2016). Importantly, corticosterone-induced behavioural and molecular changes can be attenuated with antidepressant treatments (Dwivedi et al., 2006; David et al., 2009; Fukumoto et al., 2017).

It should be highlighted that the chronic corticosterone model presents a higher homogeneity than models submitted to different stressors (*see review* Sterner and Kalynchuk, 2010).

2. Cannabidiol

The *Cannabis sativa* plant has been used since ancient times for recreational and therapeutic purposes. In 4000 BC, in China, it was documented the use of this plant's preparations to treat several ailments, such as pain, constipation, menstrual cramps and

malaria. In fact, it has been used as an analgesic, antiemetic, anticonvulsant, antibiotic, anti-inflammatory, anaesthetic, antispasmodic, diuretic, digestive, appetite stimulant, antitussive, expectorant and aphrodisiac (*see review* Zlebnik and Cheer, 2016).

Currently, more than a hundred phytocannabinoids have been identified in the *Cannabis sativa* plant. Among them, the Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychotropic component of the plant, and cannabidiol (CBD), the main non-psychotomimetic component, are the most abundant and studied.

Specifically, CBD has been proposed as a potential pleiotropic therapeutic drug for several diseases, acting on a wide variety of targets that go beyond the endocannabinoid system (*see review* Silote et al., 2019). The use of CBD may be of great interest in the treatment of multiple illnesses, including psychiatric diseases. Several preclinical and clinical studies have reported broad therapeutic actions of CBD as anxiolytic, antidepressant, antipsychotic, anticonvulsant, neuroprotective, antiemetic, antioxidant, anti-inflammatory, antiarthritic, antiapoptotic, analgesic and antineoplastic (*see reviews* Ligresti et al., 2016; Levinsohn and Hill, 2020). Indeed, the Food and Drug Administration (FDA) in 2018 and the *Agencia Española de Medicamentos y Productos Sanitarios* (AEMPS) in 2019, approved Epidiolex® (CBD) to treat seizures associated with specific rare and severe epilepsy syndromes in children.

2.1 Antidepressant effect of cannabidiol

Preclinical studies

Zanelati et al. (2010) reported, for the first time, the potential antidepressant effect of CBD. They reported the antidepressant-like effect of acute CBD administration, comparable to the effect of imipramine, in male Swiss naïve mice. CBD presented an inverted U-shape effect mediated by the activation of the 5-HT_{1A} receptor (Zanelati et

al., 2010; Rock et al., 2012). CBD action over the 5-HT_{1A} receptor has also been associated with its anxiolytic (Campos and Guimarães, 2008), panicolytic (Soares et al., 2010), neuroprotective (Pazos et al., 2013), and anti-emetic (Rock et al., 2012) effects and the prevention of the physiological and behavioural response to acute stress (Resstel et al., 2009).

CB_1 receptors in the PFCx are also involved in the antidepressant effect of CBD (Sartim et al., 2016) (figure 6), although CBD presents low affinity for CB_1 and CB_2 receptors (*see review* Pertwee, 2008). CBD can increase anandamide levels, by inhibiting its uptake in the ventromedial PFCx and decreasing the expression of the fatty acid amide hydrolase (FAAH) enzyme in the hippocampus (Bisogno et al., 2001; Leweke et al., 2012; Fogaça et al., 2018). Anandamide activates cannabinoid receptors and disinhibits serotonergic neurotransmission, favouring the effects mediated by the 5-HT_{1A} receptor (Bambico et al., 2007; Sartim et al., 2016). The mechanism that could account for the neurogenesis induced by CBD, as well as some of its anxiolytic-like effects, have been attributed to the activation of the cannabinoid receptors (Campos et al., 2013; Sartim et al., 2016; Fogaça et al., 2018). In addition, the inhibition of FAAH may contribute to the antidepressant-like effect of CBD and the enhancement of synaptic plasticity through the activation of the BDNF-TrkB-mTOR pathway (Sartim et al., 2018; Sales et al., 2019; Carnevali et al., 2020).

Other targets less studied related to the antidepressant and anxiolytic effect of CBD, are the transient receptor potential vanilloid ion channels 1 ($TRPV_1$) and the peroxisome proliferator-activated receptor gamma (PPARγ) (figure 6). The activation of $TRPV_1$ receptors by anandamide contributes to the inverted U-shaped dose-response curve of the anxiolytic-like effects of CBD (Rubino et al., 2008; Campos and Guimarães, 2009). Moreover, the activation of $TRPV_1$, CB_1, and 5-HT_{1A} receptors, and the antagonism of the orphan G protein-coupled receptor 55 (GPR55) has been proposed to underlie the homeostatic-induction of the glutamatergic system exerted by CBD (Xing and Li, 2007;

Navarrete and Araque, 2008; Linge et al., 2016; Rosenberg et al., 2022). On the other hand, the activation of PPARγ receptors is involved in the anti-inflammatory properties of CBD (Esposito et al., 2011; Vallée et al., 2017). Moreover, PPARγ activation elicits antidepressant effects (*see review* Tufano and Pinna, 2020). However, it has not been reported if the antidepressant effect of CBD is PPARγ-dependent.

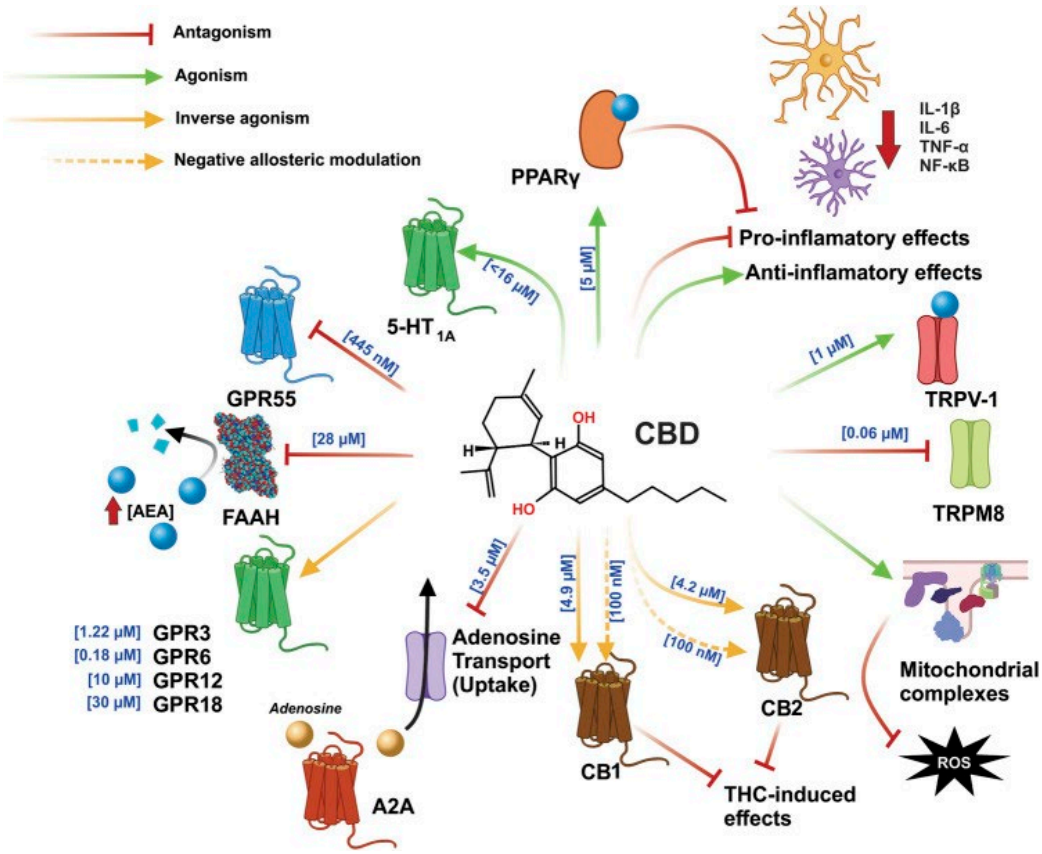

Figure 6. Principal targets of cannabidiol. 5-HT$_{1A}$: 5-hydroxytryptamine receptor 1A; A2A: adenosine A2A receptor; AEA: anandamide; CB: cannabinoid receptor; CBD: cannabidiol; FAAH: fatty acid amide hydrolase; GPR: G protein coupled receptor; IL: interleukin; NF-Kβ: nuclear factor Kβ; PPARγ: peroxisome proliferator activated receptor γ; ROS: reactive oxygen species; THC: tetrahydrocannabinol; TNF-α: tumour necrosis factor α; TRPM8: transient receptor potential cation channel subfamily M member 8; TRPV1: transient receptor potential vanilloid ion channels 1. Obtained from Patricio et al. (2020).

The acute administration of CBD has shown antidepressant- and anxiolytic-like effects in naïve animals (Moreira et al., 2006; Zanelati et al., 2010; Sales et al., 2019) and in chronic stress (Resstel et al., 2009; Sales et al., 2019), olfactory bulbectomy (Linge et al., 2016), neuroinflammatory (Florensa-Zanuy et al., 2021), and genetic (Shbiro et al., 2019) models of depression, revealing its potential as fast-acting antidepressant. Moreover, Sales et al. (2019) reported the sustained antidepressant-like effect of a single dose of CBD in the learned helplessness stress-based rat model, a selective breeding rat model of depression and in naïve mice.

The subchronic administration of CBD exerted an antidepressant-like effect in the olfactory bulbectomy mouse model of depression, while the anxiolytic-like effect was only observed after the first injection (Linge et al., 2016). Moreover, it has been described a sustained antidepressant-, but not anxiolytic-like effect, after subchronic CBD treatment (Bis-Humbert et al., 2020). Interestingly, both studies reported an anorexigenic effect after subchronic CBD treatment (Linge et al., 2016; Bis-Humbert et al., 2020). The chronic CBD treatment also displayed antidepressant-like and anxiolytic-like effects in several animal models of depression (Campos et al., 2013; Linge et al., 2016; Fogaça et al., 2018).

Clinical studies

No clinical studies have specifically evaluated CBD as an antidepressant drug in patients diagnosed with depression. However, several studies point, indirectly, towards a potential benefit of CBD in MDD, as depressive symptomatology has been evaluated as a secondary outcome of other pathologies. In this regard, CBD improved anxiety, depressive and cognitive symptoms displayed by patients with cannabis use disorder (Crippa et al., 2013; Beale et al., 2018; Solowij et al., 2018), post-traumatic stress disorder (Elms et al., 2019) and treatment-resistant epilepsy (Gaston et al., 2019). In addition, in adolescents with multiple substance abuse, that show social anxiety and

depression that did not respond to antidepressant drugs, CBD treatment reduced their depressive and anxiety symptoms and avoided the withdrawal syndrome as they quit illegal drugs (Laczkovics et al., 2021). Moreover, Notcutt et al. (2004) reported that the co-administration of CBD with Δ^9-THC might be more effective to treat depressive symptomatology in patients suffering chronic pain than CBD as a monotherapy.

Lately, CBD has gained huge popularity in society, and people have started to self-medicate with CBD, especially with depressive and anxious symptoms. Several authors conducted surveys were most of the people diagnosed with depression or anxiety disorder affirmed that CBD made them feel better (Corroon and Phillips, 2018; Fortin et al., 2021; Moltke and Hindocha, 2021; Wieckiewicz et al., 2022). Curiously, 88% of those surveyed by Wieckiewicz et al. (2022), would rather be treated with CBD than with any other drug prescribed.

There are no ongoing clinical studies evaluating the antidepressant potential of CBD in patients with MDD. In fact, the only study that was evaluating CBD for treatment-resistant MDD patients was withdrawn due to lack of funding (NCT04732169). However, several studies are currently assessing the effect of CBD on anxiety and depression present in other pathologies (*reviewed in* ClinicalTrials.gov, 2022a).

On the other hand, the anxiolytic effect of CBD in humans was reported for the first time in healthy volunteers while speaking in public (Zuardi et al., 1993). Since then, several clinical trials have followed this line of research, evaluating the anxiolytic effect of CBD in different cohorts, such as healthy volunteers (Zuardi et al., 2017; Linares et al., 2019) and patients diagnosed with social anxiety (Bergamaschi et al., 2011; Crippa et al., 2011), general anxiety and sleep disorders (Shannon et al., 2019). It has been reported that the anxiolytic effect induced by CBD follows an inverted U-shape dose-response (Linares et al., 2019). Recently, Souza et al. (2022) performed two studies simultaneously, where they administered CBD orally to healthcare workers for 28 days during the COVID-19 pandemic. They observed beneficial effects on anxiety, emotional

exhaustion, and depressive symptoms, which lasted up to 1 month after the end of the treatment. Currently, there is a great variety of ongoing clinical trials assessing the anxiolytic effect of CBD (*reviewed in* ClinicalTrials.gov, 2022b).

3. Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are endopeptidases, which catalytic active site is dependent on calcium and a highly conserved zinc finger motif. These enzymes are synthesised as inactive pro-peptides. Once released in the extracellular medium, the pro-domain can be removed by other MMPs, serine proteases, plasmin, or furin. They can be inhibited by tissue inhibitors of metalloproteinases (TIMPs) (*see review* Cui et al., 2017). Their principal function is to degrade components from the extracellular matrix and basement membrane, cleave cell adhesion molecules, cell surface receptors, growth factors, cytokines, and other proteases, and induce the regulation of intracellular signalling cascades (*see reviews* McCawley and Matrisian, 2001; Xie et al., 2017; Laronha and Caldeira, 2020). Moreover, MMPs are the only enzymes with the ability to break down collagen, the main compound of the extracellular matrix (*see review* Laronha and Caldeira, 2020). In line with these functions, MMPs have been related to a wide variety of physiological functions, such as angiogenesis, inflammation, apoptosis, cell survival, and migration, among others (*see reviews* Cui et al., 2017; Li et al., 2022). MMPs family is composed of over 20 enzymes in humans and rodents, which are subcategorized regarding their specificity of the substrates: collagenases, gelatinases, stromelysin, matrilysins, membrane-type MMPs, and other MMPs, that differ in structure and substrate from the other groups (*see review* Lattanzi et al., 2020). Among them, matrix metalloproteinase 9 is probably the most studied in the brain (*see review* Beroun et al., 2019).

3.1 Matrix Metalloproteinase 9

Matrix metalloproteinase 9 (MMP-9) or Gelatinase B is a gelatinase that targets denatured collagen such as gelatine. It is expressed in a wide variety of cells, like neutrophils, macrophages, leucocytes, and endothelial cells (*see review* Laronha and Caldeira, 2020) and in neurons and glia (*see review* Beroun et al., 2019). MMP-9 is expressed in both, the central and peripheral nervous systems, and it is released into the synaptic cleft from glutamatergic neurons upon activation of NMDA receptors (*see review* Vafadari et al., 2016). This protein is predominantly found in hippocampus, cerebellum, and cerebral cortex. However, MMP-9 levels in the adult brain are low and are up-regulated in processes such as neuronal plasticity or under pathological conditions (*see review* Beroun et al., 2019). The expression of these proteins is tightly regulated (from protein transcription to activation). One of the proteins responsible for maintaining physiological levels of MMP-9 is TIMP-1 (Roderfeld et al., 2007).

MMP-9 plays an important role in synaptic plasticity, learning and memory, allowing the growth and maturation of dendritic spines, which contributes to the formation of new and more efficient synaptic connections. MMP-9 cleave cell adhesion molecules, like nectins, and participate in the accumulation and immobilization of AMPA receptors, and the trafficking of NMDA receptors (Michaluk et al., 2009; Kaczmarek, 2016). Moreover, MMP-9 can catalyse the activation of BDNF and pro-inflammatory cytokines, cleaving its inactive precursor form, and participating in processes of neurogenesis and inflammation, respectively (*see review* Vafadari et al., 2016) (see figure 7).

The overexpression of MMP-9 can lead to aberrant plasticity, forming longer and thinner immature spines, and silencing of excitatory synapses by lowering the AMPA/NMDA receptor ratio (Wilczynski et al., 2008). Moreover, elevated levels of MMP-9 also alter the production of inflammatory cytokines (de Pinho et al., 2014). Therefore, it has become the focus of study in several neuropathologies, such as

epilepsy, multiple sclerosis, schizophrenia, Alzheimer's, Parkinson's and Huntington's disease (*see review* Vafadari et al., 2016).

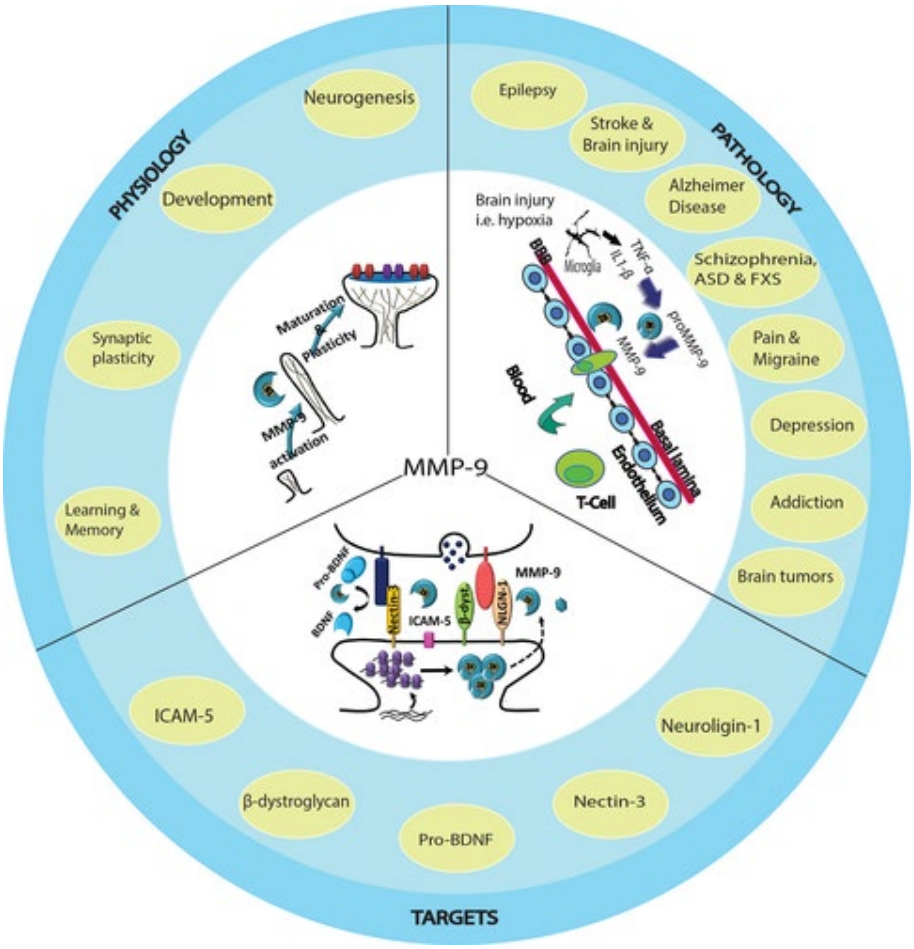

Figure 7. MMP-9 brain targets involved in physiological functions and its implication in neurological and psychiatric diseases. ASD: autism spectrum disorder; BBB: blood-brain barrier; BDNF: brain-derived neurotrophic factor; FXS: Fragile X syndrome; ICAM-5: intercellular adhesion molecule-5; IL1-β: interleukin 1β; MMP-9: matrix metalloproteinase 9; NLGN-1: neuroligin-1; TNF-α: tumor necrosis factor α. Obtained from Vafadari et al. (2016).

Implication in depression

Elevated MMP-2, MMP-7 and MMP-9 levels were reported in blood samples of MDD patients (Domenici et al., 2010; Bobińska et al., 2016a; Bobińska et al., 2016b). Curiously, elevated levels of MMP-9 were also detected in the tears of MDD patients (Krajčíková et al., 2021). Other studies reported no differences in MMP-9 serum levels (Yoshida et al., 2012; Shibasaki et al., 2016; Shibasaki et al., 2018). Patients that showed a therapeutic response to the electroconvulsive therapy presented a reduction of MMP-9 serum levels compared to pre-treatment levels, while those that did not respond presented no differences (Shibasaki et al., 2018). However, discrepancies in the modulation of MMP-9 by antidepressant drugs have been reported in several human studies (*see review* Li et al., 2022). Nonetheless, a positive correlation between MMP-9 levels in the serum of MDD patients and the severity of their symptomatology has been reported (Garvin et al., 2009; Yoshida et al., 2012; Shibasaki et al., 2016; Che et al., 2019; Talarowska et al., 2019).

Currently, only two studies have assessed MMP-9 in *post-mortem* brain samples of patients with MDD. Alaiyed et al. (2020) reported no differences in MMP-9 protein levels in the PFCx, while Bijata et al. (2022) found higher MMP-9 activity in the hippocampus of MDD patients.

On the other hand, preclinical studies have also suggested the implication of MMP-9 in the etiopathogenesis of depression. In fact, different animal models of depression including the chronic restraint stress model (van der Kooij et al., 2014), a neuroinflammation model (Shishkina et al., 2020), the chronic unpredictable stress model in mice (Bijata et al., 2022) and the chronic corticosterone model of depression (Breviario et al., 2023), displayed increased MMP-9 expression or activity in the brain. In addition, the administration of an MMP-9 inhibitor reverted the depressive-like phenotype of the chronic restrain stress rat model (van der Kooij et al., 2014).

Moreover, antidepressant drugs induce an increase of MMP-9 in animal stress models of depression (Alaiyed et al., 2020) and naïve rats (Benekareddy et al., 2008).

All these findings highlight MMP-9 as a potential biomarker and as a therapeutic target for depression (*see review* Li et al., 2022).

HYPOTHESIS AND OBJECTIVES

Based on previous preclinical and clinical studies, we **postulate** an altered expression and/or activity of matrix metalloproteinase 9 (MMP-9) in the chronic corticosterone mouse model of depression, as well as, in *post-mortem* brain samples of patients diagnosed with depression. We also hypothesize that cannabidiol would produce a fast antidepressant effect through the modulation of MMP-9.

Therefore, the **main objective** of this thesis is to provide further knowledge regarding the role of MMP-9 in major depressive disorder and as a potential therapeutic target for the disease.

To answer this hypothesis, the following **specific objectives** were established:

1. To study the effect of a subchronic treatment with cannabidiol in the chronic corticosterone-induced mouse model of depression in depressive- and anxiety-like behaviour and MMP-9 expression and activity in the brain.

2. To characterise the behavioural (depression/anxiety paradigms), molecular (synaptic plasticity markers), and neurochemical (monoamine levels) phenotype of MMP-9 transgenic mice.

3. To evaluate the expression and activity of MMP-9 and neuroplasticity markers in *post-mortem* human brain samples from depressed patients.

MATERIAL AND METHODS

1. Animals

C57BL/6J (Janvier, France), MMP-9 knockout (The Jackson Laboratory, Maine, USA), and MMP-9 overexpression (kindly given by Dr A.K. Tzinia) mice, 2 - 3 months old, and 25 - 30 g, were used. The animals were group-housed (4 - 5 mice per cage) and kept under controlled temperature (21 ± 1°C), relative humidity (60 - 70%) and light-dark cycle (12:12h, lights on 08:00 – 20:00 hours) conditions, with food and water provided *ad libitum*. The transgenic animals were maintained in the animal house of the Faculty of Medicine, whereas the non-transgenic mouse lines were maintained in the Specific-Pathogen-Free (SPF) from the Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC).

All procedures were carried out with the previous approval of the Animal Care Committee of the University of Cantabria and according to the Spanish legislation (Real Decreto 1386/2018) and the European Communities Council Directive on "Protection of Animals Used in Experimental and Other Scientific Purposes" (86/609/EEC).

Transgenic mice

1.1. MMP-9 knockout mice

B6.FVB(Cg)-Mmp9^{tm1Tvu}/J or MMP-9 KO mice (#007084, The Jackson Laboratory, Maine, USA) were generated by inserting a neomycin resistance gene driven by the mouse phosphoglycerate kinase promoter. This cassette is replacing most of exon 2 and all of intron 2, disrupting the functionality of MMP-9.

1.2. MMP-9 overexpressing mice

TgMMP9 or MMP-9 OE mice were kindly given by Athina K. Tzinia (Institute of Biosciences and Applications - National Centre of Scientific Research "Demokritos", Greece). MMP-9 OE mice were generated with PDGF-B/MMP9 transgene of human origin, a gene encoding for human pro-MMP-9 under the control of the human neuron-specific PDGF-B promoter (Fragkouli et al., 2012).

2. *Post-mortem* human brain samples

Human brain samples from the prefrontal cortex (Brodmann´s area 9) were obtained at autopsy in the Basque Institute of Legal Medicine, Bilbao, Spain. Patients were diagnosed with major depression disease, with and without antidepressant blood levels at the time of death. Control subjects were free of psychiatric or neurological disorders based on medical history, *post-mortem* tissue examinations and a negative toxicological screening on blood at autopsy. Both groups were matched for sex, age and *post-mortem* interval (table 1). All samples were kindly given by Brain Bank of the Instituto de Salud Carlos III, Universidad del País Vasco (UPV/EHU).

Table 1. Characteristics of the subjects diagnosed with MDD and their matched controls by gender, age and post-mortem interval. C: control; CRF: cardiorespiratory failure; F: female; M: male; MDD: major depressive disorder; PMI: post-mortem interval; THC: Tetrahydrocannabinol.

Code	Gender (M/F)	Age (years)	PMI (h)	Cause of death	Diagnostic	Toxicology
C 1	M	62	9	Natural (CRF)	Control	(-)
MDD 1	M	62	15	Suicide (knife)	Depression	Citalopram Trazodone Benzodiazepine
C 2	F	50	10	Natural (CRF)	Control	(-)
MDD 2	F	49	19	Suicide (precipitation)	Depression	Mirtazapine Benzodiazepine
C 3	F	36	20	Accident (train)	Control	(-)
MDD 3	F	36	32	Suicide (toxics)	Depression	Venlafaxine Paroxetine Benzodiazepine
C 4	F	71	22	Accident (traffic)	Control	(-)
MDD 4	F	70	7	Suicide (precipitation)	Depression	Mirtazapine Sertraline Benzodiazepine
C 5	M	64	22	Natural (heart attack)	Control	(-)
MDD 5	M	65	12	Suicide (hanging)	Depression	Venlafaxine
C 6	M	51	19	Accident (traffic)	Control	(-)
MDD 6	M	53	22	Suicide (hanging)	Depression	Mirtazapine Venlafaxine Benzodiazepine

Table 1 (Continued).

Code	Gender (M/F)	Age (years)	PMI (h)	Cause of death	Diagnostic	Toxicology
C 7	M	47	17	Natural (CRF)	Control	(-)
MDD 7	M	47	18	Suicide (hanging)	Depression	Citalopram
C 8	F	45	12	Natural (CRF)	Control	(-)
MDD 8	F	43	24	Natural (CRF)	Depression	Venlafaxine
C 9	M	67	22	Natural (CRF)	Control	(-)
MDD 9	M	65	11	Suicide (toxics)	Depression	Mirtazapine Sertraline Benzodiazepine
C 10	F	45	8	Natural (haemorrhage)	Control	(-)
MDD 10	F	49	18	Suicide (submersion)	Depression	Fluoxetine
C 11	M	56	24	Natural (heart attack)	Control	(-)
MDD 11	M	57	21	Suicide (precipitation)	Depression	(-)
C 12	F	43	3	Accident (traffic)	Control	(-)
MDD 12	F	45	21	Suicide (hanging)	Depression	(-)
C 13	M	54	23	Accident (precipitation)	Control	(-)
MDD 13	M	51	24	Suicide (hanging)	Depression	(-)
C 14	M	56	13	Natural (CRF)	Control	(-)
MDD 14	M	56	3	Natural (thromboembolism)	Depression	(-)
C 15	M	61	23	Accident (traffic)	Control	(-)
MDD 15	M	60	24	Suicide (precipitation)	Depression	(-)
C 16	M	73	10	Accident (precipitation)	Control	(-)
MDD 16	M	73	11	Suicide (hanging)	Depression	(-)
C 17	M	48	10	Natural (heart attack)	Control	THC Benzoylecgonine
MDD 17	M	50	24	Suicide (firearm)	Depression	(-)
C 18	M	55	22	Natural (heart attack)	Control	(-)
MDD 18	M	56	22	Suicide (firearm)	Depression	(-)
C 19	F	56	25	Natural (CRF)	Control	(-)
MDD 19	F	58	27	Natural (haemorrhage)	Depression	(-)
C 20	M	67	12	Natural (aorta dissection)	Control	(-)
MDD 20	M	68	20	Suicide (precipitation)	Depression	(-)

Table 1 (Continued).

Code	Gender (M/F)	Age (years)	PMI (h)	Cause of death	Diagnostic	Toxicology
C 21	F	80	16	Accident (run over)	Control	(-)
MDD 21	F	79	14	Natural (CRF)	Depression	Amitriptiline
C 22	F	55	13	Natural (CRF)	Control	(-)
MDD 22	F	55	24	Suicide (precipitation)	Depression	(-)
C 23	F	51	12	Natural (CRF)	Control	(-)
MDD 23	F	50	13	Natural (heart attack)	Depression	(-)
C 24	F	74	16	Natural (heart attack)	Control	(-)
MDD 24	F	73	26	Suicide (precipitation)	Depression	Benzodiazepine
C 25	F	59	24	Accident (precipitation)	Control	(-)
MDD 25	F	60	3	Suicide (precipitation)	Depression	Mirtazapine Benzodiazepine
C 26	F	55	32	Accident (run over)	Control	ethanol
MDD 26	F	57	27	Natural (CRF)	Depression	Mirtazapine Olanzapine Benzodiazepine

3. Drugs and reagents

All chemicals and reagents were of analytical grade. The chemicals, drugs, reagents and commercial suppliers are listed in table 2.

Table 2. Drugs, reagents and their suppliers.

REAGENT	SUPPLIER
2-mercaptoethanol	Sigma-Aldrich, Missouri, USA
40% Acrylamide/Bis solution, 37.5:1	Bio-Rad, California, USA
Acetic acid	Probus SA, Badalona, Spain
Aprotinin	Affymetrix - USB® Products, Ohio, USA
APS (ammonium persulfate)	Sigma-Aldrich, Missouri, USA
Brij35®	Sigma-Aldrich, Missouri, USA

Table 2 (Continued).

REAGENT	SUPPLIER
$CaCl_2$	Panreac, Barcelona, Spain
(-) Cannabidiol	Tocris, Bristol, UK
Coomassie Blue R-250	Thermo Fischer Scientific S.L., Madrid, Spain
Corticosterone (4-pregnen-11beta, 21-diol-3, 20-dione 21-hemisuccinate)	Steraloids, Inc., Rhode Island, USA
DC™ Protein Assay	Bio-Rad, California, USA
DreamTaq Green PCR Master Mix	Thermo Fischer Scientific S.L., Madrid, Spain
EDTA (ethylenediaminetetraacetic acid)	Sigma-Aldrich, Missouri, USA
Ethanol	Scharlab S.L., Barcelona, Spain
FBS (fetal bovine serum)	Biowest, Pays de la Loire, France
Gelatin	Sigma-Aldrich, Missouri, USA
Glycine	Sigma-Aldrich, Missouri, USA
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Usb, Ohio, USA
Isopropanol	Sigma-Aldrich, Missouri, USA
Laemmli buffer 2x/4x	Bio-rad, California, USA
Methanol	Scharlab S.L., Barcelona, Spain
$MgCl_2$	Scharlab S.L., Barcelona, Spain
NaCl	BDH Prolabo/VWR, Tingalpa, Australia
NaF	Sigma-Aldrich, Missouri, USA
Na_3VO_4	Sigma-Aldrich, Missouri, USA
PageRuler Plus Prestained Protein Ladder	Thermo Fischer Scientific S.L., Madrid, Spain
PMSF (Phenylmethylsulfonyl fluoride)	Sigma-Aldrich, Missouri, USA
Powder skimmed milk	Central lechera asturiana, Asturias, Spain
Propylenglycol®	Panreac, Barcelona, Spain
Protease inhibitors cocktail	Sigma-Aldrich, Missouri, USA

Table 2 (Continued).

REAGENT	SUPPLIER
SDS (sodium dodecyl sulfate)	Thermo Fischer Scientific S.L., Madrid, Spain
1×SYBR® Safe DNA Gel Stain	Thermo Fischer Scientific S.L., Madrid, Spain
Proteinase K	Sigma-Aldrich, Missouri, USA
Sucrose	Scharlab S.L., Barcelona, Spain
TEMED (tetramethylethylenediamine)	Sigma-Aldrich, Missouri, USA
Tris-HCl	Sigma-Aldrich, Missouri, USA
Triton X-100	Sigma-Aldrich, Missouri, USA
Triton X-114	Acros Organics, New Jersey, USA
Tween-20	Sigma-Aldrich, Missouri, USA
Tween 80®	Scharlab S.L., Barcelona, Spain
$ZnCl_2$	Sigma-Aldrich, Missouri, USA

Table 3. Primary and secondary antibodies and dilutions used for protein detection by Western Blot, with their corresponding reference and supplier. BDNF: brain-derived neurotrophic factor; g: goat; m: mouse; MMP-9: matrix metalloproteinase 9; mTOR: mammalian target of rapamycin; PSD95: postsynaptic density protein 95; rb: rabbit.

ANTIBODY	DILUTION	REFERENCE	SUPPLIER
β-tubulin III (rb, m)	1:20000	T2200/T8660	Sigma-Aldrich
BDNF (rb)	1:500	Ab108319	Abcam
MMP-9 (rb)	1:6000	#102-16681	RayBiotech Life
mTOR (m)	1:1000	#4517	Cell signaling
p-Ser2448-mTOR (rb)*	1:1000	#2971	Cell signaling
Nectin-3 (m)	1:800	sc-271611	Santa Cruz Biotechnology
Nectin-3 (rb)	1:10000	JM-3821	MBL International
PSD95 (g)	1:200	sc-8575	Santa Cruz Biotechnology
Synapsin I (m)	1:200	sc-390867	Santa Cruz Biotechnology
700CW anti-mouse	1:15000	926-68072	LI-COR®
800CW anti-rabbit	1:15000	926-32213	LI-COR®
800CW anti-goat	1:15000	926-32214	LI-COR®

* The phosphorylation form of mTOR corresponds to the active form of the proteins.

4. Corticosterone mouse model

Male C57BL/6J mice were treated with corticosterone dissolved in drinking water (45 mg/l, equivalent to a dose of 6 - 10 mg/kg/day) in opaque bottles to avoid degradation, for 4 weeks following the protocol previously described by Gourley and Taylor (2009). The corticosterone solution was changed every 7 days. Control mice were provided with water.

5. Pharmacological treatment with cannabidiol

Cannabidiol (CBD) was dissolved in a vehicle solution (5% Propylenglycol®, 2% Tween 80®, in saline) and administered intraperitoneally (i.p.), at a dose of 50 mg/kg/day, for 4 consecutive days. The dose and route of administration of CBD were based on a previous study of the antidepressant-like effect of CBD in mice by Linge et al. (2016) (see experimental design, figure 8).

6. Genotyping

6.1 DNA extraction

DNA was obtained from the mouse tail. Briefly, the tissue was incubated overnight in tail buffer (10 mM Tris-HCl, pH 8.3, 50 mM EDTA, 100 mM NaCl, 0,5% SDS), containing 750 mU proteinase K, at 55°C. Then, NaCl 5 M was added, and samples were centrifuged for 10 min at 16,000xg at room temperature. The supernatant was collected, mixed with isopropanol, and centrifuged for 10 min at 16,000xg at 4°C. The pellet was resuspended in ethanol 70%, and centrifuged for 5 min at 16,000xg at 4°C. The pellet was dried and finally resuspended in TE buffer (10 mM Tris-HCl, pH 8.3, and 1 mM EDTA). The samples were stored at -20°C until use.

6.2 PCR amplification

MMP-9 KO

To perform the polymerase chain reaction (PCR) the following primers were used: a common (forward) primer (5'-TCCTCCATCCACAGGCATAC-3'), a wild type (reverse) primer (5'- TCCCACTTGAGGCCTTTGA-3') and a mutant (reverse) primer (5'-CCTTCTATCGCCTTCTTGACG-3') (Sigma-Aldrich, Missouri, USA). After the amplification, a band of 223 bp would correspond to WT mice, a band of 400 bp would correspond to MMP-9 KO mice and both bands to heterozygous mice. The PCR mixture contained: 0.53 µM of each primer, DreamTaq Green PCR Master Mix (containing Taq polymerase, 0.4 mM of each dNTPs, 4 mM $MgCl_2$), 5 µl RNAsa free water, 10 µM $MgCl_2$ and 1 µl DNA. The touchdown PCR (table 4) were performed in a 2720 Thermal Cycler (Applied Biosystems, California, USA).

Table 4. Touchdown PCR conditions for MMP-9 KO genotyping.

