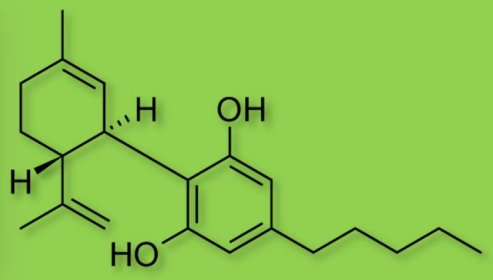
UNIVERSIDAD DE CANTABRIA

PROGRAMA DE DOCTORADO EN
BIOLOGÍA MOLECULAR Y BIOMEDICINA



TESIS DOCTORAL

Cannabidiol como antidepresivo de acción rápida: identificación de los mecanismos moleculares implicados en su efecto antidepresivo

PHD THESIS

Cannabidiol as a fast-acting antidepressant: identification of the molecular mechanisms implicated in its antidepressant effect

Realizada por: **Eva Ariadna Florensa Zanuy**

Dirigida por: Fuencisla Pilar Cuéllar y Álvaro Díaz Martínez

Escuela de Doctorado de la Universidad de Cantabria

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ÁLVARO DÍAZ MARTÍNEZ, Profesor Titular de la Universidad de Cantabria y **FUENCISLA PILAR CUÉLLAR**, Profesora Asociada Doctor de la Universidad de Cantabria.

CERTIFICAN:

Que la Tesis Doctoral titulada "Cannabidiol como antidepresivo de acción rápida: identificación de los mecanismos moleculares implicados en su efecto antidepresivo", "Cannabidiol as a fast-acting antidepressant: identification of the molecular mechanisms implicated in its antidepressant effect", ha sido realizada por Eva Ariadna Florensa Zanuy, 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.

Santander, Febrero de 2021

Fdo: Álvaro Díaz Martínez Fuencisla Pilar Cuéllar

Este trabajo de tesis doctoral ha sido realizado en el laboratorio de "*Bases neurobiológicas del mecanismo de acción de compuestos que actúan a nivel del Sistema Nervioso Central*", del Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC) y del Departamento de Fisiología y Farmacología, de la Universidad de Cantabria, bajo la dirección de los Drs. Fuencisla Pilar Cuéllar y Álvaro Díaz Martínez, entre septiembre de 2016 y febrero de 2021.

Para la realización de este trabajo, la doctoranda ha disfrutado de una beca de la "Convocatoria 2016 de Ayudas a la formación para la iniciación a la investigación CIBER en el área temática de salud Mental", así como de un contrato predoctoral de la "Convocatoria de Ayudas del Programa de Personal Investigador en Formación Predoctoral en el Área de Biomedicina, Biotecnología y Ciencias de la Salud 2016", de la Universidad de Cantabria.

Este trabajo ha sido financiado por:

- Ministerio de Economía y Competitividad (SAF2015-67457-R MINECO/FEDER).
- Ministerio de Ciencia, Innovación y Universidades (RTI2018-097534-B-I00).
- Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), Instituto de Salud Carlos III.

La doctoranda ha realizado una estancia científica en el laboratorio del profesor John Cryan, Professor & Chair, en el Dept. of Anatomy & Neuroscience, University College Cork, realizada en el APC Microbiome - University College Cork (Cork, Irlanda), del 01/09/2019 al 30/11/2019.

Al pare

En primer lugar, quiero agradecerle inmensamente a Fuen las mil y una horas que me ha dedicado durante estos 4 años y pico. Por enseñarme el trabajo de laboratorio, a pensar las cosas por mí misma, a solucionar problemas, y a hacer las cosas bien (entre muchas otras cosas). También porque más que una jefa, has sido una compañera y amiga, ¡y por aguantar largas listas de reproducción sin rechistar! jaja Y, sobre todo, porqué ha hecho posible que hoy estéis leyendo esta tesis. También es imprescindible agradecerles a Álvaro, a Elena (la Castro), y a Albert toda la ayuda y conocimiento que han compartido conmigo, como también las horas de ensayos y sobre todo de discusión y preguntas, además de todos los buenos momentos que hemos pasado durante incontables cafés y comidas.

Además, un aspecto clave para la realización de esta tesis ha sido formar parte de un grupo de investigación que, más que simples compañeros de trabajo, han sido como una familia. Desde el día que llegué a Santander me ofrecieron su ayuda para lo profesional y lo personal, y me acogieron con los brazos bien abiertos. Considero que el buen ambiente en el trabajo es esencial para poder trabajar bien y a gusto, y en este aspecto me he sentido muy afortunada. Fuen, Álvaro, Elena, Ángel, Albert, Raquel y Víctor, gracias por haber sido una acogedora familia para mí.

Ellos han sido los que siempre han estado ahí, pero también ha sido super importante la gente que ha estado "de paso" en nuestro grupo. También fuisteis piezas clave de esta gran familia, y ojalá hubieseis estado conmigo durante todo el proceso. Primero fueron Emilio y Josep. Los pillé pretésicos y poco pudimos coincidir en esta primera etapa, pero fue un placer empezar la tesis a vuestro lado. ¡Crecía el clan Catalán! Y os hacía liar otra vez entre los dos idiomas jaja El Dr. Garro se quedó después de la tesis, y pude disfrutar durante más tiempo de su peculiar forma de ser que tanto nos encanta a todos. Con él, ¡la alegría y las risas están aseguradas! Marta también estuvo en mis inicios, y fue clave para integrarme y empezar a hacer planes. Aparte de ser muy simpática y divertida, siempre te estaré agradecida por invitarme esa noche a ir con vosotros a una clase de bachata en la Black Bird. ¡Ese día cambió mi vida! Encontré un hobbie increíble que me llevó a encontrar muchas cosas más.

Después, empezamos a recibir erasmus italianas, ¡y la cosa se animó mucho! La primera fue Deborah (Gina), con la que congenié al instante y empezamos a pasar momentos geniales juntas, tanto de día como de noche. Aparte de ser divertida, tiene una visión para los detalles y las personas que le permite dar unos consejos geniales, ¡qué bien haberla tenido durante unos meses! Luego… llegó un enorme torbellino. Annamaría vino con toda la energía que le faltaba a Dehorah jaja ¡Ella es un show andante! Pasamos increíbles

momentos juntas, y me alegro infinitamente de haberla conocido y poder tenerla como amiga. Siempre serán mis dos italianas favoritas.

Luego vino Laura, y también nos caímos genial enseguida. Super trabajadora y super fiestera, ¡qué buena combinación! Nos entendíamos en todo y lo compartíamos todo, y pasamos momentos geniales que nunca se me olvidarán. Como por ejemplo la noche de San Juan y tu momento estrella en el lab. Fue un placer ser compañeras de laboratorio. Y la última incorporación fue cosa del destino… ¡otra catalana! Conocí a Júlia en un *blablacar*, y por mi culpa acabó haciendo nuestro máster y el doctorado en nuestro grupo. La verdad es que se está super a gusto con ella en el lab, y aunque la lie un poco a veces, lo compensa con su simpatía y su forma de ser tan graciosa jaja (¡es Emilio-like!). También hemos compartido muchos momentos geniales fuera del lab, desde excursiones de montaña, turísticas, y noches de fiesta, y todo ello en conjunto ha sido simplemente perfecto.

Otras dos personitas que siempre han estado allí son Rocío y Laurita. La verdad es que cuando nos juntamos es digno de ver, y no puedo pasármelo mejor con vosotras. Mil gracias por todos los momentos vividos, desde las noches de fiesta, las actividades varias, hasta las largas charlas de confesiones y consejos. No sé qué habría hecho yo sin vosotras. Para los que no lo sepan ya, ¡son de las mejores personas que conozco, y son de las amistades que nunca quiero perder!

Los doctorandos de la facultad de medicina han sido otro pilar clave, especialmente Oriol. La verdad es que estaré eternamente agradecida de que se juntaran nuestros caminos. No podía haber una persona más ideal con la que empezar mi aventura cántabra. Por casualidades de la vida, le conocí a los pocos días de mudarme a Santander, ¡y eso que era Lleidatà! Desde el minuto 0 de conocernos, ya empezó a formarse una bonita amistad. No conozco una persona más buena en este mundo, ni más divertida, ni más buen amigo. Los momentos que pasamos juntos son inestimables e inigualables, igual que su amistad. Sin él esto no habría sido lo mismo, estoy segura. Y si hay alguna actividad a destacar (aparte de la fiesta, como no) es el surf, mi otro gran hobbie descubierto durante estos años. Gracias por engancharte tanto al surf como yo, y compartir tantos días de aventuras conmigo. De la mano de Oriol conocí a otros doctorandos de la facultad de medicina con los que formamos un grupito muy guay. Raquel, Jorge (x2), Nacho, Cris, Alicia, Ana Belén, María, Vero, y Jesús, gracias por tantos momentos geniales e incontables cenas y noches de diversión juntos.

A todos vosotros, os llevaré siempre en mi corazón.

Antes decía que el baile me ha cambiado la vida. Primero, por ser algo a lo que me enganché sin remedio, que me llena tantísimo, me hace conocer a un montón de personas geniales, y que me ayuda a desconectar de todo y recargar las pilas. Y lo más importante, porqué ha sido gracias al baile que he conocido a Marlon. Él es importantísimo en mi vida y me ha apoyado y ayudado muchísimo en todo, sobre todo durante estos últimos meses tan difíciles. Gracias por los infinitos buenos momentos, por animarme en los malos, y por tu paciencia y comprensión. ¡¡¡Gracias por tanto!!!

Y, por último, pero no menos importante, quiero agradecerle a mi familia que siempre esté allí, apoyándome en cada decisión que tomo y ayudándome en lo que sea. En especial a mis padres, ya que gracias a ellos soy la persona que soy hoy en día, y he conseguido todo lo que me he propuesto. Mi esfuerzo también ha sido el vuestro, y os estaré por siempre agradecida. Gràcies mare per ensenyar-me les coses importants de la vida, a ser forta, i a seguir vingui el que vingui. A ti, Dusky, por tantos años estando incondicionalmente a mi lado, dando vueltas conmigo por todas partes, siendo el perro perfecto, y dándome tantas alegrías y tantísimo amor. Y también a Mariona y a Alba, que son prácticamente familia. Gracias por estar allí siempre a pesar de la distancia.

Santander también va a estar siempre en mi corazón, y siempre será mi segunda casa. Llegué aquí sin conocer absolutamente nada de esta ciudad, y ella, y sobre todo sus costas, consiguieron enamorarme muy fácilmente. Aunque a veces tiene un carácter difícil (incluido el clima), estoy encantada de vivir en Cantabria, de los millones de actividades que ofrece, y de la belleza natural que tiene. Ciertamente, ¡Cantabria es infinita!

ABBREVIATION LIST

2-AG: 2-arachidonoylglycerol

3-HK: 3-Hydroxy kynurenine

5-HT: 5-Hydroxytryptamine (serotonin)

AEA: N-arachidonoylethanolamine / anandamide

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

AP: Anteroposterior

APS: Ammonium persulfate

BBB: Blood brain barrier

BDNF: Brain derived neurotrophic factor

BNST: Bed nucleus of the stria terminalis

BSA: Bovine serum albumin

cAMP: Cyclic adenosine monophosphate

$CB_{1/2}$: Cannabinoid receptor 1/2

CBD: Cannabidiol

cDNA: Complementary deoxyribonucleic acid

CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

CNS: Central nervous system

CREB: Cyclic AMP response element-binding protein

CSF: Cerebrospinal fluid

CUMS: Chronic unpredictable mild stress

Cx: Cortex

DAB: Diaminobenzidine

DBS: Deep brain stimulation

DIV: Days *in vitro*

dlPAG: Dorsolateral portion of periaqueductal gray

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethyl sulfoxide

DPBS: Dulbecco's phosphate-buffered saline

DRN: Dorsal raphe nucleus

DTT: Dithiothreitol

DV: Dorsoventral

EAAT: Excitatory amino acid transporter

ECS: Endocannabinoid system

ECT: Electroconvulsive therapy

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol tetraacetic acid

ELISA: Enzyme linked immunosorbent assay

FAAH: Fatty acid amide hydrolase

FBS: Fetal bovine serum

fMRI: Functional magnetic resonance imaging

FST: Forced swimming test

GABA: γ-aminobutyric acid

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GPCR: G-protein coupled receptor

GPR55: G protein-coupled receptor 55

HBSS: Hanks' Balanced Salt solution

HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

Hp: Hippocampus

HPA: Hypothalamic-pituitary-adrenal

HPLC: High performance liquid chromatography

IDO: Indoleamine 2,3-dioxygenase

IκBα: Inhibitor of nuclear factor kappa B

IL: Infralimbic

IL-6: Interleukin 6

i.p.: Intraperitoneal

KAT: Kynurenine amino-transferase

KMO: Kynurenine 3-monooxygenase

KO: Knock-out

KYN: Kynurenine

KYNA: Kynurenic acid

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinase

MDD: Major depressive disorder

ML: Mediolateral

mPFC: Medial prefrontal cortex

mRNA: Messenger ribonucleic acid

mTOR: Mammalian target of rapamycin

NA: Noradrenaline

NAc: Nucleus accumbens

NaCl: Sodium chloride

NAM: negative allosteric modulator

NDS: Normal donkey serum

NF-κB: Nuclear factor kappa B

NMDA: N-methyl-D-aspartate

NSF: Novelty suppressed feeding test

OBX: Olfactory bulbectomy

O.D.: Optical density

OPA: O-phtalaldehyde

OSA: Octanesulfonic acid

OFT: Open field test

PAM: Positive allosteric modulator

PBS: Phosphate buffered saline

PCNA: Proliferating cell nuclear antigen

PDL: Poly-D-Lysine

PFA: Paraformaldehyde

PFC: Prefrontal cortex

PG: Propilenglicol

PI3K: Phosphatidylinositol 3-kinase

PKA: Protein kinase A

PLL: Poly-L-Lysine

PMSF: Phenylmethylsulfonyl fluoride

PPARγ: Peroxisome proliferator-activated receptor gamma

qPCR: Quantitative polymerase chain reaction

QUIN: Quinolinic acid

RT: Room temperature

SDS: Sodium dodecyl sulfate

S.E.M.: Standard error mean

SPF: Specific pathogen free

SPT: Sucrose preference test

SSRI: selective serotonin reuptake inhibitor

SNRI: serotonin/norepinephrine reuptake inhibitor

TDO: Tryptophan 2,3-dioxygenase

TEMED: N,N,N′,N′-Tetramethylethylenediamine

THC: Δ^9**-**tetrahydrocannabinol

TLR4: Toll-like receptor 4

TNFα: Tumour necrosis factor alpha

TRD: treatment resistant depression

TRP: Tryptophan

TRPV: Transient receptor potential vanilloid

TST: Tail suspension test

vmPFC: Ventromedial prefrontal cortex

w/v: Weight/volume

WB: Western blot

INDEX

INTRODUCTION 1

1. Major Depressive Disorder 3
   1.1 General aspects 3
   1.2 Aetiology of depression 4
      1.2.1 Genetic and environmental factors 4
      1.2.2 Brain areas and circuits implicated in depression 6
      1.2.3 Aetiopathological hypotheses 11
         1.2.3.1 Monoaminergic hypothesis 11
         1.2.3.2 Neurotrophic/neuroplastic hypothesis 14
         1.2.3.3 Glutamatergic hypothesis 17
         1.2.3.4 Neuroinflammatory hypothesis 21
         1.2.3.5 New comprehensive hypothesis: interconnection between depression hypotheses 24
      1.2.4 Endocannabinoid system and depression 26
2. Animal models of depression: focus on gender and the LPS model 30
   2.1 Gender bias in preclinical studies 33
   2.2 Neuroinflammatory model induced by lipopolysaccharide 34
3. Cannabidiol 37
   3.1 Pharmacokinetic aspects 38
      3.1.1 Absorption 38
      3.1.2 Distribution 38
      3.1.3 Metabolism and elimination 39
   3.2 Pharmacodynamic aspects 39
      3.2.1 Pharmacological effects 39
      3.2.2 Mechanisms of action 41
   3.3 Evidence of the antidepressant effect of cannabidiol 44
      3.3.1 Preclinical studies 44
      3.3.2 Clinical studies 46
   3.4 Safety of cannabidiol in humans 48