STEP	T (°C)	TIME (s)	NOTE
1	94	120	
2	94	20	
3	65	20	-0.5°C per cycle decrease
4	68	30	
5	repeat steps 2-4 for 10 cycles		
	(Touchdown)		
6	94	20	
7	60	20	
8	72	30	
9	repeat steps 6-8 for 28 cycles		
10	72	300	
11	4	∞	

MMP-9 OE

For the PCR of the MMP-9 OE mice, forward (5'- GCACCACCACAACATCACCTAT-3') and reverse (5'-AAACTGGATGACGATGTCTGCG-3') primers were used (Sigma-Aldrich, Missouri, USA). After the amplification, a band of 200 bp correspond to MMP-9 OE mice. The PCR mixture contained: 0.53 µM of each primer, DreamTaq Green PCR Master Mix (contains Taq polymerase, 0.4 mM of each dNTPs, 4 mM $MgCl_2$, 5.4 µl RNAsa free water, 10 µM $MgCl_2$ and 1 µl DNA extracted. The PCR conditions were: 95°C for 5 min, 30 cycles at 95°C for 1 min, 60°C for 1 min and 72°C for 1 min, and finally, a cycle at 72°C for 10 min, maintaining the samples at 4°C until use. The PCR was performed in the 2720 Thermal Cycler (Applied Biosystems, California, USA).

Once the amplification was over, 15 µl of the PCR reaction was run in a 1.5% agarose gel in TAE 1X (40 mM Tris, 20 mM acetic acid and 1 mM EDTA). The gel was stained with 0.01% of 1×SYBR® Safe DNA Gel Stain to visualize the bands in the Chemidoc XRS (Bio-Rad, California, USA).

7. Experimental design

7.1 Corticosterone mouse model treated with cannabidiol

The number of animals used per experimental group was 10. After 4 weeks of corticosterone administration, and the confirmation of the depressive-, anxious-like behaviour, vehicle or CBD (50 mg/kg/day, i.p.) were administered, and the behavioural effect on depressive/anxiety tests was evaluated (figure 8).

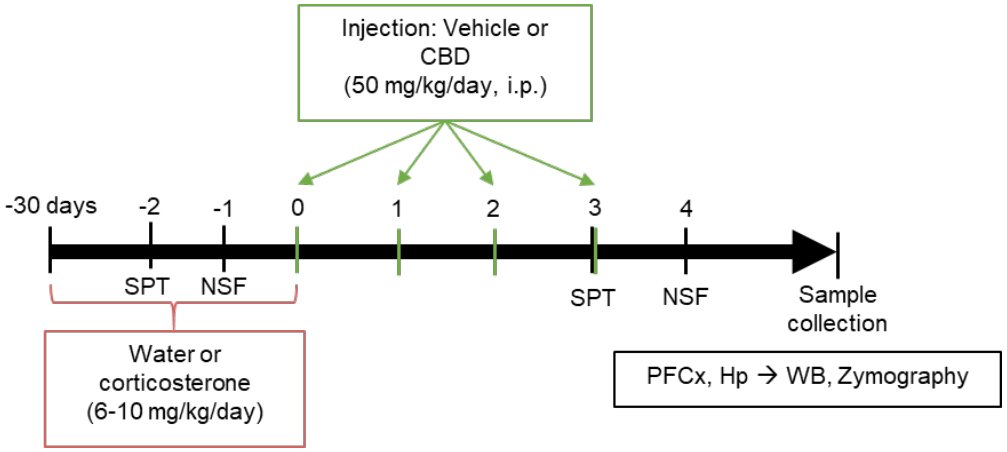

Figure 8. Schedule of the behavioural and molecular study of the subchronic treatment with CBD or vehicle in the corticosterone model performed in male mice. CBD: cannabidiol; Hp: hippocampus; NSF: novelty suppressed feeding; PFCx: prefrontal cortex; SPT: sucrose preference test; WB: Western blot.

7.2 Behavioural characterisation of transgenic mice

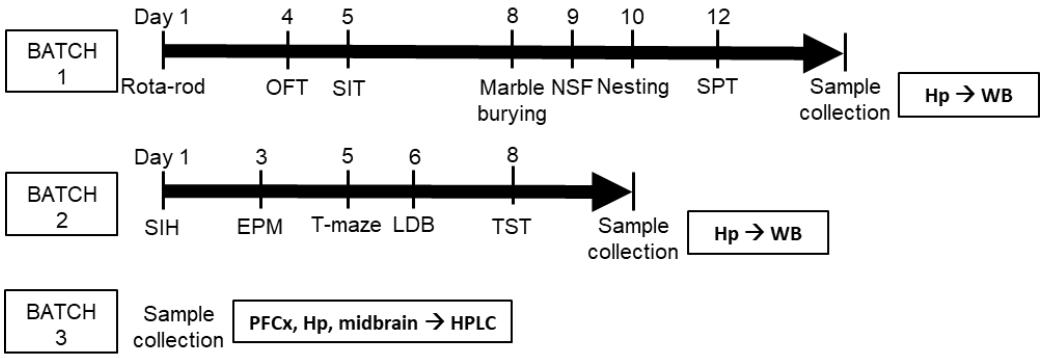

Figure 9. Schedule of the behavioural characterisation of the MMP-9 KO and MMP-9 OE transgenic male and female mice. EPM: elevated plus maze; Hp: hippocampus; HPLC: high performance liquid chromatography; LDB: light-dark box; NSF: novelty suppressed feeding; OFT: open field test; PFCx: prefrontal cortex; SIH: stress-induced hyperthermia; SIT: social interaction test; SPT: sucrose preference test; TST: tail suspension test; WB: western blot.

To characterise the behavioural phenotype of the transgenic MMP-9 KO and MMP-9 OE mice, the following experimental design was performed (figure 9). Two different batches of mice were used to avoid an overload of stress produced due to the battery of behavioural tests assessed. The number of animals used per experimental group was 6 - 10.

8. Behavioural tests

All the behavioural tests were performed during the light phase, except for the sucrose preference and the nesting tests. Mice were placed in the experimental room 1 hour before the behavioural tests to let them acclimatize. The behavioural tests were performed from the least to the most stressful.

Anxiety-related behavioural tests

8.1 Open Field Test (OFT)

The open field test is used to evaluate the innate anxiety and the locomotor activity of animals, considering that mice tend to avoid open environments (Garro-Martínez *et al.*, 2021). The apparatus consists of a squared arena (40 x 40 x 30 cm) with an illuminated (50 lx) centre (20 x 20 cm). Mice were placed in a corner of the open field, and the central time and total distance travelled were video-tracked (Any-Maze^{TM} tracking software-version 4.99, Stoelting Co., Wood Dale, USA) for 5 min. The open field was cleaned with ethanol 70% between trials to avoid olfactory cues.

8.2 Elevated plus maze (EPM)

The elevated plus maze evaluates the anxiety-like behaviour, based on the natural exploratory behaviour of mice in novel environments and their aversion to open and elevated spaces (Komada *et al.*, 2008). The apparatus (50 cm off the floor) consists of

two open arms (25 x 5 x 0.5 cm), and two perpendicular closed arms (25 x 5 x 16 cm), with a central region (5 x 5 x 0.5 cm). Mice were placed in the centre of the apparatus and were allowed to freely explore for 5 min. The time spent in each arm was video-tracked (Any-Maze^{TM}).

8.3 Light-Dark Box (LDB)

The light-dark box is a conventional test used to study anxious-like behaviour in mice, based on their innate fear of illuminated and open spaces (*see review* Bourin and Hascoët, 2003). The apparatus consists of a square box (40 x 40 x 20 cm), divided into two halves, one illuminated (50 lx) area and one dark area, connected by an opening. Mice were placed in a corner of the illuminated compartment and the animal was allowed to freely explore (Vidal et al., 2019). The time spent in each area was video-tracked (Any-Maze^{TM}) for 5 min.

8.4 Novelty Suppressed Feeding (NSF)

The novelty suppressed feeding is a conflict-based anxiety test, as mice experience a motivational conflict between eating a pellet in an open and illuminated place and avoiding the central zone (*see review* Planchez et al., 2019).

The NSF was performed as previously described by Vidal *et al.* (2019). Mice were food deprived for 24 hours, and then they were placed in a corner of an arena (40 x 40 x 30 cm) covered with woodchip. A food pellet was placed in the centre that was illuminated (50 lx). The latency to eat the pellet was recorded using video-tracking software (Any-Maze^{TM}), with a maximum duration of the test of 10 min. Once the test was completed, mice were placed back in their home cage and the amount of food eaten in 5 min was measured. Those mice that did not eat in the post-test were excluded.

Depression-related behavioural tests

8.5 Social Interaction Test (SIT)

The social interaction test assesses the social behaviour in animals. The reduction of social interest is considered a depressive-like behaviour, related to apathy (*see review* Planchez *et al.*, 2019). Mice were placed in an open field arena (40 x 40 x 30 cm) with an empty cylinder, with thick openings, placed in a corner. Experimental mice were allowed to freely explore the environment for 2.5 min. Immediately after, an unfamiliar mouse of a different strain was placed inside the cylinder and the experimental mice were allowed to explore for 2.5 min. The time in the interaction zone (20 cm diameter) was assessed by video-tracking software (Any-Maze^{TM}).

8.6 Sucrose Preference Test (SPT)

The sucrose preference test assesses anhedonia, which is the loss of interest or the inability to experiment pleasure, which is associated with depressive-like behaviour. This test is used to evaluate the brain reward system and the effect of antidepressant drugs and genetic modifications on the hedonic state.

First, mice were given a free choice between a bottle of water and another bottle with a 1% sucrose solution (Vidal *et al.*, 2019). The position of the bottles was exchanged every 24 hours for 7 days, to avoid any place preference. Mice were individualized and the sucrose preference was calculated as the percentage of sucrose solution consumed compared to the total amount of liquid intake for 24 hours.

8.7 Tail Suspension Test (TST)

The tail suspension test is widely used to assess depressive-like behaviour, specifically behavioural despair (Florensa-Zanuy *et al.*, 2021). Mice were suspended by the tail with adhesive tape for 6 min. Normally, animals struggle to free themselves, but as they

cannot escape, they will develop immobility episodes (*see review* Abelaira *et al.,* 2013). A video-tracking software (Any-Maze™) recorded the test and the time spent immobile was determined manually for the last 4 minutes by an observer blind to the experimental group.

Other general behavioural tests

8.8 Rota-rod

The rota-rod is a test used to evaluate motor coordination and balance. This test was performed using a previously described protocol (Luong et al., 2011) with minor modifications. The apparatus consists of a rotating rod of 3 cm in diameter, and 16 cm high. The rod is divided into 5 equal spaces, separated by flanges to avoid visual contact. The day before the test, mice were trained for 60 s at 20 r.p.m., in up to 3 trials. Mice were tested 24 h later, and the latency to fall was measured, with a maximum time of 60 s. The mice that fell off in the first 5 s, due to an incorrect placing by the experimenter, were placed back in a new trial.

8.9 Marble Burying Test

The marble burying test is used to assess compulsive-like behaviour in mice (Thomas *et al.*, 2009). 15 marbles where geometrically distributed in a cage (40 x 20 x 20 cm), previously covered with abundant woodchip. Then, a mouse was placed in a corner and allowed to explore for 30 min. At the end of the test, the number of marbles buried (marbles covered more than 50%) was counted.

8.10 Nesting test

The nesting is a general well-being test, but it has been shown to be sensitive to hippocampal lesions (Chiu *et al.*, 2014; Deacon *et al.*, 2002). The nesting test was

performed following Deacon (2006) protocol. Mice were individualized 1 h before the beginning of the dark phase. In every cage, a 5 cm square of pressed cotton wafer was placed, and the nests were assessed the next morning following these scoring criteria: 1 (untouched material), 2 (partially torn material), 3 (torn material, but nest not identifiable), 4 (flat nest) and 5 (perfect nest) (figure 10).

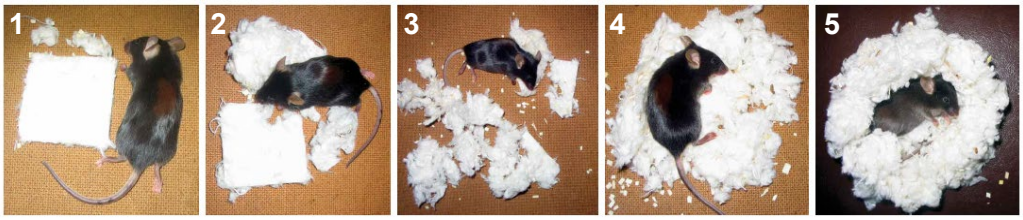

Figure 10. Scoring criteria of the nesting test (Deacon et al., 2006).

8.11 T-maze

The T-maze is used to assess spatial memory, based on the natural instinct of mice to explore novel environments (Wenk, 2001). The apparatus consists of three arms, two opposite (30 x 5 x 15 cm), and the third one perpendicular (35 x 5 x 15 cm), creating a "T" form. The T-maze was adapted from a previously described protocol (Davis *et al.*, 2017). First, a training trial was assessed blocking the left arm of the T-maze. Mice were placed at the end of the perpendicular arm and were allowed to explore for 8 min. After the training trial, mice were removed from the apparatus and left to rest in an inter-trial interval of 1 h. Then, for the experimental trial, the left arm was unlocked, recovering the "T" shape and a 3 min test was performed by placing the mice at the end of the perpendicular arm and allowing them to freely explore the apparatus. Mice were video-tracked (Any-Maze^{TM}) and the percentage of alternation was calculated by the first arm entered, which was scored 100 (new arm) or 0 (right arm).

8.12 Stress-Induced Hyperthermia (SIH)

The stress-induced hyperthermia is used to evaluate the physiological increase in the temperature induced by an acute stressor. Moreover, SIH is inhibited by anxiolytic drugs (*see review* Bouwknecht et al., 2007).

The SIH was performed as previously described by Garro-Martínez et al. (2020). The procedure consists of introducing a thermosensitive probe (2 cm), linked to a digital thermometer, in the rectum of the animal, until the temperature is stable (30 s). This procedure induces an acute stress response in mice. Therefore, the temperature was measured at time 0, and 15 minutes later.

9. Tissue collection

Mice were sacrificed by cervical dislocation 30-60 min after the last behavioural test. Brains were extracted and kept in cold and the prefrontal cortex (PFCx), cortex (Cx), and hippocampus (Hp) for molecular techniques. For neurochemical studies, naïve animals were sacrificed and PFCx, Hp and midbrain were dissected. Samples were immediately stored at -80°C until used.

10. Molecular techniques

10.1 Western Blot (WB)

Protein extraction: synaptoneurosomal fraction

According to the protocol previously described by Li et al. (2010), an enriched synaptoneurosomal fraction containing both, presynaptic (synaptosome) and postsynaptic (neurosome) elements, was used.

The brain areas were homogenized 1:15 w/v with homogenization buffer (0.32 M sucrose, 20 mM HEPES, pH 7.4, 1 mM EDTA, 1:100 protease inhibitor cocktail, 5 mM

NaF and 1 mM Na_3VO_4), using a pellet pestle motor. Subsequently, samples were centrifuged at 800xg for 10 minutes, at 4°C. The pellet (nuclear fraction) was removed, and the supernatant was centrifuged at 15,300xg for 10 minutes, at 4°C. The supernatant (cytosolic fraction) was discarded, and the pellet (synaptoneurosomal fraction) was resuspended in 150 µl of protein lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF and 1:100 protease inhibitor cocktail) and sonicated for 1 minute. The samples were stored at -20°C until used.

The protein amount was quantified using the DC™ Protein Assay, according to the manufacturer's protocol, based on the Lowry method (Lowry et al., 1951). Samples were prepared using 2x Laemmli buffer containing 2-mercaptoethanol, boiled at 100°C for 5 min, cooled down in ice for 3 minutes and centrifuged at 1000xg for 5 min, at 4°C. The supernatant was stored at -20°C until used.

Western blot protocol

8.5% acrylamide gels were used for the separation of proteins with a molecular weight higher than 40 kDa, whereas 15% acrylamide gels were used for lower molecular weight proteins. The gels were placed in the electrophoresis tank, and filled with 1x migration buffer (20 mM Tris, 0.2 M glycine, 1% SDS). Then, 50 µg of protein were loaded in duplicate, and a protein ladder (PageRuler Plus Prestained) was also loaded to identify their molecular weight. The electrophoresis consists of a first step at 100 V for 15 min, followed by a 160 V step for 50 min. Before the protein transfer, the separation gels and nitrocellulose membranes (Bio-Rad, California, USA), were incubated in transference buffer (20 mM Tris, 0.2 M glycine and 20% methanol) for 30 min. Then, the proteins were transferred to the membranes at 100 V, and 4°C. Proteins with a molecular weight higher than 40 kDa were transferred for 90 min, and proteins with a molecular weight lower than 40 kDa, for 45 min.

The nonspecific antibodies binding sites of the membranes were blocked for 1 h at room temperature. The main blocking solution used was 5% powder skimmed milk in TBS-T (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20). For phosphorylated proteins, the blocking solution used was 3% powder skimmed milk in TBS-T supplemented with phosphatase inhibitors (1 mM Na_3VO_4 and 1 mM NaF). Then, membranes were incubated overnight at 4°C, with the primary antibody (table 3) diluted in blocking solution. The next day, the membranes were washed 3 times with TBS-T for 15 min and incubated with the corresponding secondary antibody conjugated to a fluorophore (table 3) diluted in the incubation solution for 1 h at room temperature in the dark. Finally, the membranes were washed 3 times with TBS-T for 15 min in the dark, and the fluorescent signal was detected using an Odyssey® CLx Imaging System (LI-COR Biosciences, Nebraska, USA) at 700 and 800 nm.

Western Blot quantification

The signal obtained with the Odyssey® CLx Imaging System, was quantified using the Image Studio™ Software (LI-COR Biosciences, Nebraska, USA). The densiometric values were normalized with respect to the housekeeping protein β-tubulin III.

10.2 Gel Zymography

We used this technique to evaluate the gelatinase activity of MMP-9 and MMP-2.

Protein extraction: synaptoneurosomal fraction

To study the MMP-9 activity, the synaptoneurosomal fractionation in the cortex, was performed as previously described by Szklarczyk et al. (2002). In brief, the tissue was homogenized 1:20 w/v with sample buffer (10 mM $CaCl_2$, 0.25% Triton X-100), using a stirrer (Heidolph Instruments, Schwabach, Germany). Next, samples were centrifuged at 6,000xg for 30 min, at 4°C. The supernatant was discarded, and the pellet was resuspended in 150 µl of pellet buffer (50 mM Tris-HCl, pH 7.4, 0.1 M $CaCl_2$). Then, the samples were heated at 60°C for 15 min and centrifuged at 10,000xg for 30 min, at 4°C,

which releases the MMPs bound to the extracellular matrix. Finally, 4 µl of pellet buffer supplemented with 10% Triton X-114 was added to the supernatant, to increase the resolution of the digested bands.

The protein amount was quantified using the DC™ Protein Assay, based on the Lowry method (Lowry et al., 1951). Samples were prepared using 4x Laemmli buffer, to load 100 µg of protein per well.

As a positive control, 2% FBS (Fetal Bovine Serum) was prepared in extraction buffer (2% Triton X-114, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 µl/ml aprotinin, 10 µl/ml PMSF) and 4x Laemmli buffer.

Gel zymography protocol

The acrylamide gels (8.5%), containing 0.1% gelatin were placed in the electrophoresis tank, and filled with 1x migration buffer (20 mM Tris, 0.2 M glycine, 1% SDS) at 4°C. The samples (100 µg/well) were loaded per duplicate, as well as the positive control and the protein ladder (PageRuler Plus Prestained). Two steps were performed to separate the proteins: a first step at 100 V for 15 min, followed by a 160 V step for 50 min.

The gels were washed twice with washing buffer (2.5% Triton X-100) for 15 min, at room temperature. Then, gels were washed with developing buffer (50 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 6.7 mM $CaCl_2$, 1 µM $ZnCl_2$; 0.2% Brij35®) for 30 min, and finally, incubated in fresh developing buffer for 48 h for mouse samples and 7 days for human samples, at 37°C.

Zymography quantification

After the incubation, the gels were washed 3 times with distilled water for 5 min and were stained for 1 h with staining solution (0.5% Coomassie Blue R-250, 5% methanol and 10% acetic acid). Then, gels were distained with a distaining solution (10% methanol, 5% acetic acid), until areas of proteolytic activity are clearly visible. Finally, gels were scanned using an Odyssey® CLx Imaging System (LI-COR Biosciences, Nebraska,

USA). The bands were quantified with the Image Studio™ Software (LI-COR Biosciences, Nebraska, USA).

11. High-Performance Liquid Chromatography (HPLC)

This technique was used to analyse the levels of monoamines in the PFCx, Hp and midbrain of MMP-9 transgenic mice.

Sample preparation

Samples were placed in nuclease-free tubes and 400 µl of 0.1% sodium metabisulphite, 0.01% EDTA, 0.1% cysteine, and 0.4 M perchloric acid were added. Then, samples were homogenized with a sonicator ultrasonic homogenizer (Braun, California, USA), at the maximum power, for 30 seconds and centrifuged at 21000xg, for 30 minutes, at 4°C. The supernatant was collected and filtered through 0.45 µm filters. Samples were immediately stored at -80°C, until use.

HPLC system

The HPLC system was an ALEXYS® Neurotransmitter Analyzer (Antec Scientific, Leiden, the Netherlands), which consists of an OR 110 degasser unit with pulse damper, LC 110S pump, a DECADE Elite electrochemical detector, Clarity chromatography software of DataApex (Prague, Czech Republic) and an AS 110 autosampler.

Monoamines detection

The levels of serotonin (5-HT), dopamine (DA) and noradrenaline (NA) were determined using an Acquity UPLC® BEH C18, 1.7 µm, 1 x 100 mm column (Waters, Massachusetts, USA). The mobile phase consisted of 100 mM citric acid, 100 mM phosphoric acid, pH 6.0, 0.1 mM EDTA, 950 mg/L octanesulfonic acid, and 5% acetonitrile. The injection volume was 5 µl. The temperature was set at 42°C for the separation and detection steps, the flow rate was 75 µl/min and the oxidation potential used was 0.46 V.

HPLC quantification

The neurotransmitter levels were quantified using a calibration standard curve. The results were expresed as pmol/mg of tissue.

12. Data analysis and statistics

For molecular studies, the mean of the sample replicates was normalized *versus* the vehicle (objective 1), WT mice (objective 2), or control group (objective 3) (100%). All the results are expressed as the mean ± standard error of the mean (S.E.M.).

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., California, USA). The statistical analyses were performed using two-way ANOVA for the data obtained in the **corticosterone mice model** (objective 1) treated with cannabidiol experiments, followed by Newman-Keuls *post-hoc* test. The correlation between the results obtained in the molecular experiments and the behaviour was done using Pearson's correlation coefficient (r). Unpaired Student *t*-test was used to compare the behavioural phenotype of **transgenic mice** (objective 2), while paired Student *t*-test was used in the SIH and a chi-squared test was used to analyse the percentage of alternation of the T-maze. Paired Student *t*-test was used in the molecular results in the **human samples** (objective 3). The outliers were identified using GraphPad Prism 6 (GraphPad Software Inc., California, USA). The type of statistical analysis for each experiment is specified in the Results section. The statistical significance was considered when $p < 0.05$.

RESULTS

Objective 1. Effect of a subchronic treatment with cannabidiol in the corticosterone animal model of depression

1.1 Behavioural effect of cannabidiol in a corticosterone-induced mouse model of depression

To confirm the mouse model of depression induced by the chronic administration of corticosterone (CORT) were performed a sucrose preference test (SPT) and a novelty suppressed feeding test (NSF) (*see figure 8 in Materials and methods*). A lower sucrose preference in the SPT (51.8 ± 2.7% in CORT *vs* 85.7 ± 0.9% in the vehicle group, $p<0.001$) and a higher latency to feed in the NSF (441.2 ± 32.4 s in CORT *vs* 151.2 ± 17.9 s in the vehicle group, $p<0.001$) were observed in this model, which is predictive of a depressive- and anxious-like phenotype.

Sucrose preference test

Both naïve and CORT groups were divided into vehicle and cannabidiol (CBD) subgroups. The cannabidiol groups were administered 50 mg/kg/day, i.p. CBD (*see figure 8 in Materials and methods*). After three days of cannabidiol treatment, the anhedonic state of the animals was evaluated. In the CORT group, a lower sucrose preference (54.6 ± 6.8% in CORT *vs* 78.1 ± 2.4% in the vehicle group, $p<0.01$; figure 1A) was observed. A two-way ANOVA showed a significant effect of the CORT administration [$F_{(1,35)} = 21.45$, $p<0.001$] in the anhedonia. No statistical differences were observed in the sucrose preference in the CORT+CBD group compared to the CORT group (52.4 ± 7.7% in CORT+CBD *vs* the CORT group, *ns*, figure 11A).

However, data showed two different populations in the CORT+CBD group: responders (figure 11B) and non-responders (figure 11C). A reversion of the anhedonic state in the CORT+CBD responder group was observed compared to the CORT group (70.2 ± 3.8% in CORT+CBD *vs* the CORT group, $p<0.05$, figure 11B). A two-way ANOVA showed a significant effect of the CORT model [$F_{(1,31)} = 13.18$, $p<0.001$] in the SPT with the

responder group. By contrast, the CORT+CBD non-responder group exhibited a further decrease in the sucrose preference compared to the CORT model group (25.7 ± 3.1% in CORT+CBD *vs* the CORT group, $p<0.001$, figure 11C). A two-way ANOVA showed an effect of the CORT model [$F_{(1,29)} = 62.03$, $p<0.001$], of the CBD treatment [$F_{(1,29)} = 7.94$, $p<0.01$] and of the interaction of both [$F_{(1,29)} = 9.50$, $p<0.01$] in the SPT with the non-responder group.

Finally, in naïve animals, the treatment with CBD had no effect in the sucrose preference test (figures 11A, 11B and 11C).

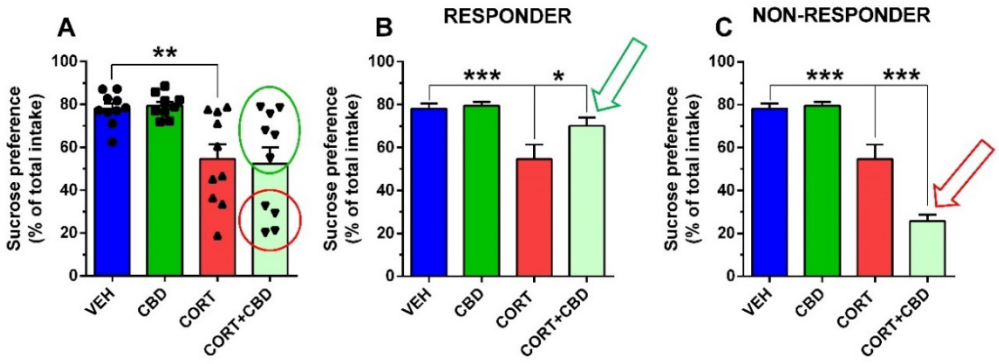

Figure 11. Effect of the subchronic treatment with CBD in the corticosterone-induced mouse model of depression in the sucrose preference test. (A) Percentage of sucrose intake. (B) Sucrose preference in the responder group. (C) Sucrose preference in the non-responder group. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by a Newman-Keuls *post hoc* test *$p<0.05$, **$p<0.01$, ***$p<0.001$. n = 9 – 10 animals per group. VEH: vehicle, CBD: cannabidiol, CORT: corticosterone.

Novelty suppressed feeding test

After 4 days of cannabidiol treatment, an higher latency to feed in the NSF (578.5 ± 11.6 s in CORT *vs* 405.5 ± 45.2 s in the vehicle group, $p<0.05$, figure 12A) was observed in the CORT group. Moreover, the treatment with CBD reversed the increased latency to feed in this model (420.5 ± 50.2 s in CORT+CBD *vs* the CORT group, $p<0.05$, figure 12A). A

two-way ANOVA showed a significant effect of the interaction between the model and the treatment [$F_{(1,31)}$ = 13.34, p<0.001] in the NSF.

The CORT+CBD group also showed two different populations in the NSF: responder (figure 12B) and non-responder (figure 12C). A significant reversion was observed in the latency in the CORT+CBD responder group compared to the CORT group (249.1 ± 32.7 s in CORT+CBD *vs* the CORT group, p<0.001, figure 12B). A two-way ANOVA showed a tendency to an effect of the CBD treatment [$F_{(1,25)}$ = 6.699, p = 0.0158], and a significant effect of the interaction of the model and the treatment [$F_{(1,25)}$ = 40.02, p<0.001] in the responder group. No effect of the CBD administration was observed in the non-responder group (figure 12C). A two-way ANOVA with the non-responder group data showed an effect of the CORT model [$F_{(1,27)}$ = 6.02, p<0.05] and the interaction of both factors [$F_{(1,27)}$ = 7.37, p<0.05].

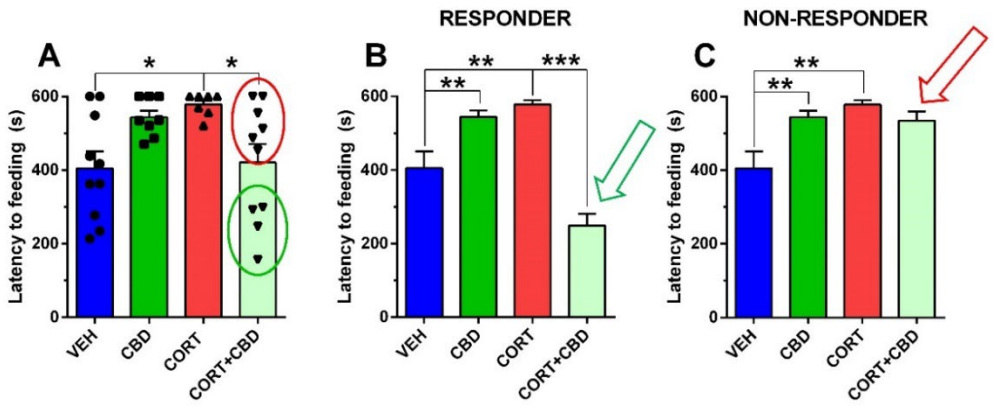

Figure 12. Effect of the subchronic treatment with CBD in the corticosterone-induced mouse model of depression in the novelty suppressed feeding test. (A) Latency to feeding. (B) Latency to feeding in the responder group. (C) Latency to feeding in the non-responder group. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by a Newman-Keuls *post hoc* test *p<0.05, **p<0.01, ***p<0.001. n = 7 – 10 animals per group. VEH: vehicle, CBD: cannabidiol, CORT: corticosterone.

Surprisingly, the treatment with CBD induced an increase in the latency to feed in naïve animals (543.5 ± 18.3 s in CBD *vs* the vehicle group, $p<0.01$) (figures 12B and 12C).

Additionally, the amount of food consumed in the home cage was assessed after the behavioural test, to exclude the possible interference of the lack of appetite. The amount of food eaten was not modified in the corticosterone animal model, nor by the treatment with CBD in naïve or CORT animals (data not shown).

1.2 MMP-9 modulation induced by cannabidiol treatment in the corticosterone mouse model

MMP-9 levels were studied in the synaptoneurosomal fraction of two crucial brain areas implicated in the neurobiology of depression and the antidepressant response: the prefrontal cortex and the hippocampus.

MMP-9 expression

In the PFCx, a significant increase of MMP-9 levels was detected in the CORT group (165.1 ± 17.8%) compared to the vehicle group (100.0 ± 2.1%, $p<0.01$). A two-way ANOVA showed a significant effect of the CORT model [$F_{(1,31)} = 21.27$, $p<0.001$] (figure 13A).

In the hippocampus, although the two-way ANOVA *post hoc* test showed no statistical differences, an unpaired Student's *t*-test revealed that there was a significant increase in MMP-9 levels of the CORT compared to the vehicle group (135.0 ± 12.5% in CORT *vs* 100.0 ± 6.8% in the vehicle group, $p<0.05$). A two-way ANOVA showed a significant effect of the CORT model [$F_{(1,31)} = 8.40$, $p<0.01$] (figure 13B).

No significant effect of the 4-day treatment with cannabidiol was observed in none of the experimental conditions (CORT or naïve group), and the areas studied (figures 13A and 13B).

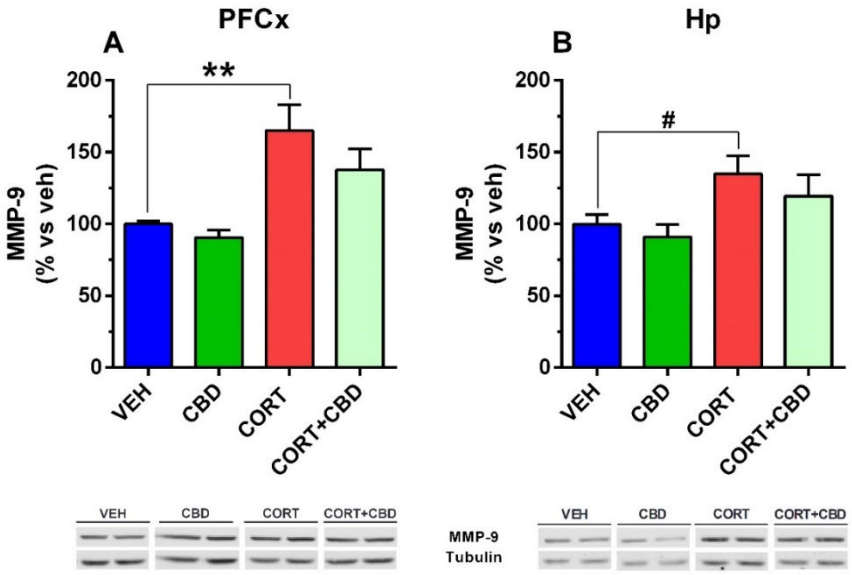

Figure 13. Effect of CBD in MMP-9 expression in (A) the prefrontal cortex and (B) the hippocampus in the corticosterone model. Representative western blot bands are shown. Results are expressed in percentage *vs* the vehicle group and as mean ± S.E.M. Two-way ANOVA followed by a Newman-Keuls *post hoc* test $^{**}p<0.01$. Unpaired Student's *t*-test $^{\#}p<0.05$ vs vehicle group. n = 8 - 10 animals per group. VEH: vehicle, CBD: cannabidiol, CORT: corticosterone, PFCx: prefrontal cortex, Hp: hippocampus.

MMP-9 functionality

Following the molecular expression study, the activity of MMP-9 was assessed in the cortex and the hippocampus by gel zymography technique. In the cortex, a two-way ANOVA *post hoc* test showed no significant differences, although an unpaired Student's *t*-test showed a significant increase in MMP-9 activity of the CORT compared to the vehicle group (144.1 ± 16.2% in CORT *vs* 100.0 ± 6.7% in the vehicle group, $p<0.05$).

Moreover, the CBD treatment in this mouse model displayed a reversion of the gelatinase activity compared to the CORT model (93.2 ± 14.2% in CORT+CBD *vs* the CORT group, $p<0.05$). Also, a two-way ANOVA showed a pronounced tendency of the effect of the CBD treatment [$F_{(1,30)} = 4.035$, $p = 0.054$] (figure 14A).

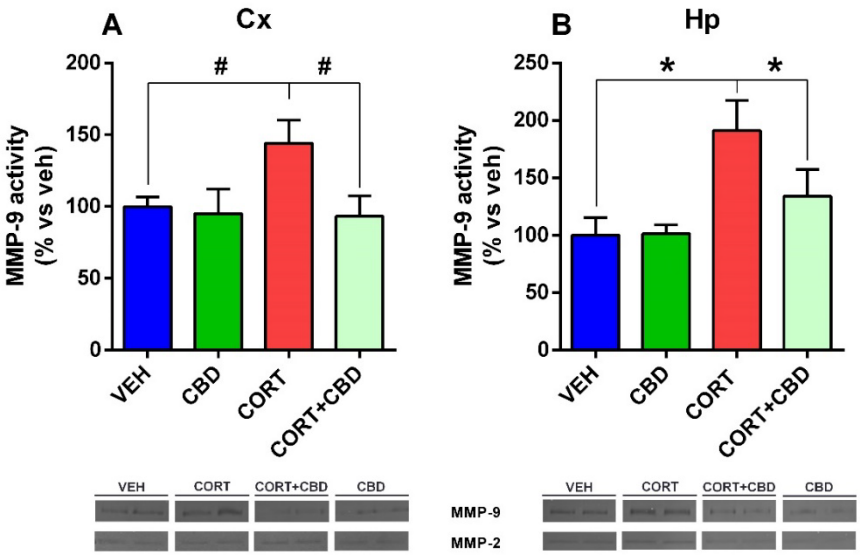

Figure 14. MMP-9 activity assessed by gel zymography in (A) cortex and (B) hippocampus. Representative bands from the gel zymography are shown. Results are expressed in percentage *vs* the vehicle group, as mean ± S.E.M. Unpaired Student's *t*-test $^{\#}p<0.05$ *vs* vehicle group. n = 8 - 10 animals per group. VEH: vehicle, CBD: cannabidiol, CORT: corticosterone, Cx: cortex, Hp: hippocampus.

Regarding the MMP-9 activity in the hippocampus, a two-way ANOVA *post hoc* test showed a significant increase in the activity of MMP-9 (191.2 ± 26.3%, $p<0.05$) in the CORT group compared to the vehicle group (100.0 ± 15.6%). Normalization of the MMP-9 activity was observed in the CORT+CBD group compared to the CORT model (134.1 ± 23.1% in CORT+CBD *vs* the CORT group, $p<0.05$). Moreover, a two-way ANOVA showed a significant effect of the CORT model [$F_{(1,32)} = 9.295$, $p<0.01$] (figure 14B).

In naïve animals, the treatment with CBD had no effect on the MMP-9 activity (figures 14A and 14B).

Finally, MMP-2 was also analysed in both brain areas. No statistical differences were observed in any experimental group (data not shown).