HYPOTHESIS AND OBJECTIVES 49

MATERIAL AND METHODS 53

1. Animals 55

2. Drugs and reagents 55
3. Experimental design 58
3.1 *In vivo* studies 58
3.2 *In vitro* studies 60
4. Pharmacological treatments 61
5. Stereotaxic surgery 62
6. LPS-induced neuroinflammatory model 63
7. Behavioural tests 63
7.1 Open field test (OFT) 63
7.2 Forced Swimming Test (FST) 64
7.3 Tail Suspension Test (TST) 64
7.4 Sucrose Preference Test (SPT) 65
8. Sample collection 65
9. Primary neuronal cultures 66
10. Primary microglial cultures 68
11. Molecular techniques 69
11.1 Quantitative Polymerase Chain Reaction (qPCR) 69
11.2 Western Blot (WB) 71
11.3 Enzyme-Linked Immunosorbent Assay (ELISA) 74
11.4 Immunohistochemistry (IHQ) 75
11.5 High-Performance Liquid Chromatography (HPLC) 76
12. Statistical analysis 77

RESULTS 79

OBJECTIVE 1. Cannabidiol antidepressant-like effects: site and mechanism of action. 81
1.1 Acute behavioural and molecular effects of the bilateral infralimbic cannabidiol infusion 81
1.1.1 Evaluation of the depressive-like, anxious-like and locomotor behaviour 81
1.1.2 Plasticity markers' expression in the prefrontal cortex and hippocampus 82
1.1.3 Neurotransmitter levels in the prefrontal cortex and dorsal raphe nucleus 83
1.1.4 Neuronal activation in the prefrontal cortex and dorsal raphe nucleus: effect of cannabidiol in *naïve* and stressed animals 85

1.2 Behavioural and molecular effects of the bilateral infralimbic cannabidiol infusion 24 hours after its administration 86
1.2.1 Evaluation of the depressive-like, anxious-like and locomotor behaviour 86
1.2.2 Plasticity markers' expression in the prefrontal cortex 87
1.3 Behavioural and molecular effects of the blockade of presynaptic 5-HT_{1A} receptors 24 hours after the bilateral infralimbic cannabidiol infusion 88
1.3.1 Evaluation of the depressive-like behaviour 88
1.3.2 Plasticity markers' expression in the prefrontal cortex 89
1.3.3 Neurotransmitter levels in the prefrontal cortex and dorsal raphe nucleus 91
1.4 Acute behavioural and molecular effects of the dorsal raphe nucleus cannabidiol infusion 92
1.4.1 Evaluation of the depressive-like, anxious-like and locomotor behaviour 92
1.4.2 Plasticity markers' expression in the prefrontal cortex 93
1.4.3 Neurotransmitter levels in the prefrontal cortex and dorsal raphe nucleus 94
1.5 Study of the effects of cannabidiol in rat primary neuronal cultures 95
1.5.1 Determination of the optimal cannabidiol concentration for cell cultures: dose-response assays 95
1.5.2 Effect of cannabidiol on plasticity markers in primary neuronal cultures from rat cortex 96

OBJECTIVE 2. Effects of cannabidiol in experimental lipopolysaccharide models 99
2.1 Evaluation of the anti-inflammatory effect of cannabidiol in primary microglia cell cultures activated with lipopolysaccharide 99
2.2 Behavioural and molecular effects of cannabidiol in mice subjected to the neuro-inflammatory model induced by lipopolysaccharide: influence of gender 100
2.2.1 Optimization of the lipopolysaccharide model 100
2.2.2 Evaluation of the influence of gender in the depressive- and anxious-like behaviour in the mouse LPS model 102

2.2.3 Evaluation of the depressive- and anxious-like behaviour following cannabidiol administration to male mice subjected to the LPS model 104
2.2.4 Evaluation of the depressive- and anxious-like behaviour following cannabidiol administration to female mice subjected to the LPS model 106
2.2.5 Role of 5-HT_{1A} receptors in the antidepressant-like effect of cannabidiol in the LPS model 108
2.2.6 NF-κB, IκBα and PPARγ levels in nuclear and cytoplasmic fractions of cortical samples from LPS mice: effect of cannabidiol 109
2.2.7 TNFα and IL-6 levels in plasma and prefrontal cortex of mice injected with LPS: effect of cannabidiol 111
2.2.8 Kynurenine pathway metabolites and neurotransmitters in hippocampus and cortex of LPS mice: effect of cannabidiol 112
2.2.9 Correlation analysis between experimental findings 117

DISCUSSION 123

1. Cannabidiol antidepressant-like effects: site and mechanism of action. 125
1.1 Bilateral cannabidiol infusion into the infralimbic cortex: acute effects 125
1.1.1 Depressive- and anxious-like behaviour 125
1.1.2 Plasticity markers in the prefrontal cortex and hippocampus 127
1.1.3 Neurotransmitters in the prefrontal cortex and dorsal raphe nucleus 130
1.1.4 Cannabidiol effects on neuronal activation in prefrontal cortex and dorsal raphe nucleus 132
1.2 Bilateral cannabidiol infusion into the infralimbic cortex: effects after 24 h 135
1.2.1 Depressive- and anxious-like behaviour 135
1.2.2 Plasticity markers in the prefrontal cortex 136
1.3 Effects of the bilateral cannabidiol infusion into the infralimbic cortex in the presence of a presynaptic 5-HT_{1A} receptor blockade 139
1.3.1 Depressive-like behaviour 138
1.3.2 Plasticity markers in the prefrontal cortex 140
1.3.3 Neurotransmitters in the prefrontal cortex and dorsal raphe nucleus 141

1.4 Cannabidiol infusion into the dorsal raphe nucleus: acute effects 142
1.4.1 Depressive- and anxious-like behaviour 142
1.4.2 Plasticity markers in the prefrontal cortex 144
1.4.3 Neurotransmitters in the prefrontal cortex and dorsal raphe nucleus 145
1.5 Cannabidiol effects on plasticity markers in primary neuronal cultures 146

2. Effects of cannabidiol in experimental lipopolysaccharide models. 151
2.1 Effect of cannabidiol in LPS-activated microglial cells 151
2.2 Behavioural effects of cannabidiol in the LPS model: influence of gender 151
2.2.1 LPS-induced depressive-like behaviour: effect of cannabidiol and gender 152
2.2.2 LPS-induced anxiety-like behaviour: effect of cannabidiol and gender 154
2.2.3 Gender differences in the behavioural effects 156
2.2.4 Role of postsynaptic 5-HT_{1A} receptors in the antidepressant-like effect of cannabidiol in the LPS model 158
2.3 Molecular effects of cannabidiol in the LPS model 159
2.3.1 NF-κB pathway 159
2.3.2 Pro-inflammatory cytokines 160
2.3.3 Kynurenine pathway 162
2.3.4 Glutamate and GABA 165

CONCLUSIONS 169

BIBLIOGRAPHY 173

ANNEXES 237

Annexe 1. Statistical analysis
Annexe 2. Resumen
Annexe 3. Publications

INTRODUCTION

1. Major Depressive Disorder

1.1 General aspects

Major Depressive Disorder (MDD) is a disabling chronic and recurrent disease associated with premature death due to comorbid medical illness and suicide (Laursen et al., 2016; Pratt et al., 2016), as it is indeed one of the most relevant risk factors for suicide (Bachmann, 2018). Among the comorbidities of MDD there are other psychiatric diseases (Rush et al., 2005) –being the co-occurrence of anxiety-related disorders especially common (Fava et al., 2004; Rush et al., 2005; Choi et al., 2020)–, and physical illnesses, such as cardiovascular disease, cancer, diabetes, and chronic pain (Krishnan et al., 2002; Thom et al., 2019). The average age of onset of MDD is around 35 years, but the disease can be developed at any age, having this led to the subclassification in early- and late-onset depression (Tondo et al., 2010; Charlton et al., 2013; Yalin and Young, 2019).

Depression is the most prevalent psychiatric disorder worldwide (GBD, 2017). Recently, the World Health Organization (WHO) reported that more than 264 million people of all ages suffer from depression around the world (WHO, 2020), being women affected by the disease at roughly twice the rate of men (Holden, 2005; Marcus et al., 2005; Grigoriadis and Robinson, 2007). The prevalence of depression has been increasing significantly over the years (GBD, 2017), and nowadays the situation is further worsened by the increase in mental health problems such as depression provoked by the COVID-19 pandemic (Raony et al., 2020; Vindegaard and Benros, 2020).

Regarding its socioeconomic impact, MDD is a leading cause of disability worldwide and one of the major contributors to the overall global burden of disease (GBD, 2017), and it is predicted to be the leading cause of disease burden globally by 2030 (WHO, 2011). In addition, it causes great socioeconomic costs (Sicras-Mainar et al., 2010; Gustavsson et al., 2011).

MDD symptoms are heterogeneous –comprising somatic, emotional, and cognitive manifestations– which can greatly vary from one patient to another. According to Diagnostic and Statistical Manual of Mental Disorders (DSM-5, 2013), a diagnostic of MDD is made when five or more of the symptoms listed below are reported for longer than two weeks, and when these symptoms disturb the normal social and occupational performances of the patient. In addition, at least one of the symptoms should be (1)

depressed mood or (2) anhedonia. The diagnostic criteria of DSM-V for Major Depressive Disorder are the following:

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.*

Currently, the diagnosis of MDD is uniquely clinic. As it is not a simple task, the disease is commonly underdiagnosed. Moreover, other mental psychiatric pathologies as bipolar disorder and schizophrenia also present depressive symptoms, which sometimes leads to incorrect diagnoses (Hirschfeld, 2014; Rahim and Rashid, 2017). Although multiple efforts have been made in the search for molecular biomarkers of the disease to overcome these limitations (Hacimusalar and Eşel, 2018; Yuan et al., 2019; Kennis et al., 2020), the high variability of symptoms in MDD patients, together with the shared symptomatology and molecular footprints with other psychiatric and non-psychiatric diseases, hinders the discovery of specific biomarkers for MDD. In consequence, it has been argued that a dimensional view of psychiatric disorders could favour the finding of new reliable biomarkers (Venkatasubramanian and Keshavan, 2016) and that the development of a biomarker panel would be more useful in the search for a biological signature of MDD (Schmidt et al., 2011).

1.2 Aetiology of depression

1.2.1 Genetic and environmental factors

Despite intense research, the aetiology of depression is still poorly understood. In an attempt to unravel its genetic basis, a huge number of studies have been performed. The strongest evidence for the contribution of genetic factors in depression comes from family and twin studies, which have estimated a heritability around 37%, and an almost threefold increase in the risk of MDD in their offsprings (Sullivan et al., 2000). However, the same

studies evidence that environmental factors play a crucial role. Using a candidate gene approach, some genetic variants of genes implicated in the physiopathological pathways described for depression have been associated with a higher risk for suffering the disease. For instance, in the serotonergic system, the polymorphisms in the serotonin transporter (5-HTT) (SLC6A4), the rate-limiting enzyme in serotonin synthesis (tryptophan hydroxylase, TPH2), and the 5-HT receptors have been reported to increase the risk of depression (Lesch et al., 1996; Lemonde et al., 2003; Zill et al., 2004). The association of depression with the single nucleotide polymorphism Val66Met of brain-derived neurotrophic factor (BDNF) has also been extensively studied, leading to divergent results (Schumacher et al., 2005; Verhagen et al., 2010).

In recent years, the approach to study the genetics of depression changed from candidate genes towards genome-wide association studies (GWAS), a technique that tests millions of polymorphisms simultaneously, and genome-wide sequencing (GWS). Although these may be better tools due to the polygenic nature of the disease, most of the studies did not find an association between susceptibility genes and MDD (Athira et al., 2020). The main problems regarding these studies are the large heterogeneity of the samples and the need to include a huge number of participants. More recent studies have identified several genetic risk loci for depression (Mullins and Lewis, 2017; Howard et al., 2018; Wray et al., 2018). Unfortunately, despite all the efforts, there is a high inconsistency within the genetic association studies of MDD and, to date, the major genes implicated in depression have not been identified. The existing evidence shows that depression is a polygenic disorder, in which each susceptibility gene makes a small contribution to the risk for developing the disease (Ebmeier et al., 2006; Lohoff, 2010).

The environment, defined as all the exogenous factors that influence the individual's social, emotional, and physical world (including stress, trauma, nutrition, lifestyle, etc.), is thought to account for 2/3 of the risk for depression (Saveanu and Nemeroff, 2012; Duclot and Kabbaj, 2015). In fact, it is the interaction between genes and the environment which increases the risk to develop the disease. In this regard, several authors have reported that stress, by its interaction with a specific genetic background, is an important trigger of MDD (Caspi et al., 2003; Wang et al., 2018a; Zhao et al., 2018). The mechanism proposed for the interaction between environmental factors and genes are epigenetic modifications (Duclot and Kabbaj, 2015). These epigenetic mechanisms include DNA methylation and histone modification by methylation and acetylation, and differences in these patterns have been associated with MDD (Sabunciyan et al., 2012; Cruceanu et al., 2013; Davies et al., 2014; Aberg et al., 2020).

In all, it is commonly accepted that depression is originated from a complex interplay between genetic, epigenetic, and environmental factors (Shadrina et al., 2018). Future studies would be needed to find an efficient way of integrating the study of all these contributors to the risk of MDD to gain a better understanding of the disease and to improve its prevention and treatment.

1.2.2 Brain areas and circuits implicated in depression

Clinical and preclinical studies have linked depression to different functional and morphologic alterations in cortico-limbic brain regions controlling mood and emotions (Price and Drevets, 2010; Duman and Aghajanian, 2012). Here we present a brief overview of the function and findings in the brain areas most relevant for depression.

Prefrontal cortex

The prefrontal cortex (PFC) processes sensory input and the emotional stimuli originated in the limbic system, mediates executive motor functions, and regulates the appropriate emotional response. It is connected with important areas of the limbic system such as the amygdala, the hippocampus (Hp), the nucleus accumbens (NAc), and the aminergic nuclei in the brain stem. The PFC function has been associated with decision-making, personality, social behaviour, and hedonic responses (Price, 1999; Mitterschiffthaler et al., 2003). In MDD patients, the volume of multiple PFC areas is reduced (Drevets, 2000; Bremner et al., 2002; Grieve et al., 2013; Chen et al., 2018).

In particular, the mPFC has been strongly implicated in depression and antidepressant treatment (*reviewed in* Hare and Duman, 2020). In rodents, the mPFC is subdivided into infralimbic (IL), prelimbic (PL), and anterior cingulate (ACC) cortex (Vertes, 2004). The dorsal regions in rodents (PL cortex and ACC) appear to correspond to the human dorsal ACC, although this is still a matter of debate (Seamans et al., 2008; Heilbronner et al., 2016). There is more consensus about the rodent IL cortex, which appears to correspond to the subgenual cortex area (Brodmann area 25) in humans, an important area involved in emotion processing conserved across species (Heilbronner et al., 2016).