Correlation analysis: sucrose preference vs MMP-9 expression

In view of the results, the correlation between the behavioural tests and the MMP-9 data was assessed. A strong negative correlation between the sucrose preference and MMP-9 synaptoneurosomal levels of the PFCx (r = -0.4633, $p<0.01$, figure 15) was observed. Higher levels of MMP-9 in the PFCx correlate with more severe anhedonia, while lower MMP-9 levels corresponds to a higher sucrose preference.

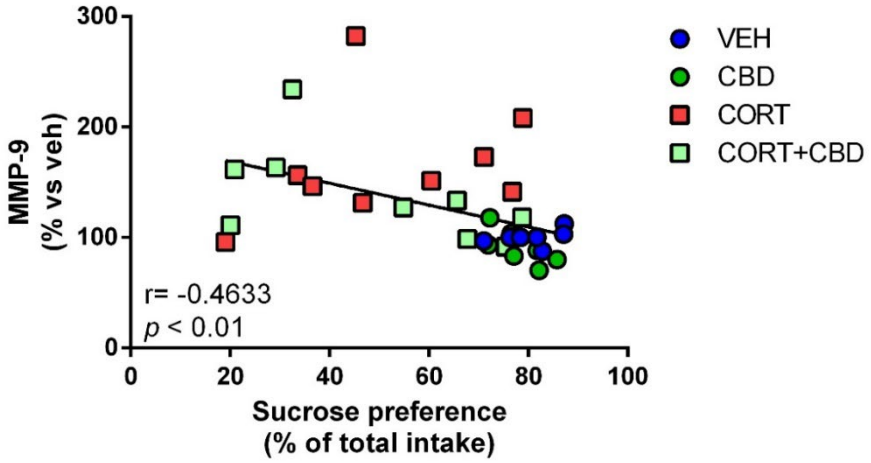

Figure 15. Correlation between the sucrose preference and the MMP-9 expression in the prefrontal cortex. Results are shown as the individual values obtained for each experiment. Statistical analysis was performed using a Pearson correlation (r). n = 8 – 9 animals per group. VEH: vehicle, CBD: cannabidiol, CORT: corticosterone.

No correlation was found between sucrose preference and MMP-9 expression in the hippocampus. In both areas, no correlation was observed between behaviour and MMP-9 functionality (data not shown).

Objective 2. Characterisation of MMP-9 transgenic mice

In order to study the implication of MMP-9 to the development of a depressive/anxious-like phenotype, two transgenic mice models were used: MMP-9 knockout (MMP-9 KO) mice, with a non-functional MMP-9 protein, and mice overexpressing human MMP-9 (MMP-9 OE) in the central nervous system. The effect on both genders, male and female, was studied.

First, a behavioural characterisation was assessed, performing a battery of tests to evaluate the general phenotype (hyperthermic response to stress, locomotion, compulsivity, well-being and spatial memory), and the anxious- and depressive-like related phenotype (*see figure 9 in Materials and methods*). Then, we analysed the expression of different neuroplasticity markers in the hippocampus and the levels of neurotransmitters in the prefrontal cortex, hippocampus and midbrain. The results obtained in this objective are compiled in Annexe 1.

2.1 MMP-9 knockout mice

2.1.1 Behavioural characterisation

Evaluation of the anxious-like behaviour

An open field test (OFT), an elevated plus maze (EPM) test and a light-dark box (LDB) test were used to evaluate the phenotype of MMP9 KO mice in paradigms of innate anxiety.

In the OFT, MMP-9 KO male mice presented an increase in the time spent in the centre of the arena compared to their wildtype (WT) counterparts (18.1 ± 1.8 s in MMP-9 KO *vs* 11.6 ± 1.8 s in WT group, $p<0.05$, figure 16A). In contrast, MMP-9 KO female mice spent less time in the centre than the WT female group (26.0 ± 2.7 s in MMP-9 KO *vs* 39.6 ± 5.2 s in WT group, $p<0.05$, figure 16A). MMP-9 KO, neither male nor female,

showed differences in the total distance travelled when compared with their corresponding WT counterparts (figure 16B).

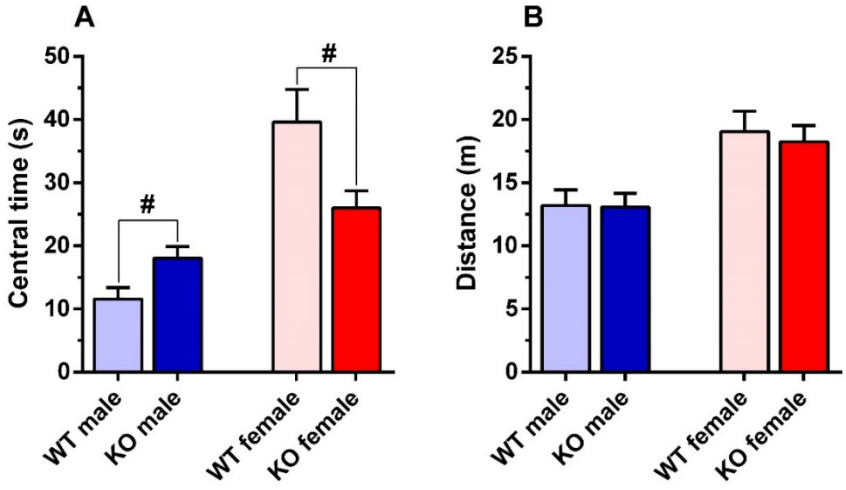

Figure 16. Open field test. (A) Central time and (B) total distance travelled in wildtype (WT) and MMP-9 knockout (KO) mice. Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test $^{\#}p<0.05$ *vs* WT group. n= 8 - 10 animals per group.

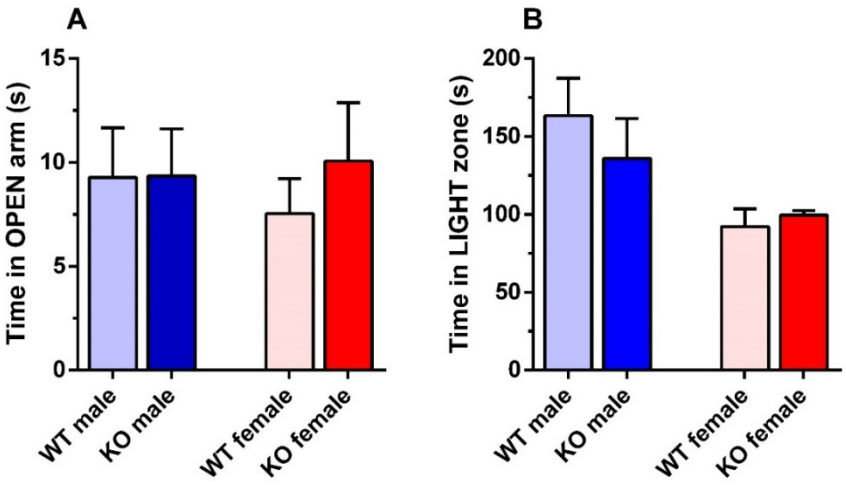

Figure 17. Elevated plus maze and light-dark box tests. (A) Time spent in the open arms of the EPM and (B) time spent in the light zone of the LDB of wildtype (WT) and MMP-9 knockout (KO) mice. Results are expressed as mean ± S.E.M. n= 8 - 10 animals per group.

Regarding the EPM and the LDB tests, no significant differences were found in the time spent in the open arms in the EPM (figure 17A), or the time spent in the light zone of the LDB (figure 17B) of male or female MMP-9 KO mice, compared to their WT group.

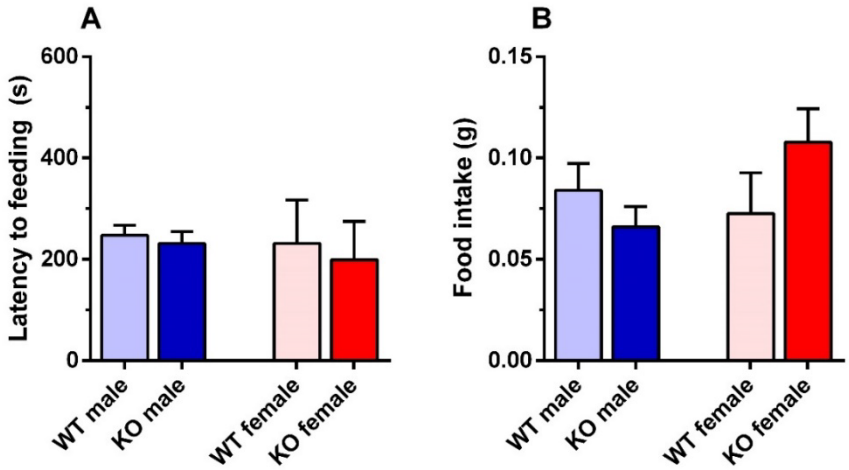

Figure 18. Novelty suppressed feeding test. (A) Latency to feeding in the NSF test and (B) food intake in the NSF post-test of wildtype (WT) and MMP-9 knockout (KO) mice. Results are expressed as mean ± S.E.M. n= 8 - 10 animals per group.

The novelty suppressed feeding (NSF) test was performed to study the conflict-based anxiety of MMP-9 KO mice. No significant differences were observed in the latency to feed in this test, neither in male nor in female mice (figure 18A). Additionally, the amount of food consumed after the test did not show statistical differences (figure 18B).

Evaluation of the depressive-like behaviour

Apathy and anhedonia were evaluated using the social interaction test (SIT) and the sucrose preference test (SPT), respectively.

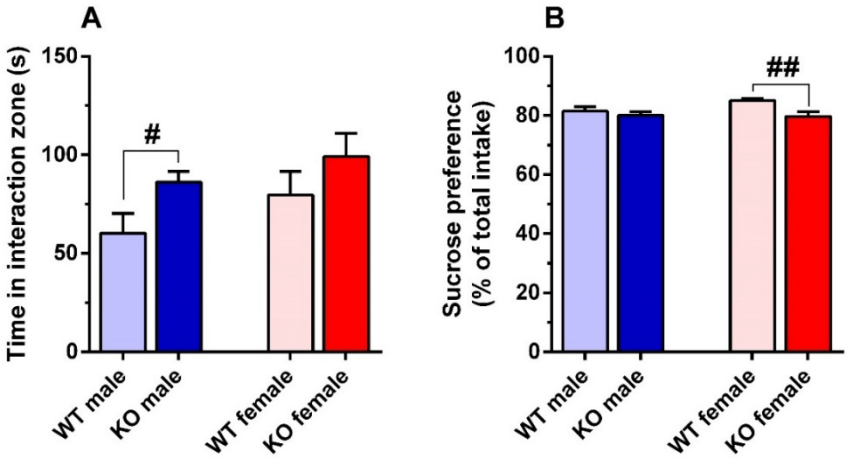

Figure 19. Social interaction and sucrose preference tests. (A) Time spent in the interaction zone in the SIT and (B) sucrose preference in the SPT of wildtype (WT) and MMP-9 knockout (KO) mice. Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* WT group. n= 8 - 10 animals per group.

In the SIT, MMP-9 KO male mice were more sociable than the corresponding WT mice, as evidenced by the higher time spent in the interaction zone (86.2 ± 5.3 s in MMP-9 KO *vs* 60.3 ± 10.0 s in WT group, $p<0.05$, figure 19A). No differences were observed in MMP-9 KO female mice compared to their WT group (figure 19A).

In the SPT, male mice did not show significant differences between MMP-9 KO and WT group (figure 19B). On the other hand, statistical differences had been seen comparing MMP-9 KO and WT females (79.7 ± 1.6% in MMP-9 KO *vs* 85.0 ± 0.7% in WT group, $p<0.01$, figure 19B).

The behavioural despair of MMP-9 KO mice was evaluated using the tail suspension test (TST). The lack of MMP-9 in male mice did not alter the immobility time compared to the WT group (figure 20). However, in female mice, the absence of MMP-9 exhibited lower immobility time compared to their WT counterparts (98.1 ± 10.1 s in MMP-9 KO *vs* 133.8 ± 9.3 s in WT group, $p<0.05$, figure 20).

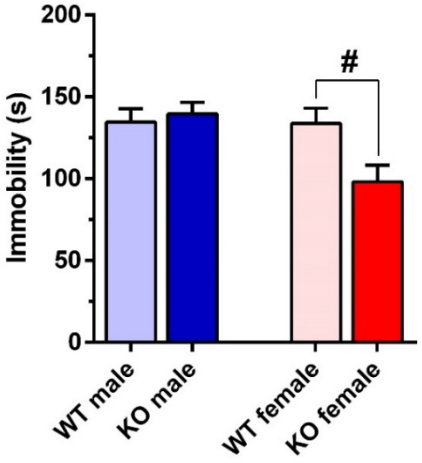

Figure 20. Tail suspension test. Immobility time of wildtype (WT) and MMP-9 knockout (KO) mice. Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test $^{\#}p<0.05$ *vs* WT group. n= 7 - 10 animals per group.

Evaluation of the general behaviour

Table 5. Rota-rod, marble burying, nesting and T-maze tests in wildtype (WT) and MMP-9 knockout (KO) mice. Results are expressed as mean ± S.E.M. n= 8 - 10 animals per group.

		WT ♂	***KO*** ♂	***WT*** ♀	***KO*** ♀
TESTS	**PARAMETERS EVALUATED**	**Mean ± S.E.M.**	**Mean ± S.E.M.**	**Mean ± S.E.M.**	**Mean ± S.E.M.**
ROTA-ROD	Time (s)	56.4 ± 3.6	59.1 ± 0.9	56.0 ± 4.0	56.4 ± 2.8
MARBLE BURYING	n of marbles buried	2.7 ± 0.5	2.2 ± 0.5	2.7 ± 0.6	3.3 ± 1.0
NESTING	Nesting score	4.8 ± 0.2	4.9 ± 0.2	4.8 ± 0.1	4.9 ± 0.1
T-MAZE	Alternation (%)	70.0 ± 15.3	70.0 ± 15.3	66.7 ± 16.7	50.0 ± 19.0

For a more general behavioural characterisation, the following parameters were studied: motor coordination using a rota-rod test, the compulsive-like behaviour using the marble burying test, the evaluation of welfare or health state (i.e.: the presence of a hippocampal injury) using the nesting test and the spatial memory using a T-maze test.

No significant differences were observed in male or female MMP-9 KO mice compared to their WT counterparts (table 5).

Afterwards, stress-induced hyperthermia (SIH) was assessed to study the effect of the lack of MMP-9 protein on the physiological response to stress. In male mice, WT presented the expected increase of temperature 15 minutes after stress exposure (36.2 ± 0.2°C at t = 0 min *vs* 37.1 ± 0.2°C at t = 15 min, $p<0.001$, figure 21A). The MMP-9 KO did not show this hyperthermic response. No significant differences were observed in the basal and t = 15 min temperature between MMP-9 KO and WT male mice.

Similarly, WT female mice displayed hyperthermia 15 minutes after the stressor exposure (36.9 ± 0.1°C at t = 0 min *vs* 37.3 ± 0.1°C at t = 15 min, $p<0.01$, figure 21B), while MMP-9 KO female mice did not show alterations in their temperature. No significant differences were observed in the basal and t = 15 min temperature between MMP-9 KO and WT female mice.

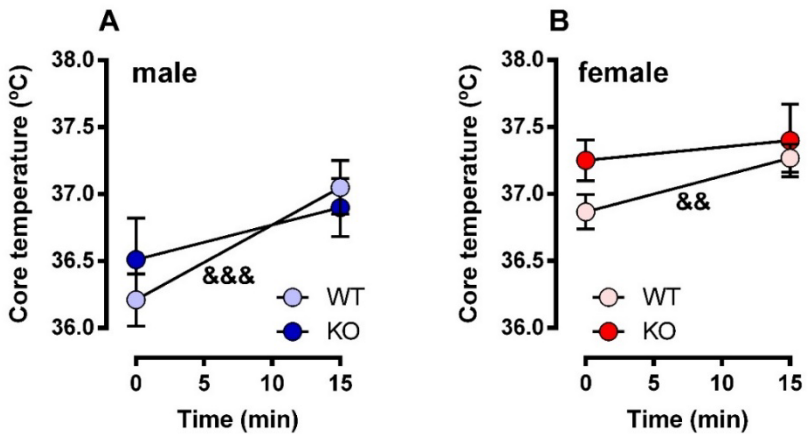

Figure 21. Stress-induced hyperthermia. Wildtype (WT) and MMP-9 knockout (KO) mice basal temperature (t = 0) and 15 minutes after a stressor in (A) male and (B) female mice. Results are expressed as mean ± S.E.M. Paired Student's *t*-test $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ *vs* basal (t = 0). n = 8 - 10 animals per group.

2.1.2 Molecular characterisation

MMP-9 activity was assessed in the cortex by gel zymography technique to verify the lack of functionality of the protein. In WT mice, the band corresponding to gelatine degradation by MMP-9 was visible, while in MMP-9 KO mice samples no MMP-9 activity was observed. No differences were observed in the MMP-2 activity (figure 22).

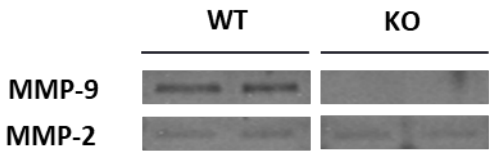

Figure 22. MMP-9 and MMP-2 activity assessed by gel zymography in wildtype (WT) and MMP-9 knockout (KO) mice. Representative bands from the gel zymography are shown.

Expression of neuroplasticity markers in the hippocampus

Different neuroplasticity markers (nectin-3, mTOR and p-mTOR, BDNF, PSD95 and synapsin I) were analysed in the synaptoneurosomal fraction of the hippocampus of WT and MMP-9 KO mice to study the effect driven by the lack of MMP-9 in the expression of those proteins in both genders.

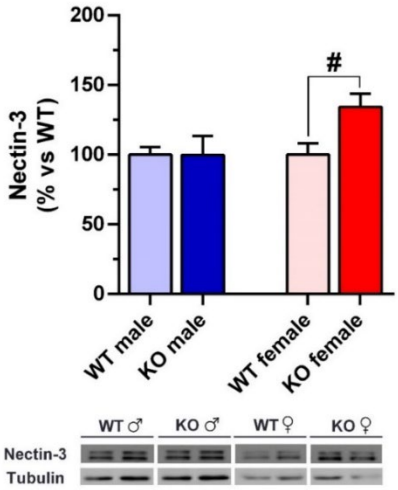

Figure 23. Nectin-3 expression in the hippocampus of wildtype (WT) and MMP-9 knockout (KO) mice**.** Representative western blot bands are shown. Results are expressed in percentage *vs* the WT group and as mean ± S.E.M. Unpaired Student's *t*-test $^{\#}p<0.05$ *vs* WT group. n = 8 animals per group.

Nectin-3 levels in MMP-9 KO male mice were similar to those observed in WT male mice. MMP-9 KO female mice presented higher levels of nectin-3 than their corresponding WT mice (134.3 ± 9.5% in MMP-9 KO *vs* 100.0 ± 8.1% in WT group, $p<0.05$, figure 23).

In the hippocampus, no significant differences in BDNF levels were observed between MMP-9 KO and WT male mice. However, MMP-9 KO female mice showed higher expression levels of BDNF than their WT counterparts (126.9 ± 6.8% in MMP-9 KO *vs* 100.0 ± 12.5% in WT group, $p<0.05$, figure 24A). No statistical differences were observed between MMP-9 KO and WT mice in pro-BDNF levels (figure 24B) and pro-BDNF/BDNF ratio (figure 24C), neither in males nor females.

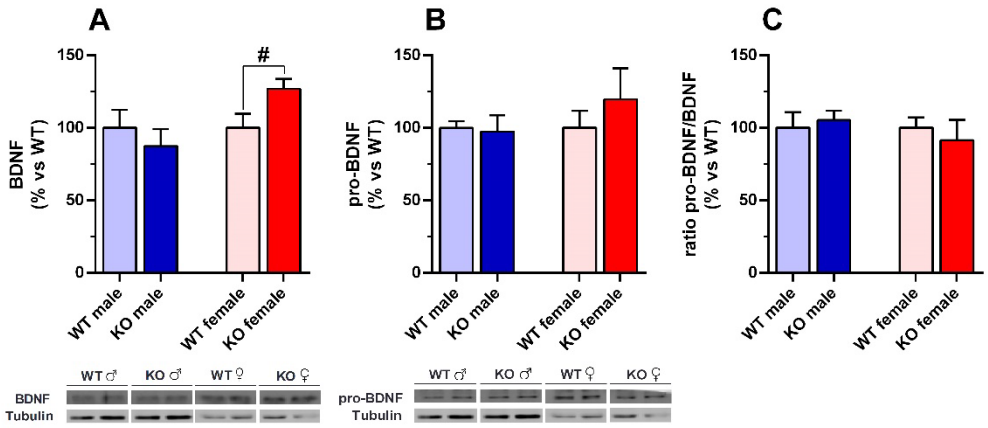

Figure 24. (A) BDNF, (B) pro-BDNF and (C) ratio pro-BDNF/BDNF expression in the hippocampus of wildtype (WT) and MMP-9 knockout (KO) mice**.** Representative western blot bands are shown. Results are expressed in percentage *vs* the WT group and as mean ± S.E.M. Unpaired Student's *t*-test $^{\#}p<0.05$ *vs* WT group. n = 8 animals per group.

Regarding mTOR protein levels, male mice did not show significant differences between MMP-9 KO and WT groups. On the other hand, MMP-9 KO female mice showed significantly higher levels of mTOR than WT female mice (123.0 ± 9.0% in MMP-9 KO *vs* 100.0 ± 5.4% in WT group, $p<0.05$, figure 25A). A similar pattern was observed in phospho-mTOR (p-mTOR) expression levels. No statistical differences were observed in

p-mTOR levels between MMP-9 KO and WT male mice, while increased levels of the phosphorylated protein were observed in the hippocampus of MMP-9 KO female mice (140.1 ± 17.7% in MMP-9 KO *vs* 100.0 ± 5.9% in WT group, $p<0.05$, figure 25B).

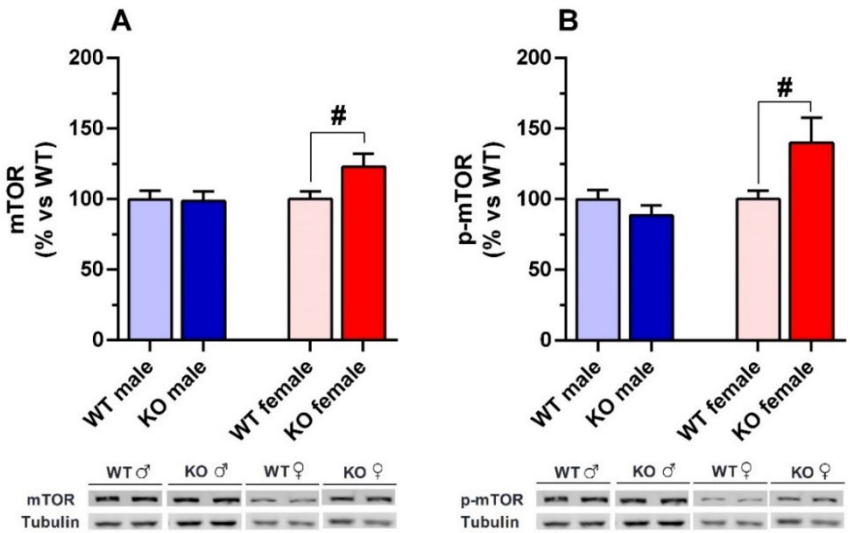

Figure 25. (A) mTOR and (B) p-mTOR expression in the hippocampus of wildtype (WT) and MMP-9 knockout (KO) mice**.** Representative western blot bands are shown. Results are expressed in percentage *vs* the WT group and as mean ± S.E.M. Unpaired Student's *t*-test $^{\#}p<0.05$ *vs* WT group. n = 8 animals per group.

Hippocampal levels of PSD95 were not altered in transgenic male mice compared to their WT counterparts, while MMP-9 KO females showed higher PSD95 expression levels than their WT mice (134.2 ± 8.9% in MMP-9 KO *vs* 100.0 ± 4.2% in WT group, $p<0.01$, figure 26A).

Finally, higher levels of synapsin I were observed in transgenic MMP-9 KO mice, compared to their respective WT mice, in both males (115.8 ± 4.7% in MMP-9 KO *vs* 100.0 ± 4.3% in WT group, $p<0.05$, figure 26B) and females (125.2 ± 7.8% in MMP-9 KO *vs* 100.0 ± 7.1% in WT group, $p<0.05$, figure 26B).

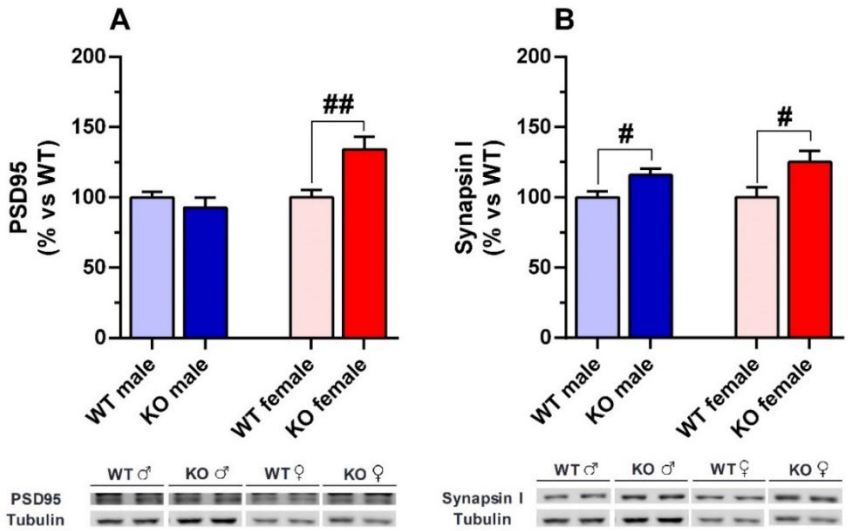

Figure 26. (A) PSD95 and (B) synapsin I expression in the hippocampus of wildtype (WT) and MMP-9 knockout (KO) mice**.** Representative western blot bands are shown. Results are expressed in percentage *vs* the WT group and as mean ± S.E.M. Unpaired Student's *t*-test $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* WT group. n = 8 animals per group.

2.1.3 Neurochemical characterisation: neurotransmitter brain levels

Serotonin (5-HT), dopamine (DA) and noradrenaline (NA) play an important role in the pathology of depression. These neurotransmitters were determined by HPLC in different brain areas involved in depression.

MMP-9 KO males exhibited higher 5-HT levels in the PFCx (6.4 ± 0.2 pmol/mg in MMP-9 KO *vs* 5.2 ± 0.3 pmol/mg in WT group, $p<0.01$) and the Hp (6.5 ± 0.6 pmol/mg in MMP-9 KO *vs* 4.4 ± 0.6 pmol/mg in WT group, $p<0.05$), while no differences were observed in the midbrain (figure 27). In contrast, MMP-9 KO female had lower 5-HT levels in the PFCx (5.2 ± 0.3 pmol/mg in MMP-9 KO *vs* 6.3 ± 0.4 pmol/mg in WT group, $p<0.05$) and the Hp (5.6 ± 0.2 pmol/mg in MMP-9 KO *vs* 7.2 ± 0.4 pmol/mg in WT group, $p<0.01$), with no differences in the midbrain levels, compared to their WT counterparts (figure 27).

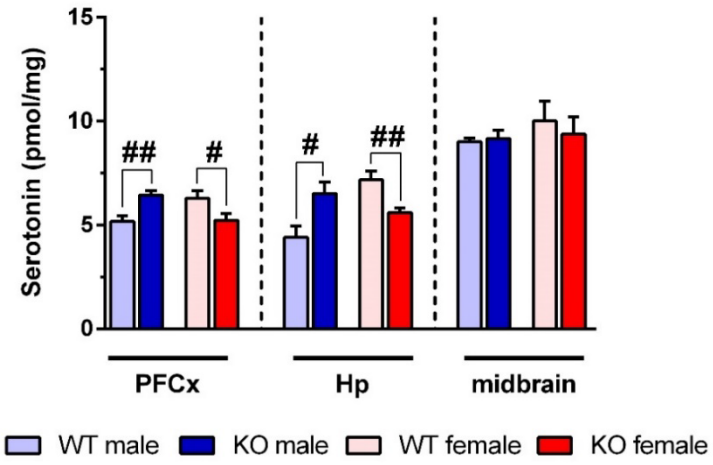

Figure 27. Serotonin levels in the prefrontal cortex (PFCx), hippocampus (Hp) and midbrain of wildtype (WT) and MMP-9 knockout (KO) mice. Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs WT group. n = 6 - 7 animals per group.

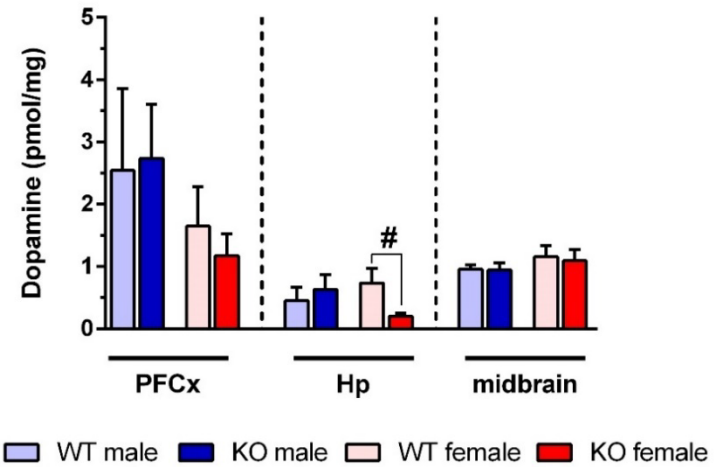

Figure 28. Dopamine levels in the prefrontal cortex (PFCx), hippocampus (Hp) and midbrain of wildtype (WT) and MMP-9 knockout (KO) mice**.** Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test $^{\#}p<0.05$ vs WT group. n = 6 - 7 animals per group.

Levels of DA (figure 28) were similar for MMP-9 KO and WT male mice in all the brain areas studied. On the other hand, MMP-9 KO females presented lower levels of DA (0.2 ± 0.05 pmol/mg in MMP-9 KO *vs* 0.7 ± 0.2 pmol/mg in WT group, $p<0.05$, figure 28) in the Hp, without differences in the PFCx and the midbrain.

Regarding NA levels, MMP-9 KO males present a trend to higher levels in the Hp (4.6 ± 0.8 pmol/mg in MMP-9 KO *vs* 2.9 ± 0.3 pmol/mg in WT group, $p = 0.07$, figure 29), while no differences were observed in the other brain areas studied. Any differences were observed in NA levels in MMP-9 KO female mice (figure 29).

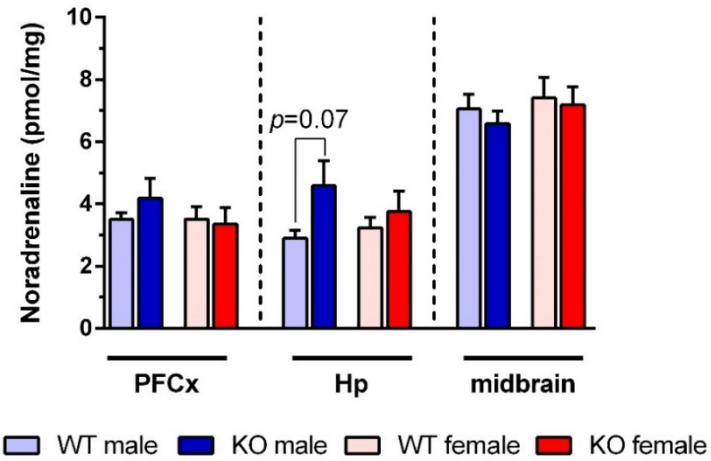

Figure 29. Noradrenaline levels in the prefrontal cortex (PFCx), hippocampus (Hp) and midbrain of wildtype (WT) and MMP-9 knockout (KO) mice**.** Results are expressed as mean ± S.E.M. n = 7 animals per group.

2.2 MMP-9 overexpressing mice

2.2.1 Behavioural characterisation

Evaluation of the anxious-like behaviour

In the OFT, MMP-9 OE male mice did not show differences in the time spent in the centre of the arena compared to WT male mice. In contrast, MMP-9 OE female mice showed a tendency to spend more time in the centre than the WT female group (22.8 ± 3.7 s in MMP-9 OE *vs* 16.2 ± 1.0 s in WT group, $p = 0.063$, figure 30A). Moreover, both male and female MMP-9 OE mice showed hypoactivity, measured as the total distance, compared to their respective WT mice (male: 14.0 ± 1.7 m in MMP-9 OE *vs* 19.1 ± 1.7 m in WT group, $p<0.05$, figure 30B; and female 17.0 ± 1.2 m in MMP-9 OE *vs* 21.2 ± 1.1 m in WT group, $p<0.05$, figure 30B).

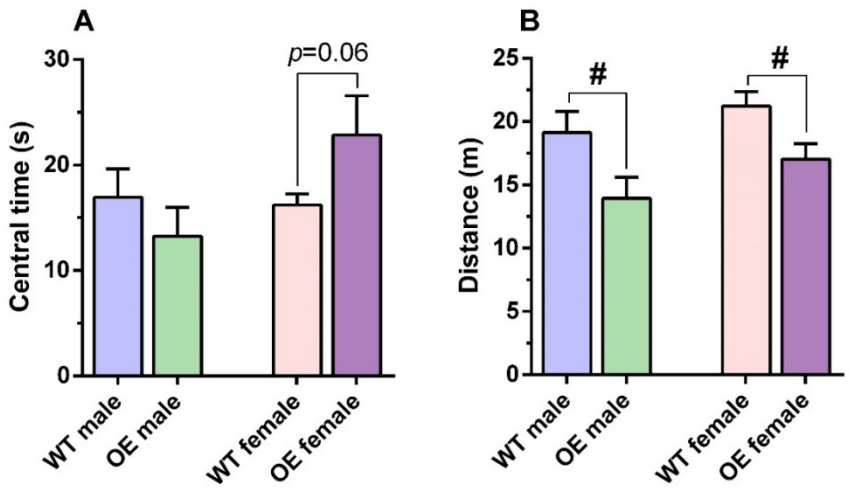

Figure 30. Open field test. (A) Central time and (B) total distance travelled in wildtype (WT) and MMP-9 overexpression (OE) mice. Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test $^{\#}p<0.05$ vs WT group. n= 7 - 10 animals per group.

In the EPM test, MMP-9 OE male mice did not show differences in the time spent in the open arms compared to the WT male group. However, MMP-9 OE female mice displayed higher time spent in the open arms than the WT female (94.3 ± 22.5 s in MMP-9 OE *vs* 28.9 ± 4.0 s in WT group, $p<0.01$, figure 31A).

In the LDB test, similar results to the OFT and EPM were obtained. While MMP-9 OE male displayed a similar time in the light zone compared to the WT male group, MMP-9 OE female spent more time in the light zone than WT female (165.0 ± 15.9 s in MMP-9 OE *vs* 101.2 ± 11.0 s in WT group, $p<0.01$, figure 31B).

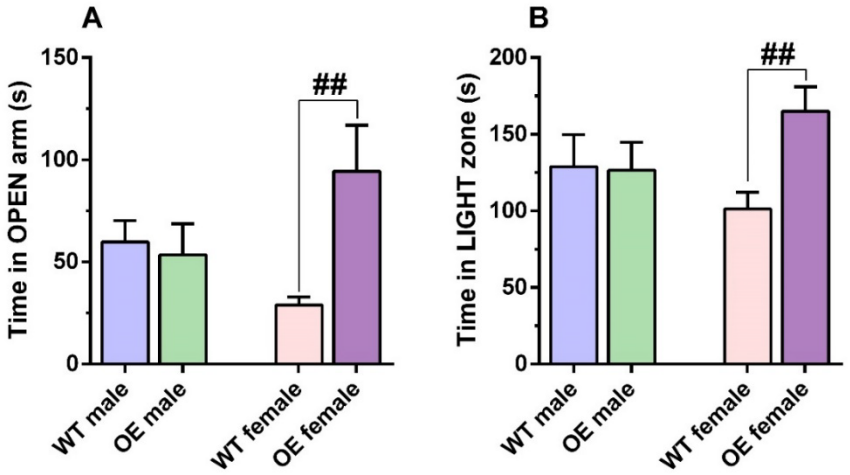

Figure 31. Elevated plus maze and light-dark box tests. (A) Time spent in the open arms of the EPM and (B) time spent in the light zone of the LDB of wildtype (WT) and MMP-9 overexpression (OE) mice. Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test $^{\#\#}p<0.01$ vs WT group. n= 7 - 10 animals per group.

In the NSF test, MMP-9 OE male mice presented higher latency to feed than WT males (196.5 ± 19.4 s in MMP-9 OE *vs* 104.0 ± 13.3 s in WT group, $p<0.001$, figure 32A). Regarding MMP-9 OE females, no differences were observed. Additionally, no differences were observed in the food consumed after the test (figure 32B).

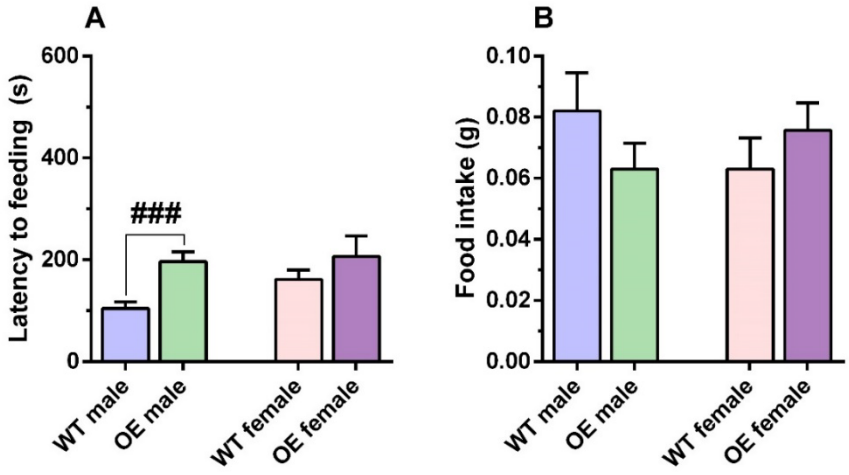

Figure 32. Novelty suppressed feeding test. (A) Latency to feeding in the NSF test and (B) food intake in the NSF post-test in wildtype (WT) and MMP-9 overexpression (OE) mice. Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test $^{###}p<0.001$ vs WT group. n= 7 - 10 animals per group.

Evaluation of the depressive-like behaviour

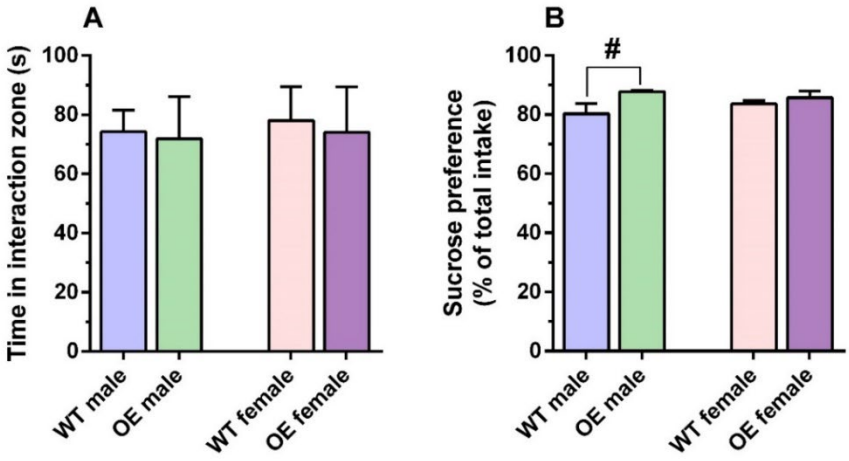

Figure 33. Social interaction and sucrose preference tests. (A) Time spent in the interaction zone in the SIT and (B) the percentage of sucrose intake in the SPT of wildtype (WT) and MMP-9 overexpression (OE) mice. Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test $^{#}p<0.001$ *vs* WT group. n= 7 - 10 animals per group.

In the SIT, neither male nor female MMP-9 OE mice showed significant differences compared to their WT counterparts (figure 33A). In the SPT, the sucrose preference was higher in MMP-9 OE male mice than in the WT group (87.8 ± 0.4% in MMP-9 OE *vs* 80.3 ± 3.5% in WT group, $p<0.05$, figure 33B), whereas MMP-9 OE female mice did not show differences compared to the WT group (figure 33B).

Finally, the behavioural despair of MMP-9 OE mice was evaluated using the tail suspension test (TST), where no differences were observed in the immobility time in both male and female mice (figure 34).

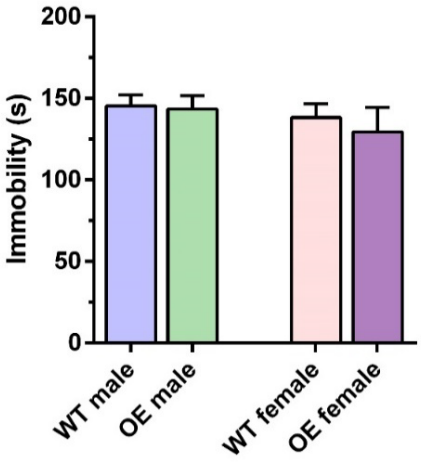

Figure 34. Tail suspension test. Immobility time of wildtype (WT) and MMP-9 overexpression (OE) mice. Results are expressed as mean ± S.E.M. n= 7 - 10 animals per group.

Evaluation of the general behaviour

Both, MMP-9 OE male and female mice did not display alterations in the rota-rod test, the marble burying test, or the nesting test (table 6). However, in the T-maze, MMP-9 OE male and female mice exhibited a lower percentage of correct alternation, suggesting a deficit in spatial memory (table 6).

Table 6. Rota-rod, marble burying, nesting and T-maze tests of wildtype (WT) and MMP-9 overexpression (OE) mice. Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test $^{\#}p<0.05$ *vs* WT group. Chi-square test $^{\xi}p<0.05$ *vs* WT group. n= 7 - 10 animals per group.

		WT ♂	***OE*** ♂	***WT*** ♀	***OE*** ♀
TESTS	**PARAMETERS EVALUATED**	**Mean ± S.E.M.**	**Mean ± S.E.M.**	**Mean ± S.E.M.**	**Mean ± S.E.M.**
ROTA-ROD	Time (s)	60.0 ± 0.0	57.2 ± 2.3	60.0 ± 0.0	59.0 ± 1.0
MARBLE BURYING	n of marbles buried	4.2 ± 0.8	4.8 ± 0.7	6.2 ± 1.2	5.4 ± 1.1
NESTING	Nesting score	4.9 ± 0.1	4.8 ± 0.1	4.9 ± 0.1	4.6 ± 0.2
T-MAZE	Alternation (%)	**100.0 ± 0.0**	**50.0 ± 16.7$^{\xi}$**	**100.0 ± 0.0**	**57.1 ± 20.2$^{\xi}$**

The stress-induced hyperthermia (SIH) was assessed to evaluate the impact of the overexpression of MMP-9 protein in the physiological response to stress. MMP-9 OE male mice presented significant hyperthermia induced by stress (35.9 ± 0.2°C at t = 0 min *vs* 37.4 ± 0.1°C at t = 15 min, $p<0.001$, figure 35A), similar to the effect observed in WT mice (36.4 ± 0.2°C at t = 0 min *vs* 37.6 ± 0.1°C at t = 15 min, $p<0.001$, figure 35A). MMP-9 OE male mice exhibit significantly lower basal temperature compared to WT mice ($p<0.05$, figure 35A).

Regarding female mice, similar stress-induced hyperthermia was observed in MMP-9 OE mice (35.4 ± 0.2°C at t = 0 min *vs* 36.6 ± 0.1°C at t = 15 min, $p<0.01$, figure 35B), and WT mice (36.1 ± 0.1°C at t = 0 min *vs* 36.9 ± 0.1°C at t = 15 min, $p<0.001$, figure 35B). A lower basal temperature was observed in MMP-9 OE females compared to WT mice ($p<0.05$, figure 35B).

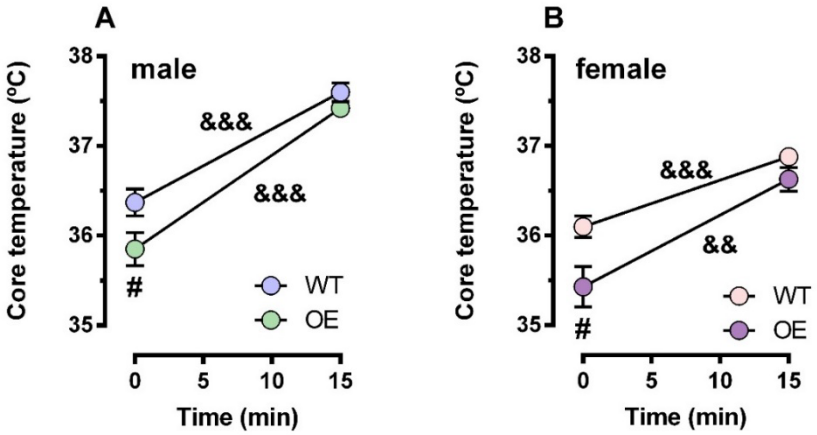

Figure 35. Stress-induced hyperthermia. Wildtype (WT) and MMP-9 overexpression (OE) mice basal temperature (t = 0) and 15 minutes after a stressor in (A) male and (B) female. Results are expressed as mean ± S.E.M. Paired Student's *t*-test $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ *vs* t=0 min. Unpaired Student's *t*-test $^{\#}p<0.05$ *vs* WT group. n= 7 - 10 animals per group.

2.2.2 Molecular characterisation

To confirm that the activity of MMP-9 in OE mice was higher than that observed in their WT counterparts we performed a gel zymography. The bands in figure 36 show the increased activity of MMP-9 and similar activity levels in MMP-2.

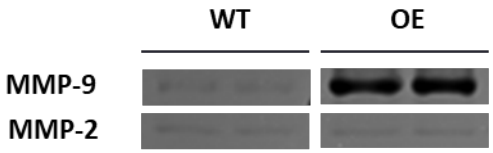

Figure 36. MMP-9 and MMP-2 activity assessed by gel zymography in wildtype (WT) and MMP-9 overexpression (OE) mice. Representative bands from the gel zymography are shown.

Expression of neuroplasticity markers in the hippocampus

Nectin-3 levels in MMP-9 OE mice were similar to those observed in their WT groups in both sexes (figure 37).

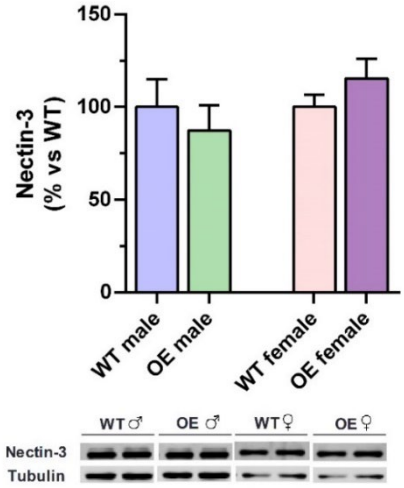

Figure 37. Nectin-3 expression in the hippocampus of wildtype (WT) and MMP-9 overexpression (OE) mice. Representative western blot bands are shown. Results are expressed in percentage *vs* the WT group and as mean ± S.E.M. n = 6 - 7 animals per group.

The hippocampal levels of BDNF (figure 38A) and pro-BDNF (figure 38B) showed no statistical differences between MMP-9 OE and WT mice in both males and females. However, MMP-9 OE male mice presented a higher pro-BDNF/BDNF ratio than WT male mice (140.1 ± 14.2% in MMP-9 OE *vs* 100.0 ± 10.4% in WT group, $p<0.05$, figure 38C), while MMP-9 OE female did not display differences compared to WT female group (figure 38C).

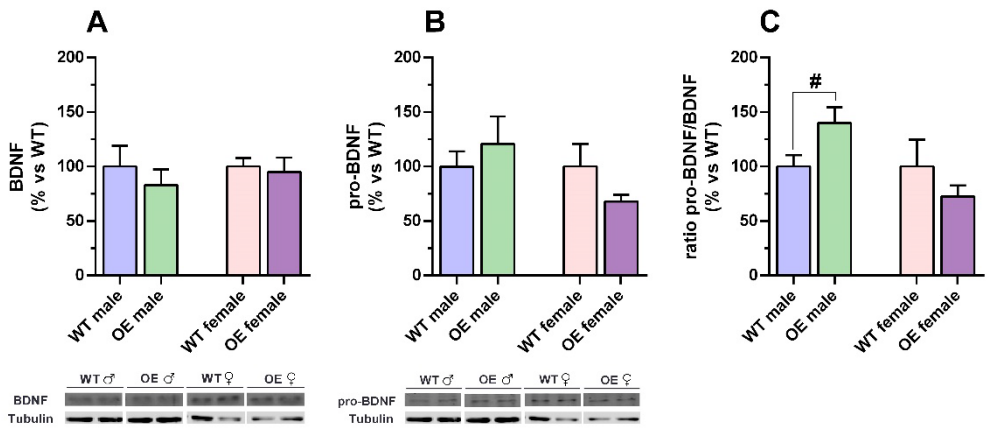

Figure 38. (A) BDNF, (B) pro-BDNF and (C) ratio pro-BDNF/BDNF expression in the hippocampus of wildtype (WT) and MMP-9 overexpression (OE) mice. Representative western blot bands are shown. Results are expressed in percentage *vs* the WT group and as mean ± S.E.M. Unpaired Student's *t*-test $^{\#}p<0.05$ *vs* WT group. n = 6 - 7 animals per group.

The overexpression of MMP-9 did not modify the hippocampal mTOR levels in transgenic mice compared to their WT counterparts, in males and females (figure 39A). Regarding p-mTOR expression, MMP-9 OE male mice showed a tendency to decrease compared to their WT counterparts (59.1 ± 12.8% in MMP-9 OE *vs* 100.0 ± 13.2% in WT group, $p = 0.05$, figure 39B). No differences were observed in females (figure 39B).

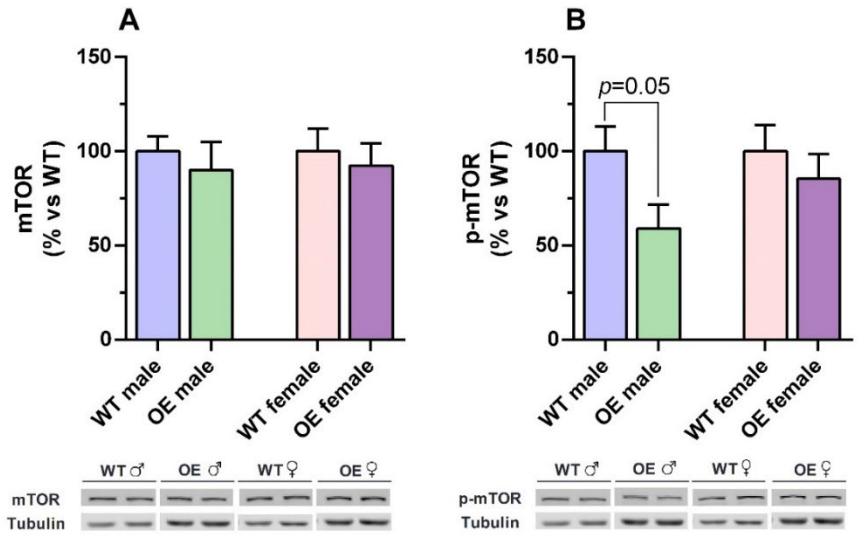

Figure 39. (A) m-TOR and (B) p-mTOR expression in the hippocampus of wildtype (WT) and MMP-9 overexpression (OE) mice. Representative western blot bands are shown. Results are expressed in percentage *vs* the WT group and as mean ± S.E.M. n = 6 - 7 animals per group.

PSD95 expression was higher in MMP-9 OE males compared to their WT mice (134.0 ± 9.2% in MMP-9 KO *vs* 100.0 ± 4.7% in WT group, $p<0.01$, figure 40A), while no differences were found in female mice. Finally, hippocampal levels of synapsin l (figure 40B) were not altered in the different groups studied.

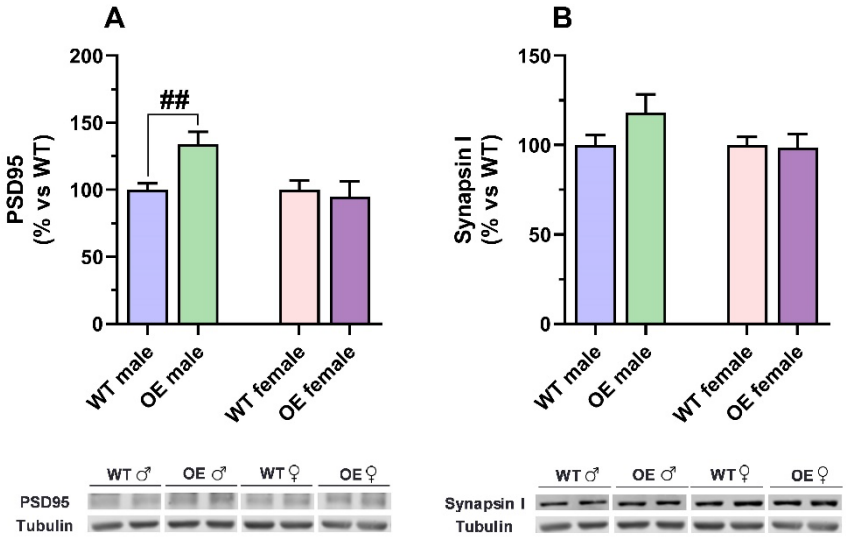

Figure 40. (A) PSD95 and (B) synapsin I expression in the hippocampus of wildtype (WT) and MMP-9 overexpression (OE) mice. Representative western blot bands are shown. Results are expressed in percentage *vs* the WT group and as mean ± S.E.M. Unpaired Student's *t*-test $^{\#\#}p<0.01$ *vs* WT group. n = 6 - 7 animals per group.

2.2.3 Neurochemical characterisation: neurotransmitter brain levels

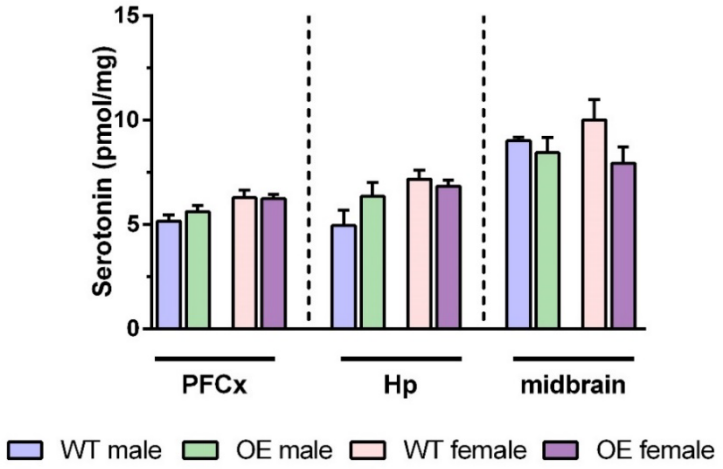

Figure 41. Serotonin levels in prefrontal cortex (PFCx), hippocampus (Hp) and midbrain of wildtype (WT) and MMP-9 overexpression (OE) mice. Results are expressed as mean ± S.E.M. n = 6 - 7 animals per group.

MMP-9 OE male and female mice did not display statistical differences in 5-HT levels (figure 41), in any of the areas studied, compared to their corresponding WT counterparts.

High DA levels were observed in the PFCx of MMP-9 OE male (9.6 ± 2.6 pmol/mg in MMP-9 OE *vs* 2.5 ± 1.3 pmol/mg in WT group, $p<0.05$), and female (10.9 ± 2.8 pmol/mg in MMP-9 OE *vs* 1.6 ± 0.6 pmol/mg in WT group, $p<0.05$), with no changes in the other brain areas for both genders (figure 42).

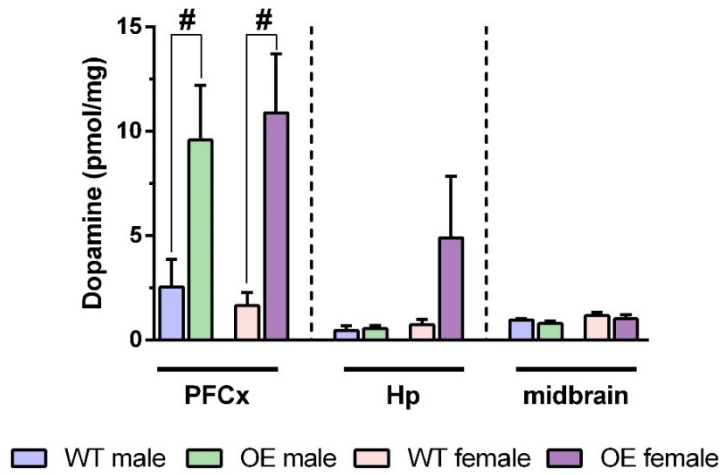

Figure 42. Dopamine levels in prefrontal cortex (PFCx), hippocampus (Hp) and midbrain of wildtype (WT) and MMP-9 overexpression (OE) mice. Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test $^{\#}p<0.05$ vs WT group. n = 6 - 7 animals per group.

Levels of NA in MMP-9 OE mice, both male and female, were similar in all the brain areas studied, compared to their WT group (figure 43).

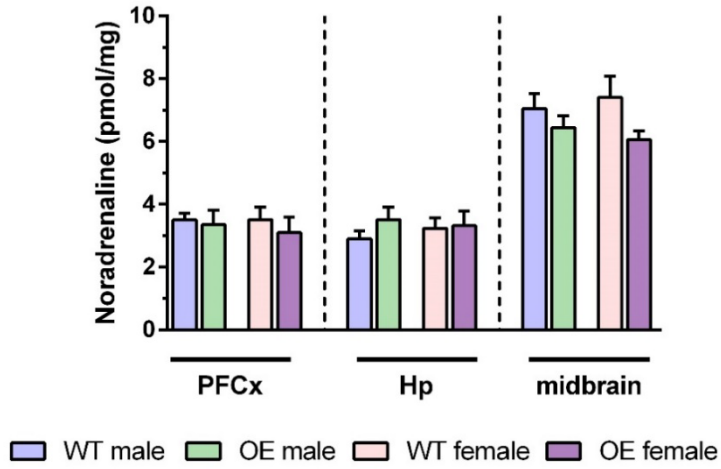

Figure 43. Noradrenaline levels in prefrontal cortex (PFCx), hippocampus (Hp) and midbrain of wildtype (WT) and MMP-9 overexpression (OE) mice. Results are expressed as mean ± S.E.M. n = 6 - 7 animals per group.

Objective 3. Evaluate the expression of MMP-9 and neuroplasticity markers in *post-mortem* human brain samples from depressed patients

The last objective of this thesis was to study the expression levels and activity of MMP-9 and the expression levels of neuroplasticity markers in the synaptoneurosomal fraction of *post-mortem* cortical (Brodmann area 9) samples of patients diagnosed with major depression disease (MDD). MDD patients were classified as treated (AD treated) and non-treated (AD free) with antidepressant drugs, according to the *post-mortem* toxicology report. Matched controls were used and chosen by gender, age and *post-mortem* interval similarity *(see table 1 in Materials and methods*). The study was carried out with all the patients as a unique cohort and afterward, gender differences were also evaluated. For WB studies, only samples 1-20 were used.

3.1 MMP-9 expression and functionality

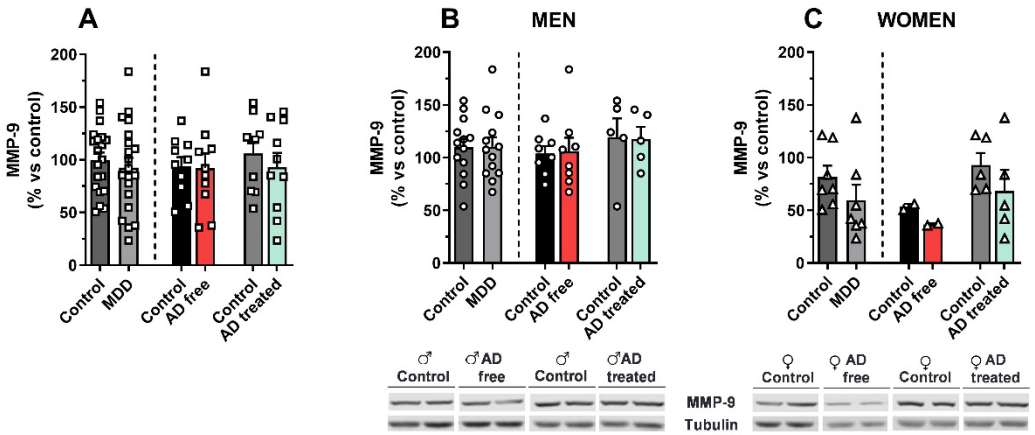

Figure 44. MMP-9 expression in cortical *post-mortem* samples of treated and non-treated depressed patients. (A) All patients together, (B) men and (C) women patients. Representative western blot bands are shown. Results are expressed in percentage *vs* the control MDD matched group and as mean ± S.E.M. n = 20 samples per group.

The study of MMP-9 levels in the PFCx showed no statistical differences in any experimental group (MDD, AD free and AD treated) compared to their matched controls, when the complete cohort (figure 44A), men (figure 44B) and women (figure 44C) patients were studied.

The analysis of MMP-9 functionality showed increased activity of MMP-9 in the complete cohort of MDD patients (130.6 ± 10.9% in MDD *vs* 100.0 ± 7.8% in the control group, $p<0.05$, figure 45A). While no differences were observed between AD free patients and their matched controls, increased activity of MMP-9 was observed in AD treated patients (137.7 ± 16.5% in AD treated *vs* 93.6 ± 13.4% in the control group, $p<0.05$, figure 45A).

When the MMP-9 activity was studied only in men, no significant differences were observed in any of the experimental groups (figure 45B). On the other hand, women diagnosed with MDD displayed higher MMP-9 activity in the PFCx (147.3 ± 14.5% in MDD *vs* 97.7 ± 12.1% in the control group, $p<0.05$, figure 45C). Moreover, increased activity of MMP-9 was observed in AD treated women (155.8 ± 22.0% in AD treated *vs* 94.5 ± 17.4% in the control group, $p<0.05$, figure 45C), with no differences in non-treated patients.

MDD patients were also classified by their cause of death into those who committed suicide and those who suffered a natural death (non-suicide). A higher MMP-9 activity was found in the PFCx of those MDD patients that committed suicide (130.7 ± 13.8% in suicide *vs* 95.9 ± 9.2% in the control group, $p<0.05$, figure 45D), with no differences in non-suicide patients.

In men, no significant differences were observed in the MMP-9 activity regarding their cause of death (figure 45E). In contrast, a strong tendency to increase MMP-9 activity was observed in those MDD women who committed suicide (150.3 ± 27.0% in suicide

vs 85.3 ± 15.5% in the control group, $p = 0.056$, figure 45F), with no differences in non-suicide women patients.

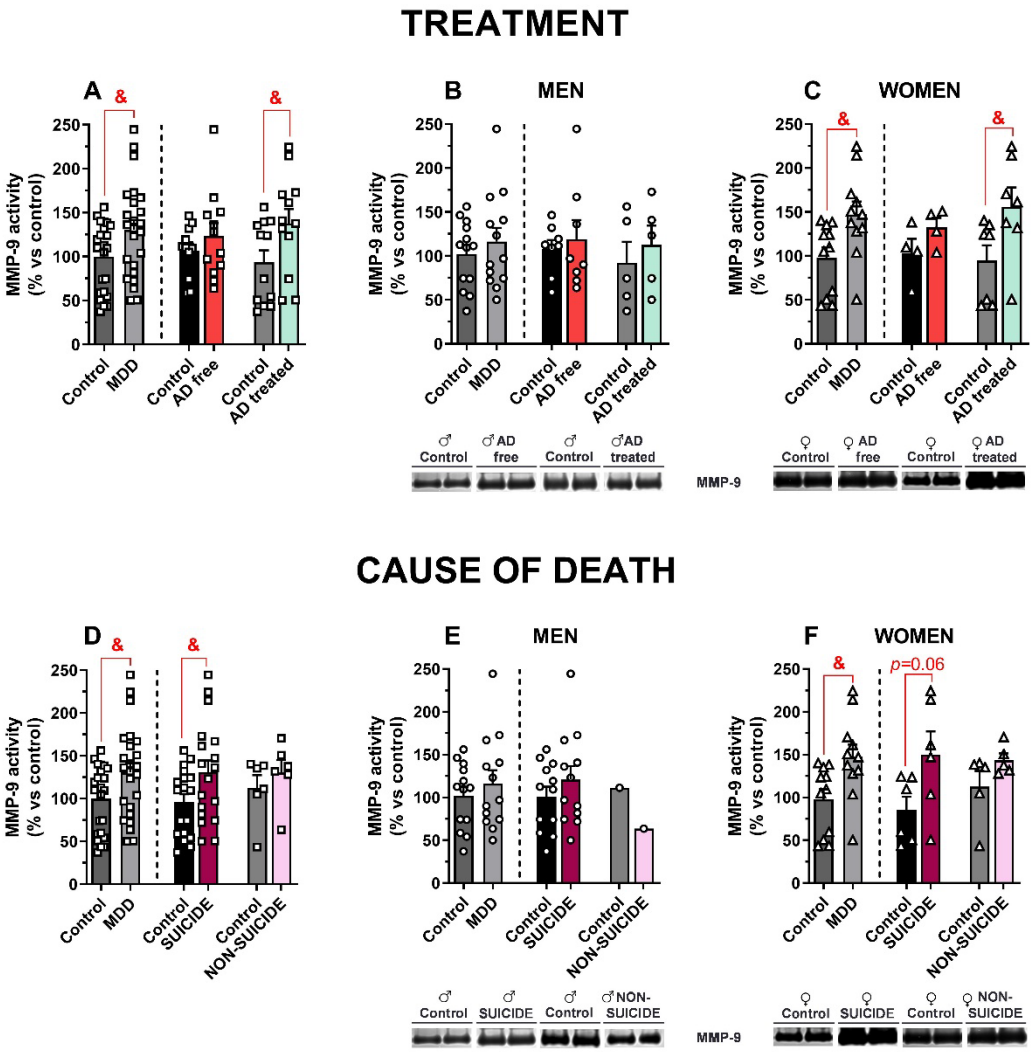

Figure 45. MMP-9 activity assessed by gel zymography in cortical *post-mortem* samples of treated and non-treated depressed patients. (A) All patients together, (B) men and (C) women patients. **In addition, the cause of death was considered.** (D) All patients together, (E) men and (F) women patients. Representative bands from the gel zymography are shown. Results are expressed in percentage *vs* the control MDD matched group and as mean ± S.E.M. Paired Student's *t*-test $^{\&}p<0.05$ *vs* control group. n = 24 samples per group.

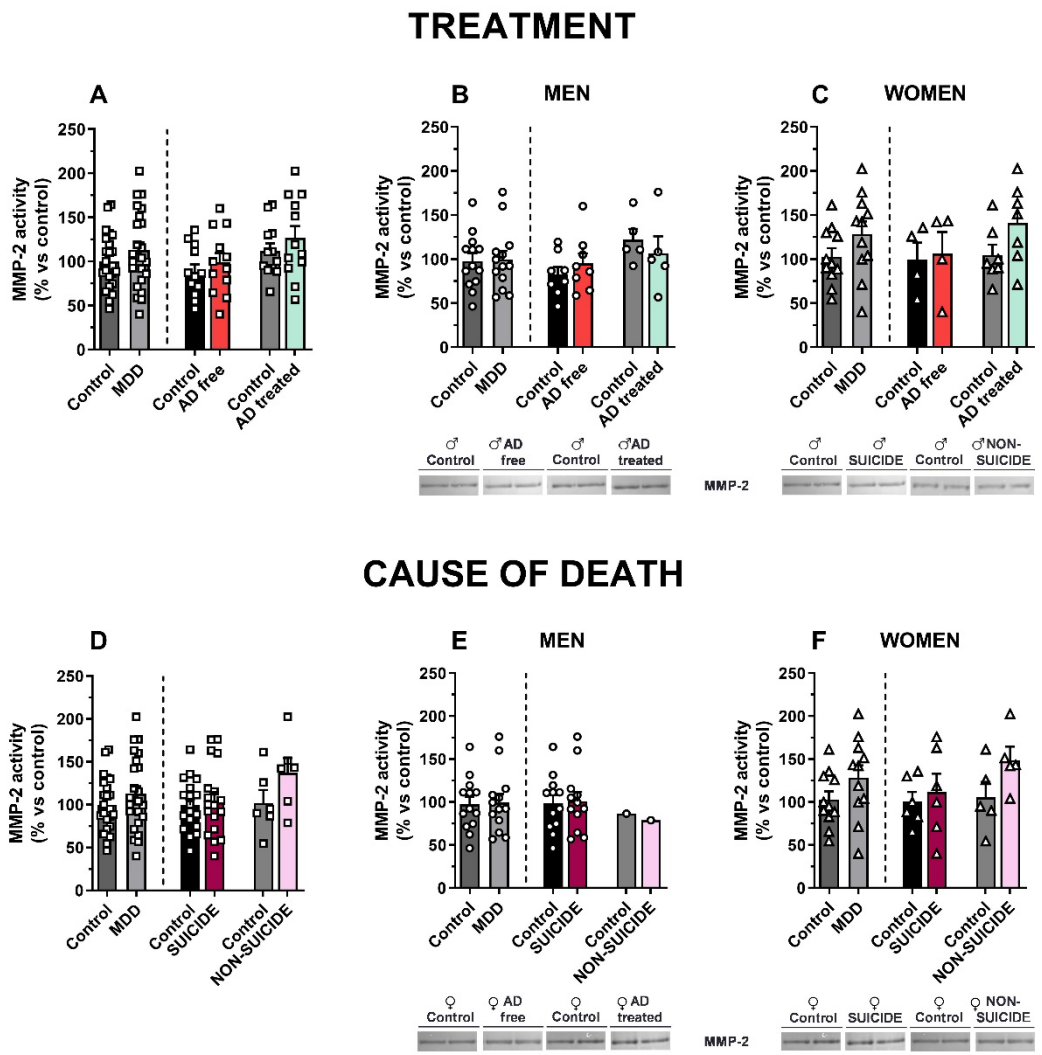

Figure 46. MMP-2 activity assessed by gel zymography in cortical *post-mortem* samples of treated and non-treated depressed patients. (A) All patients together, (B) men and (C) women patients. **In addition, the cause of death was considered.** (D) All patients together, (E) men and (F) women patients. Representative bands from the gel zymography are shown. Results are expressed in percentage *vs* the control MDD matched group and as mean ± S.E.M. n = 24 samples per group.

Besides MMP-9, MMP-2 is also capable to degrade gelatine and its activity can be assessed by the gel zymography technique. In this sense, no statistical differences were observed in any experimental group, neither in the complete cohort (figures 46A and

46D), in men (figures 46B and 46E) nor women (figures 46C and 46F) patients compared to their matched controls, regardless of being classified by their treatment or their cause of death.

3.2 Expression of neuroplasticity markers in the cortex

Different neuroplasticity markers were studied in the synaptoneurosomal fraction of the PFCx of depressed patients and their matched controls, analysed as a complete cohort, and differentiated by gender.

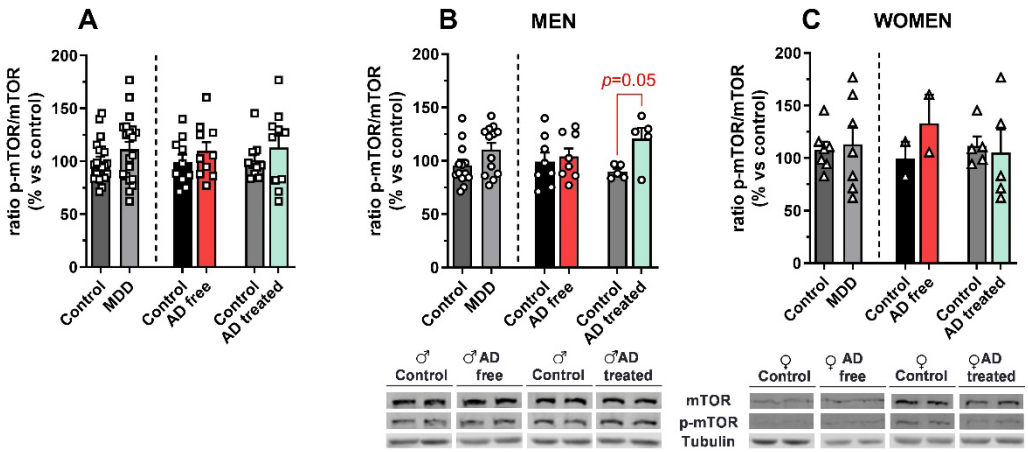

Figure 47. Ratio p-mTOR/mTOR expression in cortical *post-mortem* samples of treated and non-treated depressed patients. (A) All patients together, (B) men and (C) women patients. Representative western blot bands are shown. Results are expressed in percentage *vs* the control MDD matched group and as mean ± S.E.M. n = 20 samples per group.

The ratio between p-mTOR and mTOR levels was not modified in MDD patients compared to their matched controls, regardless of their blood antidepressant levels (figure 47A). In men, no statistical differences were observed in the whole cohort of MDD patients or patients without treatment. A great tendency to an increased ratio p-mTOR/mTOR was observed in MDD men with antidepressant treatment (120.8 ± 10.2%

in AD treated *vs* 89.9 ± 2.9% in the control group, $p = 0.055$, figure 47B). Instead, women did not present differences in the p-mTOR/mTOR levels in any of the experimental groups studied (figure 47C). mTOR levels were not altered in any of the experimental groups.

Finally, decreased levels of BDNF were found in all MDD patients (62.1 ± 10.7% in MDD *vs* 100.0 ± 13.3% in the control group, $p<0.01$) and in patients that were not following the AD treatment at the moment of death (51.8 ± 12.3% in AD free *vs* 106.2 ± 20.6% in the control group, $p<0.05$). No differences were observed in the AD treated group (figure 48A).