The activation state of the different subdivisions of the PFC has been widely studied in MDD patients, and although some inconsistencies have been reported, in general, MDD patients present a hypoactivity of the dorsolateral PFC (dlPFC) and hyperactivity of the ventromedial PFC (vmPFC) (*reviewed in* Koenigs and Grafm, 2009; Liu et al., 2017a; Levy et al., 2018). The dlPFC mediates cognitive functions such as goal-directed action, attentional control, and intention formation, while the vmPFC regulates affection,

including the generation of negative emotion (Miller and Cohen, 2001). Apart from the hyperactivity described in the vmPFC, this area is especially important for the antidepressant treatment, as is one of the brain areas selected for deep brain stimulation (DBS) that has been reported to produce antidepressant effects in rodents (Hamani et al., 2010; Veerakumar et al., 2014; Jiménez-Sánchez et al., 2016; Torres-Sánchez et al., 2018) and humans, representing a milestone in the treatment of treatment-resistant depression (TRD) (Dandekar et al., 2018; Khairuddin et al., 2020).

Although a few authors reported that drug administration into the rodent IL and PL cortices had the same effects (Sartim et al., 2016; León et al., 2017), there is accumulating evidence proving the distinct effect of some drugs in these brain regions (Lemos et al., 2010; Sierra-Mercado et al., 2011; Fuchikami et al., 2015; Gasull-Camós et al., 2017a). In particular, the IL cortex has been described as a crucial area for the pathophysiology of depression and the effect of fast-acting antidepressants (Fuchikami et al., 2015; Jiménez-Sánchez et al., 2016; Gasull-Camós et al., 2017b). For instance, the activation of the IL cortex reverses changes in the dorsal raphe nucleus of animals subjected to the chronic social defeat depression model (Veerakumar et al., 2014), reinforcing the critical role of this brain area and its outputs to the midbrain in the regulation of emotion and stress responses.

Hippocampus

The hippocampus exerts a critical role in diverse cognitive processes such as learning and the formation and consolidation of memory, and in the regulation of emotion, fear, anxiety, and stress. The high degree of hippocampal neuroplasticity may confer to this limbic area an increased vulnerability to stress and other environmental factors (Bartsch and Wulff, 2015).

In MDD patients, the reduction of the hippocampal volume is a solid finding in magnetic resonance imaging (MRI) studies (Bremner et al., 2000; Frodl et al., 2007; Cole et al., 2011). Besides, a negative correlation between the total duration of MDD and hippocampal volume was reported (Sheline et al., 1996, 1999). Inversely, antidepressant drug administration (Sheline et al., 2003; Boldrini et al., 2013; Fu et al., 2013) and electroconvulsive therapy (Nordanskog et al., 2010; Gbyl et al., 2021) increased the hippocampal volume in depressed patients.

Regarding the activity of this area in MDD patients, mixed results have been obtained (*reviewed in* Levy et al., 2018), with studies showing increased (Toki et al., 2014; Jaworska et al., 2015; Johnston et al., 2015) or decreased hippocampal activation (Milne et al., 2012; Toki et al., 2014). Despite this, a meta-analysis reported that the antidepressant treatment

decreased the hypersensitivity to negative stimuli in depressed patients, which was attributed to a decrease in hippocampus hyperactivation (Delaveau et al., 2011).

Furthermore, functional magnetic resonance imaging (fMRI) studies have revealed a disrupted functional hippocampal connectivity with the PFC and parietal cortex in MDD patients (Delaveau et al., 2011; Milne et al., 2012; Toki et al., 2014; Jaworska et al., 2015). In this regard, the effects of the manipulation of the mPFC-hippocampus connectivity on anxious- and depressive-like behaviour has been examined in preclinical studies, which led to both pro-depressant- and antidepressant-like effects (*reviewed in* Hare and Duman, 2020). Interestingly, it has been recently reported that the activation of the ventral hippocampus-mPFC pathway is both necessary and sufficient for the behavioural effects of a fast-acting antidepressant (Carreno et al., 2016).

Dorsal raphe nucleus

The serotonergic system is highly involved in depression. In the CNS, serotonergic neurones are located in the brainstem raphe nuclei and extend their projections throughout the brain. Among these nuclei, research has shown that the dorsal raphe nucleus (DRN) plays a crucial role in MDD (*see 1.2.3.1 in the Introduction section*). The DRN, through its projections to forebrain structures, modulates behavioural and emotional functioning, including mood regulation, response to stress, memory, and sleep-wake cycle (Walker and Tadi, 2020).

Some alterations in the raphe nuclei have been found in depressed patients. A morphometric study in *postmortem* samples reported a reduction in DRN area in depressed patients, while it was increased in suicide victims (Matthews and Harrison, 2012). *In vivo* transcranial sonography studies have shown that the raphe nuclei hypoechogenicity or interruption is highly prevalent in depressive patients as well as in depression associated with neurodegenerative diseases (Mijajlovic et al., 2014). In fact, the hypoechogenicity of the raphe nuclei has been proposed as a biomarker for depression in Parkinson's disease and migraine (Richter et al., 2018; Tao et al., 2019a).

The connectivity of the DRN with other brain areas implicated in depression is also altered in MDD patients. For instance, a resting-state fMRI study showed a decreased DRN connectivity with the prefrontal and mid-cingulate cortex in medication-free young adults with MDD. In addition, an increased DRN connectivity with the hippocampus and the amygdala was positively correlated with the severity of depression (Anand et al., 2019). A similar study performed with later-life depression patients reported lower functional connectivity between the DRN and bilateral posterior cingulate cortex (Ikuta et al., 2017).

In addition, the manipulation of the mPFC-DRN circuitry results in changes in depressive-like behaviour in preclinical studies (*reviewed in* Hare and Duman, 2020).

Amygdala

The amygdala is a brain area implicated in cognitive and emotional processing and is in particular involved in fear and anxiety (Aggleton, 1993; LeDoux, 2000). Although contrasting results have been obtained in volumetric studies in MDD patients (Frodl et al., 2003; Kronenberg et al., 2009; Lange and Irle, 2004; Lorenzetti et al., 2009), most of the fMRI studies have reported increased activity of the amygdala in depressed patients during the encoding of negative stimuli (Sheline et al., 2001; Peluso et al., 2009; Yang et al., 2010), being this another of the robust findings in MDD patients. This amygdala hyperactivity is associated with the characteristic higher encoding and remembering of negative rather than positive information in MDD patients, contributing to the negative bias described in this pathology (Hamilton and Gotlib, 2008; Groenewold et al., 2013). In addition, antidepressant drugs normalize amygdala activity in MDD patients (Sheline et al., 2001; Victor et al., 2010).

Interestingly, abnormal connectivity between amygdala and medial and supragenual PFC has been described in depression (Matthews et al., 2008; Arnold et al., 2012), suggesting that the top-down control during emotion processing exerted through the PFC-amygdala projections is compromised in MDD (Pezawas et al., 2005; Matthews et al., 2008). In line with these findings, the modulation of this circuit in preclinical studies induced changes in anxious- and depressive-like behaviour (*reviewed in* Hare and Duman, 2020).

Nucleus accumbens

The dopaminergic connections between the ventral tegmental area (VTA) and the NAc are a fundamental part of the neural reward system and are believed to mediate the anhedonic symptoms of depression (Nestler and Carlezon, 2006; Russo and Nestler, 2013). The activation of the VTA-NAc pathway or the NAc itself has been found attenuated in fMRI studies of MDD patients (Epstein et al., 2006; Pizzagalli et al., 2009), and in animal models of depression (Shirayama and Chaki, 2006; Friedman et al., 2008).

The antidepressant treatment has been shown to normalize this hyporesponsiveness in depressed patients (Stoy et al., 2012), and DBS targeting the NAc exerted antidepressant, hedonic, and anxiolytic effects in TRD (Schlaepfer et al., 2008; Bewernick et al., 2010) that were long-lasting (Bewernick et al., 2012). In preclinical studies, the modulation of the mPFC-NAc and the mPFC-VTA networks has also led to changes in depressive-like behaviour (*reviewed in* Hare and Duman, 2020).

The alterations described in the mentioned brain areas in MDD patients are represented in figure 1.

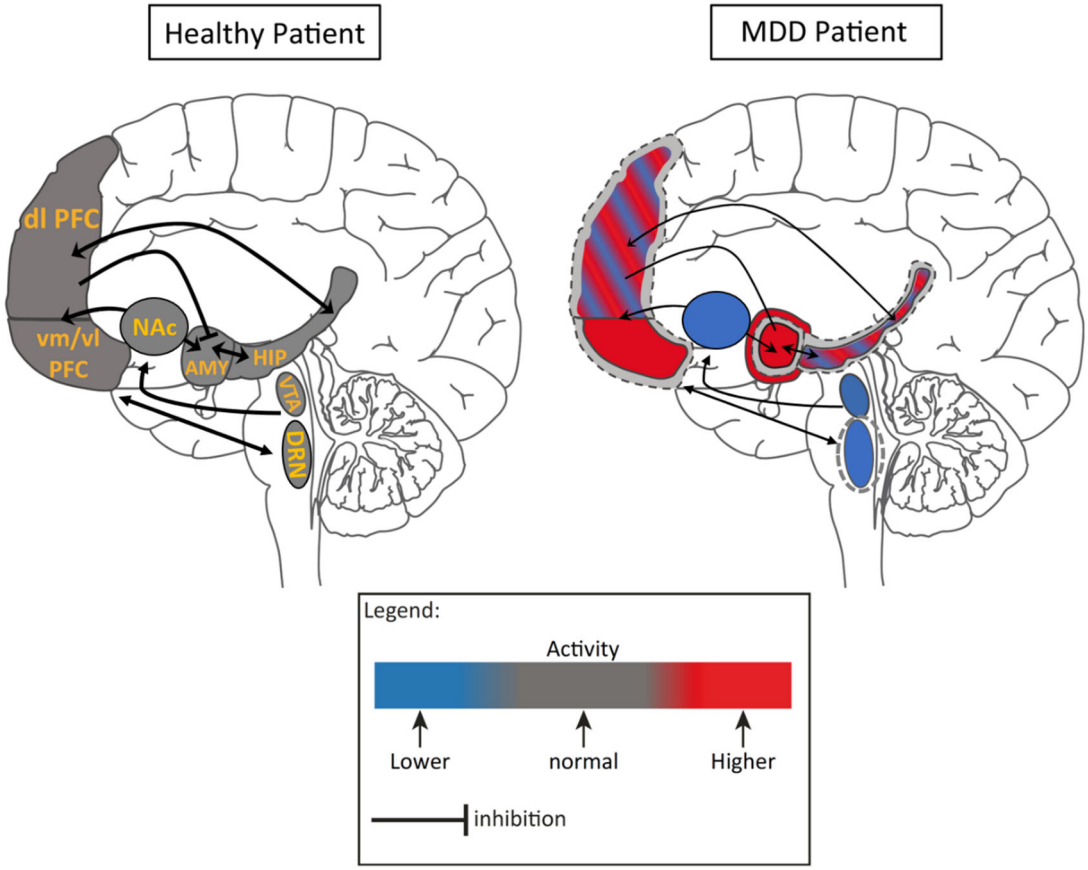


Figure 1. Summary of the neuroanatomical and functional changes observed in MDD patients. In the MDD brain, the regions that present volume changes are surrounded by dotted lines. The alternation of blue and red colours represents the discrepancies reported in the literature. The reduced connectivity is depicted with thinner lines. Modified from Levy et al. (2018).

Given these findings, in the last years, depression is starting to be considered as a brain circuit pathology or connectopathy, suggesting that the physiopathology underlying the disease is an aberrant connection between key brain areas (*reviewed in* Drevets et al., 2008; Pandya et al., 2012; Duman et al., 2019a; Hare and Duman, 2020).

1.2.3 Aetiopathological hypotheses

Since the aetiology of depression is still partially unknown, different hypotheses have been proposed regarding the molecular mechanisms that could underlie the disorder. Far from being mutually exclusive, these hypotheses complement each other. The discovery of links between these theories has led to the proposal of a new comprehensive hypothesis in the very last years, which starts to draw a clearer picture of the neurobiology of depression. The following sections contain a brief overview of the most relevant hypothesis for this thesis. As the antidepressant treatment is inseparably bound to the different hypotheses, the most used antidepressant therapies are also explained in this section.

1.2.3.1 *Monoaminergic hypothesis*

The monoaminergic hypothesis of depression was the first to be postulated. It proposes that MDD is a consequence of the lower activity of the brain monoaminergic systems, namely the serotonergic, noradrenergic, and dopaminergic systems (Schildkraut, 1967; Coppen, 1967). The monoaminergic neurones are mainly located in three brainstem nuclei: the raphe nuclei (serotonin), the locus coeruleus (noradrenaline), and the ventral tegmental area (dopamine). These neurones send their projections throughout the brain and exert a major influence on the brain areas involved in mood regulation and therefore in depression (Berton and Nestler, 2006; Hamon and Blier, 2013). Although the three monoamines have been related to depression, serotonin has gathered most of the attention, followed by noradrenaline, and finally by dopamine.

The monoaminergic hypothesis was based on the observation in the 1950s that reserpine, a drug used as antihypertensive that produces a catecholamine depletion, induced depressive states. A few years later, the antidepressant efficacy of two distinct types of drugs used for other pathologies was discovered by serendipity (Kuhn, 1958; Pare and Sandler, 1959). This led to the first two types of antidepressant drugs: the monoamine oxidase inhibitors (MAOIs) and the tricyclic antidepressants (TCAs), which exert their effects by inhibiting the degradation of monoamines or blocking their reuptake, finally leading to an increase in monoamines in the synaptic cleft. Based on the antidepressant effects of these drugs and their mechanism of action, research led to the development of more selective drugs with fewer side effects, which constitute the first line of treatment for MDD nowadays: serotonin reuptake inhibitors (SSRIs) and serotonin/norepinephrine reuptake inhibitors (SNRIs) (Gorman and Kent, 1999; Gautam et al., 2017).

Despite the hypothesis of the deficiency of monoamines has been the basis of the antidepressant treatment since it was postulated, it has been questioned several times.

First, the study of monoamine levels and their metabolites in various biological fluids and *postmortem* brain samples of MDD patients has provided inconsistent findings (Ricci and Wellman, 1990; Ashcroft et al, 1966; Asberg, 1997). Second, although the mentioned drugs increase monoamine levels in the brain in a matter of hours (Bel and Artigas, 1992), their therapeutic effect is not observed until 2 - 4 weeks of treatment (*reviewed in* Gardier et al., 1996; Duman et al., 2016).

Because of this discordancy, research started to focus on the study of the state of monoaminergic receptors and the changes induced by the chronic administration of antidepressants. An important discovery was that after the initial rapid increase in monoamines induced by classical antidepressants (the ones targeting the monoaminergic systems), the activation of the auto-receptors located in the soma of these neurones produces an inhibitory effect that downregulates the release of monoamines, which has been specially described for the serotonergic neurones in the DRN (Gartside et al., 1995; Rutter et al., 1995; Gardier et al., 1996). Thus, it has been postulated that it is not until these auto-receptors are desensitized that the antidepressant treatment is effective (Artigas et al., 1996; Gardier et al., 1996; Blier et al., 1998; Albert and François, 2010). Indeed, the time needed for this desensitization coincides with the appearance of the therapeutic effect.