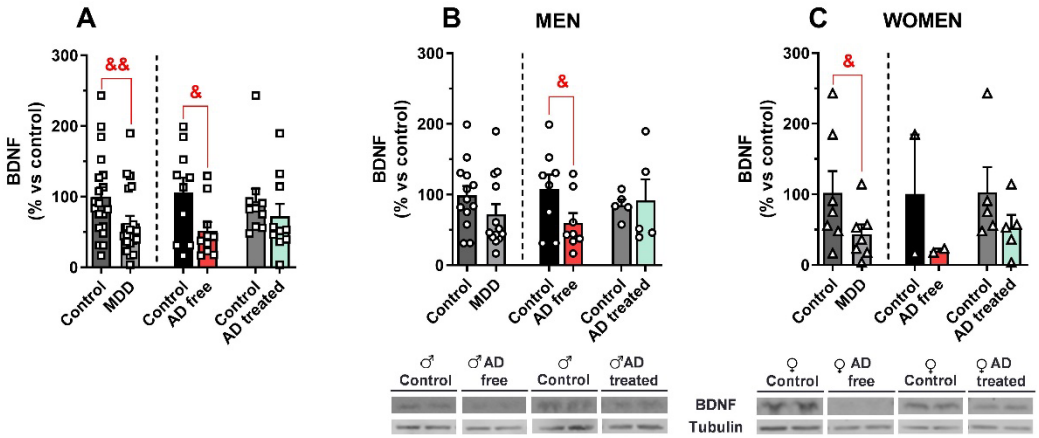

Figure 48. BDNF expression in cortical *post-mortem* samples of treated and non-treated depressed patients. (A) All patients together, (B) men and (C) women patients. Representative western blot bands are shown. Results are expressed in percentage *vs* the control MDD matched group and as mean ± S.E.M. Paired Student's *t*-test $^{\&}p<0.05$, $^{\&\&}p<0.01$ vs control group. n = 20 samples per group.

When only men patients were analysed, no alterations of BDNF levels were observed in the whole cohort of MDD patients or AD-treated. However, lower levels of BDNF were seen in MDD men AD free, compared to their matched control subjects (59.7 ± 14.1% in AD free *vs* 107.6 ± 20.7% in the control group, $p<0.05$, figure 48B). MDD women

displayed decreased BDNF levels, compared with the control group (43.6 ± 13.8% in MDD *vs* 102.1 ± 30.8% in the control group, $p<0.05$), without statistical differences in the AD-free or AD treated groups (figure 48C).

DISCUSSION

Objective 1. Effect of a subchronic treatment with cannabidiol in the corticosterone animal model of depression

Despite the availability of pharmacological treatments, one-third of patients develop treatment resistance depression (Rush et al., 2006; Al-Harbi, 2012). This evidences the requirement to find new treatments and targets. In this thesis, we aimed to study the implication of MMP-9 in the etiopathology of depression and the antidepressant-like effect induced by subchronic treatment with CBD, in a corticosterone-induced mouse model of depression. The chronic administration of corticosterone is a widely used murine model for the study of depression and anxiety (Ardayfio and Kim, 2006; David et al, 2009; Gourley and Taylor, 2009; Pascual-Brazo et al., 2012). It induces the impairment of the HPA axis and mimics some of the molecular alterations described in depressed patients, associated with anxious and depressive-like symptomatology (Gourley and Taylor, 2009; Rainer et al., 2012). There are several preclinical (Zanelati et al., 2010; Linge et al., 2016; Florensa-Zanuy et al., 2021; Hou et al., 2022; Martín-Sánchez et al., 2022; Silote et al., 2022) and growing clinical (Solowij et al., 2018; Elms et al., 2019; Laczkovics et al., 2021) evidence about CBD as a potential anxiolytic and antidepressant drug. In this objective, a subchronic treatment with CBD was selected based on previous data (Linge et al., 2016), where they observed a fast, but not maintained anxiolytic-like effect, as well as anorectic actions of acute CBD administration, together with the anxiolytic and antidepressant-like effect of chronic CBD administration. Here we performed a 4-day subchronic treatment with CBD in the corticosterone model of depression and MMP-9 protein levels and activity was evaluated in two main areas implicated in MDD: the prefrontal cortex and the hippocampus (see *review* Belleau et al., 2019). MMP-9 plays an important role in neuroplasticity, in several neuropathologies such as Alzheimer's, Parkinson's and Huntington's disease, multiple sclerosis, schizophrenia (*see review* Vafadari et al., 2016), and in other diseases that can present comorbidity with MDD like cancer, diabetes and

coronary heart diseases (Hu et al., 2020; Mondal et al., 2020; Moroianu et al., 2022). Therefore, we decided to focus on the role of this protein.

1.1 Behavioural effect of cannabidiol in a corticosterone-induced mouse model of depression

To our knowledge, this is the first study of the CBD-induced behavioural effect on depression/anxiety paradigms in a corticosterone-induced animal model. We have shown that the chronic administration of corticosterone in the drinking water in C57BL/6J male mice produces an anxious and depressive-like behaviour as evidenced by an induction of anhedonia (decreased sucrose preference) and anxious/depressive-like behaviour (increased latency to feeding in the novelty suppressed feeding test). These results are in line with previous studies carried out in the same murine model (Gourley and Taylor, 2009; Vidal et al., 2019; Garro-Martínez et al., 2020; Amigo et al., 2021), following other systemic administrations of corticosterone (Zhao et al., 2008), and with other animal models of depression (Linge et al., 2016; Fogaça et al., 2018; Florensa-Zanuy et al., 2021).

The subchronic treatment with CBD reverted the anxious/depressive-like behaviour observed in the novelty suppressed feeding test in the corticosterone model, but it was not able to revert the anhedonia in this model. In the novelty suppressed feeding test, the chronic treatment (3-4 weeks) with fluoxetine (David et al., 2009; Rainier et al., 2012; Samuels et al., 2014; Amigo et al., 2021), and agomelatine (Rainier et al., 2012) in mice also show a reduction in the latency to feeding in this animal model. Moreover, in naïve rats a decrease in the latency to feeding was described after 7, but not 3 days, treatment with the 5-HT_4 partial agonist RS67,333 (Pascual-Brazo et al., 2012).

In agreement with our results, chronic CBD treatment displayed an anxiolytic-like effect in the light/dark box and elevated plus maze tests in a post-traumatic stress disorder

model (Long et al., 2010; Gasparyan et al., 2021). In addition, the anxious behaviour observed in the novelty suppressed feeding test was reverted with different CBD treatments in other animal models of mood disorders, such as the chronic unpredictable stress model (Campo et al., 2013; Fogaça et al., 2018), the olfactory bulbectomy model (Linge et al., 2016), and other pathologies as the neuropathic pain model (De Gregorio et al., 2019). Moreover, clinical trials have also assessed the anxiolytic effect of CBD in patients diagnosed with anxiety and sleep disorder (Shannon et al., 2019), social anxiety disorder (Bergamaschi et al., 2011), and post-traumatic stress disorder (Elms et al., 2019).

Regarding anhedonia, 3-day treatment with CBD did not revert the sucrose preference in the corticosterone model when considering all the animals. However, we found two mice populations, "responders" and "non-responders", attending to their behaviour in this test. The subchronic CBD treatment reverted the anhedonia in more than half of the animals studied. The anti-anhedonic effect exerted by a CBD treatment has been described in other mouse models of depression such as the acute LPS administration (Florensa-Zanuy et al., 2021) and the olfactory bulbectomy (Linge et al., 2016), and the chronic unpredictable mild stress model in the rat (Gáll et al., 2020). In addition, our results are in line with the antidepressant-like effect on behavioural despair produced by an analogue of CBD in a corticosterone-induced model of depression in female rats (Wróbel et al., 2020). The antidepressant-like effect of the subchronic CBD administration is comparable with the antidepressant-like effect observed in this model when chronically treated with classical antidepressant drugs such as fluoxetine (Pascual-Brazo et al., 2012; Ali et al., 2015), and paroxetine (Herbet et al., 2019).

However, it is important to remark that the "responder" and "non-responder" populations are also present in the novelty suppressed feeding test. In fact, these populations have been also described following the treatment with fluoxetine in the chronic corticosterone animal model of depression in this behavioural test (*see review* Samuels et al., 2011). In addition, this phenomenon was reported in clinical studies after

antidepressant drug treatment (Ontiveros-Sánchez de la Barquera, 2017; Desmidt et al., 2022; Diep et al., 2022).

Finally, we cannot assure that the "non-responder" group is resistant to the cannabidiol effect. In fact, we have previously described for RS67,333 that the fast-acting antidepressant effect on anhedonia is observed after 7 but not 3 days of treatment (Pascual-Brazo et al., 2012). In this sense, a longer duration of the treatment with CBD may be of interest to clarify if these animals could be considered "treatment resistant" or "non-responders". However, different individual sensitivity to the anorectic effect induced by the CBD subchronic treatment cannot be discarded, as evidenced by the lower food consumption following acute CBD treatment (50 mg/kg) in the olfactory bulbectomy model (Linge et al., 2016). Nevertheless, contradictory studies have been published, about the antidepressant- and anxiolytic-like effects of CBD, suggesting a strain, gender, and dose-dependent effect of the CBD administration (*see review* García-Gutiérrez et al., 2020).

1.2 MMP-9 modulation induced by cannabidiol treatment in the corticosterone mouse model

To determine the potential role of MMP-9 in the etiopathology of MDD and the subchronic effect of CBD, we studied the protein expression and activity of MMP-9 in two crucial brain areas implicated in the neurobiology of depression and the antidepressant response: the prefrontal cortex and the hippocampus (*see review* Belleau et al., 2019).

We found an increased protein expression and activity of MMP-9 in the cortex and hippocampus of the corticosterone model of depression. This is in line with previous reports in our group (Breviario et al., 2023), and others describing a high proteolytic activity of MMP-9 in the CA1 region of the hippocampus in a chronic stress rat model

(van der Kooij et al., 2014), and in the chronic unpredictable mild stress mouse model of depression (Bijata et al., 2022). A study carried out by Alaiyed et al. (2020) reported that MMP-9 levels were not altered in the prefrontal cortex of a corticosterone-induced mouse model of depression, assessed by the ratio of pro-MMP-9/TIMP-1, a finding that is inconsistent with our data. This discrepancy might be associated with the different corticosterone doses, the duration of the corticosterone administration, and with the different techniques used to assess MMP-9 levels (Alaiyed et al., 2020).

Several studies have associated peripheral MMP-9 levels with MDD and the severity of its symptomatology. A clinical trial with 558 Chinese patients with ischemic stroke, reported that elevated MMP-9 serum levels, but not TIMP-1 levels, are correlated with a higher risk to develop post-stroke depression in the acute phase (Che et al., 2019). Another clinical trial, with 245 depressed patients, 229 schizophrenic patients, and 254 controls of Caucasian origin, revealed increased MMP-9 levels in the blood of both, MDD and schizophrenic patients (Domenici et al., 2010). Then, Yoshida et al. (2012) described a positive correlation between MMP-9 levels and MDD severity, although they do not find differences in MMP-9 serum levels between MDD patients and healthy controls. In contrast, decreased serum mRNA and protein levels of MMP-9 in patients with recurrent depressive disorder were observed (Bobińska et al., 2016c). Nevertheless, the same authors found an association between MMP-9 (T-1702A and C1562T) polymorphisms and depression (Bobińska et al., 2016b). These polymorphisms induce an increase in the MMP-9 transcription and protein levels (Bobińska et al., 2016a).

Herein, the subchronic CBD treatment, in parallel to the reversal of the anxious- and depressive-like behaviour, normalized the increase of the proteolytic activity of MMP-9 observed in the corticosterone model, without modifying its expression. In this sense, HeLa cells treated with CBD increased TIMP-1 levels release, in a dose-dependent manner, without altering the MMP-9 levels, by targeting CB_1, CB_2, and $TRPV_1$ receptors (Ramer et al., 2010). Taking this into consideration, CBD could be inducing TIMP-1

expression in our animal model of depression, explaining the reduction of the MMP-9 activity observed in our study, but not its protein expression.

Several studies have also described a CBD-induced normalization of MMP-9 levels in the periphery in different animal models. The high MMP-9 levels observed in the LPS-induced rat model were reduced after CBD treatment in the blood, the liver and the kidney (Trivedi et al., 2022). The systemic administration of CBD also prevented the increased expression of MMP-9 in the heart of mice treated with doxycycline (Hao et al., 2015). Finally, the TNFα-induced MMP-9 release in HaCaT cells (an immortalized human keratinocyte cell line), was inhibited by CBD treatment in a dose-dependent manner (Sangiovanni et al., 2019).

Besides, the microinfusion of an MMP-9 inhibitor in the CA1 region of the hippocampus prevented the social and cognitive deficit characteristic of a chronic stress rat model (van der Kooij et al., 2014). In the current study, we observe a positive correlation between MMP-9 levels in the prefrontal cortex and the severity of anhedonia, according to data previously reported by Yoshida et al. (2012) in MDD patients. Interestingly, in parallel to our results, the electroconvulsive therapy decreased the MMP-9 levels found in the serum of depressive patients (Shibasaki et al., 2016), although the MMP-9 levels were not modified in patients who relapsed (Shibasaki et al., 2018).

Finally, in naïve animals, subchronic CBD treatment induces an anxiogenic-like effect evaluated in the novelty suppressed feeding test. Although CBD can induce an anorectic effect (Linge et al., 2016), we did not observe changes in the food consumed in the post-test, suggesting that the increased latency observed in this test is exclusively due to increased conflict-based anxiety. An anxiogenic effect has been also observed with antidepressants such as the SSRI fluoxetine using the open field test (Amigo et al., 2021), the elevated plus maze (Baek et al., 2015), and the hole-board test in rats (Gomez and García-García, 2017). Interestingly, the appearance of adverse effects of fluoxetine treatment has been linked with the decrease of MMP-9 in the central amygdala (Puścian

et al., 2021). Thus, it would be of interest to study the effect of CBD treatment on MMP-9 activity in other brain areas.

Objective 2. Characterisation of MMP-9 transgenic mice

2.1 Behavioural characterisation

Taking into account the results obtained in the objective 1, we hypothesised that MMP-9 KO mice may display a similar behaviour than WT mice or a less anxious/depressive-like phenotype, whereas MMP-9 OE mice may present an anxious/depressive-like phenotype.

Anxious-like behaviour

Anxiety and depression are comorbid diseases (Sartorius et al., 1996). Thus, we evaluated whether the genetic inhibition of MMP-9 activity or its overexpression would influence the anxious-like behaviour of mice using different paradigms to discriminate between innate and conflict-based anxiety. To address this issue, we evaluated the innate anxiety using the open field, the light-dark box, and the elevated plus maze tests.

In the **open field test**, MMP-9 KO male mice present less innate anxiety, while MMP-9 KO female mice exhibited higher innate anxiety, without differences in locomotor activity. Instead, MMP-9 OE male mice behaved similarly to WT male mice, and MMP-9 OE females displayed less innate anxiety, opposite to the behaviour observed in MMP-9 KO females. Both MMP-9 OE males and females displayed hypoactivity probably associated to a lower exploratory drive and seems not to be influencing the mobility parameters in behavioural tests as the tail suspension test.

Our results suggest that the MMP-9 activity is related to innate anxiety in a sex-dependent manner. To the best of our knowledge, this is the first time that an anxious-related behaviour has been observed in these transgenic mice, using the open field test. Our findings contrast with other authors that did not report differences in anxiety in the open field test in MMP-9 KO mice either in males (Mizoguchi et al., 2010; Sidhu et al.,

2014; Ringland et al., 2021) and females (Knapska et al., 2013; Ringland et al., 2021), and MMP-9 OE mice in both male and female (Fragkouli et al., 2012).

Spite these discrepancies observed in the transgenic mice, the contribution of MMP-9 in the performance of the open field test is still unclear. In line with our findings in MMP-9 KO male mice, Kelestemur et al. (2020) reported a reduction of the cortical MMP-9 levels and a lower anxious-like behaviour in rats subjected to newborn hypoxia-ischemia and treated with hyperoxia. In contrast, the lower gene expression of MMP-9 observed in the COX2-deficient knockin male, but not female mice is associated with anxious-like behaviour and hyperactivity in the open field test (Wong et al., 2019).

Regarding MMP-9 overexpression, Yoo et al. (2016) found an overactivation of MMP-9 in males but not females in zinc transporter 3 null mice, which leads to an anxious-like behaviour in the open field test, in contrast with our findings in male mice. Moreover, some authors reported that the pharmacological inhibition of MMP-9 in naïve young male mice (Pirbhoy et al., 2020), and the overexpression of MMP-9 by an enriched environment (Cao et al., 2014) did not modify the anxious behaviour in the open field test.

It is worth mentioning that the above-mentioned discrepancies might be associated with different experimental conditions used to assess this behavioural test. Here we have tested the mice under non-aversive conditions, to induce the minimum stress possible to mice and not affect their basal behaviour in other tests. However, the above-mentioned studies were tested under a bright light that induces a more aversive environment that may induce latent anxiety (Gould et al., 2009).

In the **light-dark box test**, no differences were observed in anxious-like behaviour in both male and female MMP-9 KO mice. These data are in concordance with the only previous report using this behavioural paradigm in MMP-9 KO mice, although those animals were not separated by gender (George et al., 2019). Similar results were

observed in the **elevated plus maze test**, with no differences in anxiety in male nor female MMP-9 KO mice, as previously reported by Ringland et al. (2021). Moreover, MMP-9 KO mice did not develop an anxious-like behaviour after chronic treatment with corticosterone (Alaiyed et al., 2020), suggesting that these mice may be resilient to develop an anxious-like phenotype.

On the other hand, MMP-9 OE males did not show an anxious phenotype in the light-dark box and the elevated plus maze tests, which is consistent with the findings in the open field test. However, female MMP-9 OE mice presented a lower anxious phenotype in the three different behavioural paradigms. Our results in innate anxiety in MMP-9 OE male mice are not in line with our initial hypothesis, as the results obtained in the chronic corticosterone model of depression (see Objective 1 and Breviario et al., 2023), point to increased anxiety associated with high MMP-9 brain levels. In fact, different authors report that an increased MMP-9 activity in cortical and hippocampal samples in the fragile X transgenic mouse model presents higher innate anxiety (Bilousova et al., 2009; Pirbhoy et al., 2020). This anxious behaviour is reverted by the pharmacological inhibition of MMP-9 with minocycline, an MMP-9 non-selective inhibitor (Bilousova et al., 2009), and SB-3CT, a selective gelatinase inhibitor (Pirbhoy et al., 2020). Also, a male rat model of chronic stress that present an increased MMP-9 activity in the CA1 region of the hippocampus (van der Kooij et al., 2014), showed an innate anxious-like behaviour (Beery et al., 2012). In other brain areas as the amygdala, implicated in stress-induced anxiety, high levels of MMP-9 have been related to an anxious-like behaviour observed in a neuroinflammation male rat model (Shishkina et al., 2020).

In contrast, it has been described in male rats in an early-enriched environment that presents an increase of MMP-9 levels in the hippocampus an anxiolytic effect in the elevated plus maze test (Cao et al., 2014), as observed in our MMP-9 OE female mice. In this sense, MMP-9 OE females display lower innate anxiety than WT females, in the elevated plus maze and the light-dark box test. In this regard, Kennedy-Wood et al.

(2021) proposed the involvement of MMP-9 in the basal anxiety phenotype in females. In fact, these authors found that C57BL/6J had higher basal levels of MMP-9 in the brain and a lower anxiety-like behaviour performed in the EPM, whereas BALB/c strain presented lower basal levels of MMP-9 in the brain and a higher anxiety-like behaviour. Furthermore, the different anxious response between female and male mice has been previously reported in response to chronic corticosterone administration (Berger et al., 2019; Yohn et al., 2019), and lipopolysaccharide (Florensa-Zanuy, 2021), showing higher resilience in female mice. Moreover, the Fmr1 knockout (KO) mice with high levels of MMP-9 in the brain (Wen et al., 2018), exhibit lower anxious behaviour in the open field and the light/dark box test, in both male and female mice (Ding et al., 2014). All these findings reinforce the idea that MMP-9 activity levels produce a sex-dependent effect on innate anxious-like behaviour.

It is worth mentioning that these behavioural tests partially measure the same anxiety-related characteristics, but they also maintain some idiosyncrasies (see *review* Ramos, 2008). In fact, Carola et al. (2002) reported a correlation between four out of nine factors for the elevated plus maze and open field tests. Moreover, it has been proved that different strains, gender, pharmacological treatments, and genetic modifications differ in the performance of the anxiety-related paradigms in the open field, elevated plus maze, and light-dark box tests (Salas et al., 2003; Bhatnagar et al., 2004; Ramos et al., 2008). We should also consider that, although the behavioural tests were performed from the least to the most stressful, previous experiences may have an impact on basal anxiety, leading to differences in the behavioural outcome.

The **novelty suppressed feeding test** is used to assess conflict-based anxiety in basal conditions (Planchez et al., 2019). To the best of our knowledge, there are no previous studies on MMP-9 transgenic animals that report this behavioural outcome. In our MMP-9 KO mice, both male and female, we did not observe conflict-based anxiety

evaluated in this behavioural paradigm. Similar results have been reported in male Wistar rats treated with minocycline (Amani et al., 2019).

Regarding MMP-9 OE mice, male but not female, showed conflict-based anxiety. These findings in MMP-9 OE male mice are supported by several evidences. First, the results obtained in Objective 1 in the chronic corticosterone model, and data previously published in our group (Breviario et al., 2023). Second, two animal models with high cortical MMP-9 levels as the traumatic brain injury (Watanabe et al., 2013), and obese male rats (Lorena et al., 2021), also exhibited a conflict anxiety phenotype in the novelty suppressed feeding test, and not innate anxiety. Third, clinical studies describe a significant association between the MMP-9 SNP (rs3918242) and the susceptibility to anxiety disorders (McGregor et al., 2018). All this evidence reinforce the role of MMP-9 in conflict-based anxiety.

Depressive-like behaviour

Regarding the influence of MMP-9 on depressive-like behaviour, the social interaction and the sucrose preference test were performed to assess an anhedonia-related phenotype, whereas the tail suspension test was used to study behavioural despair in mice.

In the **social interaction test**, MMP-9 KO male mice, but not female, were more sociable compared to WT. However, Sidhu et al. (2014) did not observe altered sociability of MMP-9 KO male mice. Other studies using gender-matched MMP-9 KO mice (Ringland et al., 2021), and MMP-9 KO heterozygous mice (Vafadari et al., 2019) also reported no alterations in social interaction. It is important to remark that here we present the data divided by gender, while other authors as Vafadari et al. (2019) and Ringland et al. (2021) pooled gender data, which may explain the apparent discrepancies observed. Moreover, it has been described that the MMP-9 inhibition after curcumin (Bhandari and Kuhad, 2015) and resveratrol (Bhandari and Kuhad, 2017) treatment restore the

increased MMP-9 levels and the lower social interaction in a rat model of autism. All these data suggest that a normalization or decrease in MMP-9 levels could improve social interaction behaviour, at least, according to our data in MMP-9 KO male mice.

In MMP-9 male and female OE mice, a social interaction comparable to their WT counterparts was observed. Our findings contrast with the impaired sociability reported in other animal models that present high MMP-9 levels in the brain. These animal models include the chronic stress mouse model (van der Kooij et al., 2014), the transgenic fragile X syndrome mouse model (Gkogkas et al., 2014), the zinc transporter 3 knockout male mice (Yoo et al., 2016), and a model of autism induced by the administration of propionic acid (Bhandari and Kuhad, 2015; 2017). The apparent discrepancy between our findings and others may be a consequence of the different protocols used, as other authors use the three-chamber paradigm test, to assess sociability, compared to our one-chamber protocol.

In the **sucrose preference test**, MMP-9 KO male mice did not show anhedonia compared to their WT mice, while in MMP-9 KO females, a statistically lower sucrose preference was detected. However, we may take into account that anhedonia is considered when the percentage of sucrose preference is under 65-70% (Strekalova et al., 2011; Bijata et al., 2022), thus we can consider that our MMP-9 female mice are not anhedonic. In MMP-9 OE mice, females did not present differences in sucrose preference, while males exhibited a statistically significant increase in sucrose preference. This statistically significant difference was detected by the small deviation between the samples in both MMP-9 KO and OE mice. In our opinion, these small differences do not have any biological relevance.

To date, the role of MMP-9 in anhedonia is still unclear. Although we do not observe a clear phenotype regarding anhedonia in our MMP-9 transgenic mice, recent studies seem to highlight the potential role of MMP-9 in anhedonia. In this regard, different stress models presented increased hippocampal (Bijata et al., 2022) and cortical

(Breviario et al., 2023) MMP-9 activity, in line with the positive correlation between MMP-9 levels in the PFCx and the severity of anhedonia reported in this thesis (*see Discussion 1.2*). In contrast, naïve male rats exposed to an enriched environment showed an increased sucrose preference and less despair-like behaviour, associated with increased levels of MMP-9 and active BDNF in the hippocampus (Cao et al., 2014). Moreover, Puścian et al. (2021) described that in female naïve animals, the chronic treatment with fluoxetine induced an anhedonic-like behaviour, together with a reduction of MMP-9 activity in the central amygdala. However, MMP-9 KO mice treated chronically with fluoxetine did not present anhedonia, suggesting that anhedonia is developed in an MMP-9-dependent manner (Puścian et al., 2021).

Our results suggest that either the deletion of MMP-9 in males or the overexpression of MMP-9 in both sexes is not altering the behavioural despair in the **tail suspension test**. Interestingly MMP-9 KO females presented lower immobility, compared to the WT group, suggesting lower behavioural despair. The lack of differences in male MMP-9 KO mice is in agreement with those previously reported in MMP-9 KO mice (Bijata et al., 2022), and in MMP-9 heterozygous mice (Vafadari et al., 2019). The lower immobility time in the tail suspension test of MMP-9 KO female is in concordance with the antidepressant-like effect elicited by minocycline, an MMP-9 inhibitor (Vandooren et al., 2017), either in male mice (O'Connor et al., 2009; Gong et al., 2018), or rat (Saravi et al., 2016; Yang et al., 2020; Maras et al., 2022). Minocycline also induces a reduction in behavioural despair in animal models of different mental disorders comorbid with depression including schizophrenia (Monte et al., 2013; Zhu et al., 2014a; Zhu et al., 2014b), stress (Tong et al., 2017; Han et al., 2019), and autism (Kumar and Sharma, 2016; Yang et al., 2021). In the forced swimming test, which also measures behavioural despair, different authors also report an antidepressant-like effect induced by the treatment with resveratrol (Bhandari and Kuhad, 2017), and curcumin (Bhandari and Kuhad, 2015) in animal models that present depressive-like behaviour, in parallel to the reduction of MMP-9 levels.

In contrast to the lack of differences in the behavioural despair observed in the MMP-9 OE mice, some authors report an increased MMP-9 activity in the hippocampus in anhedonic mice (Bijata et al., 2022). Moreover, in the chronic unpredictable mild stress model, brain MMP-9 levels correlate with the susceptibility (high levels) or resilience (low levels) to develop anhedonia (Bączyńska et al., 2022; Bijata et al., 2022). However, MMP-9 heterozygous mice present more susceptibility to developing depressive-like behaviour when they are subjected to chronic psychosocial stress (Vafadari et al., 2019). In this regard, it would be of interest to induce a model of depression in the adult transgenic MMP-9 KO and OE mice to evaluate their resilience or susceptibility to develop an anxious-depressive-like phenotype.

Therefore, our findings confirm our hypothesis for MMP-9 KO male mice in social interaction and MMP-9 KO female in tail suspension test, pointing to the resilience to develop depressive-like behaviour. However, the overexpression of MMP-9 in mice did not induce a depressive-like phenotype.

Some of the discrepancies between our findings in anxious- and depressive-like behaviour and previous reports may be associated with the experimental approach used (genetic *versus* pharmacological manipulations, or genetic *versus* non-genetic animal model of depression). In addition, we and other authors report the association between depressive-like behaviour and high MMP-9 levels in animal models of different pathologies. However, the regulation of MMP-9 levels appears not to be the only factor responsible for the modulation of depressive-like manifestations.

The impact of the genetic manipulation of MMP-9 results in differences in depressive/anxious-like behaviour depending on gender. This fact supports the importance to include females in research, especially in depression, as the incidence of this disease is two-fold higher in women than in men.

Other general effects on behaviour

A study of a more general behaviour was used to evaluate if the inhibition or the overexpression of MMP-9 may induce an impaired behaviour. With this aim, motor coordination (rota-rod test), compulsive-like behaviour (marble burying test), health and welfare (nesting test), and spatial memory (T-maze test) were assessed.

In the **rota-rod test**, no deficits in motor coordination in MMP-9 KO or MMP-9 OE mice, male or female, were observed. These data are in good concordance with two previous studies carried out in MMP-9 KO mice (Wang et al., 2000; Knapska et al., 2013). In fact, different studies also demonstrate that the MMP-9 deletion and/or normalization of MMP-9 had a positive influence on motor coordination. For example, Wang et al. (2000) reported that MMP-9 KO mice displayed less motor impairment after a brain injury. In addition, the genetic deletion of MMP-9 in a transgenic model of amyotrophic lateral sclerosis rescued the motor impairments (Kaplan et al., 2014). Finally, it is important to mention that different pharmacological treatments reverted the motor impairments by partly inhibiting the increased MMP-9 activity in several murine models of stroke (Jang et al., 2014; Gupta et al., 2017), autism spectrum disorder (Bhandari and Kuhad, 2015; Bhandari and Kuhad, 2017), neonatal hypoxia-ischemia (Kelestemur et al., 2020) and spinal cord compression injury (Hong et al., 2015). Our results also showed that the overexpression of MMP-9 did not affect motor coordination. In fact, it has been described that an increase in MMP-9 is not enough to alter motor coordination (Kaplan et al., 2014).

To further investigate the probability that altered MMP-9 activity would be related to compulsive behaviour we performed the **marble burying test**. Here, our MMP-9 transgenic mice, both males, and females showed similar behaviour to their WT counterparts, pointing to a non-obsessive-compulsive-like behaviour. However, using a different paradigm to evaluate compulsive behaviour, Gkogkas et al. (2014) reported an increase in the time spent self-grooming in transgenic mice that overexpress MMP-9.

Moreover, in two mouse models that present high cortical MMP-9 levels, animals given microbiota from mice under a high-fat diet (Bruce-Keller et al., 2015), and knockout male mice of the zinc transporter 3 (Yoo et al., 2016), showed a compulsive behaviour. In fact, the treatment with minocycline (Yoo et al., 2016), curcumin (Bhandari and Kuhad, 2015), and resveratrol (Bhandari and Kuhad, 2017) reverted the compulsive behaviour. Finally, clinical studies showed that minocycline, used as an adjuvant, decreased the Yale–Brown Obsessive Compulsive Scale in patients diagnosed with obsessive-compulsive disorder (Esalatmanesh et al., 2016). The differences observed between our results and previously reported data may be associated with the methods used to evaluate this compulsive-like behaviour: marble test *vs* self-grooming test, and/or the animal model used, including transgenic mice.

In order to evaluate the integrity of the hippocampus, we used two experimental approaches: the nesting and the T-maze tests. MMP-9 transgenic mice obtained an appropriate score in the **nesting test**. The nesting test is sensitive to hippocampal lesions (Cunningham et al., 2003), and also to the general well-being state, and the apathetic-like behaviour (Filali et al., 2009). In this sense, MMP-9 KO and MMP-9 OE mice exhibit a normal well-being state and a non-apathetic-like behaviour. To the best of our knowledge, there are no published data regarding the influence of brain MMP-9 in this behaviour. Thus, our data suggest that the modulation of MMP-9 expression may not be directly involved in this behavioural outcome. However, previous studies have associated low peripheral MMP-9 levels with well-being state of women diagnosed with cancer. Lower MMP-9 gene expression in leukocytes has been associated with better social well-being in women diagnosed with non-metastatic breast cancer (Jutagir et al. 2017). It should be highlighted that the few studies that associate the well-being state with MMP-9, are analysing only peripheral MMP-9. Considering that cancer presents comorbidity with depression and that peripheral MMP-9 levels could reflect the MMP-9 protein present in the central nervous system, it may be expected that a reduction in MMP-9 levels could be related to a well-being state.

It is known that spatial memory and learning are related to hippocampal integrity (Andrews-Zwilling et al., 2012), although other brain areas are also implicated. Our findings in the **T-maze test** suggest that MMP-9 KO mice did not exhibit spatial memory deficits, whereas MMP-9 OE mice showed impaired spatial memory. The normal performance of MMP-9 KO mice in the T-maze is in line with our findings in the nesting test, suggesting no hippocampal deficit or lesion. Our results are also in line with previous data in male and female MMP-9 KO mice, not displaying impaired working or reference memory in the radial arm water maze test (Ringland et al., 2021). Furthermore, MMP-9 KO mice are protected against corticosterone-induced cognitive impairment (Alaiyed et al., 2020). In the same line, in naïve animals, the pharmacological inhibition of MMP-9 with SB-3CT (Semple et al., 2015) and with minocycline (Bilousova et al, 2009; Monte et al., 2013; Kumar and Sharma, 2016), do not affect spatial memory. Moreover, the MMP-9 inhibition reverts cognitive deficits in an autistic animal model (Kumar and Sharma, 2016), or associated to subarachnoid haemorrhage (Sherchan et al., 2011).

Cognitive impairment is characteristic of the depressive symptomatology of patients (Du et al., 2021; Vance and Winther, 2021) and animal models of depression (Alaiyed et al., 2020). The impaired spatial memory observed in our MMP-9 OE mice, is in line with the negative correlation between the hippocampal MMP-9 expression levels and the spatial memory assessed in the T-maze (Sherchan et al., 2011) and the Morris water maze (Sherchan et al., 2011; Adeli et al., 2019; Dupré et al., 2020). Also, β-amyloid i.c.v. infusion induces cognitive deficits and MMP-9 overexpression (Mizoguchi et al., 2009). This cognitive impairment is prevented by the pharmacological MMP-9 inhibition and in MMP-9 KO mice (Mizoguchi et al., 2009). All this evidence and our data point to a detrimental effect of high MMP-9 levels in spatial memory performance.

In contrast to the above-mentioned, other studies are showing that an enhanced spatial memory may be due to increased MMP-9 functionality. Fragkouli et al. (2012) observed

that MMP-9 OE mice exhibited a superior performance in spatial memory evaluated in the Morris water maze. In addition, in a transgenic model of Alzheimer and overexpression of MMP-9, a restored alternation rate in the T-maze was reported (Fragkouli et al., 2014). In this regard, increased levels of MMP-9 in the hippocampus and the prefrontal cortex were found after performing the Morris water maze test (Wright et al., 2004; Wright et al., 2007), while the pharmacological inhibition of MMP-9 impaired the performance in this test (Wright et al., 2007). Similarly, the administration of nicotine in the ventral tegmental area improves spatial working memory through the activation of MMP-9 in the dorsal hippocampus and the hippocampal infusion of an MMP inhibitor reverts the memory enhancement in the T-maze (Shu et al., 2015).

Finally, we evaluated **stress-induced hyperthermia (SIH)**. The physiological response to acute stress is an increase in body temperature, as observed in MMP-9 OE mice. However, MMP-9 KO male and female mice did not present this stress-induced hyperthermia response. This is the first time that an impaired hyperthermia response to acute stress is associated with MMP-9 deletion.

In line with our findings in MMP-9 KO mice, Taylor et al. (2018) reported that minocycline attenuated the hyperthermia induced by a moderate cortical concussion injury. On the other hand, it has been reported that anticipatory anxiety is associated with hyperthermic response (Borsini et al., 1989). In fact, anxiolytic drugs (such as chlordiazepoxide, diazepam, clobazam, oxazepam, and buspirone), but not anti-depressive drugs, can reduce this hyperthermia (*see review* Borsini et al., 2002; Spooren et al., 2002). Also, mGluR7 (Cryan et al., 2003) and mGluR5 (Brodkin et al., 2002) knockout mice presented lower stress-induced hyperthermia associated with anxiolytic behaviour. The association between the absence of stress-induced hyperthermia and MMP-9 activity and the presence of anxiolytic behaviour is in line with our results in MMP-9 KO male mice, but not in females, which exhibit increased innate anxiety. As

previously discussed, gender differences have been reported in two different animal models of depression (Berger et al., 2019; Yohn et al., 2019; Florensa-Zanuy, 2021), showing more resilience in females.

Regarding MMP-9 OE mice, the physiological response to stress is not altered, but they present a lower basal temperature than WT mice. Differences in the regulation of the core temperature have been described in some transgenic animals (Adriaan Bouwknecht et al., 2007).

2.2 Molecular characterisation

Expression of neuroplasticity markers in the hippocampus

We found high **nectin-3** levels in female MMP-9 KO mice, while MMP-9 KO male and MMP-9 OE mice did not present differences. The results obtained in MMP-9 KO females are in line with studies reporting that nectin-3 overexpression in the hippocampus can promote resilience to stress, reverting early-life stress (Wang et al., 2013) and chronic stress-induced alterations (van der Kooij et al., 2014). This evidence is in line with our hypothesis that proposed the resilience to present a depressive-like phenotype in the MMP-9 KO mice. In fact, we have observed a significant correlation between MMP-9 levels and depressive-like behaviour in the corticosterone model of depression (Breviario et al., 2023). However, the MMP-9 KO female mice present higher innate anxiety that may not be associated with the nectin-3 levels, as we have previously reported a lack of correlation between anxiety and nectin-3 levels in the corticosterone model of depression (Breviario et al., 2023). Moreover, other authors have reported that alteration in hippocampal nectin-3 levels does not correlate with anxiety behaviour (Wang et al., 2013; Wang et al., 2017; LIU et al., 2019). Surprisingly, MMP-9 KO males did not present altered nectin-3 expression. Although MMP-9 is the only enzyme known to digest nectin-3 in the brain, the differences observed in male and female mice may

be associated with alternative proteases replacing the role of MMP-9 in these knockout animals.