The major somatodendritic auto-receptor on serotonergic neurones is the 5-HT_{1A} receptor (Sotelo et al., 1990), which is a G protein-coupled receptor that couples to inhibitory G proteins (Gi/Go) (Raymond et al., 1999). The 5-HT_{1A} receptor is also located postsynaptically all over the brain (Pazos and Palacios, 1985), with high densities in corticolimbic areas that are implicated in mood regulation (Albert et al., 1996; Albert and Lemonde, 2004). The activation of the 5-HT_{1A} receptor leads to different outcomes depending on its location, as the activation of the auto-receptor hinders the antidepressant effect, while the activation of the postsynaptic receptors is implicated in the therapeutic effect (Blier et al., 1997; Artigas, 2013; David and Gardier, 2016). This may explain why, among the 14 subtypes of serotonin receptors that have been described (Hoyer et al., 1994), the 5-HT_{1A} receptor is the most studied in depression.

Among the neural networks in which the DRN is involved, the reciprocal connectivity between the DRN and the mPFC has been demonstrated to be crucial for mood regulation and antidepressant effect (Celada et al., 2001; Challis and Berton, 2015; Fullana et al., 2020). The mPFC is one of the few forebrain areas projecting densely to the DRN and regulates the activity of the serotonergic neurones in the DRN through two distinct pathways. In the direct or monosynaptic pathway, glutamatergic neurones in the mPFC send direct projections to the serotonergic neurones in the DRN. When these pyramidal

neurones are activated, the released glutamate stimulates post-synaptic AMPA receptors on 5-HT neurones, which induces the release of 5-HT at nerve terminals such as the mPFC. In the indirect or disynaptic pathway, the mPFC pyramidal neurones synapse onto GABAergic neurones in the DRN, which in turn synapse onto 5-HT neurones. Thus, the activation of the mPFC pyramidal neurones activates the GABAergic neurones in the DRN, and the released GABA will activate post-synaptic $GABA_A$ receptors located on 5-HT neurones to inhibit their activity (Celada et al., 2001; *reviewed in* Pham and Gardier, 2019). The connectivity between the mPFC and DRN as well as the main receptors implicated are depicted in figure 2.

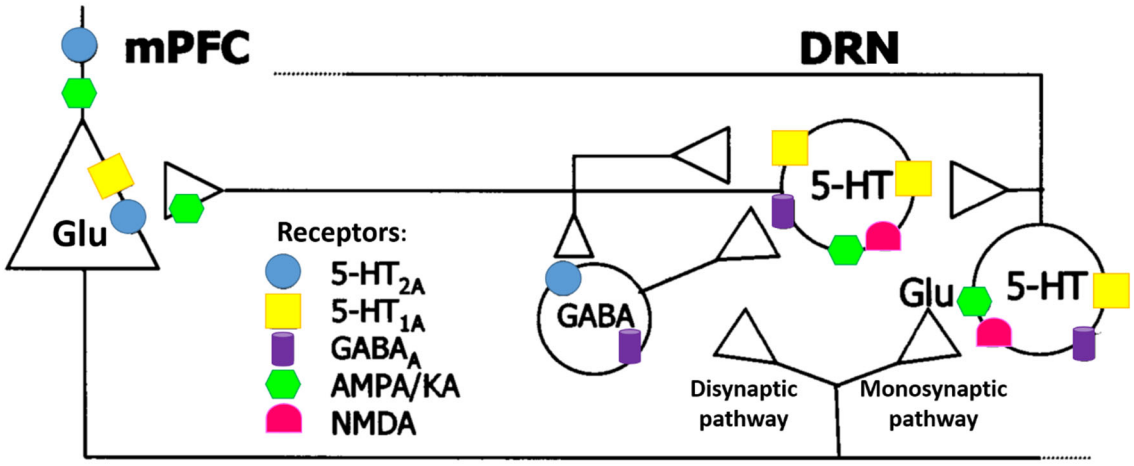


Figure 2. Schematic representation of the neuronal connectivity between the medial prefrontal cortex (mPFC) and the dorsal raphe nucleus (DRN), and the main receptors implicated in its regulation. AMPA/KA and NMDA: ionotropic glutamate receptors. Modified from Celada et al. (2001).

Although classical antidepressants are still the first-line treatment for MDD, their effectiveness is far from optimal, as the remission rates are low (Pigott et al., 2010; Fornaro et al., 2019) and about a third of patients are resistant to the treatment (Holtzheimer and Mayberg, 2011; Johnston et al., 2019), a rate that reaches the 50% of patients over time, as some patients that responded finally develop TRD (McIntyre et al., 2014; Akil et al., 2018). This added to their adverse effects –such as sexual dysfunction, drowsiness, weight gain, and dry mouth (Cascade et al., 2009)– and the long delay in the therapeutic response (Gardier et al., 1996; Duman et al., 2016), may also demoralize patients and contribute to the risk of suicide. Thus, there is an urgent need to develop faster and more effective treatments for MDD.

Despite the evidence supporting the monoaminergic hypothesis, the fact that the treatments based on this hypothesis are suboptimal, and that it fails to explain all the physiopathological findings in MDD patients, has led to the postulation of other hypotheses.

1.2.3.2 *Neurotrophic/neuroplastic hypothesis*

Several authors described a decrease in the volume of brain areas implicated in mood regulation and depression in MDD patients, especially in the hippocampus (Hp) and the prefrontal cortex (PFC) (Drevets et al., 2008; Kronmüller et al., 2009; Kandilarova et al., 2019). Exposure to stress, the most significant susceptibility factor for depression, leads to atrophy and degeneration of neurones and glia, which may account for the decreased volume observed in these brain areas in MDD (Warner-Schmidt and Duman, 2006; Duman and Aghajanian, 2012; McEwen et al., 2012). These findings served as the basis for the neurotrophic or neuroplastic hypothesis of depression, which postulates that a decrease in brain neurotrophin levels and neuronal plasticity could be the underlying cause of MDD (Duman et al., 1997).

Neurotrophic factors have a critical role in the maintenance and survival of neurones in the adult brain (Huang and Reichardt, 2001), and in neuronal plasticity (D'Sa and Duman, 2002). Brain-derived neurotrophic factor (BDNF), by far the most studied neurotrophin in depression (Park and Poo, 2013), induces its effects acting on the tropomyosin receptor kinase B (TrkB). Several studies have shown that BDNF and TrkB levels are decreased in *postmortem* brain tissue from MDD patients and suicide victims, and there is also a decrease in BDNF levels in the serum and plasma of depressed patients (*reviewed in* Castrén and Kojima, 2017). By contrast, both pharmacological and non-pharmacological antidepressant strategies reverse these changes (Chen et al., 2001; Aydemir et al., 2006; Zanardini et al., 2006). In fact, BDNF has been proposed as a biomarker for the effectiveness of the antidepressant treatment (Hashimoto, 2010), and it has been claimed to be necessary for the antidepressant action of both classical and fast-acting antidepressants (Björkholm and Monteggia, 2016). BDNF is essential for the two types of neuronal plasticity that have been related to depression and the mechanism of action of antidepressant therapies: neurogenesis and synaptic plasticity.

Neurogenesis, the process in which progenitor cells proliferate, mature, and integrate into the functional neuronal network, is limited to two regions in the adult brain: the subventricular zone, and the subgranular zone of the dentate gyrus of the hippocampus (Altman and Das, 1965). Basic research has shown that some stimulus like stress, which is used to induce depressive-like behaviour in animal models, reduce neurogenesis in the adult hippocampus, which could contribute to the reduced volume and the atrophy of this

brain area observed in MDD (Schmidt and Duman, 2007). One mechanism that could account for this effect is the dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis by stressful experiences, as the hippocampus is rich in glucocorticoid receptors. In contrast, chronic antidepressant treatment up-regulates hippocampal neurogenesis, which could reverse the stress-induced atrophy in this area (Warner-Schmidt and Duman, 2006). In addition, as the up-regulation of neurogenesis is only seen after chronic treatment, it has been suggested as an alternative explanation to the delay of the therapeutic effect of classical antidepressants (Malberg et al., 2000).

The neurotrophic/neuroplastic hypothesis of depression complements the monoaminergic hypothesis, as serotonin and noradrenaline regulate intracellular signalling pathways downstream to its receptors such as the cyclic adenosine monophosphate (cAMP) - cyclic AMP response element-binding protein (CREB) cascade (Warner-Schmidt and Duman, 2006), which regulates adult neurogenesis (Nakagawa et al., 2002). In brief, the second messenger cAMP stimulates protein kinase A (PKA), which phosphorylates and activates the transcription factor CREB (Yan et al., 2016). This pathway is altered in depressed patients (Dwivedi et al., 2003, 2004, 2006), and is up-regulated after chronic antidepressant treatment (Nibuya et al., 1996; Thome et al., 2000).

The Wnt/glycogen synthase kinase-3β (GSK3β)/β-catenin pathway is another key regulator of adult neurogenesis (Adachi et al., 2007; Wexler et al., 2009). Briefly, when the pathway is not activated, GSK3β phosphorylates β-catenin, labelling it for degradation. Wnt ligands activate the pathway, which leads to the phosphorylation and inhibition of GSK3β. This enables the cytosolic accumulation of β-catenin, a transcription factor, and its translocation to the nucleus, where it induces gene transcription. Besides its role in intracellular signalling, β-catenin has also a structural function in the cell membrane (*reviewed in* Pilar-Cuéllar et al., 2014). Decreased levels of β-catenin and phospho-GSK3β have been found in the hippocampus and PFC of animal models of depression and MDD patients (Chen et al., 2012; Karege et al., 2012; Liu et al., 2012a), whereas the chronic administration of classical antidepressants (Chen et al., 2012; Liu et al., 2012a) and other antidepressant strategies (Madsen et al., 2003; Wexler et al., 2008) have been shown to revert these changes.

The other type of neuronal plasticity affected in depression is synaptic plasticity or synaptogenesis, which is the ability to sense, assess and store complex information, and make appropriate, adaptative responses to subsequent related stimuli, and represents one of the most fundamental brain functions (Duman et al., 2016). Together with the hippocampus, the PFC is another brain area especially sensitive to stress, which leads to a reduction in the dendrites branch length and number, and the number of dendritic spines

in this brain area (Cook and Wellman, 2004; Radley et al., 2004; Qiao et al., 2016). This reduction in synaptic plasticity has also been associated with the vulnerability to suffering depression (Blugeot et al., 2011), and is reverted by antidepressant treatments (Magariños et al., 1999; Hajszan et al., 2005; Moda-Sava et al., 2019; Zhang et al., 2019a).

The mammalian target of rapamycin (mTOR) pathway participates in different physiological functions and has a key role in synaptic plasticity through the regulation of protein synthesis translation (Hay and Sonenberg, 2004). The mTOR protein is a large serine/threonine kinase activated by phosphorylation. mTOR acts as a signal unifier inside the cells, and is regulated by many signalling pathways: the stimulation of several receptors by neurotransmitters, trophic factors, and mitogens leads to the activation of phosphatidylinositol 3-kinase (PI3K) - protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) pathways, which in turn activate mTOR (Swiech et al., 2008). *Postmortem* studies have shown that the mTOR pathway and its downstream signaling targets, as well as the levels of prominent postsynaptic proteins, are decreased in the PFC of MDD patients (Jernigan et al., 2011). Similar results have been reported in the hippocampus and PFC of animal models of depression as the chronic unpredictable mild stress (Zhu et al., 2013; Zhong et al., 2014; Yao et al., 2020).

In the last years, the mTOR pathway has gained great importance due to its implication in the mechanism of action of fast-acting antidepressant drugs, and it is considered a new target for antidepressant treatment. The most revolutionary discovery of the last decades in the treatment of depression was that a single administration of ketamine, an N-methyl-D-aspartate (NMDA) receptor antagonist, produced antidepressant effects in TRD patients as fast as 2 h after treatment, that lasted for one week (Berman et al., 2000; Zarate et al., 2006). A huge number of clinical and preclinical studies have replicated this finding. The mechanism of action proposed for fast-acting antidepressants as ketamine is the rapid increase in BDNF release, and the increase in mTOR signalling via activation of AKT and ERK pathways, which increases synaptic number and function in the PFC (Li et al., 2010; Duman et al., 2012; Miller et al., 2014; Zhou et al., 2014a).

A compilation of the signaling pathways implicated in neurogenesis and synaptic plasticity is depicted in figure 3.

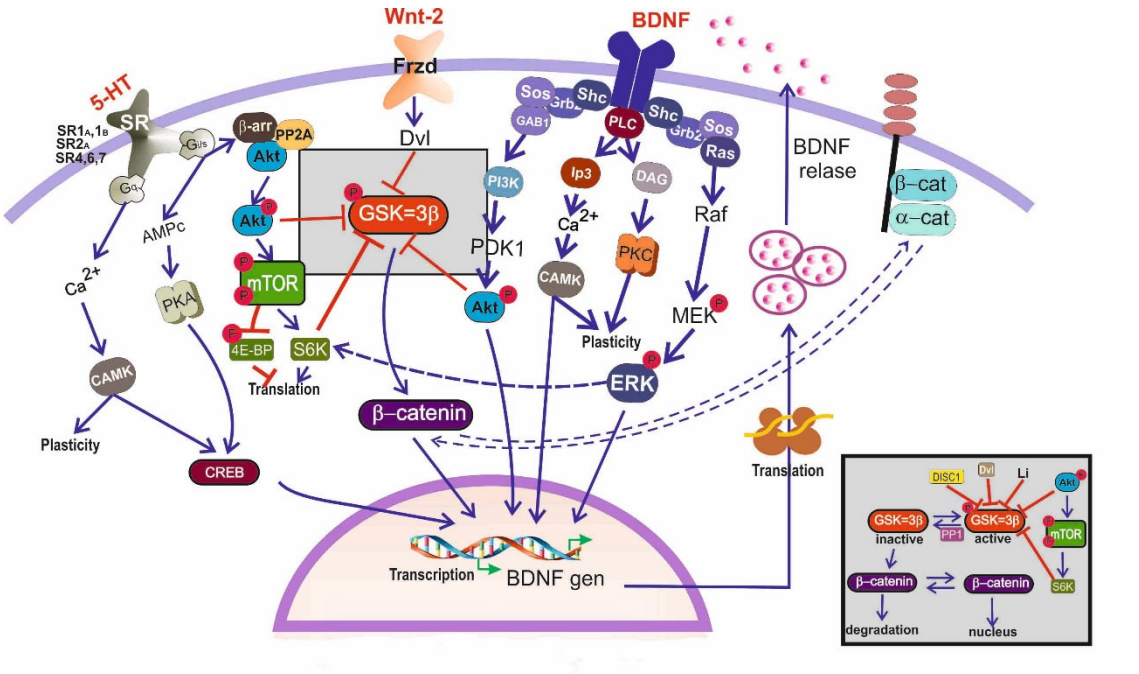


Figure 3. Main signalling pathways implicated in neurogenesis and synaptic plasticity. Modified from Pilar-Cuéllar et al. (2014).

Despite strong evidence supporting the neurotrophic/neuroplastic hypothesis of depression, not all the findings fit well with this theory (*reviewed in* Schmidt and Duman, 2007). Interestingly, while a reduction in neuronal plasticity is described in some brain areas (PFC and hippocampus), the opposite scenario seems to take place in other areas such as the amygdala and the nucleus accumbens, as MDD patients present higher volume and increased glucose metabolism in the amygdala (Drevets et al., 1992; Frodl et al., 2002), and the blockade of BDNF signaling in the nucleus accumbens produces antidepressant-like effects (Eisch et al., 2003). Thus, neurotrophic and neuroplastic alterations in depression are region-specific. Moreover, some reports demonstrate that the ablation of neurogenesis does not induce depressive-like effects in rodents and that neurogenesis is not always required for the antidepressant effect (*reviewed in* Petrik et al., 2012).