Nectin-3 expression is not modified in our MMP-9 OE mice. These findings are in contrast with the lower nectin-3 levels observed in the chronic corticosterone model of depression that exhibit high MMP-9 levels (Breviario et al., 2023) and in the chronic restraint stress model (van der Kooij et al., 2014) in relevant areas implicated in depression as cortex and hippocampus. Low nectin-3 seems to be associated with a decrease in dendritic spine density and the development of cognitive deficits in animal models of social defeat (Wang et al., 2011; Wang et al., 2013; van der Kooij et al., 2014; Wang et al., 2017), and the nectin-3 knockdown in the hippocampus (Wang et al., 2013; Wang et al., 2017; Liu et al., 2019). However, nectin-3 does not seem to be the only protein responsible for these cognitive deficits since our MMP-9 OE mice present cognitive impairment, as observed in the T-maze, but not alterations in nectin-3 levels. We suggest that due to the constitutive condition of our transgenic mice, compensatory mechanisms may induce the synthesis of nectin-3 as a consequence of an increased degradation rate in MMP-9 OE.

Decreased **BDNF** signalling has been found in *post-mortem* brain samples of MDD patients (Dunham et al., 2009; Guilloux et al., 2012; Tripp et al., 2012; Ray et al., 2014; see review in Duman et al., 2016). Conversely, typical antidepressant drugs increase BDNF expression in depressed patients (see review in Duman et al., 2016).

Our MMP-9 KO female mice exhibited increased hippocampal BDNF levels. In fact, electroconvulsive therapy increases levels of BDNF in the serum of patients diagnosed with MDD (Vanicek et al., 2019), and restores the increase of MMP-9 levels (Shibasaki et al., 2016). All this evidence point to an inverse correlation between BDNF and MMP-9 levels in depression. Moreover, the higher BDNF levels and the innate anxiety observed in the open field test in our female MMP-9 KO are in line with previous studies that associate high hippocampal BDNF levels with an anxiogenic-like behaviour (Deltheil

et al., 2009; Casarotto et al., 2012). High BDNF levels are also linked to an antidepressant-like effect (Shirayama et al., 2002; Deltheil et al., 2009), which is in good agreement with lower behavioural despair elicited by our female MMP-9 KO mice. In fact, high levels of hippocampal BDNF induce a decrease in 5-HT levels (Deltheil et al., 2008), similar to the low hippocampal and cortical levels of this neurotransmitter observed in female MMP-9 KO mice (discussed below in section 2.3).

Male MMP-9 KO mice did not exhibit differences in hippocampal BDNF expression. Nevertheless, we cannot associate the low anxious behaviour in these animals with BDNF levels, although it is well known the negative influence of BDNF on anxiety (Deltheil et al., 2009). Moreover, the lack of differences in BDNF levels agrees with the findings reported by Mizoguchi et al. (2011) on male MMP-9 KO mice. In addition, pro-BDNF levels in the hippocampus were not altered in our male and female MMP-9 KO mice, as previously reported (Mizoguchi et al., 2011).

Regarding the influence of MMP-9 overexpression, there are not previous studies analysing BDNF basal levels. In patients diagnosed with MDD, it has been reported high MMP-9 levels (Bobińska et al., 2016c) and low BDNF levels in serum (Molendijk et al., 2011). A negative correlation of BDNF has been reported with the severity of MDD (Shimizu et al., 2003). However, our MMP-9 OE mice did not present altered BDNF levels, which may be underlying the lack of clear depressive-like phenotype; although we and other authors have reported an association between depressive-like behaviour and high levels of MMP-9 in animal models (Bijata et al., 2022; Breviario et al., 2023). One of the roles of MMP-9 is to cleave the inactive precursor form of BDNF, converting it into its mature form (Hwang et al., 2005; Mizoguchi et al., 2011). In fact, a positive correlation between MMP-9 and mature BDNF, in a tyrosine kinase receptor B-dependent manner, has been reported (Kuzniewska et al., 2013). Moreover, upregulation of both BDNF and MMP-9 levels in the hippocampus has been reported in animal models of epilepsy (Mizoguchi et al., 2011), Alzheimer's disease (Fragkouli et al., 2014), and naïve animals exposed to environmental enrichment (Cao et al., 2014).

Our MMP-9 OE mice show an increased hippocampal ratio pro-BDNF/BDNF in male but not in female mice, while no differences were observed in BDNF and pro-BDNF values. It has been described that patients diagnosed with MDD present high pro-BDNF and low BDNF levels in blood samples, which correlate with MDD severity (Zhou et al., 2013). As above-mentioned, we did not observe a clear depressive-like phenotype in our MMP-9 OE mice, although our male mice exhibited increased conflict-based anxiety in the novelty suppressed feeding test. However, it has been described that this conflict-based anxiety is characteristic of animal models of depression as the chronic corticosterone model (Objective 1 of this thesis; David et al., 2009; Breviario et al., 2023). Thus, the differences observed in innate and conflictive anxiety between male and female MMP-9 OE mice might be linked to the different regulation of the pro-BDNF/BDNF ratio.

MMP-9 has been reported to induce the phosphorylation of **mTOR**, probably, through the activation of BDNF and integrins as reported in hippocampal neuronal cultures (Sidhu et al., 2014). However, Sidhu et al. (2014) did not see alterations in the ratio p-mTOR/mTOR or downstream signalling in the hippocampus of MMP-9 KO male mice, in good agreement with our results, as MMP-9 KO males did not show differences in mTOR and p-mTOR. This lack of changes in mTOR could be responsible for the normal **PSD95** levels, although high **synapsin I** levels were detected. We have not a plausible explanation for the increased levels of synapsin I in male MMP-9 KO mice. However, it has been described that increased serotonin levels, as observed in our animals (see Results section 2.1.3), can promote the expression of synapsin I (Hart et al., 2011).

Regarding MMP-9 KO female mice, the lack of functionality of MMP-9 induced an increase in mTOR and p-mTOR levels in the hippocampus. The high p-mTOR levels are in line with the subsequent high levels of PSD95 and synapsin l, as the activation of the mTOR pathway induces the transcription of these neuroplasticity proteins (Lee et al., 2005; Li et al., 2010; *see review* Duman et al., 2012; Luo et al., 2021; Xia et al., 2021). The upregulation of different neuroplasticity markers (BDNF, mTOR, PSD95, and synapsin I) may contribute to the lower behavioural despair observed in our MMP-9 KO

female mice. These findings are in line with the effect elicited by fast-acting antidepressant drugs such as ketamine (Li et al., 2010) or cannabidiol (Sales et al., 2019). A plausible explanation for the differences observed between MMP-9 KO males and females could be due to the opposite serotonin levels observed in the cortex and hippocampus in both genders (discussed in section 2.3).

Regarding, MMP-9 OE mice, male animals displayed a decreased activation of the mTOR pathway, which could be a consequence of the dysregulated pro-BDNF/BDNF ratio. As mTOR is involved in the regulation of the transcription of MMP-9 (Chandrika et al., 2016; Kou et al., 2016; Van Skike et al., 2018), the decrease in mTOR pathway could be a compensatory mechanism to MMP-9 overexpression. Surprisingly, MMP-9 OE males presented high levels of hippocampal PSD95, but no differences in synapsin l levels, in contrast to the decreased expression expected due to the low mTOR pathway activation, and in different animal models of depression (Cohen et al., 2011; Liu et al., 2015; Pazini et al., 2016; Xu et al., 2018; de Almeida et al., 2020; Xu et al., 2020; Yao et al., 2020).

Our findings in the hippocampus of MMP-9 OE male mice agree with studies in cortical *post-mortem* samples of patients diagnosed with major depression, showing a lower mTOR signalling (Jernigan et al., 2011), and no changes in synapsin l expression (Schmidt et al., 2015). Regarding PSD95 levels, Feyissa et al. (2009) reported lower cortical PSD95 levels in patients with MDD, while in the hippocampus these protein levels were not modified (Toro and Deakin, 2005). Previous reports have indicated that the binding of proBDNF to the p75 neurotrophin receptor promotes the expression of apoptosis-related proteins as PSD95 (Yang et al., 2021). Considering that we observe increased PSD95 levels together with the increased ratio pro-BDNF/BDNF in male MMP-9 OE mice, we may suggest that these findings could be associated with apoptosis and not neural plasticity. However, we cannot discard that besides mTOR and proBDNF pathways, other pathways as PLC, PI3K, and MAPK/ERK may be also responsible for PSD95 expression regulation (Yoshii and Constantine-Paton, 2014). Finally, MMP-9 OE females

showed no alterations in mTOR pathway activation, and BDNF, PSD95, and synapsin I levels supporting the importance of the studies including both genders.

Interplay MMP-9, behaviour and neuroplasticity molecular markers

It is well known that several murine models of depression present reduced hippocampal levels of mTOR, PSD95 and synapsin I, which are restored by antidepressant drug treatments, such as SSRIs and ketamine (Liu et al., 2015; Pazini et al., 2016; Xu et al., 2018; de Almeida et al., 2020; Xu et al., 2020; Yao et al., 2020). In fact, in the chronic unpredictable mild stress (Liu et al., 2015) and the chronic social defeat stress models (Xu et al., 2018), fluoxetine and paroxetine treatments normalize the ratio p-mTOR/mTOR, and PSD95 and synapsin I levels in the hippocampus, but not in the prefrontal cortex. These studies support our findings in MMP-9 KO females that display increased levels of mTOR, p-mTOR and synaptic proteins, together with low immobility in the TST.

MMP-9 OE male mice showed conflict-based anxiety in the NSF test, in line with several studies that reported a role of reduced mTOR activation in the behavioural outcomes assessed in this test (Ran et al., 2018; Camargo et al., 2019; Neis et al., 2020; Pazini et al., 2020). Finally, the high ratio of pro-BDNF/BDNF observed in these animals has been described in MDD patients (Zhou et al., 2013), reinforcing the putative role of MMP-9 in depression.

2.3 Neurochemical characterisation: neurotransmitter brain levels

According to the monoaminergic hypothesis that proposes a hypofunctionality of the monoaminergic systems as a cause of MDD, we evaluated the basal tissue levels of serotonin, dopamine and noradrenaline. These neurotransmitters were evaluated in

the midbrain, containing the monoaminergic neuronal somas —dorsal raphe nucleus, substantia nigra and ventral tegmental area—, and in the prefrontal cortex and hippocampus that receive innervations of those neurotransmitter systems. All these areas are implicated in the aetiology of major depression.

In order to provide a correct interpretation of these results, we must consider that here we analyze tissular and not cerebrospinal fluid samples. Tissular samples include the neurotransmitters released into the synaptic cleft plus the ones stored in vesicles, compared to the released neurotransmitters evaluated by *in vivo* microdialysis studies.

In **serotonin**, the lack of functionality of MMP-9 in mice seems to induce opposite effects in males and females, as males presented higher 5-HT levels, while females showed lower levels, in the prefrontal cortex and the hippocampus. The increased 5-HT levels in MMP-9 KO male is in good agreement with a previous study where MMP-9 KO male mice showed high mRNA expression of tryptophan hydroxylase 1, the enzyme involved in the synthesis of 5-HT, in pancreas samples (Christoffersson et al., 2015). The higher 5-HT levels observed in our MMP9 male mice may underlie their higher sociability. In this sense, the genetic silencing of the serotonin transporter in male mice induces increased serotonergic neurotransmission (Ferrés-Coy et al., 2013; 2016), and reverts the depressive-like behaviour in the corticosterone model of depression (Ferrés-Coy et al., 2016). Surprisingly, MMP-9 KO female mice exhibited lower 5-HT levels. A plausible explanation might be related to their BDNF levels, as it has been described a reduction in 5-HT extracellular levels after an intra-hippocampal injection of BDNF (*see review* Deltheil et al., 2008). Interestingly, the intra-hippocampal acute infusion of BDNF is associated with an antidepressant-like effect in behavioural despair and increased anxiety in the open field test (Deltheil et al., 2009), similar to our finding in MMP-9 KO female mice, but not male. We must be cautious, as those studies use acute but not chronic BDNF infusion, while our data have been obtained from constitutive transgenic animals, which may show compensatory changes.

On the other hand, male and female MMP-9 OE displayed similar serotonin levels to their respective wildtype counterparts, which is in concordance with their normal despair behaviour.

Regarding the **dopaminergic** neurotransmission, MMP-9 KO mice showed an unaltered dopaminergic activity in the midbrain, in good agreement with Annese et al. (2015) that did not find differences in the number of dopaminergic neurons in the substantia nigra *pars compacta* in MMP-9 KO male mice. Moreover, in line with our findings in MMP-9 KO mice cortical dopamine levels, no differences have been reported in the functionality of the dopamine transporter (Mizoguchi et al., 2007a), nor in the D_1 and D_2 receptor-mediated G protein activation in the frontal cortex (Mizoguchi et al., 2007b) in MMP-9-deficient mice. All these data suggest that MMP-9 KO animals do not present disrupted dopaminergic neurotransmission in the mesocortical pathway. Moreover, MMP-9 KO male mice did not present differences in the mesolimbic dopaminergic pathway, although female mice presented lower hippocampal dopamine levels. We do not have a plausible explanation for these lower levels, taking into consideration that they present reduced behavioural despair and higher plasticity markers.

MMP-9 OE male and female mice tripled the amount of cortical dopamine compared to their respective wildtype counterparts. An increase in MMP-9 and MMP-2 activity in cortical areas have been also observed following the chronic administration of methamphetamine (Mizoguchi et al., 2007a). The repeated exposure to methamphetamine in rat mesocorticolimbic slices co-cultures also induces an augmentation in dopamine release (Nakagawa et al., 2011). Thus, a plausible explanation of the data obtained in our MMP-9 OE mice could be that increased MMP-9 levels are indirectly modulating dopamine levels.

It is known that the overexpression of MMP-9 induces NMDA trafficking (Michaluk et al., 2009) and a decrease in the ratio of AMPA/NMDA receptors (Kaczmarek, 2016). The activation of NMDA receptors promotes the release of dopamine in the synapse, in

areas such as the prefrontal cortex (Feenstra et al., 1995; Takahata et al., 1998; Grilli et al., 2009). A plausible mechanism by which the increase of dopamine in our MMP-9 OE mice is triggered may be a higher presence of NMDA receptors. To contrast this hypothesis, additional studies are needed.

Furthermore, the high dopamine levels observed in the prefrontal cortex of our MMP-9 OE mice could contribute to the impaired working memory observed in the T-maze. In this regard, early works in rats have reported that either the elevation or the depletion of dopamine in the frontal cortex alters spatial working memory (Bubser and Schmidt, 1990; Murphy et al., 1996; *see review* Puig et al., 2014). In fact, in the prefrontal cortex, dopamine displays an inverted U-shaped influence on working memory abilities (*see review* Arnsten et al., 2009).

Several reports also indicate an increased dopaminergic firing in the prefrontal cortex after acute stress, which induces the activation of the mesocortical system (*see review* Delva and Stanwood, 2021). Chronic treatment with antidepressant drugs (Dazzi et al., 2001a) and acute treatment with anxiolytic drugs (Dazzi et al., 2001b) prevented the cortical dopamine release after foot-shock stress. Studies in healthy humans show that exposure to psychosocial stress also promotes the release of dopamine in the prefrontal cortex (Lataster et al., 2011). However, there are discrepancies in the role of cortical dopamine in anxiety, since anxiogenic and anxiolytic actions have been reported (*see review* Sullivan, 2004). Considering that cortical dopamine levels in our male and female MMP-9 OE mice are elevated and that these animals present an opposite phenotype in anxiety tests, we cannot attribute this behaviour exclusively to dopamine.

The drug-induced decrease of dopamine, in humans, could contribute to the appearance or worsening of depressive symptoms (*see review* Yadid and Friedman, 2008). In contrast, most antidepressant drugs induce an increase in cortical dopamine levels (Lavergne and Jay, 2010). Therefore, we can speculate that the high dopamine

levels observed in our MMP-9 OE mice may underlie the lack of a depressive-like phenotype.

Regarding the **noradrenaline** brain levels, MMP-9 KO male, but not female mice, presented a trend to higher levels in the hippocampus. It has been reported that noradrenergic neurotransmission promotes MMP-9 protein expression (Yang et al., 2006; Maolood et al., 2008; Alaiyed et al., 2020). Thus, although the differences observed in MMP-9 male noradrenaline levels are slight, we could speculate the involvement of compensatory mechanisms trying to increase MMP-9 activity. Instead, the lack of differences in noradrenaline levels in MMP-9 KO females, may be a consequence of sexual dimorphism related to a higher vulnerability of females to develop stress-related psychiatric disorders (Curtis et al., 2006).

The MMP-9 overexpression in male and female mice seems to have no effect on noradrenaline levels. Therefore, the behavioural manifestations studied do not seem to be related to noradrenaline levels.

In spite of all these possible explanations, *in vivo* microdialysis studies would be needed to specifically analyse the neurotransmitter levels released in the synaptic cleft and confirm our hypothesis. In addition, the functionality of the receptors could also display an important role in the neurotransmission of our transgenic mice. Finally, whether the outcomes observed in these transgenic mice are a consequence or a compensatory mechanism regarding the non-functionality or the overexpression of MMP-9 is an issue that remains to be elucidated.

Objective 3. Evaluate the expression of MMP-9 and neuroplasticity markers in *post-mortem* human brain samples from depressed patients

3.1 MMP-9 expression and functionality

Our cohort of patients did not present differences in prefrontal **MMP-9 protein** levels in none of the experimental groups analysed. These results are in agreement with Alaiyed et al. (2020), as they reported no differences in the prefrontal MMP-9 levels between *post-mortem* human samples of patients diagnosed with MDD and healthy controls. However, we did not find differences in MMP-9 levels between samples of MDD patients treated with antidepressants and healthy controls, in contrast to the increased MMP-9 levels reported by these authors (Alaiyed et al., 2020). These discrepancies may be due to (1) different doses/type of antidepressants used, and (2) the different experimental techniques used to analyse the protein levels (Western blot *vs* ELISA).

In addition, several studies report no differences in serum MMP-9 levels between MDD patients (Yoshida et al., 2012; Shibasaki et al., 2016; Shibasaki et al., 2018), or bipolar patients with an ongoing major depressive episode (Shibasaki et al., 2016; Shibasaki et al., 2018), and healthy volunteers. Moreover, Shibasaki et al. (2018) reported that those patients who relapsed did not show a reduction of peripheral MMP-9 after electroconvulsive therapy. Taking into account that we did not find differences in MMP-9 protein levels, we could speculate that this is associated with their resistance to the antidepressant treatment, as 11 out of 14 treated patients committed suicide. However, other studies reported increased MMP-9 levels in the periphery (Domenici et al., 2010; Bobińska et al., 2016a; Bobińska et al., 2016b; Krajčíková et al., 2021), and in *postmortem* brain samples (Alaiyed et al., 2020) of MDD patients treated with antidepressant drugs.

In our study, all the patients diagnosed with MDD, independently of whether they presented or not antidepressant drugs in the toxicological screening, exhibited an increased **MMP-9 activity** in the prefrontal cortex. Our findings are in line with the previous report on another brain area as the CA1 subfield of the hippocampus of MDD patients (Bijata et al., 2022). The increased MMP-9 activity in our cohort is observed in the antidepressant-treated, but not in the antidepressant-free MDD subjects. An increase in pro-MMP-9/TIMP-1 ratio was reported in the PFCx of MDD-treated patients (Alaiyed et al., 2020), which points towards a lower inhibition of MMP-9.

The higher MMP-9 activity in MDD patients treated with antidepressant drugs may seem contradictory to our hypothesis. However, we have observed high MMP-9 activity in CBD non-responder mice (see Objective 1), which could be parallel to the high MMP-9 activity levels observed in our cohort of suicide patients. Also, several reports indicate a positive correlation between MMP-9 levels and the severity of depression in humans (Garvin et al., 2009; Yoshida et al., 2012; Shibasaki et al., 2016; Che et al., 2019; Talarowska et al., 2019). Thus, we further studied the possible association between suicide and MMP-9 activity, as it has been previously described that suicide could be considered indicative of MDD severity and/or resistance to the antidepressant treatment (Bijata et al., 2022). Our findings show that patients that committed suicide presented an over-activated MMP-9 compared to their corresponding control subjects, while patients that died in natural circumstances did not show differences. Increased levels of MMP-9 have been also reported in the cerebrospinal fluid of MDD patients (Ventorp et al., 2016) and serum of bipolar disorder patients (Chandrasekaran et al., 2020), related to suicide attempts. We must highlight that a high percentage of patients that were under antidepressant treatment committed suicide, supporting the association between high MMP-9 activity and resistance to antidepressant drug treatment. Finally, it is important to consider that the increased MMP-9 activity observed in MDD and suicide patients is only evident in women and not in men, pointing to a sex-dependent regulation of this metalloproteinase.

The **MMP-2 activity** was not altered in the prefrontal cortex of MDD cohort independently of the presence or absence of antidepressant drugs in the toxicological screening. This agrees with previous data in the hippocampus of MDD patients who committed suicide (Bijata et al., 2022). In contrast, increased MMP-2 protein levels were described in the cerebrospinal fluid of patients diagnosed with MDD (Omori et al., 2020).

In peripheral samples of MDD patients, contradictory data have been reported. First, no differences in MMP-2 levels (Hüfner et al., 2015); second, an increased MMP-2 activity (Bobińska et al., 2016a); and third, decreased MMP-2 mRNA and protein levels (Domenici et al., 2010; Bobińska et al., 2016b; Bobińska et al., 2016c; Shibasaki et al., 2016; Shibasaki et al., 2018) in peripheral samples. All these pieces of evidence indicate that the role of MMP-2 MDD is still unclear.

Our data point to a role of cortical MMP-9 activity, but not MMP-2, in the severity of symptomatology of MDD specifically in women.

3.2 Expression of neuroplasticity markers in the cortex

No differences were observed in **p-mTOR/mTOR ratio** or total mTOR levels in the PFCx of MDD patients. The fact that total mTOR levels were unaltered is in good agreement with a previous report assessing mTOR pathway in prefrontal areas of MDD patients (Salort et al., 2020). However, Jernigan et al. (2011) found lower mTOR levels and suggested a decreased activation of the mTOR pathway in MDD, in this brain area. On the contrary, Salort et al. (2020) describe an increased p-mTOR/mTOR ratio in the prefrontal cortex of both MDD antidepressant-treated and antidepressant-free patients. Those discrepancies may be related to the different types of antidepressant drugs prescribed which could be playing an impact on mTOR pathway (*see review* Abelaira et al., 2014). In fact, escitalopram and paroxetine, but not fluoxetine, sertraline

and imipramine, increase mTOR activation in hippocampal cell cultures (Park et al., 2014).

This is the first time that the activation of mTOR pathway is studied in human brain samples in a sex-dependent manner. It is important to highlight that in our cohort only the group of MDD men treated with antidepressant drugs present an increased activation of the mTOR pathway in the PFCx. These findings are in line with the higher mTOR activation observed in plasma samples of three men diagnosed with MDD and treated with ketamine (Yang et al., 2013). Moreover, a study using the CUMS model of depression in male mice also reported the reversion of the stress-induced decrease of mTOR pathway activation by fluoxetine (Liu et al., 2015). To the best of our knowledge, there are few data on the antidepressant-dependent mTOR pathway activation in females/women. Thus, we cannot discard that the different antidepressant drugs may have a different impact on mTOR activation depending on the sex.

Regarding **BDNF,** we found lower protein levels in the PFCx of MDD patients in agreement with previous reports (Dwivedi et al., 2003; Karege et al., 2005). Lower BDNF mRNA expression in the PFCx (Dwivedi et al., 2003), BDNF protein levels in other brain areas (Dwivedi et al., 2003; Dunham et al., 2009) and periphery (Yoshida et al., 2012; Qi et al., 2015) have been also described in MDD patients. In contrast, one study reported no differences in cortical BDNF levels in MDD patients (Nunes et al., 2018).

Interestingly, in our cohort, patients with undetectable antidepressant blood levels presented lower BDNF levels, while AD-treated patients showed no differences compared to their matched controls, in agreement with Karege et al. (2005). In addition, Sheldrick et al. (2017) reported a significant increase of cortical BDNF levels in AD-treated patients, without alterations in non-treated patients, and in the same line, Chen et al. (2001) observed a trend to increased hippocampal BDNF levels in AD-treated MDD patients. Several studies also reported that chronic AD treatments upregulated peripheral BDNF expression levels (Gonul et al., 2005; Wolkowitz et al., 2011; Ghosh et

al., 2015), while other studies reported no differences following chronic AD treatment in MDD patients in brain areas such as the prefrontal cortex (Dwivedi et al., 2003) or the hippocampus (Dunham et al., 2009).

To the best of our knowledge, this is the first study analysing the effect of antidepressant treatment on BDNF levels of men and women separately. Herein, no differences are observed in the complete cohort of MDD men. Nevertheless, antidepressant-free, but not antidepressant-treated men presented lower BDNF levels. When considering only women, the MDD group presented lower BDNF levels. However, due to the low number of antidepressant-free women, we cannot discuss if there is a similar or different pattern in BDNF expression compared to men.

Our results should be taken with caution, due to the relatively reduced number of patients, as well as the heterogeneity in the antidepressant drugs, which may lead to different regulation of the proteins assessed. Moreover, the normalization of BDNF in the PFCx, as well as the activation of mTOR pathway in men, seems not to be enough to induce an antidepressant effect, considering that 9 out of 10 of the antidepressant-treated patients committed suicide. In conclusion, our results point to important sex differences in MDD and/or the response to antidepressant treatment, which remains poorly understood and shall be deeply investigated in future studies.

CONCLUSIONS

1. Subchronic cannabidiol administration induces an anxiolytic and antidepressant-like effect in the chronic corticosterone mouse model of depression.

2. MMP-9 is up-regulated in the prefrontal cortex and hippocampus of the chronic corticosterone model.

3. Subchronic cannabidiol administration reverts the increased MMP-9 activity in the corticosterone model, without altering its expression.

4. MMP-9 protein levels correlate with anhedonia severity in the chronic corticosterone model.

5. MMP-9 KO male mice exhibit lower innate anxiety, higher sociability, higher synapsin I levels, and higher cortical and hippocampal serotonin levels.

6. MMP-9 KO female mice exhibit higher innate anxiety, lower behavioural despair, an up-regulation of different neuroplasticity markers, and lower serotonin and dopamine levels in different brain areas.

7. MMP-9 OE male mice exhibit higher conflictive anxiety, lower spatial memory, an up-regulation of PSD95 and pro-BDNF/BDNF ratio, and down-regulation of phospho-mTOR, together with higher dopamine levels in the prefrontal cortex.

8. MMP-9 OE female mice exhibit lower innate anxiety, lower spatial memory, no alterations in synaptic plasticity markers, and higher dopamine levels in the prefrontal cortex.

9. MMP-9 activity in the prefrontal cortex is higher in women diagnosed with major depression treated with antidepressant drugs, and depressed women that committed suicide, whereas it is not altered in depressed men. MMP-9 protein expression is not altered in depressed patients.

10. The mTOR pathway is not altered in the prefrontal cortex of depressed patients. The activation of the mTOR pathway is observed in depressed men treated with antidepressants, but not in women.

11. Depressed patients present lower BDNF levels in the prefrontal cortex.

12. Our findings in animals and humans suggest that alterations in MMP-9 have a sex-dependent impact.

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RESUMEN

INTRODUCCIÓN

El Trastorno Depresivo Mayor (TDM) se caracteriza por el sentimiento de tristeza y culpabilidad, la pérdida de interés y la incapacidad de disfrutar (anhedonia), alteraciones en el sueño y el apetito, baja autoestima, dificultad de concentración y en los casos más graves, pensamientos suicidas. Para ser diagnosticado de TDM el paciente debe padecer al menos 5 síntomas incluidos en el *Diagnostic and Statistical Manual of Mental Disorders* (DSM-V) (American Psychiatric Association, 2013) durante al menos 2 semanas. El TDM es muy frecuente y afecta a más de 250 millones de personas en todo el mundo, especialmente a mujeres, en una proporción de 2:1 (Salk et al., 2017; GBD, 2022).

La etiopatogenia del TDM es todavía desconocida en la que intervienen múltiples factores de riesgo, que incluyen factores biológicos, genéticos y psicosociales. Se han propuesto diferentes hipótesis, no excluyentes entre sí. La primera propuesta fue la hipótesis monoaminérgica, la cual postula que la causa del TDM es un déficit de los sistemas de neurotransmisión aminérgicos como el de serotonina, dopamina y noradrenalina (Schildkraut, 1965). Los hallazgos posteriores han dado pie a la formulación de nuevas hipótesis. La hipótesis glutamatérgica propone que el estrés produciría una sobreactivación del sistema glutamatérgico y su posterior desregulación, conduciendo a alteraciones en estructuras que modulan el comportamiento cognitivo-emocional (*revisado en* Sanacora et al., 2012). Esta hipótesis se postuló al observar el efecto antidepresivo producido por un antagonista del receptor NMDA (Trullas y Skolnick, 1990). La evolución de la hipótesis glutamatérgica dio paso a la hipótesis neurotrófica. La hipótesis neurotrófica defiende que la causa del trastorno tiene relación con una elevada muerte neuronal y una disminución de la plasticidad sináptica en el hipocampo y en la corteza prefrontal (*revisado en* Duman et al., 1997). La exposición crónica al estrés activa el eje hipotalámico-pituitaria-adrenal (HPA), el cual promueve la secreción de glucocorticoides. Se postula que el aumento de

glucocorticoides y glutamato reduciría la expresión de factores tróficos en el sistema límbico, especialmente del factor neurotrófico derivado de cerebro (BDNF), produciendo atrofia neuronal, un acortamiento de las dendritas y la disminución de la densidad de las espinas dendríticas (*revisado en* Duman and Li, 2012; Herman et al., 2016). Por último, la hipótesis neuroinflamatoria propone que un proceso inflamatorio puede ser causante del TDM. Una elevación de los niveles de las citoquinas pro-inflamatorias está asociada con una disminución de serotonina y que la activación de la microglía induce el aumento de los niveles de glutamato (*revisado en* Maes, 1999; Miller et al., 2010; Miller y Raison, 2016).

Los fármacos antidepresivos actualmente utilizados presentan importantes limitaciones, como el elevado tiempo requerido para la aparición de su efecto terapéutico o el alto porcentaje de pacientes resistentes al tratamiento (Commons y Linnros, 2019; Desmidt et al., 2022). Cabe destacar que la mayoría de los fármacos antidepresivos disponibles actúan sobre el sistema de neurotransmisión de las aminas, más concretamente sobre los sistemas serotonérgico y noradrenérgico (Gautam et al., 2017; Shelton, 2019). Teniendo en cuenta las limitaciones que presentan los tratamientos disponibles, la investigación actual se centra en la búsqueda de nuevas dianas/estrategias terapéuticas, mediante las cuales se consiga un efecto antidepresivo rápido y/o disminuir la resistencia al tratamiento.

El cannabidiol (CBD) es el mayor componente no psicomimético del *Cannabis sativa*. En diferentes estudios preclínicos y clínicos se ha observado un efecto ansiolítico y antidepresivo del CBD (*revisado en* Ligresti et al., 2016; Levinsohn and Hill, 2020), asociado a diversas dianas como los receptores 5-HT_{1A}, CB_1, CB_2, $TRPV_1$, GPR55 y PPARγ, además de un aumento en los niveles de anandamida e inhibición de la hidrolasa de amidas de ácidos grasos (FAAH) (*revisado en* Patricio et al., 2020). Estos procesos facilitan la señalización glutamatérgica, serotonérgica y la cascada BDNF-mTOR,

favoreciendo la neurogénesis y la sinaptogénesis hipocampal y cortical (Campos et al., 2013; Linge et al., 2016; Sales et al., 2019).

Por otro lado, las metaloproteinasas de matriz (MMP) son endopeptidasas con un motivo en dedo de zinc conservado en el centro catalítico activo, dependiente de calcio (*revisado en* Cui et al., 2017). Sus funciones principales son cortar o descomponer los componentes de la matriz extracelular y de la membrana basal, las moléculas de adhesión celular, los receptores de superficie celular, los factores de crecimiento, las citoquinas y otras proteasas, además de la regulación de diferentes cascadas de señalización intracelular (*revisado en* Xie et al., 2017).

Una de las metaloproteinasas más estudiadas es la MMP-9, una gelatinasa expresada tanto en el sistema nervioso central como en el periférico. La MMP-9 es liberada al espacio sináptico desde las neuronas glutamatérgicas como consecuencia de la activación de los receptores NMDA (*revisado en* Vafadari et al., 2016). La MMP-9 tiene un importante papel en la plasticidad sináptica, permitiendo el crecimiento y maduración de las espinas dendríticas, catalizando la rotura de moléculas de adhesión celular, como las nectinas, y participando en la acumulación e inmovilización de los receptores AMPA y en el tráfico de los receptores NMDA (Michaluk et al., 2009; Kaczmarek, 2016). La MMP-9 también es responsable de la activación del factor neurotrófico BDNF y las citoquinas inflamatorias, participando así en los procesos de neurogénesis e inflamación, respectivamente (*revisado en* Vafadari et al., 2016). La sobreexpresión de MMP-9 puede provocar una plasticidad aberrante, al formarse espinas inmaduras, más largas y estrechas y con una ratio de receptores AMPA/NMDA baja (Wilczynski et al., 2008). Niveles elevados de MMP-9 también pueden alterar la producción de citoquinas inflamatorias (de Pinho et al., 2014).

En pacientes con TDM se han descrito niveles elevados de MMP-2, MMP-7 y MMP-9 en sangre (Domenici et al., 2010; Bobińska et al., 2016a; Bobińska et al., 2016b). Estos niveles de MMP-9 se correlacionan positivamente con la severidad de la sintomatología

depresiva (Garvin et al., 2009; Yoshida et al., 2012; Shibasaki et al., 2016; Che et al., 2019; Talarowska et al., 2019). Además, se ha descrito una disminución de los niveles de MMP-9 en suero de los pacientes respondedores a la terapia electroconvulsiva (Shibasaki et al., 2018). Por todo ello, la MMP-9 se propone como un potencial biomarcador de la depresión y de la respuesta a fármacos antidepresivos, además de una posible diana terapéutica (*revisado en* Li et al., 2022).

HIPÓTESIS Y OBJETIVOS

En base a las evidencias previas, postulamos que tanto en el modelo de depresión por la administración crónica de corticosterona, como en muestras cerebrales *post-mortem* de pacientes diagnosticados con depresión, existe una alteración en la expresión y/o actividad de la MMP-9. Además, postulamos que el cannabidiol ejercerá su efecto antidepresivo rápido a través de la modulación de esta metaloproteinasa.

Por tanto, el principal objetivo de esta tesis es profundizar en la implicación de la MMP-9 en el trastorno depresivo mayor y como posible diana terapéutica de la enfermedad. Para ello se establecieron los siguientes objetivos específicos:

1. Estudiar el efecto del tratamiento subcrónico con cannabidiol en el modelo de depresión inducido por la administración crónica de corticosterona sobre el comportamiento de tipo depresivo/ansioso, así como sobre la expresión y actividad de MMP-9 en el cerebro.

2. Realizar una caracterización conductual (paradigmas de depresión/ansiedad), molecular (marcadores de plasticidad sináptica) y neuroquímica (niveles de aminas) de los ratones transgénicos MMP-9.

3. Evaluar la expresión y actividad de MMP-9 y marcadores de neuroplasticidad en muestras de cerebro humano *post-mortem* de pacientes con diagnóstico de depresión.

MATERIAL Y MÉTODOS

Animales y tratamientos

En esta tesis se utilizaron ratones macho C57BL/6J, ratones macho y hembra MMP-9 *knockout* (KO) (The Jackson Laboratory, Maine, USA) y que sobreexpresan (OE) la MMP-9 (proporcionado por Dra. A.K. Tzinia), de 2 - 3 meses de edad. Los animales fueron estabulados con un ciclo de luz-oscuridad de 12 h, temperatura de 22 ± 1°C, humedad relativa del 60 - 70%, y con libre acceso a la comida y a la bebida. Todos los experimentos fueron aprobados por el Comité de Bioética de la Universidad de Cantabria y fueron realizados de acuerdo con la Legislación Española (Real decreto 1386/2018) y la directiva Europea 86/609/EEC.

Para el objetivo 3 se usaron muestras de la corteza prefrontal (área de Brodmann 9) de sujetos control y pacientes diagnosticados con depresión, con y sin niveles de antidepresivos en sangre en el momento de la defunción. Ambos grupos fueron macheados atendiendo al sexo, edad e intervalo *post-mortem.* Las muestras fueron proporcionadas por el Banco de Cerebros del Instituto de Salud Carlos III, Universidad del País Vasco (UPV/EHU).

En el modelo animal de corticosterona, se administró corticosterona en el agua de bebida (45 mg/l, equivalente a una dosis de 6-10 mg/kg/día), durante 4 semanas. Se confirmó la aparición del fenotipo de tipo ansioso/depresivo y se procedió a la administración de vehículo o CBD (50 mg/kg/día, i.p.), durante 4 días consecutivos, y se evaluó el comportamiento de tipo ansioso y depresivo. Los animales se sacrificaron

treinta minutos después de finalizar el tratamiento y se diseccionó la corteza prefrontal e hipocampo, conservándolo a -80°C hasta su utilización.

En los animales transgénicos se realizó una caracterización conductual (pruebas de depresión/ansiedad y ámbito general) y posteriormente se procedió a su sacrificio y disección de diferentes áreas cerebrales para su posterior uso en técnicas moleculares. Los ensayos neuroquímicos se realizaron en animales no previamente evaluados conductualmente.

Pruebas conductuales

Se han seguido los protocolos previamente descritos por otros autores con ligeras modificaciones:

- Pruebas relacionadas con la ansiedad: test del campo abierto (OFT) (Garro-Martínez et al., 2021), laberinto elevado en cruz (EPM) (Komada et al., 2008), test de la caja clara-oscura (LDB) (Vidal et al., 2019) y test de la supresión de la ingesta inducida por la novedad (NSF) (Vidal et al., 2019).

- Pruebas relacionadas con la depresión: test de interacción social (SIT) (Planchez et al., 2019), test de ingesta de sacarosa (SPT) (Vidal et al., 2019) y test de la suspensión por la cola (TST) (Florensa-Zanuy et al., 2021).

- Pruebas generales del comportamiento: rota-rod (Luong et al., 2011), test de las canicas (Thomas et al., 2009), test del nido (Deacon, 2006), laberinto en T (T-maze) (Davis et al., 2017) e hipertermia inducida por estrés (SIH) (Garro-Martínez et al., 2020).

Técnicas moleculares

- Western Blot (WB). Se obtuvo la fracción de sinaptoneurosomas de muestras de hipocampo y corteza prefrontal (Li et al. 2010). Se procedió a la separación de proteínas mediante electroforesis SDS-PAGE y se transfirieron a membranas de

nitrocelulosa. Las membranas se incubaron con distintos anticuerpos primarios durante la noche (MMP-9, BDNF, mTOR, fosfo-mTOR, nectina-3, PSD95 y sinapsina I) y posteriormente con anticuerpos secundarios fluorescentes. La señal fue detectada con el sistema de imagen Odyssey® CLx y cuantificada con el software Image Studio™. Como proteína de control interno se usó la β-tubulina III.

- Zimografía en gel (Szklarczyk et al., 2002). Se obtuvo la fracción rica en sinaptoneurosomas de muestras de hipocampo y corteza cerebral. Se procedió a la separación de proteínas mediante electroforesis SDS-PAGE en un gel de poliacrilamida al 8,5% conteniendo 0,1% de gelatina. Los geles se incubaron durante 48 h para inducir la actividad gelatinasa. Finalmente, se procedió a la tinción del gel con Coomassie blue 0,5% y se destiñeron con una solución de metanol 40% y ácido acético 10%, hasta observar las áreas de actividad de la MMP-9 y MMP-2. Finalmente, los geles se escanearon con el sistema de imagen Odyssey® CLx y se cuantificaron las bandas con el software Image Studio™.

Cromatografía líquida de alta resolución (HPLC)

Las muestras de hipocampo, corteza prefrontal y mesencéfalo se homogeneizaron y se filtraron utilizando un filtro de 0,45 µm. Los niveles de serotonina, dopamina y noradrenalina se detectaron mediante el sistema de HPLC ALEXYS® Neurotransmitter Analyzer con detección electroquímica, utilizando una columna Acquity UPLC® BEH C18, 1.7 µm, 1 x 100 mm y una fase móvil compuesta por 100 mM ácido cítrico, 100 mM ácido fosfórico, pH 6, 0,1 mM EDTA, 950 mg/L ácido octanosulfónico y 5% acetonitrilo. Los valores obtenidos se expresaron en pmol/mg de tejido.

Expresión de los resultados y contraste estadístico

Los resultados se expresaron como media ± error estándar de la media (S.E.M.). El análisis estadístico de los resultados se realizó utilizando el software GraphPad Prism 6. Los resultados del **objetivo 1** en el modelo de corticosterona tratado con cannabidiol se analizaron utilizando un ANOVA de dos vías, seguido del test *post-hoc* de Newman-

Keuls. La correlación entre los resultados de los experimentos moleculares y conductuales se realizó mediante el coeficiente de correlación de Pearson (r). En el **objetivo 2** se utilizó la prueba *t-Student* no pareada para comparar los valores conductuales, moleculares y neuroquímicos de los ratones transgénicos, con la excepción de la prueba *t-Student* pareada en el SIH y la prueba de chi-cuadrado para analizar el porcentaje de alternancia en el T-maze. Finalmente, en el **objetivo 3** se utilizó la prueba *t-Student* pareada en los resultados obtenidos en cerebro humano. El nivel de significación se fijó en $p<0,05$.

RESULTADOS Y DISCUSIÓN

Objetivo 1. Efecto del tratamiento subcrónico con cannabidiol en el modelo animal de depresión inducido por la administración crónica de corticosterona

El tratamiento subcrónico con CBD revirtió el comportamiento de tipo ansioso/depresivo inducido por la administración crónica de corticosterona, observado en el NSF, pero no revirtió la anhedonia característica del modelo. Sin embargo, en ambas pruebas conductuales se observaban dos poblaciones, "respondedores" y "no respondedores" al tratamiento con CBD en el modelo de corticosterona. En el grupo de "respondedores", a diferencia de los "no respondedores", se observó una reversión de la anhedonia y de la conducta ansioso/depresiva, como se había descrito previamente en otros modelos animales de depresión (Linge et al., 2016; Florensa-Zanuy et al., 2021). Esta resistencia al efecto terapéutico de los fármacos antidepresivos se había descrito en estudios preclínicos utilizando el modelo de corticosterona (*revisado en* Samuels et al., 2011), y en estudios clínicos (Desmidt et al., 2022; Diep et al., 2022).

En el modelo de corticosterona se observó un incremento en los niveles de MMP-9 en la corteza prefrontal (PFCx) e hipocampo, en línea con hallazgos previos de nuestro laboratorio (Breviario et al., 2023). El tratamiento subcrónico con CBD revirtió el incremento en la actividad de la MMP-9, pero no de su expresión, inducida por el

modelo. Es la primera vez que se describe la modulación de la MMP-9 cerebral por CBD en un modelo animal de depresión. Estos datos están en línea con el aumento de los niveles del inhibidor tisular de metaloproteinasas (TIMP-1) inducido por CBD en células HeLa, lo que conlleva a una disminución de la actividad de la MMP-9, pero no de su expresión (Ramer et al., 2010).

Por último, encontramos una correlación positiva entre los niveles de MMP-9 en la corteza prefrontal y la severidad de la anhedonia, en concordancia con la correlación entre los niveles séricos de MMP-9 y la severidad de la sintomatología depresiva en pacientes descrita por Yoshida et al. (2012).

Objetivo 2. Caracterización de los ratones transgénicos MMP-9

Caracterización conductual

Inicialmente, se realizó una caracterización conductual basal de los ratones transgénicos en ambos sexos.

En cuanto a la *conducta de tipo ansiosa*, los **ratones MMP-9 KO** macho mostraron una menor ansiedad innata (OFT), contrariamente a lo observado en hembras. Nuestros hallazgos en ratones macho corroboran la menor ansiedad asociada a la disminución de los niveles de MMP-9 descrita previamente (Kelestemur et al., 2020). Sin embargo, otros autores no encontraron alteraciones en la ansiedad en ratones MMP-9 KO macho y hembra (Knapska et al., 2013; Sidhu et al., 2014; Ringland et al., 2021). Los **ratones MMP-9 OE** hembra, contrariamente a las hembras MMP-9 KO, muestran menor ansiedad innata en los tres test evaluados (OFT, EPM y LDB), en contraste con lo previamente publicado por Fragkouli et al. (2012) donde no se observaron diferencias en la ansiedad innata evaluada mediante el OFT. En un estudio reciente se ha descrito que basalmente los ratones C57BL/6J hembra presentaban mayores niveles de MMP-9 y menor ansiedad innata que otra cepa (Kennedy-Wood et al., 2021). Por otra parte, se

ha descrito que tanto en el modelo de la administración crónica de corticosterona (Berger et al., 2019; Yohn et al., 2019), como en el inducido por la administración de lipopolisacárido (Florensa-Zanuy, 2021), existen diferencias en la respuesta ansiosa entre machos y hembras, ya que las hembras presentan una mayor resiliencia al desarrollo del modelo. Estas evidencias junto con nuestros resultados sugieren que la actividad de la MMP-9 está relacionada con la ansiedad innata de forma dependiente del sexo.

La ausencia de ansiedad conflictiva observada en los ratones MMP-9 KO está en línea con los resultados descritos en ratas naïve tratadas con un inhibidor no específico de la MMP-9 (Amani et al., 2019). Por otro lado, la sobreexpresión de MMP-9 en ratones macho conlleva a una mayor ansiedad conflictiva, en paralelo con la mayor ansiedad observada en el NSF en modelos animales que presentan elevados niveles cerebrales de MMP-9 (Watanabe et al., 2013; Lorena et al., 2021; Breviario et al., 2023), así como en el Objetivo 1 de esta tesis.

En relación con las *manifestaciones de tipo depresivo*, los machos **MMP-9 KO** presentan mayor sociabilidad que sus respectivos controles, a diferencia de lo previamente descrito por Sidhu et al. (2014). En este sentido, diversos estudios realizados en modelos animales sugieren que la normalización o disminución de los niveles de MMP-9 pueden mejorar la sociabilidad (Bhandari and Kuhad, 2015; 2017). Las hembras MMP-9 KO tienen una menor desesperación conductual (TST), en línea con el efecto antidepresivo observado en diferentes modelos animales tras inhibir la MMP-9 (Gong et al., 2018; Yang et al., 2020; Maras et al., 2022), y a los resultados obtenidos en el Objetivo 1 de esta tesis.

A pesar de que un incremento de la expresión/actividad de la MMP-9 parece ejercer un impacto perjudicial sobre la conducta de tipo depresivo, nuestros **ratones MMP-9 OE** no mostraron alteraciones en dicha conducta. Esto último parece contrastar con los resultados obtenidos en el Objetivo 1, y con algunos estudios que indican que una

elevación de los niveles de MMP-9 conlleva a un comportamiento de tipo depresivo (van der Kooij et al., 2014; Bączyńska et al., 2022; Bijata et al., 2022; Breviario et al., 2023).

Nuestros ratones transgénicos MMP-9 KO y OE, no presentaron alteraciones en la coordinación motora (rota-rod), y en el bienestar o comportamiento de tipo obsesivo-compulsivo (test de las canicas). Sin embargo, los **ratones MMP-9 OE** de ambos sexos mostraron déficits en la memoria espacial evaluada mediante el T-maze. Nuestros hallazgos concuerdan con la correlación negativa que se ha descrito entre los niveles hipocampales de MMP-9 y la memoria espacial (Sherchan et al., 2011; Adeli et al., 2019; Dupré et al., 2020). Además, cabe destacar que la sintomatología depresiva va acompañada de déficits cognitivos tanto en pacientes (Du et al., 2021; Vance and Winther, 2021) como en modelos animales de depresión (Alaiyed et al., 2020).

Los **ratones MMP-9 KO** de ambos sexos no presentan la respuesta hipertérmica que se induce tras la exposición a un estrés. Esto se ha asociado previamente a una menor actividad de la MMP-9 (Taylor et al., 2018), así como a una menor conducta ansiosa (*revisado en* Borsini et al., 2002; Spooren et al., 2002).

Caracterización molecular

El estudio de los marcadores de neuroplasticidad en el hipocampo, revela que los **ratones MMP-9 KO** macho presentan niveles altos de sinapsina l, sin afectar a la expresión de nectina-3, mTOR, p-mTOR, BDNF, pro-BDNF, ratio pro-BDNF/BDNF o PSD95. No tenemos una clara explicación de por qué la eliminación de MMP-9 provoca una elevación de los niveles de sinapsina l en ratones macho, teniendo en cuenta que no se observa una activación de la cascada de mTOR. Sin embargo, se ha descrito que un incremento en los niveles de serotonina, como el observado en la corteza prefrontal y en el hipocampo de nuestros ratones MMP-9 KO, pueden inducir la expresión de sinapsina l (Hart et al., 2011). Teniendo en cuenta que la MMP-9 es la única enzima

conocida que degrada nectina-3 en cerebro, resulta sorprendente que los niveles de esta proteína no estuviesen alterados. Por todo ello, debemos considerar la posibilidad de que otras proteasas compensen la falta de MMP-9 en los MMP-9 KO macho.

En las hembras MMP-9 KO observamos un incremento en los niveles de nectina-3, y de diferentes marcadores de neuroplasticidad (mTOR, p-mTOR, BDNF, PSD95 y sinapsina I). Varias evidencias apuntan hacia una correlación inversa entre los niveles de expresión de BDNF y MMP-9 (Shibasaki et al., 2016; Vanicek et al., 2019). El incremento de los niveles de BDNF induce la activación de la vía de mTOR, con el consiguiente aumento en la transcripción de marcadores de neuroplasticidad como el PSD95, sinapsina I y BDNF, de forma similar al observado con algunos fármacos antidepresivos (Li et al., 2010; Liu et al., 2015; Xu et al., 2018). Por tanto, esta regulación al alza podría estar relacionada con la menor desesperación conductual observada en las hembras MMP-9 KO. Además, se ha descrito que niveles altos de BDNF en el hipocampo pueden inducir un comportamiento de tipo ansioso (Deltheil et al., 2009; Casarotto et al., 2012), como observamos en las hembras MMP-9 KO.

En referencia a los **ratones MMP-9 OE**, las hembras no muestran ninguna alteración en la expresión de marcadores de plasticidad. Sin embargo, los machos, a pesar de mostrar un aumento en la ratio pro-BDNF/BDNF y una menor activación de la vía de mTOR, presentan mayores niveles de PSD95 en el hipocampo. La elevación de los niveles de la nectina-3 observada en estos ratones transgénicos, podría deberse a la existencia de mecanismos compensatorios que induzcan la síntesis de esta proteína, debido al carácter constitutivo de los animales. En pacientes con TDM se ha descrito un aumento de la ratio pro-BDNF/BDNF (Zhou et al., 2013), así como la menor activación de la cascada de mTOR (Jernigan et al., 2011). Los niveles altos de PSD95 en ratones macho, además de estar relacionados con la plasticidad neuronal, podrían asociarse con procesos apoptóticos, ya que la activación de receptores p75, por la unión de pro-BDNF, induce la expresión de PSD95 como proteína asociada a la apoptosis (Yang et al., 2021).

Caracterización neuroquímica

En ratones machos y hembras **MMP-9 KO** se observó un aumento y disminución, respectivamente, de los niveles de serotonina cerebral. Los niveles altos de serotonina en la corteza prefrontal e hipocampo de los ratones macho podrían asociarse a una mayor expresión de la enzima encargada de su síntesis (Christoffersson et al., 2015). Esta elevación de los niveles de serotonina podría estar asociada a la mayor sociabilidad observada en los MMP-9 KO macho, de la misma forma que la facilitación de la neurotransmisión serotonérgica revierte la conducta de tipo depresiva en el modelo de corticosterona (Ferrés-Coy et al., 2016). Los ratones MMP-9 KO hembra presentan menores niveles de serotonina en la corteza prefrontal e hipocampo. Una posible explicación podría estar asociada con los niveles altos de BDNF que presentan estas hembras. En este sentido, se ha descrito que la infusión intra-hipocampal de BDNF provoca una reducción de los niveles extracelulares de serotonina (*revisado en* Deltheil et al., 2008), además de un efecto antidepresivo y ansiogénico (Deltheil et al., 2009), similar al fenotipo observado en nuestras hembras MMP-9 KO.

Los ratones MMP-9 KO macho no mostraron alteraciones en los niveles de dopamina, de acuerdo con un estudio previo de Annese et al. (2015). Sin embargo, las hembras MMP-9 KO presentaron niveles bajos de dopamina en el hipocampo. No tenemos una explicación para este hallazgo, ya que este resultado no está en línea con la menor desesperanza conductual y con el aumento de los marcadores de plasticidad que presentan las hembras MMP-9 KO.

Finalmente, los **ratones MMP-9 OE** mostraron un notable incremento en los niveles de dopamina en la corteza prefrontal en ambos sexos, sin cambios en serotonina y noradrenalina. Una posible explicación de los niveles elevados de dopamina podría estar asociado con la sobreexpresión de MMP-9, que estaría modulando dichos niveles. En este sentido, se ha descrito que la sobreexpresión de MMP-9 induce el *trafficking* de los receptores NMDA (Michaluk et al., 2009) y una disminución en la ratio de los

receptores AMPA/NMDA (Kaczmarek, 2016). Adicionalmente, se ha descrito que la activación de los receptores NMDA es capaz de promover la liberación de dopamina en la corteza prefrontal (Feenstra et al., 1995; Takahata et al., 1998; Grilli et al., 2009). Este incremento de dopamina observado en nuestros ratones MMP-9 OE podría ser debido a una mayor presencia de receptores NMDA en estos animales. Sin embargo, serían necesarios estudios adicionales para contrastar esta hipótesis.

Esta elevación de los niveles de dopamina podría contribuir en parte al déficit en la memoria de trabajo observado en el T-maze que presentan los ratones MMP-9 OE (*revisado en* Arnsten et al., 2009; Puig et al., 2014). Diferentes autores han descrito que tanto la elevación como la depleción de dopamina cortical, pueden estar asociadas a déficits en la memoria de trabajo (Bubser y Schmidt, 1990; Murphy et al., 1996; *see review* Puig et al., 2014). De hecho, la dopamina cortical parece ejercer un efecto en forma de U invertida sobre la memoria de trabajo (*see review* Arnsten et al., 2009).

En personas, una disminución de los niveles de dopamina inducida por ciertos fármacos puede conllevar a la aparición o empeoramiento de ciertos síntomas depresivos (*see review* Yadid y Friedman, 2008). Por el contrario, algunos fármacos antidepresivos incrementan los niveles corticales de dopamina (Lavergne y Jay, 2010). De este modo, la elevación de los niveles de dopamina cortical podría impedir en parte la aparición de un comportamiento de tipo depresivo en estos ratones MMP-9 OE.

Objetivo 3. Evaluación de la expresión de MMP-9 y marcadores de neuroplasticidad en muestras cerebrales *post-mortem* de pacientes con diagnóstico de depresión

Este estudio se realizó en muestras de corteza prefrontal en una cohorte de pacientes diagnosticados con depresión y sus respectivos controles. Posteriormente, se analizaron los resultados obtenidos atendiendo al género, tratamiento y suicidio en pacientes deprimidos.

En los pacientes con depresión sin y con tratamiento antidepresivo, no se observaron diferencias en los niveles de expresión de la proteína **MMP-9** en comparación con sus respectivos controles. Estos resultados concuerdan, en parte, con un estudio previo que describe ausencia de cambios de los niveles de MMP-9 en los pacientes no tratados, sin embargo, estos autores encontraron un incremento de la expresión de la proteína MMP-9 en los pacientes tratados con antidepresivos (Alaiyed et al., 2020). Esta discrepancia puede deberse a diferentes dosis y tratamientos, además del uso de diferentes técnicas para evaluar los niveles de MMP-9. Por otro lado, la falta de modulación de MMP-9 en nuestra cohorte de pacientes tratados podría estar vinculada a su resistencia al tratamiento (Shibasaki et al., 2018).

Nuestra cohorte de pacientes con depresión muestra un incremento en la actividad MMP-9 en la corteza prefrontal, en línea con la mayor actividad de MMP-9 descrita en el hipocampo de pacientes con TDM (Bijata et al., 2022). Se observó un aumento de la actividad MMP-9 en los pacientes con niveles de antidepresivos en sangre, y no en aquellos sin niveles de antidepresivos. Numerosos estudios describieron una correlación positiva entre los niveles de MMP-9 y la severidad de la depresión en humano (Shibasaki et al., 2016; Che et al., 2019; Talarowska et al., 2019). Además, en el objetivo 1 de esta tesis, observamos mayores niveles de actividad de la MMP-9 en aquellos animales no respondedores al tratamiento con CBD.

Evaluamos la posible asociación entre la actividad de la MMP-9 y el suicidio. La cohorte de pacientes que cometieron suicidio presenta una mayor actividad de la MMP-9, mientras que en los pacientes que fallecieron por causas naturales no se observa dicho incremento. Además, el aumento de la actividad de la MMP-9 es evidente únicamente en mujeres, apuntando a una regulación sexo-dependiente de esta metaloproteinasa.

En relación a la vía de **mTOR**, no se observa una alteración de los niveles de fosfo-mTOR/mTOR corticales en pacientes deprimidos. Cabe destacar que existe cierta controversia sobre la relación mTOR y depresión. En este sentido se ha descrito tanto

una disminución (Jernigan et al., 2011), como la ausencia de cambios (Salort et al., 2020) en los niveles de mTOR y la activación de esta vía en el cerebro de pacientes con depresión. El análisis de la influencia del género muestra una mayor activación de la vía de mTOR en la cohorte de hombres deprimidos con niveles de antidepresivos en sangre. Estos hallazgos van en paralelo a lo descrito previamente en el plasma de hombres deprimidos tratados con ketamina (Yang et al., 2013).

Nuestra cohorte de pacientes deprimidos presenta menores niveles corticales de **BDNF**. Al analizar la influencia del tratamiento, observamos una disminución solo en el grupo de pacientes libres de antidepresivos, lo que concuerda con la mayoría de los estudios publicados hasta la fecha (Dwivedi et al., 2003; Karege et al., 2005; Dunham et al., 2009). Por último, los niveles de BDNF no presentan diferencias sexo-dependientes.

CONCLUSIONES

1. El tratamiento subcrónico con cannabidiol induce un efecto de tipo ansiolítico y antidepresivo en el modelo de depresión inducido por la administración crónica de corticosterona en ratones.

2. La MMP-9 está regulada al alza en la corteza prefrontal y en el hipocampo en el modelo de corticosterona.

3. El tratamiento subcrónico con cannabidiol revierte el incremento de la actividad de MMP-9 en el modelo de corticosterona, sin alterar su expresión.

4. Los niveles de la proteína MMP-9 se asocian a la severidad de la anhedonia en el modelo de corticosterona.

5. Los ratones MMP-9 KO macho muestran menor ansiedad innata, mayor sociabilidad, mayores niveles de sinapsina I y elevados niveles de serotonina en la corteza prefrontal y en el hipocampo.

6. Los ratones MMP-9 KO hembra muestran mayor ansiedad innata, menor desesperación conductual, una regulación al alza de los diferentes marcadores de neuroplasticidad, y menores niveles de serotonina y dopamina en diferentes áreas cerebrales.

7. Los ratones MMP-9 OE macho muestran mayor ansiedad conflictiva, menor memoria espacial, una regulación al alza de PSD95 y de la ratio pro-BDNF/BDNF y una regulación a la baja de fosfo-mTOR, junto con mayores niveles de dopamina en la corteza prefrontal.

8. Los ratones MMP-9 OE hembra muestran menor ansiedad innata, menor memoria espacial, mayores niveles de dopamina en la corteza prefrontal y no presentan alteraciones en los marcadores de plasticidad sináptica.

9. La actividad de la MMP-9 en la corteza prefrontal es mayor en mujeres con TDM tratadas con fármacos antidepresivos y en mujeres con TDM que cometieron suicidio, mientras que no está alterada en hombres con TDM. La expresión proteica de MMP-9 no está alterada en pacientes con TDM.

10. La activación de la vía de mTOR se observa solo en hombres con TDM tratados con antidepresivos, pero no en mujeres.

11. Los pacientes con TDM presentan menores niveles de BDNF en la corteza prefrontal.

12. Nuestros resultados en animales y humanos sugieren que las alteraciones en MMP-9 tienen un impacto sexo-dependiente.

ANNEXES

ANNEXE 1: Objective 2.

Supplementary tables

Objective 2. Supplementary tables

Tables S1-3 show a summary of the behavioural, molecular, and neurochemical results in MMP-9 knockout (KO) and MMP-9 overexpression (OE) transgenic mice obtained in objective 2.

Table S1. Behavioural characterisation of MMP-9 knockout (KO) and MMP-9 overexpression (OE) mice. Results obtained compared *vs* the WT group. EPM: elevated plus maze, LDB: light-dark box, NSF: novelty suppressed feeding, OFT: open field test, SI: social interaction, SIH: stress-induced hyperthermia, SPT: sucrose preference test, TST: tail suspension test.

	KO ♂	***KO*** ♀	***OE*** ♂	***OE*** ♀
SIH	NO	NO	YES	YES
ROTA-ROD	=	=	=	=
MARBLE BURYING	=	=	=	=
NESTING	=	=	=	=
T-MAZE (Alternation)	=	=	↓	↓
OFT (Central time)	↑	↓	=	↑ (tendency)
EPM	=	=	=	↑
LDB	=	=	=	↑
NSF (Latency)	=	=	↑	=
SI	↑	=	=	=
SPT	=	↓	↑	=
TST	=	↓	=	=

Table S2. Hippocampal neuroplasticity markers of MMP-9 knockout (KO) and MMP-9 overexpression (OE) mice. Results obtained compared *vs* the WT group.

	KO ♂	***KO*** ♀	***OE*** ♂	***OE*** ♀
Nectin-3	=	↑	=	=
BDNF	=	↑	=	=
pro-BDNF	=	=	=	=
ratio pro-BDNF/BDNF	=	=	↑	=
mTOR	=	↑	=	=
p-mTOR	=	↑	↓ (tendency)	=
PSD95	=	↑	↑	=
Synapsin I	↑	↑	=	=

Table S3. Monoamine levels in PFCx, Hp and midbrain of MMP-9 knockout (KO) and MMP-9 overexpression (OE) mice. Results obtained compared *vs* the WT group.

		KO ♂	***KO*** ♀	***OE*** ♂	***OE*** ♀
5-HT	***PFCx***	↑	↓	=	=
	Hp	↑	↓	=	=
	midbrain	=	=	=	=
DA	***PFCx***	=	=	↑	↑
	Hp	=	↓	=	=
	midbrain	=	=	=	=
NA	***PFCx***	=	=	=	=
	Hp	↑ (tendency)	=	=	=
	midbrain	=	=	=	=

ANNEXE 2: Publication

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Brain matrix metalloproteinase-9 activity is altered in the corticosterone mouse model of depression

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ABSTRACT

Major depressive disorder is a highly prevalent psychiatric condition. Metalloproteinase 9 (MMP-9), a gelatinase involved in synaptic plasticity, learning and memory processes, is elevated in both chronic stress animal models and human peripheral blood samples of depressed patients. In this study we have evaluated the MMP-9 activity and protein expression in brain areas relevant to depression using the chronic corticosterone mouse model of depression. These mice show a depressive- and anxious-like behaviour. The MMP-9 activity and protein levels are significantly elevated in both the hippocampus and the cortex, and nectin-3 levels are lower in these brain areas in this model. In particular, these mice display an increased gelatinase activity in the CA1 and CA3 subfields of the hippocampus and in the internal layer of the prefrontal cortex. Moreover, the immobility time in the tail suspension test presents a positive correlation with the cortical MMP-9 activity, and a negative correlation with nectin-3 levels.

In conclusion, the chronic corticosterone model of depression leads to an increase in the protein expression and activity of MMP-9 and a reduction of its substrate nectin-3 in relevant areas implicated in this disease. The MMP-9 activity correlates with behavioural despair in this model of depression. All these findings support the role of MMP-9 in the pathophysiology of depression, and as a putative target to develop novel antidepressant drugs.

1. Introduction

Major depressive disorder (MDD) is a widespread psychiatric condition affecting >250 million people worldwide, and the incidence of this disease has increased by 14% in the last 10 years (COVID-19 Mental Disorders Collaborators, 2021). It is also associated with an increased risk of premature death, including a high rate of suicide, and a high economic and social cost.

The etiopathogenesis of MDD is not fully known and different neurobiological hypotheses have been proposed to date. Initially, a monoamine hypothesis was postulated, which proposes the existence of a dysfunctional brain monoaminergic system in depressed patients (Schildkraut, 1965). Then, a neurotrophic hypothesis was proposed, that associates depression with a decrease in neurotrophic factors and synaptic plasticity in limbic areas, and a reduction in cell proliferation in the hippocampus (Duman et al., 1997; Duman and Monteggia, 2006). In the last years, several studies demonstrated the correlation between inflammation and neuropsychiatric diseases, including depression (Bower et al., 2002; Meyers et al., 2005), which resulted in the development of the neuroinflammatory hypothesis of depression. Moreover, the increase in inflammatory markers is associated with a decrease in synaptic plasticity markers, such as the brain derived neurotrophic factor (BDNF) (Barrientos et al., 2003; Wu et al., 2007; Ben Menachem-Zidon et al., 2008; Koo and Duman, 2008), and with a reduction in hippocampal proliferation (Zonis et al., 2015).

Metalloproteinases (MMPs) are a group of enzymes sharing a

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conserved zinc-binding motif in their catalytic active site. They are responsible for the cleavage of the extracellular matrix proteins, cell surface receptors and cell adhesion molecules, such as nectins. Interestingly, in the last few years, they have gained importance since they act not only as regulators of extracellular signalling networks but also of intracellular ones (Xie et al., 2017). Among them, the MMP-9 is a gelatinase expressed in both the peripheral and central nervous systems (CNS), playing a pivotal role in the regulation of synaptic plasticity. MMP-9 is translated locally, and it is released from glutamatergic excitatory synapses in response to neuronal activity mediated by NMDA receptors (Vafadari et al., 2016). In the CNS, extrasynaptic MMP-9 is implicated in processes such as the growth and maturation of dendritic spines, and the accumulation and immobilization of AMPA receptors, which make the excitatory synapses more efficient in modulating the AMPA/NMDA receptors ratio (Vafadari et al., 2016). In this sense, overexpression of MMP-9 in rats promotes a higher proportion of silent synapses, lower AMPA/NMDA receptor ratio, and impaired long-term potentiation (LTP) (Nagy et al., 2006). On the contrary, the administration of MMP-9 inhibitors restores these changes (Vafadari et al., 2016). These MMPs also participate in the modulation of inflammatory processes, as well as in neurogenesis, axonal growth and regeneration, and the formation of myelin (Reinhard et al., 2015). All these biological functions justify the involvement of MMP-9 in the etiopathogenesis of certain neurodegenerative disorders such as epilepsy (Michaluk and Kaczmarek, 2007), and multiple sclerosis among others (Milward et al., 2008).

The involvement of these MMPs has been extended to the field of neuropsychiatric diseases, such as depression. Few human studies performed in peripheral blood samples report an increase in the metalloproteinases MMP-2, MMP-7, and MMP-9 (Domenici et al., 2010; Rybakowski et al., 2013; Bobińska et al., 2016), and a reduction in the tissue inhibitors of metalloproteinases (TIMPs) (Bobińska et al., 2016) in major depressive patients. The MMP-9 serum levels showed a positive correlation with the severity of depression (Yoshida et al., 2012; Shibasaki et al., 2016). In addition, electroconvulsive therapy reduces serum levels of MMP-9 in depressed patients in responders, but not in the group of patients that relapse (Shibasaki et al., 2018). However, these results must be taken with caution, since findings in peripheral samples do not always correlate with those observed in the CNS. In fact, only one study using *post-mortem* brain samples confirmed the existence of higher hippocampal MMP-9 enzymatic activity in MDD patients who committed suicide (Bijata et al., 2022). High MMP-9 activity is also associated with other diseases such as epilepsy (Konopka et al., 2013) and ovarian cancer (Lutgendorf et al., 2008) that can present comorbidity with major depression. All these findings highlight MMP-9 as a possible diagnostic marker of depression (Jönsson et al., 2014), that correlates with treatment response and disease progression.

Regarding the brain MMP levels in animal models of depression, there are only two studies reporting an increased MMP-9 activity in a chronic stress model (van der Kooij et al., 2014), and chronic unpredictable stress model (CUS) in mice (Bijata et al., 2022). Moreover, there is also one article reporting the modulation of MMP-9 expression and activity after the electroconvulsive therapy in the hippocampus *versus* the lack of effect of chronic administration of three clinically used antidepressants in naïve rats (Benekareddy et al., 2008).

A deeper understanding of the role of MMP-9 in the neurobiology of depression would help to detect novel therapeutic targets in order to obtain faster and more efficacious antidepressant drugs. Therefore, in this study, we have evaluated the protein expression and activity of the metalloproteinase MMP-9 in hippocampus and cortex using the corticosterone mouse model of depression (CORT), an experimental paradigm endowed with construct, face and predictive validity (Gourley and Taylor, 2009). The hippocampus and cortex are the main areas implicated in the regulation of mood and cognition. Several studies indicate that these areas show decreased neuronal synapses (Duman and Aghajanian, 2012), and a reduction in volume and neural plasticity in patients diagnosed with MDD and chronic stress in the hippocampus and prefrontal cortex (Belleau et al., 2019), and other cortical areas as the temporal lobe (Papmeyer et al., 2015).

2. Methods

2.1. Animals

C57BL/6J male mice, 2–3 months old, were group-housed (4–5 mice per cage) with a 12 h light-dark cycle, and with food and water *ad libitum*. All procedures were carried out with the previous approval of the Animal Care Committee of the University of Cantabria and according to the Spanish legislation (RD 53/2013) and the European Communities Council Directive on "Protection of Animals Used in Experimental and Other Scientific Purposes" (2010/63/UE).

2.2. Corticosterone model

C57BL/6J mice were randomized in two groups and treated chronically with corticosterone hemisuccinate (4-Pregnen-11β, 21-diol-3, 20-dione 21-hemisuccunate) in the drinking water for 4 weeks (45 mg/l, equivalent to a dose of 6–10 mg/kg/day per animal). The corticosterone solution was placed in opaque bottles to avoid degradation and it was changed every 4 days (Gourley and Taylor, 2009; Amigo et al., 2021) and the vehicle group received water. Then, mice were behaviourally assessed in anxiety- and depression-related paradigms (Fig. 1). Following behavioural evaluation, animals were randomized in two sets that were used for: a) gel zymography and western blot experiments; and b) *in situ* zymography experiments.

2.3. Behavioural tests

Mice were subjected to a battery of behavioural tests performed during the light phase and were transported to the experimental room 1 h before each experiment to let them acclimatize. 24 h after the sucrose preference test, the animals were sacrificed.

2.3.1. Open field test (OF)

Mice were placed in one of the corners of the arena (50 × 50 × 30 cm) illuminated with 350 lx, and video-tracked by a computerized system (Any-maze Video-Tracking software, Stoelting Co., USA) for 5 min. The parameters analysed were the total ambulatory distance and the time spent in the centre of the box *versus* total time.

2.3.2. Tail suspension test (TST)

Mice were suspended by the tail using adhesive tape placed 1 cm from the tip of the tail, and at a distance of 20–25 cm from the floor (as previously described in Garro-Martínez et al., 2021). The animals' behaviour was recorded during a 5 min session. The time spent immobile was scored by an observer blind to the experimental group.

2.3.3. Novelty suppressed feeding test (NSF)

Mice were food-deprived for 24 h before performing the test. Animals were placed in one corner of an open field arena illuminated in the centre (40 lx), with the floor covered with clean wood chip bedding, and a food pellet placed in the centre. The latency time to eat the pellet was recorded for a maximal time of 10 min, using a video tracked software

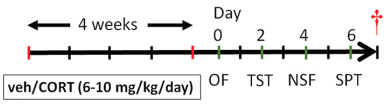

Fig. 1. Experimental time schedule: veh: vehicle; CORT; corticosterone; OF: Open Field; TST: Tail Suspension Test; NSF: Novelty Suppressed Feeding; SPT: Sucrose Preference Test. †: sacrifice.

(Any-maze Video-Tracking software, Stoelting Co., USA). After the test, mice were transferred to their home cages and a post-test of 5 min was done to check the amount of food consumed. The animals that showed no food intake in their home cages were excluded from the data analysis.

2.3.4. Sucrose preference test (SPT)

Mice were single-housed and habituated for 48 h to sucrose consumption putting two bottles in their cage, one containing sucrose in water (1% *w/v*) and one containing tap water. During the habituation period, the position of the bottles was exchanged every 12 h to avoid possible place preferences. The day after, the sucrose preference was evaluated as the percentage of sucrose solution intake *vs* total intake (sucrose+water) during a 24 h period.

2.4. Gel zymography

Mice were sacrificed by cervical dislocation and the brains were rapidly removed and dissected on an ice-cold platform to obtain cortex and hippocampus, and kept at −80 °C until used for western blot or gel zymography experiments.

2.4.1. Zymography's protein extraction

Protein extraction was performed following the protocol of Szklarczyk (Szklarczyk et al., 2002). Briefly, the tissues were homogenized (1:20 w/v) in sample buffer (10 mM $CaCl_2$; 0.25% Triton X-100 in water) and then centrifuged at 6000 x*g* for 30 min at 4 °C. The supernatant was removed, and the pellet was resuspended in 100 μl of pellet buffer (50 mM Tris pH 7.4; 0.1 M $CaCl_2$), heated for 15 min at 60 °C, and then centrifuged at 10000 x*g* for 30 min at 4 °C. The pellet was discarded, and 4 μl of pellet buffer, containing 10% triton X114 was added to the supernatant in order to increase the resolution of the digested band.