1.2.3.3 *Glutamatergic hypothesis*

The surprising antidepressant effects of ketamine (Berman et al., 2000; Zarate et al., 2006) mediated by the blockade of the glutamate NMDA receptor, originated a series of preclinical and clinical studies directed to elucidate the role of glutamate in depression. Glutamate is the most abundant excitatory neurotransmitter in the nervous system (Orrego and Villanueva, 1993). Its effects are mediated by two principal types of

receptors: the ionotropic glutamate receptors, comprising NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors; and the metabotropic glutamate receptors (mGluR1 to mGluR8). The clearance of extracellular glutamate is mainly performed by excitatory amino acid transporters (EAATs) located on neighbouring glial cells and, to some extent, on neurones (O'Shea, 2002). This process is of vital importance, as it is well-known that an excess of extracellular glutamate causes excitotoxicity, neuronal degeneration, and death (Olney, 1969; Lau and Tymianski, 2010). The excitotoxic effects of glutamate have been largely attributed to excessive activation of extrasynaptic NMDA receptors (Hardingham et al., 2002).

The glutamatergic hypothesis of depression postulates that the glutamatergic system is a primary mediator of MDD and a final common pathway for the therapeutic actions of many antidepressant drugs (*reviewed in* Sanacora et al., 2012; Thompson et al., 2015). Preclinical studies have shown that acute stress increases extracellular glutamate levels in the mPFC and hippocampus, which has led to the hypothesis that glutamate-induced excitotoxicity could produce the atrophy of these brain regions observed in stress-related pathologies (Popoli et al., 2011). This increase in extracellular glutamate could be due to a lower re-uptake provoked by a decreased number in fronto-limbic glial cells, which has been reported in preclinical stress models (Czéh et al., 2007; Banasr et al., 2010) and in MDD patients (Ongür et al., 1998; Rajkowska and Miguel-Hidalgo, 2007; Cobb et al., 2016). The excessive extracellular glutamate would have two consequences: 1) negative feedback in glutamatergic signaling through the activation of presynaptic $mGlu_{2/3}$ auto-receptors, leading to a reduction in synaptic glutamate (Kugaya and Sanacora, 2005), and 2) hyperactivation of extrasynaptic NMDA receptors inducing excitotoxicity (Vanhoutte and Bading, 2003).

The glutamatergic hypothesis of depression is closely linked to the neurotrophic/neuroplastic hypothesis, as AMPA and NMDA receptors are involved in synaptic plasticity. Long-term potentiation, the cellular substrate of learning and memory, can be elicited by the activation of NMDA receptors, which increases the number of AMPA receptors in the synaptic membrane (Lüscher and Malenka, 2012). The stimulation of AMPA receptors induces BDNF release and activates the mTOR pathway, therefore inducing synaptic plasticity (Akinfiresoye and Tizabi, 2013; Zhou et al., 2014a; Gerhard et al., 2016). Moreover, it has been reported that the activation of NMDA synaptic receptors increases CREB activity and BDNF expression, in contrast to the opposite outcomes obtained following NMDA extrasynaptic receptors activation (Hardingham et al., 2002; Vanhoutte and Bading, 2003). Thus, the decreased synaptic glutamate in depression would reduce synaptic plasticity, and the increased extracellular glutamate levels would promote excitotoxicity, in both cases leading to brain atrophy.

The fast antidepressant effects of drugs targeting the glutamatergic system further strengthened the glutamatergic hypothesis of depression. The most studied is ketamine, for which the proposed mechanism of action is the antagonism of NMDA receptors located in GABAergic interneurones, which would disinhibit glutamatergic neurones and produce a glutamate burst that activates AMPA receptors and increases synaptic plasticity (Duman et al., 2012). Despite the fast antidepressant effects of ketamine, it produces important adverse effects such as psychotomimetic and dissociative effects that vanish 2 h after administration and has a high addictive potential (Krystal et al., 2013). Even though, S-Ketamine, the S (+) enantiomer of ketamine with a higher affinity for the NMDA receptor than the R-ketamine, has been recently approved by the Food and Drug Administration (FDA) for treating TRD.

In this sense, great efforts have been directed to the development of other fast-acting antidepressant drugs targeting the glutamatergic system (*reviewed in* Gerhard et al., 2016; Murrough et al., 2017). Among them, there are $mGlu_{2/3}$ receptor antagonists (Fukumoto et al., 2016), antagonists of the GluN2B subunit of the NMDA receptor (Li et al., 2010), antagonists of the glycine site in the GluNR1 subunit of the NMDA receptor (Moskal et al., 2017), and ampakines, positive allosteric modulators (PAMs) of the AMPA receptor (Li et al., 2001; Gordillo-Salas et al., 2020). Other fast antidepressant strategies such as deep brain stimulation (DBS) in the PFC (Jimenez-Sánchez et al., 2016), the muscarinic receptor antagonist scopolamine (Wohleb et al., 2016), and cannabidiol (CBD) administration (Linge et al., 2016) also modulate glutamate levels.

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain and is responsible for the control and fine-tuning of excitatory neurotransmission. In this regard, an excitatory/inhibitory imbalance has been widely reported in depression and other psychiatric disorders (Fee et al., 2017; Lener et al., 2017; Selten et al., 2018). Although the determination of glutamate and GABA brain levels have generated mixed results –which may be in part due to region-specific alterations– (Lener et al., 2017; Duman et al., 2019a), the gathered evidence points towards a deficit in both excitatory and inhibitory neurones in depression, while fast-acting antidepressant drugs targeting those neurotransmitter systems revert these deficits (Duman et al., 2019a; Pham and Gardier, 2019). The role of GABAergic dysfunction in the pathophysiology of depression has gained increasing attention in the very last yearsand has been proposed as the primary determinant that would trigger the glutamatergic alterations and lead to depressive disorder (Fogaça and Duman, 2019; Oh et al., 2019). Drugs targeting the GABAergic system also produce rapid antidepressant effects (*reviewed in* Fogaça and Duman, 2019). For instance, negative allosteric modulators (NAMs) of $GABA_A$-α5 receptors (Zanos et al., 2017; Xiong et al., 2018), and the positive allosteric modulator of $GABA_A$ receptors brexanolone, indicated

for treating postpartum depression (Kanes et al., 2017), that has recently been approved by the FDA.

A graphical representation of the principal alterations described in the glutamatergic hypothesis and of the targets of fast-acting antidepressant drugs is shown in figure 4.

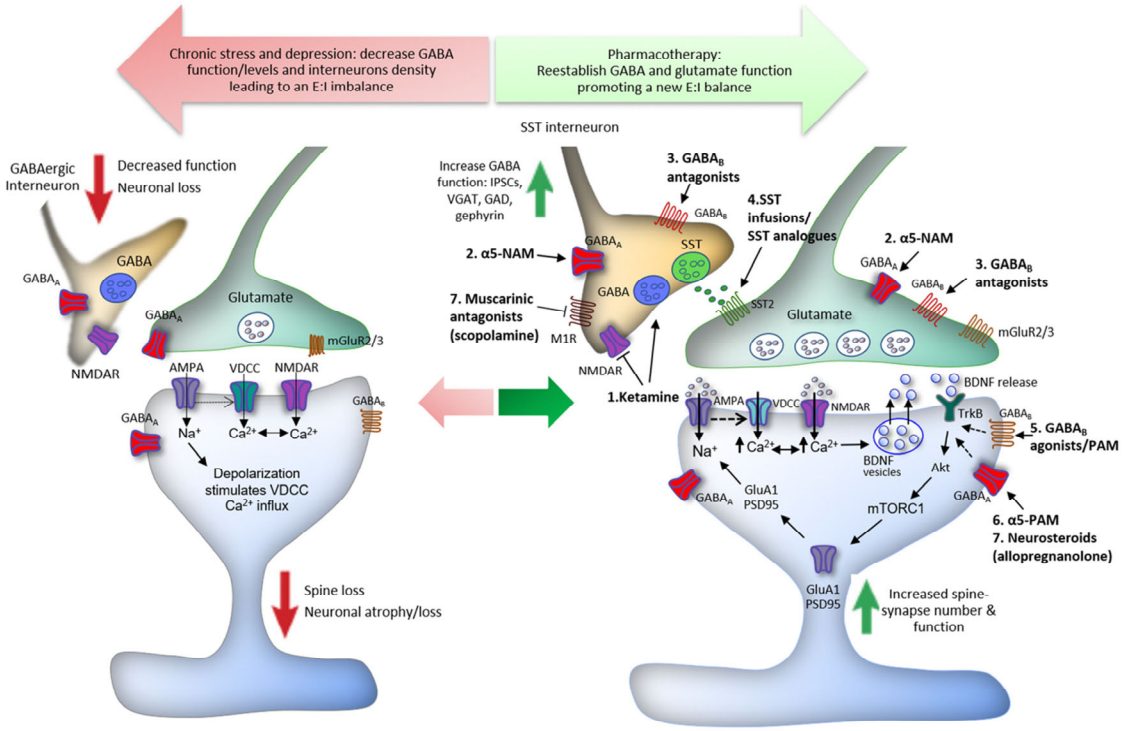


Figure 4. Glutamatergic and GABAergic alterations in depression and mechanism of action of fast-acting antidepressants. Obtained from Fogaça and Duman (2019).

Therefore, drugs targeting the glutamatergic and GABAergic systems have expanded our knowledge about the neurobiology of depression and show promising fast antidepressant effects, representing breakthrough strategies for the treatment of MDD (Fogaça and Duman, 2019). The understanding of their mechanism of action will contribute to the improvement of MDD treatment.

1.2.3.4 *Neuroinflammatory hypothesis*

The neuroinflammatory hypothesis of depression has gained evidence and popularity in the last years. It is based on three main facts. First, some inflammatory diseases such as psoriasis, systemic vasculitis, arthritis, and COVID-19 present comorbid depression (Dregan et al., 2019; Nerurkar et al., 2019; Mazza et al., 2020). In fact, a clear relationship between inflammation and depression was observed with interferon-alpha (IFNα) administration to hepatitis C patients, which induced depressive symptoms directly associated with treatment duration (Raison et al., 2005). Second, activation of the inflammatory system has been found in MDD patients (Maes, 1999; Miller et al., 2009). For instance, there is an increase in pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumour necrosis factor-alpha (TNFα) in the blood (Dowlati et al., 2010; Liu et al., 2012b; Young et al., 2014), cerebrospinal fluid (CSF) (Wang and Miller, 2018), and *postmortem* brain tissue of depressed patients (Enache et al., 2019). Moreover, an increase in microglial markers –the resident immune cells of the brain– has been reported in the brain of MDD patients, although this finding is less consistent (Enache et al., 2019). Third, some anti-inflammatory agents such as a TNFα antagonist and non-steroidal anti-inflammatory drugs produce antidepressant effects in MDD patients (Raison et al., 2013; Köhler-Forsberg et al., 2019; Bai et al., 2020), and improve the antidepressant response when co-administered with antidepressants (Akhondzadeh et al., 2009; Abbasi et al., 2012; Müller, 2013).

The inflammatory hypothesis of depression postulates that the disease is caused by chronic low-grade inflammation (Maes, 2011), and it has been suggested that inflammation is the link between chronic stress and depression (Barone, 2019). The proposed physiopathological mechanism is as follows: the exposure to different kind of stressors increases systemic and brain levels of pro-inflammatory cytokines such as TNFα and IL-6 (Zhou et al., 1993; Maes et al., 1998; Kubera et al., 2011), while reduces the levels of anti-inflammatory cytokines such as IL-10 (Voorhees et al., 2013; Labaka et al., 2017). Pro-inflammatory cytokines increase the activity of the indoleamine 2,3-dioxygenase (IDO) enzyme (Fujigaki et al., 2006; Godbout et al., 2008). In addition, stress hormones increase the expression of the tryptophan 2,3-dioxygenase (TDO) enzyme (Danesch et al., 1987).

More than 95% of the tryptophan is metabolized through the kynurenine pathway (Bender, 1983). The rate-limiting step of the kynurenine pathway is the conversion of tryptophan to kynurenine, which is mediated by the TDO and IDO enzymes. Under physiological conditions, the major conversion of tryptophan to kynurenine is performed by TDO in the periphery (mainly in the liver), while IDO activity is low (Badawy, 2017).

However, under inflammatory conditions, the activity of IDO is dramatically induced and participates significantly in kynurenine production (Cervenka et al., 2017). As tryptophan is the precursor of serotonin, the increase in IDO activity will lead to a reduction in serotonin levels (Dantzer et al., 2008), which links the neuroinflammatory and the monoaminergic hypotheses of depression. Besides, the increase in kynurenine has important neuropsychiatric implications, since it crosses the blood brain barrier (BBB) to the brain, where it is metabolized along two catabolic branches into different neuroactive compounds (*reviewed in* Dantzer et al., 2008; Barone, 2019). On the one hand, in the "neuroprotective branch" kynurenine is metabolized to kynurenic acid through the kynurenine amino-transferase (KAT) enzyme, which is mainly expressed in astrocytes in the brain. The kynurenic acid is an NMDA receptor antagonist (Birch et al., 1988), and produces neuroprotective effects by different mechanisms, such as reducing extracellular glutamate levels (Konradsson-Geuken et al., 2010). On the other hand, in the "neurotoxic branch" kynurenine is metabolized to 3-hydroxy kynurenine (3-HK) by the kynurenine 3-mono-oxygenase (KMO) enzyme, which is mainly expressed in microglial cells. Other products of this branch are quinolinic acid and nicotinamide adenine dinucleotide (NAD^+). These three metabolites produce neurotoxicity, as potent oxidative properties have been described for 3-HK and NAD^+ (Nakagami et al., 1996; Sayre et al., 2008), and quinolinic acid acts inhibiting the astrocytic glutamate reuptake (Tavares et al., 2005) and as an NMDA receptor agonist (Stone and Perkins, 1981). Importantly, the "neurotoxic branch" is mainly activated during inflammation (Guillemin, 2012), as pro-inflammatory cytokines also activate KMO (Connor et al., 2008). The weight of evidence is now starting to suggest that the principal driver of depression is an imbalance between neurotoxic and neuroprotective kynurenine metabolites (Savitz, 2017). These pieces of evidence show that the kynurenine pathway links the neuroinflammatory and the glutamatergic hypotheses of depression. A simplified diagram of the kynurenine pathway is shown in figure 5.

Supporting these proposed pathogenic mechanisms, accumulating evidence shows that the kynurenine pathway is altered in various types of depression, suggesting that the activation of this pathway could be a common marker of depressive symptoms (*reviewed in* Parrott and O'Connor, 2015; Barone, 2019). For instance, Maes et al. (2002) reported increased levels of kynurenine, and reduced tryptophan levels in plasma, and a higher kynurenine/tryptophan ratio that were positively correlated with the intensity of depressive symptoms. Also, kynurenine plasma levels were increased in suicide attempters with MDD (Sublette et al., 2011). Moreover, increased quinolinic acid and decreased kynurenic acid levels have been reported in depressive patients (Myint et al., 2007; Schwieler et al., 2016; Ogyu et al., 2018; Verdonk et al., 2019), and in the CSF of suicide attempters (Erhardt et al., 2013; Bay-Richter et al., 2015). An imbalance in the

kynurenine pathway towards the "neurotoxic branch" has been specially related to TRD and suicidality (Serafini et al., 2017; Verdonk et al., 2019). Furthermore, the kynurenic acid/quinolinic acid ratio has been negatively associated to the severity of anhedonia (Savitz et al., 2015).

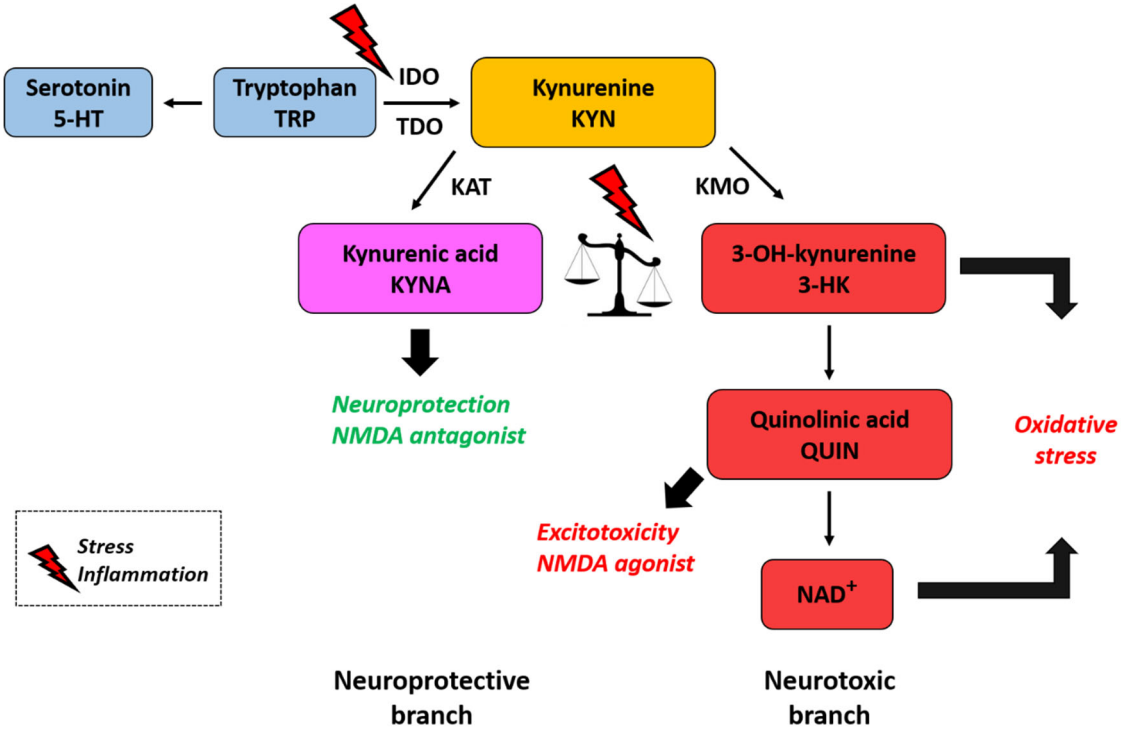


Figure 5. Crucial steps in the kynurenine pathway. Modified from Barone (2019).

Another set of findings supporting the neuroinflammatory hypothesis is that the currently used antidepressant drugs also have anti-inflammatory properties. For example, different classical antidepressants inhibited the release of pro-inflammatory cytokines and induced anti-inflammatory cytokines in preclinical and clinical studies (Maes et al., 1999; Connor et al., 2000; Kubera et al., 2001). Laugeray et al. (2016) reported that, apart from decreasing pro-inflammatory cytokines in the periphery, fluoxetine also modulated the kynurenine pathway in the brain of an animal model of depression. This has also been reported for the fast-acting antidepressant ketamine (Zunszain et al., 2013; Verdonk et al., 2019).

Interestingly, the levels of inflammation affect the antidepressant response. High levels of inflammation in MDD patients are associated with resistance to standard antidepressant

treatment with SSRIs (Lanquillon et al., 2000; Cattaneo et al., 2013) and other antidepressant drugs such as lithium (Sluzewska et al., 1997). Conversely, ketamine exerts antidepressant effects in TRD patients, who often present increased inflammation (Chamberlain et al., 2019; Strawbridge et al., 2019). Importantly, it has been recently reported that the TRD patients with the highest concentrations of serum IL-6 were the most responsive to ketamine (Yang et al., 2015). Similarly, it has been described that the kynurenic acid/quinolinic acid ratio is a predictor of ketamine response in TRD patients and that the reduction in quinolinic acid levels after ketamine administration predicts the improvement of depressive symptoms (Verdonk et al., 2019).

In all, the neuroinflammatory hypothesis could explain other alterations described in MDD (*see next section*) and has emerged as a new opportunity for the urgent need for improvement of TRD and suicidality treatment.

1.2.3.5 *New comprehensive hypothesis: interconnection between depression hypotheses*

As previously mentioned, stress has been thoroughly related to the development of depression and has been involved in the different hypotheses of depression. In fact, another postulated hypothesis is the *stress-related neuroendocrine hypothesis of depression*, which suggests that the hypothalamic-pituitary-adrenal (HPA) axis –the primary mediator of the stress response– is overactivated in MDD (Boyer, 2000) and that its normalization may be needed to achieve remission (Pariante and Lightman, 2008). The HPA axis and the immune system are closely related, as one system can activate the other (Ramírez et al., 2018). Thus, the low-grade chronic inflammation described in depressed patients could be a consequence of stress exposure, but also other stimuli inducing inflammation could hyperactivate the HPA axis. Some environmental risk factors for depression such as psychosocial stressors, poor diet, physical inactivity, obesity, and altered gut permeability have been associated with systemic inflammation (*reviewed in* Berk et al., 2013). Therefore, the interaction between gene variants and environmental factors that confer susceptibility for depression may activate both the HPA axis and the immune system, which would trigger a series of molecular cascades finally resulting in depression.

As stated before, the activation of the immune system and the kynurenine pathway could lead to the other physiopathological alterations described in MDD, such as HPA axis hyperactivity, reduced monoamine levels, neurotrophic factors, neuroplasticity, and neurogenesis, altered glutamatergic/GABAergic systems, neurotoxicity, and reduced volume of important brain areas such as PFC and hippocampus. Based on this, in the last years, an increasing number of scientific publications claim for a new and more integrative

hypothesis of depression (Maes et al., 2011; Cai et al., 2015; Miller and Raison, 2016; Dean and Keshavan, 2017; Barone, 2019).

The activation of the kynurenine pathway and the subsequent decrease in tryptophan levels is not the only way in which inflammation can decrease monoamine levels. An inflammatory state decreases the availability of tetrahydrobiopterin (BH4), a cofactor that is needed during serotonin, dopamine, and noradrenaline synthesis. Also, cytokines increase the expression and function of the presynaptic transporters of these neurotransmitters, leading to increased monoamine reuptake (*reviewed in* Miller and Raison, 2016; Felger, 2018).

Regarding the alterations in the glutamatergic system, cytokines can decrease the expression of astrocytic glutamate transporters and increase the release of glutamate from activated microglia and astrocytes (Tilleux and Hermans, 2007; Ida et al., 2008; Takaki et al., 2012), therefore leading to a glutamate/GABA imbalance. This increase in extracellular glutamate levels, together with the increased activation of the "neurotoxic branch" of the kynurenine pathway, would lead to an overactivation of extrasynaptic NMDA receptors and increased excitotoxicity. In turn, this would result in a decreased production of trophic factors such as BDNF (Hardingham et al., 2002; Vanhoutte and Bading, 2003), which could compromise important processes that are altered in depression as neuroplasticity and neurogenesis (D'Sa and Duman, 2002). In consequence, neuroinflammation generates a perfect cocktail for cell atrophy and death, which would be reflected in the decrease in the volume of susceptible brain areas such as the PFC and hippocampus (Opel et al., 2019; Green et al., 2020; Ironside et al., 2020), and would lead to altered brain connectivity (Drevets et al., 2008; Pandya et al., 2012; Duman et al., 2019a; Hare and Duman, 2020).

The dysregulation of the HPA axis can contribute to all the aforementioned physiopathological alterations in MDD. Apart from triggering inflammation (García-Bueno et al., 2008), the hyperactivation of the HPA axis can decrease monoamine levels (Ahmad et al., 2010) —as the HPA axis has complex interactions with the monoaminergic systems (Pompili et al., 2010)—, decrease BDNF levels (Kunugi et al., 2010), downregulate adult hippocampal neurogenesis (Anacker et al., 2013), and increase glutamate release (Moghaddam, 1993, 2002). A graphical representation of the "new comprehensive hypothesis of depression" is presented in figure 6.

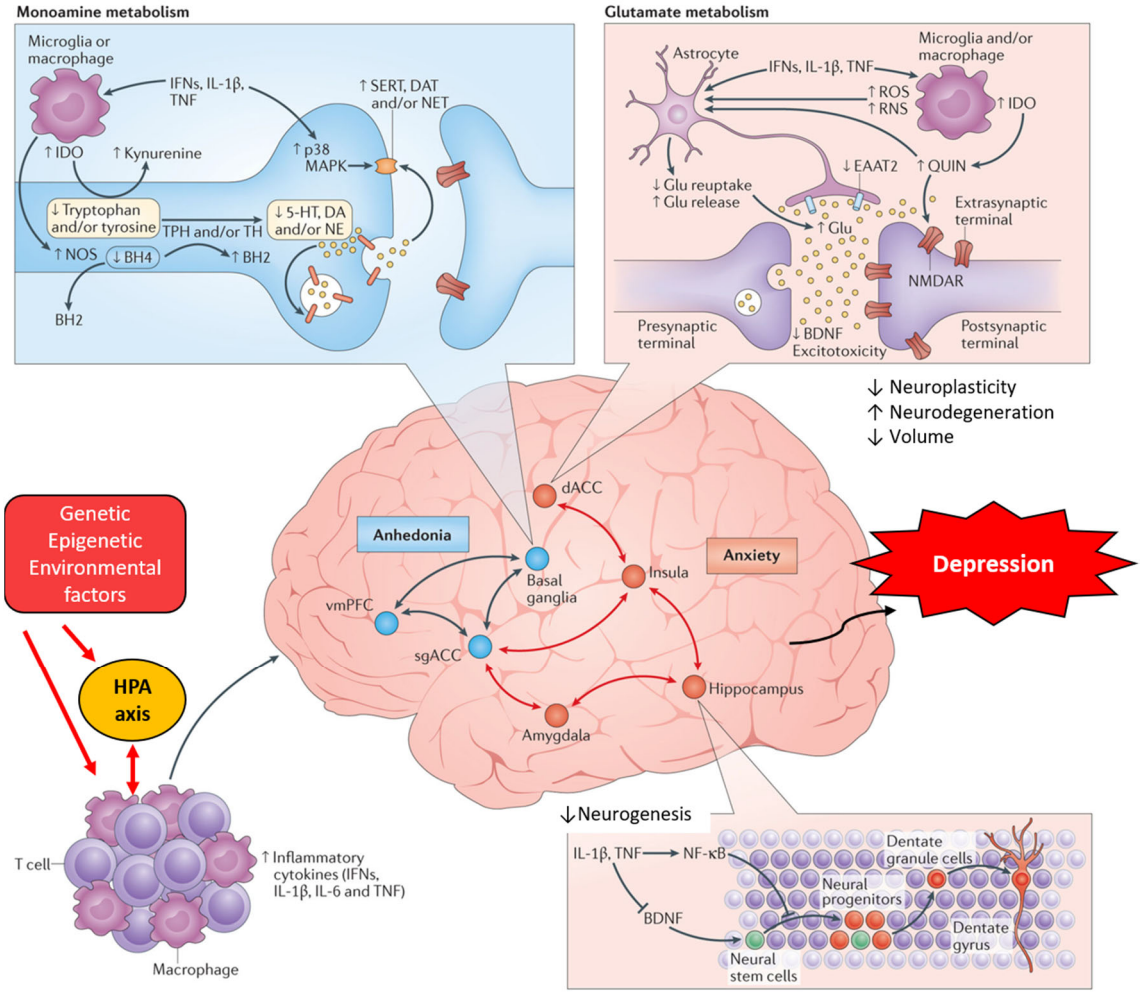


Figure 6. Overview of the "new comprehensive hypothesis of depression". Modified from Miller and Raison (2016).

This integrated view broadens our understanding of the physiopathological mechanisms implicated in depression, and at the same time highlights its complexity and its high interconnection. Although more research is needed to achieve an even deeper knowledge of the disease that enables the development of better treatments, the findings of the last years have allowed great advancements in the field.

1.2.4 Endocannabinoid system and depression

Research about the endocannabinoid system (ECS) started due to the identification of Δ^9-tetrahydrocannabinol (Δ^9-THC), the principal psychotropic component of the *Cannabis sativa* plant (Mechoulam and Gaoni, 1965). Since then, more than two decades were

needed until the discovery of the ECS, achieved with the identification of the cannabinoid receptors (Matsuda et al., 1990; Munro et al., 1993) and the endogenous ligands (Devane et al., 1992; Mechoulam et al., 1995).

The ECS is composed of two G-protein coupled receptors (GPCRs), cannabinoid receptor 1 and 2 (CB_1 and CB_2), that are mainly coupled to inhibitory G proteins (Howlett et al., 2002), and two major endogenous signaling molecules or endocannabinoids: N-arachidonoylethanolamine (anandamide/AEA) and 2-arachidonoylglycerol (2-AG). AEA is mainly synthesized by N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) and degraded by fatty acid amide hydrolase (FAAH), and 2-AG is mainly synthesized by diacylglycerol lipase (DAGL) and degraded by monoacylglycerol lipase (MAGL). A peculiar feature of the ECS is that endocannabinoids are synthesized on demand and produce retrograde signalling acting presynaptically on both cannabinoid receptors and causing the suppression of the synaptic transmission. As postsynaptic activity increases the production of endocannabinoids (Kano et al., 2009), the ECS functions as a homeostatic mechanism to terminate synaptic transmission. The main ECS components and their function are depicted in figure 7.