2.4.2. Gel zymography

The protein was quantified by the Lowry method (Lowry et al., 1951) and the samples were prepared using Laemmly buffer without β-mercaptoethanol. 75 μg of protein were loaded per duplicate on an 8.5% SDS-PAGE gel containing 0.1% gelatine. As a positive control, 10% fetal bovine serum (FBS) was prepared in extraction buffer [2% Triton X114; 10 mM Tris- HCl, pH 7.4; 150 mM NaCl; 1 μM protease inhibitors aprotinin and phenylmethylsulfonyl fluoride (PMSF)]. The gel was run at 100 V for 15 min, then 160 V for 50 min in running buffer [25 mM Tris-HCl, pH 7.4; 20 mM glycine; 0.1% sodium dodecyl sulfate, (SDS)] at 4 °C. After the electrophoresis, the gel was washed twice in washing buffer (2.5% Triton X100) at room temperature with gentle agitation for 15 min. Then the washing buffer was discarded, and the gel was incubated for 30 min at room temperature in incubation buffer (50 mM Tris-HCl, pH 7.4; 200 mM NaCl; 6.7 mM $CaCl_2$; 1 μM $ZnCl_2$; 0.2% Brij35). Later, the gel was incubated with fresh incubation buffer at 37 °C for 48 h. Following this incubation, the gel was washed 3 times with water for 5 min each at room temperature. Before staining, the gel was scanned to record the exact position of the protein standard bands. The gel was stained with Coomassie Blue R-250 for 1 h at room temperature and then was destained (10% methanol; 5% acetic acid) at room temperature until bands of proteolytic activity were clearly visible. Finally, the gel was scanned using a SnapScan 1236 AGFA scanner and the AGFA FotoLook software. The bands were quantified with the software ImageJ (NIH, USA).

2.5. In situ zymography

Brain slices (14 μm thick) were obtained with a cryostat and were kept at −80 °C until used. The *in situ* zymography was performed following previously described protocols (George and Johnson, 2010). The brain slices were dried for 1 h at room temperature and then were rehydrated in phosphate buffered saline (PBS) for 5 min. The slides were incubated with 20 μg/ml DQ™ Gelatin fluorescein-conjugated (Molecular Probes, Inc., Eugene, OR, USA), in MMP activity buffer (100 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 20 μM $ZnCl_2$, 0.2 mM sodium azide, and 0.05% Brij35, in MilliQ water), in a dark and humid chamber, at 37 °C for 18 h. As a negative control, 50 mM EDTA was added to the gelatine solution. After overnight incubation, the slides were washed with PBS 1× three times for 5 min each, with gentle agitation. Then the slides were fixed with 4% paraformaldehyde in PBS for 5 min and washed 3 × 5 min with PBS. The slides were mounted using Vectashield® (Vector Laboratories, USA). The fluorescent signal was detected using a Zeiss Axio Imager M1 fluorescence microscope, 12 bits B&W camera (AxioCam MRm). The images were analysed using the software ImageJ (NIH, USA).

2.6. Western blot

2.6.1. Synaptoneurosomal protein extraction

The protein extraction was performed following the protocol of Van der Kooij (Van der Kooij et al., 2014). Briefly, the tissues were homogenized (1:15 *w/v*) in homogenization buffer (10 mM HEPES pH 7.4; 1 mM EDTA; 2 mM EGTA; 0.5 mM DTT; 0.1 mM PMSF), containing a protease inhibitor cocktail (Sigma). The homogenate was filtered with a 40 μm filter and centrifuged at 1000 x*g* for 10 min at 4 °C. The pellet was resuspended in 100 μl of 1% SDS buffer (SDS in homogenization buffer plus inhibitors) and boiled for 1 min. The protein concentration was quantified using the Lowry method. The samples were prepared with a loading buffer containing β-mercaptoethanol, boiled at 100 °C for 5 min, and put on ice for 3 min. Then the aliquots were centrifuged at 956 x*g* for 5 min at 4 °C and the supernatant was stored at −20 °C until used.

2.6.2. Western blot

35 μg of protein per sample were loaded per duplicate on an 8,5% SDS-PAGE gel. The electrophoresis was run at 100 V for 15 min and then at 160 V for 50 min, and then transferred to a nitrocellulose membrane (GE Healthcare Europe GmbH, Munich, Germany). The membrane was blocked with 5% (*w/v*) nonfat dry milk in TBST for 1 h. Afterward, the membrane was incubated overnight at 4 °C with rabbit anti-MMP9 (1:3000) (RayBiotech, RayBiotech Life, Georgia, GA, USA) or rabbit anti-nectin-3 (1:10000) (MBL, MBL International, Woburn, MA, USA) primary antibodies in blocking solution. The day after, the membrane was washed with TBST and then incubated in fluorophore-conjugated IRDye 800CW Donkey anti-rabbit secondary antibody (LI-COR Biosciences, Lincoln, NE, USA) (1:15000 in milk 5%) for 1 h at room temperature. After washing, the specific signal was visualized using an Odyssey CLx Imaging System (LI-COR Bioscience, Lincoln, USA). The densitometric values were normalized using mouse anti-tubulin (1:20000) as housekeeping. The images were analysed with the use of Image StudioTM Lite software (LICOR Bioscience, Lincoln, USA).

2.7. Data analysis

Values are expressed as mean ± standard deviation (SD). Data normality was checked using Kolmogorov-Smirnov test for normality. When normality was confirmed, data were analysed using a two-tailed Student's *t*-test, unpaired data. For the correlation studies, the Pearson's correlation coefficient (r) was used. The statistical analyses were performed using the GraphPad Prism 6 version 8.4.3 for Windows (GraphPad Software, Inc., La Jolla, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Depressive- and anxious-like behaviour in the corticosterone model

The corticosterone-treated mice exhibited a higher immobility time compared to the control group in the TST (174.7 ± 19.6 s in the

corticosterone *vs* 148.6 ± 21.0 s in vehicle treated animals, $p < 0.05$; Fig. 2A) and showed a significant decrease in the sucrose preference compared to their control group (74.4 ± 12.5% in corticosterone *vs* 89.1 ± 2.6% in the vehicle treated animals, $p < 0.01$; Fig. 2B).

Regarding the anxious-like behaviour, the corticosterone-treated mice presented an increased latency time of feeding compared to the control group in the NSF (441.9 ± 111.2 s in corticosterone *vs* 334.2 ± 83.7 s in vehicle treated animals, $p < 0.05$; Fig. 2C). No differences were observed in the food eaten in their home-cage (Fig. 2D), in the freezing time and in the ambulation, as evidenced by the mean speed of the animals during the test (Fig. S1). Mice treated with corticosterone also presented a reduction in the time spent in the centre of the OF compared to the control group (4.4 ± 1.9 s in the corticosterone *vs* 8.3 ± 3.1 s in the vehicle treated animals, $p < 0.01$; Fig. 2E). The total distance travelled in the corticosterone treated mice was also lower compared to the vehicle treated mice (14.1 ± 2.1 m in corticosterone *vs* 17.1 ± 2.1 m in vehicle treated animals, $p < 0.01$; Fig. 2F).

No correlation was observed between the immobility time in the TST, and the distance travelled in the OF (Fig. S2).

3.2. MMP-9 and nectin-3 protein expression in cortex and hippocampus

The mice chronically treated with corticosterone showed a significant increase in MMP9 protein levels compared to the control group in

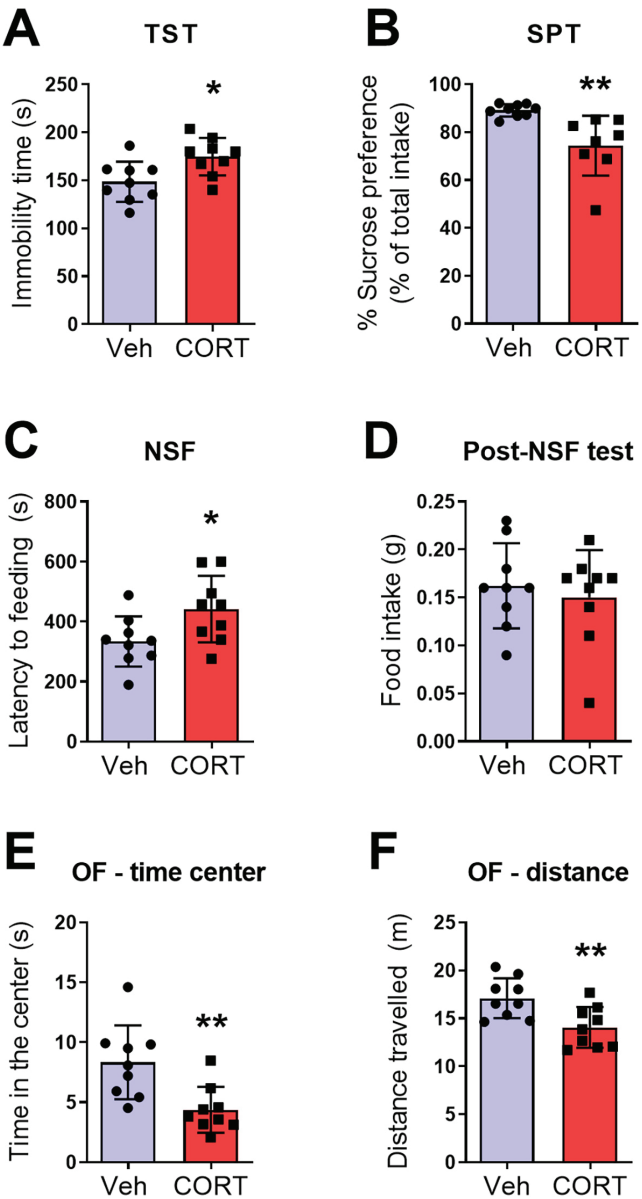

Fig. 2. Depressive- and anxious-like behaviour in CORT. Immobility time in the tail suspension test (A); percentage of the sucrose consumed *versus* total intake in the sucrose preference test (B); latency of feeding (C) and food eaten in the post-NSF test (D) of the novelty suppressed feeding test; time spent in the centre (E) and distance travelled (F) in the open field test. Data are expressed as mean ± SD. Two-tailed Student's t-test, unpaired data. *$p < 0.05$, **$p < 0.01$. $n = 9$ animals per group. Veh: vehicle group; CORT: corticosterone model; TST: tail suspension test; SPT: sucrose preference test: NSF: novelty suppressed feeding test; OF: open field test.

the cortex (117.8 ± 14.7% in corticosterone *vs* 100.0 ± 2.4% in the vehicle treated animals, $p < 0.01$) (Fig. 3A), and in the hippocampus (151.1 ± 61.0% in the corticosterone *vs* 100.0 ± 6.7% in the vehicle treated animals, $p < 0.05$) (Fig. 3B).

A significant reduction of nectin-3 protein level, one of the main substrates of MMP-9, was observed in the cortex of corticosterone treated mice compared to the control group (78.5 ± 13.9% in the corticosterone *vs* 100.0 ± 7.0% in the vehicle treated animals, $p < 0.05$) (Fig. 3C), and in the hippocampus (67.3 ± 23.7% in the corticosterone *vs* 100.0 ± 9.0% in the vehicle treated animals, $p < 0.05$) (Fig. 3D).

3.3. Metalloproteinase activity in cortex and hippocampus

Gel zymography was used to evaluate the gelatinase activity in CORT. Mice treated chronically with corticosterone displayed a significant increase in MMP-9 gelatinase activity compared to their control group in the cortex (338.3 ± 219.5% in the corticosterone *vs* vehicle treated animals, $p < 0.01$) (Fig. 4A). In the hippocampus, we also observed a tendency to increased gelatinase activity in the corticosterone model (169.0 ± 89.9% in the corticosterone *vs* vehicle treated animals, $p = 0.07$) (Fig. 4B).

MMP-2 activity was also analysed in the cortex (101.5 ± 25.5% in corticosterone *vs* 100.0 ± 36.5% in the vehicle treated animals, *ns*), and the hippocampus (159.0 ± 161.3% in corticosterone *vs* 100.0 ± 31.3% in the vehicle treated animals, *ns*), not observing statistical differences between the experimental groups. Moreover, the ratio MMP-9/MMP-2 in cortex (1.564 ± 0.681 in corticosterone *vs* 1.936 ± 1.106 in vehicle treated animals, *ns*) and hippocampus (2.754 ± 1.638 in corticosterone *vs* 2.724 ± 1.231 in vehicle treated animals, *ns*), did not present statistical differences.

An *in situ* zymography was also performed to study the regional localization of the gelatinolytic activity. Mice chronically treated with corticosterone presented a higher gelatinolytic activity compared to the control group in different regions of the hippocampus: CA1 field (178.8 ± 66.9% in the corticosterone *vs* 100.0 ± 48.2% in the vehicle treated animals, $p < 0.05$; Fig. 5A, B and C); CA3 field (179.5 ± 70.9% in the corticosterone *vs* 100.0 ± 41.0% in the vehicle treated animals, $p < 0.05$; Fig. 5D, E and F). However, no changes were observed in the dentate gyrus (Fig. 4G, H, and I).

In the cortex, the corticosterone treated animals also showed a higher gelatinolytic activity compared to their control group in layer 5 (152.1 ± 38.2% in the corticosterone *vs* 100.0 ± 45.2% in the vehicle treated animals, $p < 0.05$). The outer layers 2/3 presented a tendency to increased activity (139.4 ± 36.8% in the corticosterone *vs* 100.0 ± 37.3% in the vehicle treated animals, $p = 0.07$; Fig. 5J, K, L).

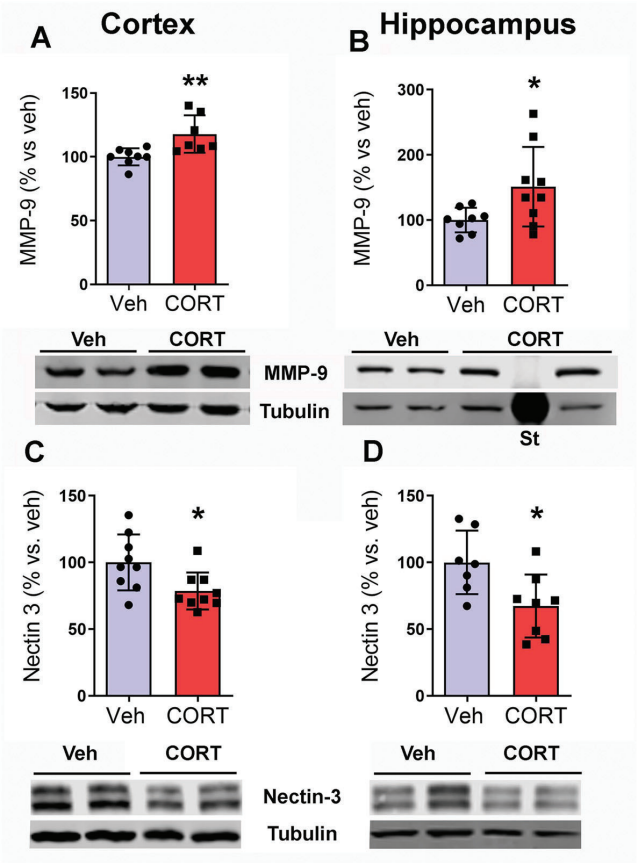

Fig. 3. MMP-9 and nectin-3 protein expression levels in the cortex (A and C, respectively) and the hippocampus (B and D, respectively) of corticosterone-treated mice. Representative images of MMP-9 and nectin-3 western blots are shown below the corresponding graphs. Data are expressed as mean ± SD. Two-tailed Student's *t*-test, unpaired data. * $p < 0.05$; ** $p < 0.01$. $n = 7$–9 animals per group. Veh: vehicle group; CORT: corticosterone model; St: protein standard.

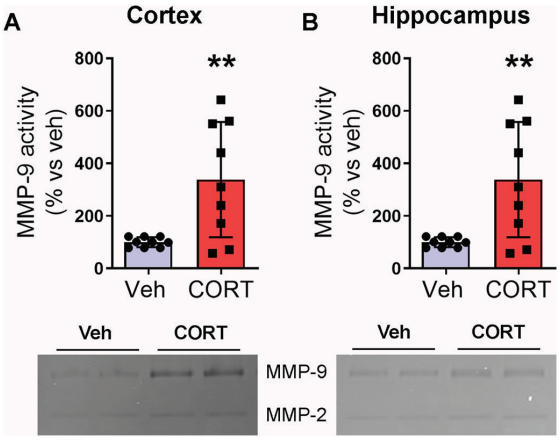

Fig. 4. MMP-9 gelatinase activity in CORT and vehicle mice in the cortex (A) and the hippocampus (B). Representative images of the gel zymography showing MMP-9 and MMP-2 bands are shown. Data are expressed as mean ± SD. Two-tailed Student's t-test, unpaired data. $^{**}p < 0.01$. n = 7–9 animals per group. Veh: vehicle group; CORT: corticosterone model; St: protein standard.

3.4. Correlation of MMP-9 protein expression/activity and behavioural despair in the TST

A correlation analysis was performed between different behavioural and molecular parameters in the cortex (Fig. 6). The immobility time in the TST positively correlated with the MMP-9 activity (Fig. 6B) and negatively correlated with nectin-3 protein levels (Fig. 6C). However, no correlation was found between the molecular markers and anhedonia (sucrose preference test) (Fig. 6D–F), or anxiety evaluated as the central time in the open field test (Fig. 6G–I) and the latency to feeding in the novelty suppressed feeding test (data not shown).

The analysis performed between the behavioural and molecular data in the hippocampus did not show any correlation (Fig. S3).

4. Discussion

This is the first study reporting an increase in the MMP-9 activity and protein expression in the chronic corticosterone animal model of depression (CORT), in brain areas relevant to the neurobiology of this disease. Moreover, we have found an association between the MMP-9 activity levels and the behavioural despair in this animal model.

It is well established that CORT is endowed with construct, face and predictive validity (Gourley and Taylor, 2009) as it resembles the impairment in the HPA axis and shows some of the manifestations of depression/anxiety disorders (Johnson et al., 2006). This model also presents neurochemical and molecular alterations similar to those reported in depressed patients (Murray et al., 2008; David et al., 2009).

In our study, the animals treated with chronic corticosterone presented anhedonia, shown as a reduction in sucrose preference (Belovicova et al., 2017), and depressive-like behaviour evidenced by increased behavioural despair in the TST (Porsolt et al., 2001; Belovicova et al., 2017). The lower ambulation found in the open field test, would difficult the interpretation of the TST. However, the lack of correlation between the immobility time and the distance travelled could be interpreted as indicative of the depressive-like behaviour, and not associated to the lower mobility in animal models of depression (Florensa-Zanuy et al., 2021). Moreover, this reduced locomotor activity is shown in the open field test, and not in the NSF. One of the factors that could contribute to this difference is the light intensity used in the arena (350 lx in the OF and 40 lx in the NSF), which may result in a higher freezing time in the open field test, as previously reported in association to conditional fear (Warthen et al., 2011).

This model also manifested conflict-based anxiety in the NSF test (Belovicova et al., 2017), and innate-anxiety in the OF test (Amigo et al., 2021), according with previous studies. These behavioural outcomes are in line with the results reported by other authors in CORT (Murray et al., 2008; David et al., 2009), and several models of chronic stress (reviewed in Willner, 2016). By contrast, Demuyser et al. (2016) reported that the CORT model does not present anxious-like manifestations, a discrepancy that may be due to differences in the methodological procedures used in their study, especially the housing conditions (individually housed animals) and the higher doses of corticosterone used during shorter periods of treatment (20 mg/kg/day, s.c, 21 days).

This is the first study reporting an increase in the gelatinolytic activity and MMP-9 protein expression in CORT in cortex. It is worth to note that this increase in cortical MMP-9 activity has been also reported in a mouse model of post-traumatic stress disorder (Chevalier et al., 2021). Moreover, the increase in MMP-9 activity was also observed in the CA1 and CA3 subregions of the hippocampus, with no changes in the dentate gyrus, confirming previous findings in other animal models of depression the chronic unpredictable stress model (Bijata et al., 2022). In contrast, other studies using a different model and species —the chronic restraint stress in rat—, only reported the MMP-9 increase in the CA1 subregion (van der Kooij et al., 2014). An MMP-9 increase in the CA1 and the dentate gyrus was also reported after 24 h of acute restraint stress (Aguayo et al., 2018). The CA1 subregion of the hippocampus appears to be associated to the chronic stress-mediated increase of MMP-9 expression by specifically activating the 5-HT_7 receptors (Bijata et al., 2022). The discrepancies found in MMP-9 activity in the different hippocampal areas may be linked to a differential activation of glutamatergic receptors in animal models of stress, since the CA3 area experiences an enhancement of NMDA-receptor dependent transmission (Kole et al., 2002), and the dentate gyrus an increase in AMPA-receptor dependent synaptic transmission (Karst and Joëls, 2003). Interestingly, our results showed a significant correlation between the immobility time in the TST and the MMP-9 activity or the nectin-3 protein expression in the cortex, but not in the hippocampus, indicating that a higher MMP-9 activity in cortical areas could lead to an increased behavioural despair.

This increased MMP-9 protein and activity observed in CORT is in line with the excitotoxicity linked to chronic stress (Popoli et al., 2011). In this sense, the enhanced glutamatergic activity mediated by NMDA activation has been associated with higher MMP-9 activity in primary cell cultures (van der Kooij et al., 2014).

Moreover, the increased MMP-9 activity observed in the CORT model is also evidenced by the reduction in nectin-3 levels —one of the substrates of this metalloproteinase— in the cortex and the hippocampus. In fact, some authors have reported a similar nectin-3 reduction in animal models associated to stress, in which this reduction was associated with alterations in social behaviour and cognition (Wang et al., 2011; van der Kooij et al., 2014). However, we did not find a significant correlation between MMP-9 activity and nectin-3 protein levels (*data not shown*), as MMP-9 degrades not only nectin-3, but also other molecules as neuroligin-1 (Peixoto et al., 2012), and β-dystroglycan (Michaluk and Kaczmarek, 2007). In addition, there are no clear evidence whether MMP-9 is the only enzyme able to degrade nectin-3 (van der Kooij et al., 2014).

The overactivation of MMP-9 has been associated to the generation of immature spines and silent synapses, lower AMPA/NMDA receptors ratio, and impaired LTP, leading to depression and other neuropathologies (Magnowska et al., 2016). These neuronal changes are in line with the neuroplasticity theory of depression that links high glucocorticoid levels, associated to chronic stress, to the atrophy of mature neurons, the shortening and the decreased density of dendritic spines in the hippocampus, leading to impaired neuronal plasticity (Boku et al., 2018).

The results obtained herein and in other animal models resemble those observed in human studies. Initial studies in peripheral blood

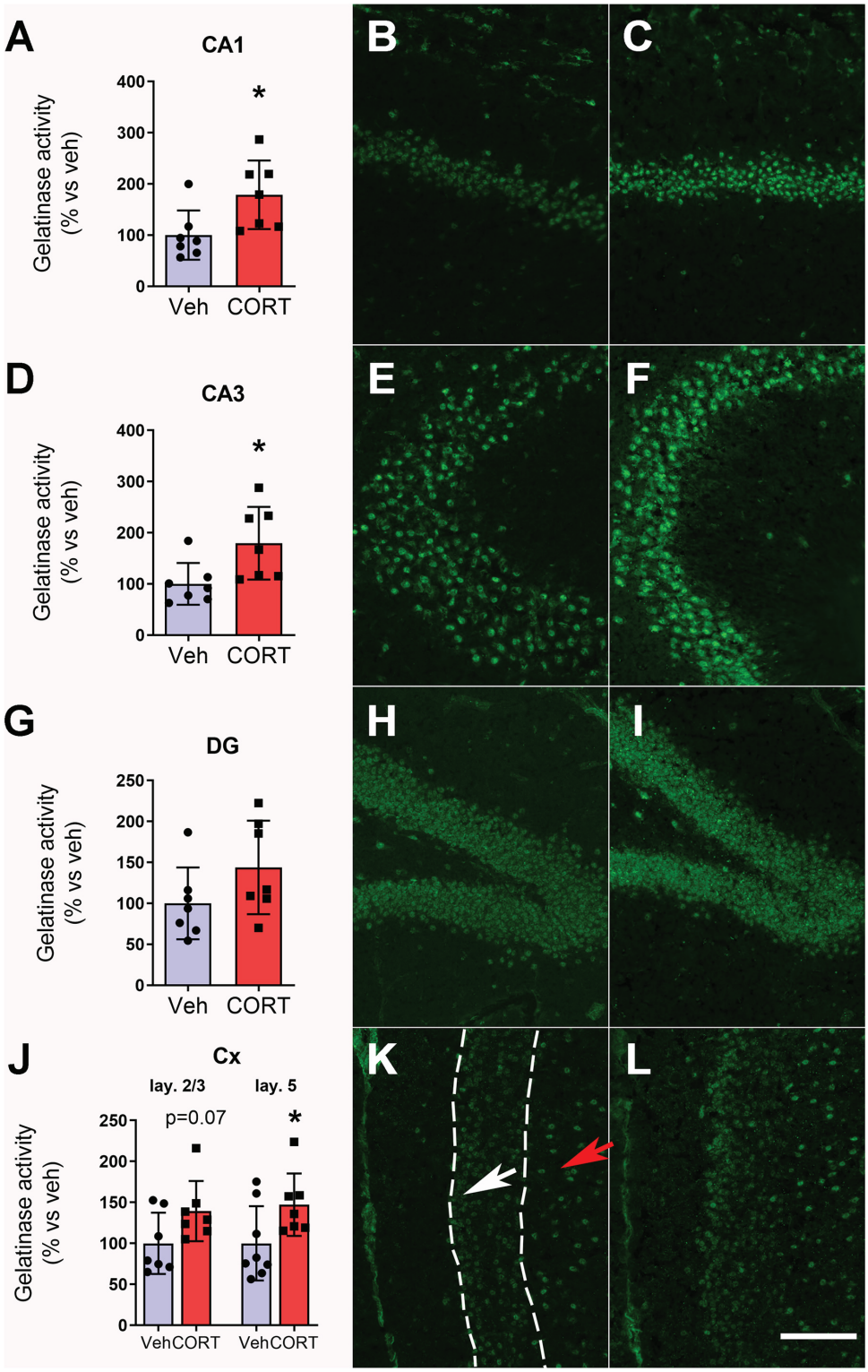

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Fig. 5. Gelatinase activity in hippocampal regions: A–C) CA1 field; D–F) CA3 field and G–I) DG field of the hippocampus, and prefrontal cortex: layers 2/3 and layer 5 (J–L). Representative images of *in situ* zymography in the CA1 field in the vehicle (B) and corticosterone-treated mice (C); in the CA3 field in the vehicle (E) and corticosterone-treated mice (F); in the dentate gyrus in the vehicle (H) and corticosterone-treated mice (I); in the prefrontal cortex in the vehicle (K) and corticosterone-treated mice (L). In K, red arrow shows layers 2–3 and white arrow shows layer 5. Data are expressed as mean ± SD. Two-tailed Student's *t*-test, unpaired. * $p < 0.05$. Veh: vehicle group; CORT: corticosterone model. Scale bar 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

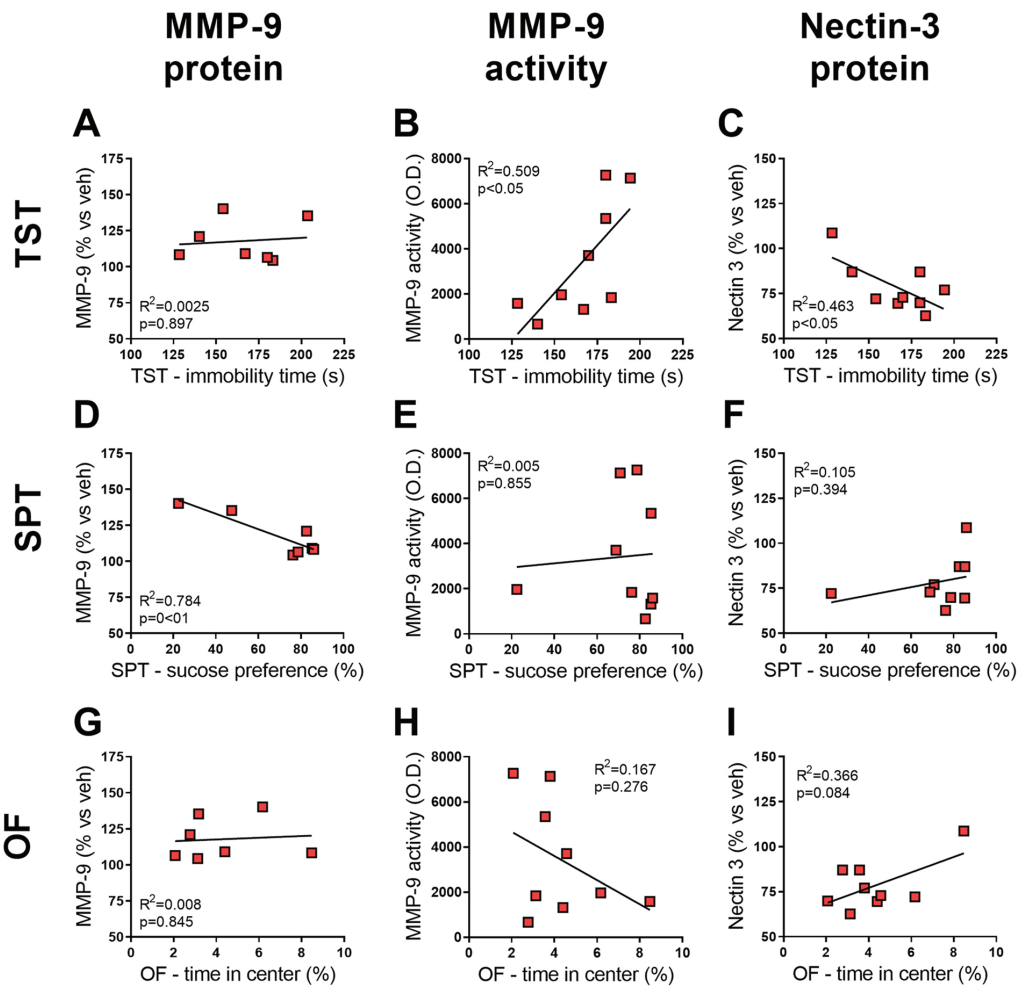

Fig. 6. Correlation in the corticosterone group between the behavioural outcomes (TST, tail suspension test; SPT, sucrose preference test; and OF, open field test) and: MMP-9 protein levels (A, D and G), MMP-9 activity (B, E and H), and nectin-3 protein levels (C, F and I) in the cortex. $n = 7–9$ animals per group. r: Pearson's correlation coefficient.

indicated higher MMP-9 levels in depressed patients compared to healthy subjects (Domenici et al., 2010; Rybakowski et al., 2013; Bobińska et al., 2016; Che et al., 2019), which were associated with the severity of the depressive symptoms (Yoshida et al., 2012; Shibasaki et al., 2016). Therefore, the correlation activity-behaviour reported here supports the importance of the MMP-9 activity level in the severity of depressive symptoms, as previously reported (Shibasaki et al., 2016). In this line, our data, together with the reduction in the level of peripheral MMP-9 after electroconvulsive therapy (Shibasaki et al., 2018), support the putative role of MMP-9 levels as a marker of the MDD severity or the responsiveness to the antidepressant-treatment. However, in our study, this correlation is only observed between MMP-9 activity and behavioural despair, and not with anhedonia or anxiety. In fact, some authors propose an association of MMP9 expression only with specific manifestations (Chevalier et al., 2021), which may account for the different correlations observed here for behavioural despair and anhedonia. These differences might be also associated to the brain area studied. In this sense, anhedonia is more associated to other brain areas such as the ventral tegmental area, nucleus accumbens (NAc), ventral striatum (VTA-NAc pathways) (Dunlop and Nemeroff, 2007; reviewed in Höflich et al., 2019), or central amygdala (Puścian et al., 2021), and anxiety to areas as the amygdala and the bed nucleus of the stria terminals

(Moreira et al., 2007; Duvarci et al., 2009; reviewed in Adhikari, 2014). In fact, a reduction of MMP-9 activity in the central amygdala induced by the chronic administration of the antidepressant drug fluoxetine to non-stressed mice, is associated to the appearance of anhedonia (Puścian et al., 2021).

One of the limitations of our study is the lack of data in areas more associated to other depressive manifestations as anhedonia and anxiety, as indicated above. Another limitation of the study is the impossibility to discriminate between MMP-9 and proMMP-9 in the gel zymography. This fact has been widely described by different authors, assuming that the enzymatic activity obtained in this technique could be associated to both enzyme forms (Cauwe and Opdenakker, 2010; Bijata et al., 2022). Other limitation in our study is the lack of specificity of the *in situ* zymography, as this technique only allows the evaluation of the gelatinase activity, which is mainly due to both MMP-9 and MMP-2 enzymes.

5. Conclusion

The chronic corticosterone model exhibits an increase in the MMP-9 protein and activity and a reduction of its substrate nectin-3 in relevant areas implicated in depression as the cortex and the hippocampus. The MMP-9 activity level correlates with behavioural despair in this model of depression. Our data support the role of MMP-9 in the pathophysiology of depression and in the severity of this illness, and act as a putative target to develop more efficacious antidepressant drugs.

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Ethical statement

We state that this work is original, and has not been submitted elsewhere for publication, completely or in part.

Authors statement

SB: performing experiments, analysing and interpreting data, writing the original draft; JS performing experiments, analysing and interpreting data, writing the original draft; EF-Z: methodology and data analysis; EG-M: methodology; AD: conceptualization and writing review; EC: conceptualization and writing review; AP: funding acquisition, writing review; and FP-C: conceptualization, funding acquisition, project administration, writing original draft.

Declaration of Competing Interest

None of the authors report potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2022.110624.

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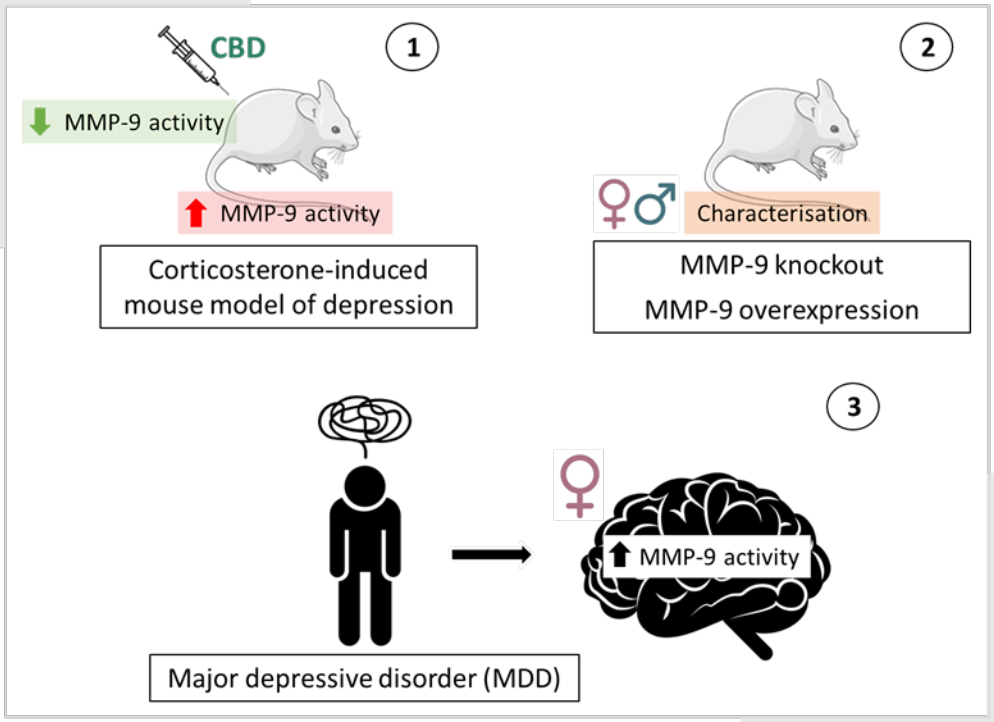

Resumen| El trastorno depresivo mayor es una enfermedad mental común, especialmente en mujeres. El cannabidiol, el mayor componente no psicomimético del *Cannabis sativa,* presenta un potencial efecto antidepresivo. Por otro lado, se han observado elevados niveles de la metaloproteinasa 9 (MMP-9) en modelos de estrés crónico y en pacientes deprimidos. En esta Tesis Doctoral, profundizamos en la implicación de la MMP-9 en la depresión. Observamos un efecto antidepresivo del tratamiento subcrónico con cannabidiol y la reversión del incremento en la actividad de la MMP-9 en un modelo de depresión inducido por la administración de corticosterona. La caracterización de ratones transgénicos MMP-9 mostraron que las alteraciones en MMP-9 tienen un impacto sexo-dependiente a nivel conductual, molecular y neuroquímico. Finalmente, detectamos una elevada actividad de MMP-9 en muestras cerebrales *post-mortem* de mujeres deprimidas que cometieron suicidio, pero no en hombres. Estos hallazgos refuerzan la implicación sexo-dependiente de MMP-9 en la etiopatología de la depresión.

Summary| Major depression is a common mental illness, especially in women. Cannabidiol, the main non-psychomimetic component of *Cannabis sativa*, presents a potential antidepressant effect. On the other hand, high levels of metalloproteinase 9 (MMP-9) have been observed in models of chronic stress and depressed patients. In this Doctoral Thesis, we delve into the involvement of MMP-9 in depression. We observed an antidepressant-like effect of subchronic cannabidiol administration and the normalization of elevated MMP-9 activity in the corticosterone-induced model of depression. The characterisation of MMP-9 transgenic mice showed that alterations in MMP-9 have a sex-dependent impact at the behavioural, molecular, and neurochemical levels. Finally, we detected elevated MMP-9 activity in *post-mortem* brain samples from depressed women who committed suicide, but not in men. These findings reinforce the sex-dependent implication of MMP-9 in the etiopathology of depression.

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