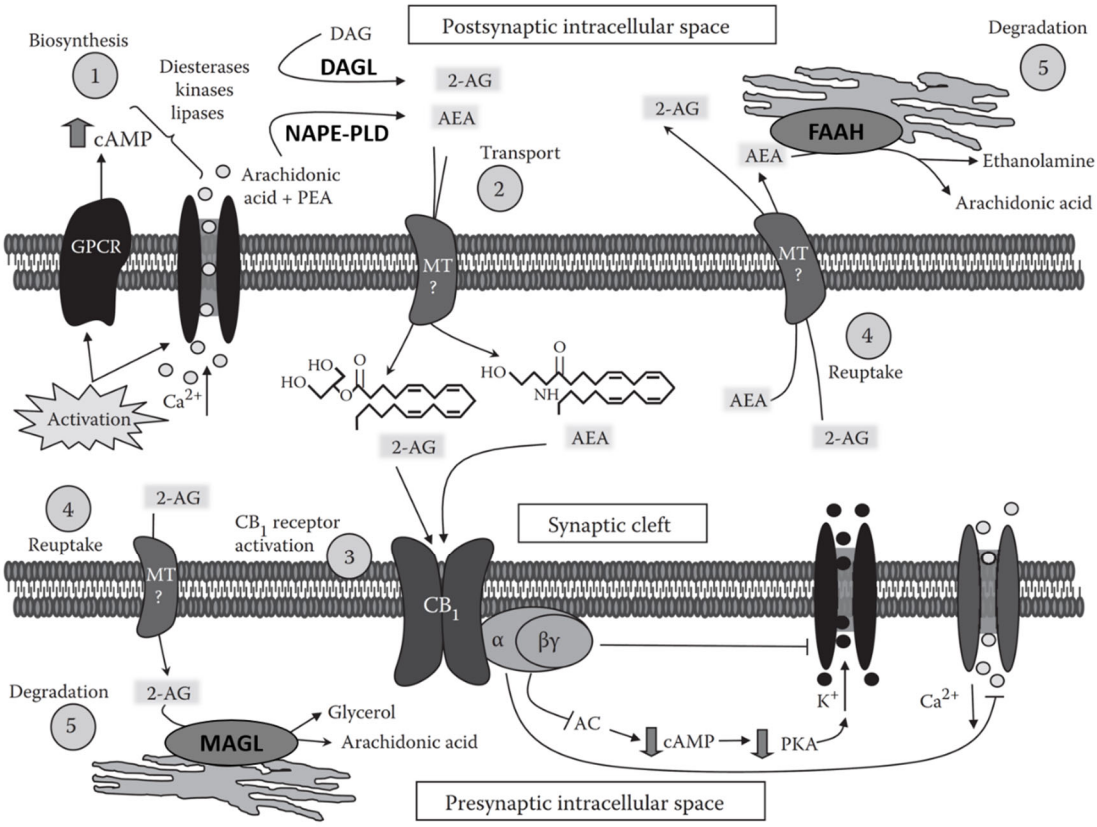


Figure 7. Simplified schematic diagram of the principal components of the endocannabinoid system and the endocannabinoid life cycle (1-5). Modified from McLaughlin et al. (2011).

The ECS is involved in a plethora of functional mechanisms in the central nervous system (CNS) including emotional regulation, motivational behaviour, and cognition (Mechoulam and Parker, 2013; Ashton et al., 2017). CB_1 receptors are the most abundant brain GPCRs and are widely expressed throughout the brain (Mechoulam and Parker, 2013), being abundant in brain areas related to the stress response and depression such as the amygdala, hypothalamus, PFC, Hp, and nucleus accumbens (Tsou et al., 1998; Hu and Mackie, 2015). As CB_1 receptors are located on virtually all types of neurones (Hu and Mackie, 2015), the ECS regulates the other neurotransmitter systems including the glutamatergic, GABAergic, and monoaminergic systems. Differently, CB_2 receptors are less numerous and are located both in the peripheral immune system and in the CNS, supporting a neuroprotective role against inflammation (Ashton and Glass, 2007). Apart from inhibiting adenylate cyclase, both $CB_{1/2}$ receptors activate the MAPK pathways, especially the ERK pathway (Galve-Roperh et al., 2002; Howlett et al., 2002), and the CB_1 receptor can regulate ionic channels (Howlett et al., 2002) and activate the PI3K-Akt pathway (Gómez del Pulgar et al., 2000). Therefore, both receptors induce changes in gene expression and have a role in neuronal plasticity.

Several studies support the involvement of the ECS in the aetiopathogenesis of depression and other mental disorders (*for reviews see* McLaughlin et al., 2011; Hill and Patel, 2013; Ibarra-Leuce et al., 2018; Navarrete et al., 2020). As the ECS regulates neurotransmission, neurogenesis (Galve-Roperh et al., 2007), synaptic plasticity (Lu and Mackie, 2016), inhibits the HPA axis activity (Gorzalka and Hill, 2009), and exerts neuroprotective effects against excitotoxicity and inflammation (Ashton et al., 2017), it is not surprising that different abnormalities in the ECS have been found in MDD patients. For instance, some genetic variants of the genes encoding for the $CB_{1/2}$ receptors and the FAAH enzyme have been reported to modulate the depressive phenotype and/or the response to antidepressant treatments, and changes in the brain expression or functionality of the $CB_{1/2}$ receptors have been described in MDD patients (*reviewed in* Ibarra-Leuce et al., 2018). Besides, lower levels of circulating endocannabinoids in depressive patients (Hill et al., 2008a, 2009), and an association between endocannabinoid levels and the duration and severity of the depressive episodes (Hill et al., 2008a) have been described. Recently, lower levels of endocannabinoids in the ventral striata of MDD female patients have been reported (Dong et al., 2020).

Much of the evidence supporting the implication of the ECS in depression comes from preclinical studies. A good example is the findings observed in knock-out (KO) animals for the CB_1 receptor, which present a higher vulnerability to stress-induced anhedonia, an anxious-like behaviour (Martin et al., 2002), and a depressive-like behaviour both in the tail suspension test (TST) and forced swimming test (FST) (Aso et al., 2008; Steiner et al.,

2008). These animals also have lower BDNF expression in the hippocampus (Aso et al., 2008; Steiner et al., 2008) and higher corticosterone levels after stress (Urigüen et al., 2004; Aso et al., 2008), which suggests hyperactivity of the HPA axis, and reproduces one of the major findings in depressed patients.

Regarding the effects of pharmacological interventions, the chronic activation of CB_1 receptors by Δ^9-THC administration also induced anhedonia and depressive-like behaviour, together with a reduction in synaptic plasticity in some brain areas (Rubino et al., 2008, 2009). Besides, the CB_1 receptor antagonism has been shown to produce antidepressant-like effects in both the FST and TST (Shearman et al., 2003; Tzavara et al., 2003; Griebel et al., 2005). Although some of these findings may seem contradictory, all of them point to a relationship between the ECS and depressive-related alterations and suggest that the ECS homeostasis is crucial for emotional stability.

In view of these pieces of evidence, the CB_1 receptor appeared as an interesting target for the treatment of mood disorders. However, its widespread expression in the brain and the fact that this receptor mediates most of the psychoactive effects of cannabinoids, discard it as a good pharmacological target. Indeed, the direct pharmacological modulation of the CB_1 receptor has provided disappointing results, and the fact that rimonabant, an inverse CB_1 receptor antagonist approved to treat obesity, was withdrawn from the market because it induced anxiety, depression, and suicidal ideation (Christensen et al., 2007; Di Marzo and Després, 2009), diverted the attention towards other targets in the ECS.

In recent years, much attention has been paid to the manipulation of endocannabinoid levels by inhibiting its degrading enzymes FAAH and MAGL, or blocking its reuptake (*reviewed in* Navarrete et al., 2020). Resembling the findings in depressed patients, the chronic unpredictable stress model in rats induced lower levels of AEA in brain areas such as the PFC and hippocampus (Hill et al., 2008b), and increased FAAH levels (Reich et al., 2009). In addition, FAAH KO mice display anxiolytic- and antidepressant-like responses (Bambico et al., 2010), present increased proliferation of hippocampal neural progenitor cells (Aguado et al., 2005), and have an increase in the firing rate of the DRN serotonergic neurones together with an enhanced 5-HT_{1A} receptor activity in the hippocampus (Bambico et al., 2010). In accordance, the administration of FAAH (Bortolato et al., 2007; Realini et al., 2011; Vinod et al., 2012) and MAGL (Zhang et al., 2015a) inhibitors induced antidepressant-like effects, which may be related to an enhancement of adult neurogenesis and synaptic plasticity (Zhang et al., 2015a) and an increase on BDNF levels (Dong et al., 2020). Consequently, the enhancement of endocannabinoid signaling has been postulated as a new promising strategy in the treatment of depression and other

stress-related disorders (Mangieri and Piomelli, 2007). In clinical studies, the beneficial effects of exercise in depression have been associated with an increase in plasma AEA levels (Heyman et al., 2012; Meyer et al., 2019), SSRI antidepressant therapy was associated with an increase in 2-AG plasma levels (Romero-Sanchiz et al., 2019), and electroconvulsive therapy (ECT) increased AEA levels in the CSF of MDD patients (Kranaster et al., 2017).

As depression has an inflammatory component (*see 1.2.3.4 in the Introduction section*), another interesting target in the ECS is the CB_2 receptor. Its activation inhibits inflammation in a range of animal models of inflammation and exerts a double positive effect upon microglia: it drives microglial proliferation and migration towards a benign phenotype and blocks their differentiation to a neurotoxic phenotype (Ashton and Glass, 2007). Although there are few available reports about the CB_2 receptor and depression, a decrease in its expression has been described in different brain areas of an animal model of depression (Onaivi et al., 2008), and its overexpression reduced the depressive-like behaviour (García-Gutiérrez et al., 2010). Moreover, it has been reported that a selective CB_2 receptor agonist exerted antidepressant-like effects (Hu et al., 2009). The lack of psychoactive effects and the increased selectivity of this pharmacological approach give the CB_2 receptor compounds a higher therapeutic appeal over CB_1 receptor compounds (Ashton and Glass, 2007).

In all, there is a wealth of evidence supporting an important role of the ECS in the physiopathology of depression and demonstrating that the ECS components are promising targets for the treatment of the disease.

2. Animal models of depression: focus on gender and the LPS model

Animal models are valuable tools to gain a better understanding of the physiopathological basis of MDD and to evaluate new potential antidepressant drugs that could improve the current treatment. They try to reproduce the genetic, behavioural, and molecular features observed in depressed patients, as well as the effects of the environment. However, the perfect animal model of depression gathering all these alterations has not been developed yet. Instead, each model reproduces a subset of alterations, allowing to study their implication in the pathophysiology of depression and their modulation by antidepressants. This may, however, result in novel therapeutic approximations not easily transferable to the clinic because of its endophenotype specificity (Cryan et al., 2002; Hasler et al., 2004). Unfortunately, not all the depressive manifestations observed in humans can be fully reproduced in animals, as some purely human symptoms such as

guilt, sad mood, and suicidality cannot be modelled (Cryan and Mombereau, 2004). In contrast, other behavioural manifestations such as behavioural despair, anhedonia, and helplessness, and neurovegetative changes such as appetite and sleep disturbances are relatively easy to model in rodents (Krishnan and Nestler, 2011).

As shown in figure 8, an ideal animal model should exhibit three basic features: 1) *construct validity*: present similar causative factors to the human disease, 2) *face validity*: present a phenotype analogous to patients with the disease, and 3) *predictive validity*: respond to pharmacological or treatment interventions that are effective in humans (Dedic et al., 2011).

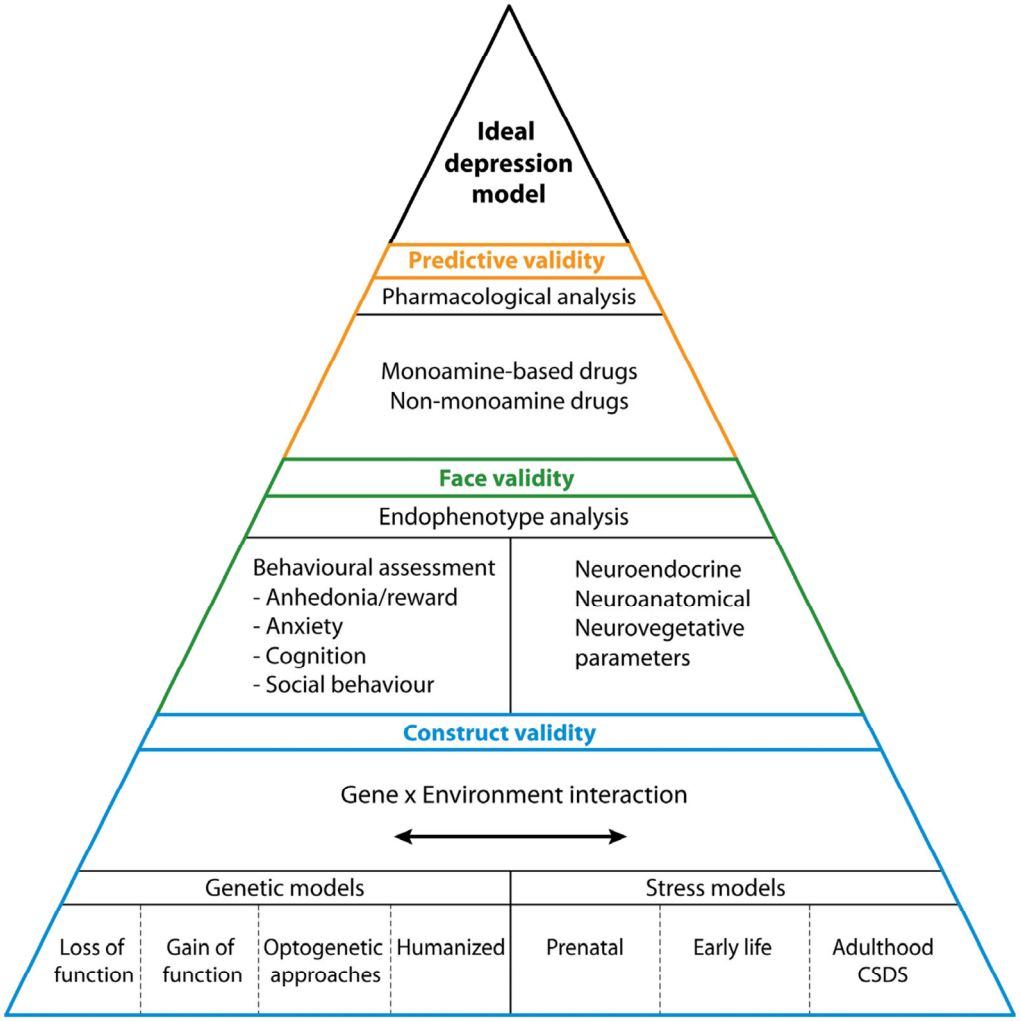


Figure 8. Basic features that would characterize an ideal animal model of depression. Obtained from Dedic et al. (2011).

There are several animal models of depression with some of the mentioned features. For instance: 1) genetic models, 2) stress models reproducing the HPA axis dysregulation observed in patients, which are induced by stressful experiences such as the chronic unpredictable mild stress (CUMS), social defeat, and maternal deprivation, or by the exogenous administration of glucocorticoids (chronic corticosterone model), 3) surgical manipulations such as the olfactory bulbectomy, 4) neuroinflammatory models such as the lipopolysaccharide (LPS) injection, and 5) optogenetic manipulations, among others (*reviewed in* Planchez et al., 2019) (figure 9).

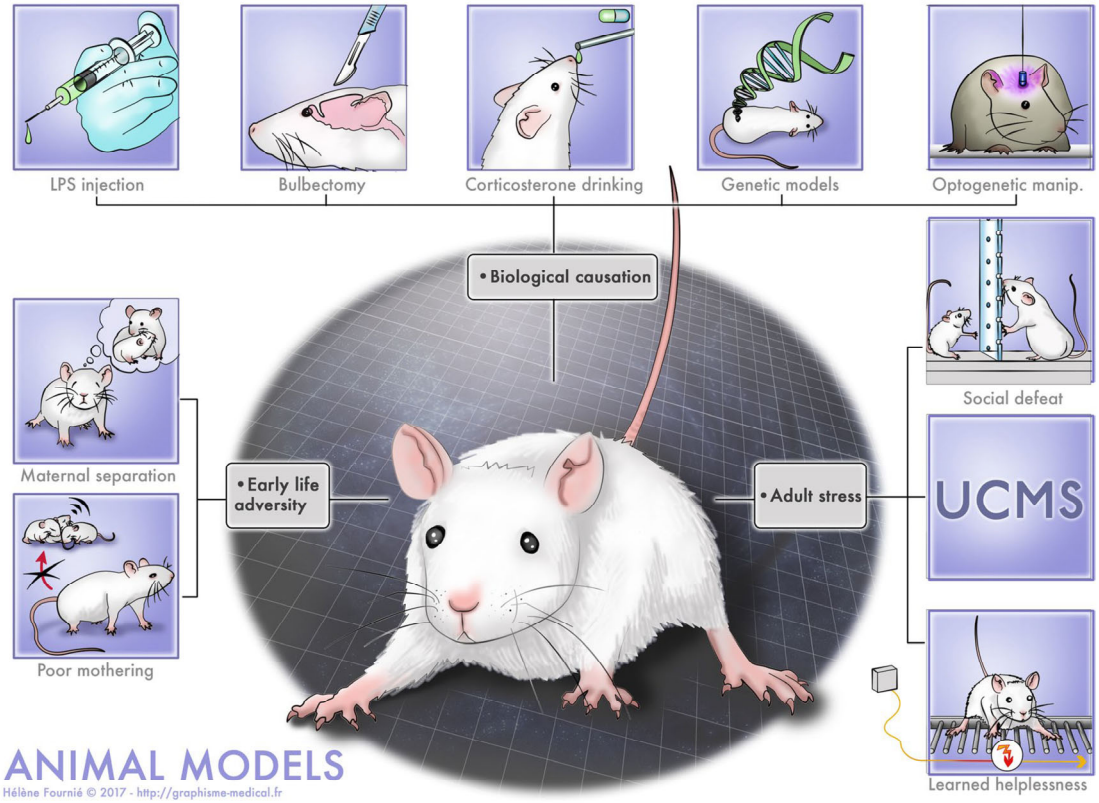


Figure 9. Animal models of depression used in preclinical research. Obtained from Planchez et al. (2019).

The behavioural tests with predictive validity are a useful tool for the study of pro-depressive manipulations and the screening of new potential antidepressant drugs. These tests are constantly used in preclinical studies of depression in both *naïve* animals and animal models. There are different tests based on the symptoms of depression that can be induced and measured in rodents, such as behavioural despair (forced swimming test/FST, tail suspension test/TST), anxiety (open field test, elevated plus maze test),

anhedonia (sucrose preference test), apathy (coat state, nest building), and social aversion (social defeat test), among others (*reviewed in* Planchez et al., 2019).

Despite the intrinsic limitations of the tests and animal models of depression, they have contributed enormously to the discovery of the neurobiological processes underlying depression and to the understanding of the mechanism of action of current and new antidepressants, thus leading to the discovery of new potential targets for the treatment of MDD.

2.1 Gender bias in preclinical studies

Strikingly, although it is largely known that the incidence of MDD in women is roughly twice that in men (Holden, 2005; Marcus et al., 2005; Grigoriadis and Robinson, 2007), most of the basic research on this pathology has been performed in male animals. The reason for this paradox is that male animals present less variability, as they do not go through the different stages within the oestrus cycle and, therefore, they are seen as easier to use in research (Blanchard and Glick, 1995; Palanza, 2001). Another observation that supports depression research in males is that many studies using animal models of depression reported that males were more susceptible to develop a depressive-like phenotype than females (Cryan and Mombereau, 2004; Kokras and Dalla, 2014).

In recent years there has been a huge criticism about the underrepresentation of the female gender in animal research (Beery, 2018), as it is also necessary to study how gender can affect the development of depression and the outcome of antidepressant treatments. Indeed, the symptomatology (Marcus et al., 2005, 2008) and treatment response (Khan et al., 2005; LeGates et al., 2019) has been reported to be different between men and women with MDD. Consequently, more research is needed to define the molecular mechanisms underlying these gender differences, which would contribute to gender-oriented prevention, diagnosis, and treatment of MDD.

The number of preclinical studies using female or both male and female animals has been increasing in the last years. These studies have shown that there are also gender differences in the behaviour and treatment response in animal models of depression (Kokras and Dalla, 2014). Besides, important gender differences in some of the systems involved in depression such as the serotonergic system and neurotrophic factors (Borrow and Cameron, 2014), the immune system (Gaillard and Spinedi, 1998), and the ECS (Reich et al., 2009), have been described in rodents. This should encourage researchers to add female animals to their studies.

2.2 Neuroinflammatory model induced by lipopolysaccharide

Lipopolysaccharide (LPS) is a component of the gram-negative bacterial wall that is commonly used as a trigger of inflammation to study immune-to-brain communication and the behavioural aspects of inflammation. The neuroinflammatory hypothesis of depression (*see 1.2.3.4 in the Introduction section*) is the basis for the use of the neuroinflammatory model induced by LPS in preclinical depression research. Besides, two clinical findings regarding LPS further support the usefulness of this animal model. First, the administration of low doses of LPS has been assessed in healthy subjects and leads to sickness, decreased mood, and increased anxiety (*reviewed in* Lasselin et al., 2018). Besides, the sickness manifestations induced by LPS include reduced appetite, slowed movements, sleep pattern alteration, and changes in cognitive functions (Lasselin et al., 2018), which are also prominent symptoms in depression. Second, increased IgA and IgM responses against LPS of different gram-negative enterobacteria (Maes et al., 2012), and LPS plasma levels (Stevens et al., 2018) have been reported in depressed patients. This could be due to an increased bacterial translocation from the gut to the blood as a consequence of gut dysbiosis or an unbalanced distribution of commensal bacteria in the gut (Stevens et al., 2018). Therefore, the immune response orchestrated against LPS could either cause or aggravate the low-grade inflammation observed in MDD (Maes et al., 2012).

The administration of LPS induces similar behavioural and molecular effects when is performed in rodents. The most common protocol is the i.p. administration of 0.83 mg/kg LPS to mice. This induces an initial sickness behaviour that peaks 2 - 6 h later and gradually wanes in less than 24 h. Some characteristic manifestations of sickness in mice are reduced body weight, decreased locomotor activity, and decreased food consumption (Dantzer, 2001; O'Connor et al., 2009). Depressive-like behaviour, as measured by increased behavioural despair (higher immobility in the TST and FST), and anhedonia (decreased sucrose preference), emerges on this sickness background (Dantzer et al., 2008), but differently, it peaks at 24 h and lasts longer than the sickness manifestations (Frenois et al., 2007).

The mechanism by which LPS induces the immune response is as follows. LPS activates toll-like receptor 4 (TLR4) in monocytes and macrophages, initiating the canonical nuclear factor kappa B (NF-κB) pathway (Kawai et al., 2001). NF-κB is a transcription factor that mediates the inflammatory response. Under basal conditions, the association of NF-κB with the inhibitor of nuclear factor kappa B (IκBα) retains the protein in the cytosol. When LPS activates TLR4, IκBα is phosphorylated and undergoes proteasome degradation. This allows the translocation of NF-κB to the nucleus and the transcription of its target genes,

which initiates the inflammatory response (Covert et al., 2005). Some of the proteins induced by NF-κB activation are pro-inflammatory cytokines such as IL-6, TNFα, and interferon-gamma (IFNγ) (Kawai et al., 2001), and IκBα, which creates a negative regulatory feedback of the pathway (Brown et al., 1993; Scott et al., 1993). In addition, LPS can downregulate anti-inflammatory pathways. The activation of the peroxisome proliferator-activated receptor gamma (PPARγ) is known to antagonize the transcription activity of NF-κB, consequently producing anti-inflammatory effects (Ricote et al., 1998). It has been reported that LPS reduces PPARγ levels in cell culture (Juknat et al., 2013; Choi et al., 2017), and rat cortex (Pérez-Nievas et al., 2010; MacDowell et al., 2013). An overview of the NF-κB pathway and PPARγ crosstalk is presented in figure 10.

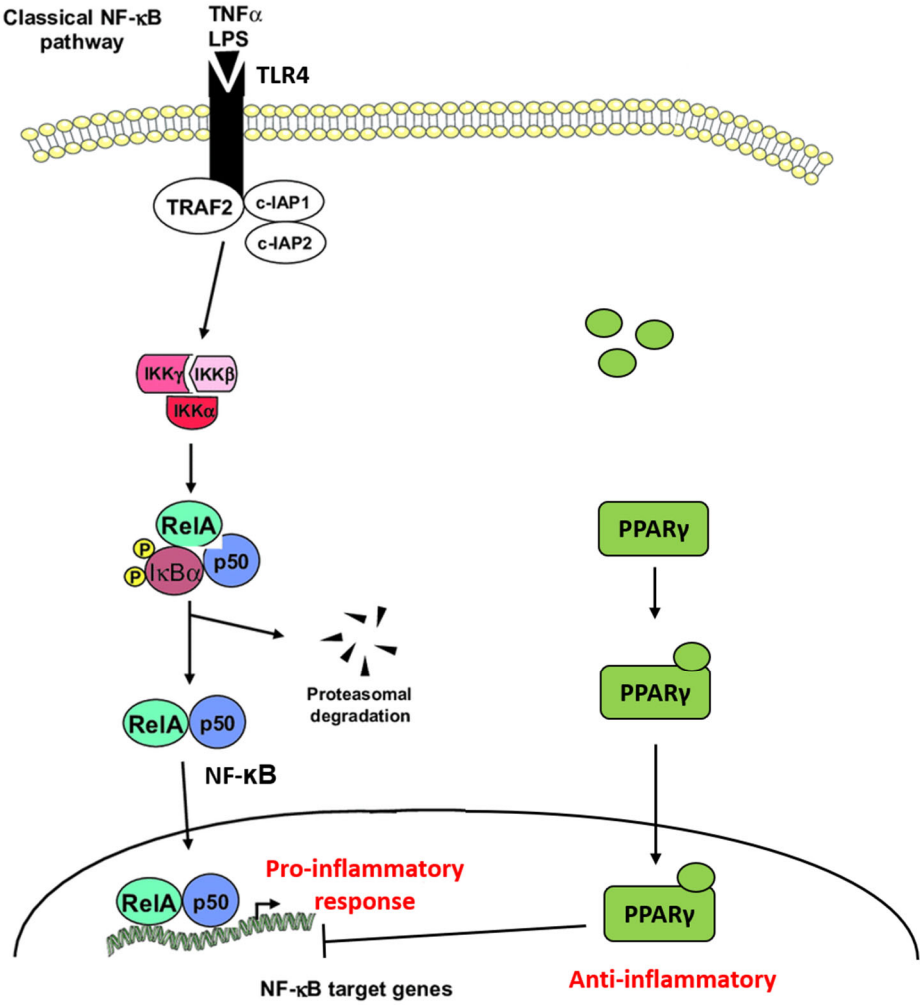


Figure 10. Representation of the classical NF-κB signalling pathway and PPARγ crosstalk. Modified from Baud and Collares (2016).

The LPS-induced increase in pro-inflammatory cytokines activates the IDO enzyme, which in turn will lead to a skewed pathway toward the formation of kynurenine instead of serotonin and an increase in excitotoxic kynurenine metabolites, further aggravating the inflammatory state (*see 1.2.3.4 in the Introduction section*). In mice, the peak of increased IDO activation has been observed 24 h after the LPS injection and has been associated with the appearance of depressive-like behavior (André et al., 2008; Dantzer et al., 2008). Pre-treatment with minocycline, a potent anti-inflammatory drug, blocks both LPS-induced sickness and depressive-like behaviour (Henry et al., 2008). In contrast, the administration of an IDO inhibitor blocks the LPS-induced depressive-like behaviour without altering the LPS-induced sickness, and the administration of kynurenine induces depressive-like behaviour without affecting locomotion (O'Connor et al., 2009). Also, the blockade of kynurenine transport through the blood brain barrier impedes the LPS-induced depressive-like behaviour but not its sickness manifestations (Walker et al., 2019). All these pieces of evidence support the notion that the LPS-induced depressive-like behaviour can be dissociated from the sickness behaviour, being the switch from sickness to a depressive-like phenotype mediated by the IDO and kynurenine pathway activation. A diagram of the behavioural and molecular effects induced by LPS is shown in figure 11.

One question that may arise is how peripheral inflammation can affect the brain. Peripheral cytokines signal the brain that immune activation has occurred through both neural and blood-borne routes (Maier, 2003). The neural route uses the vague and trigeminal nerve afferents to transfer immunological information to the brain. In the blood-borne route, released peptides and activated cells from the peripheral immune system can signal or be transported across the intact BBB. Also, the inflammatory signals can diffuse into the brain parenchyma through the CNS areas that lack BBB (Lasselin et al., 2018), and peripheral inflammation can alter the function and structure of the BBB (Ruiz-Valdepeñas et al., 2011; Varatharaj and Galea, 2017). Once the inflammatory signals have reached the brain, they activate a neural cascade that includes a *de novo* induction of pro-inflammatory cytokines. Microglial cells have been suggested as key players in translating the peripheral inflammatory signal to a central inflammatory pattern (Watkins and Maier, 2000; Maier, 2003).

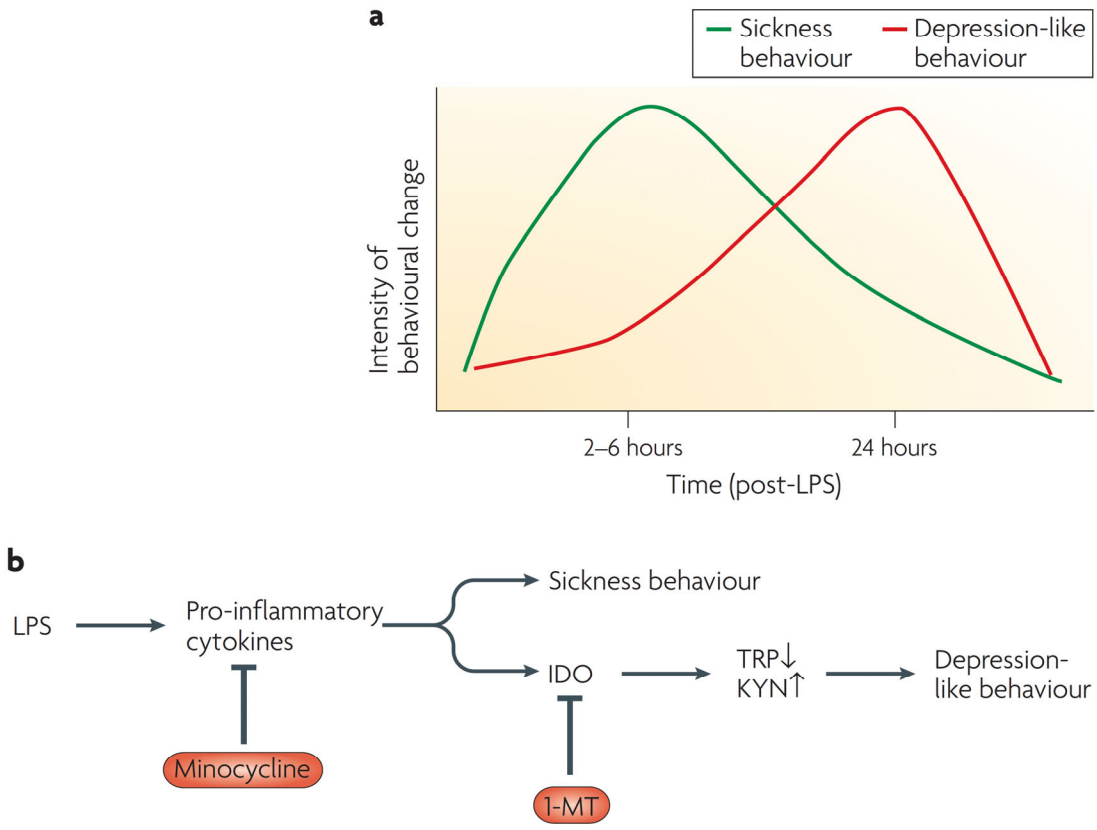


Figure 11. Behavioural changes induced by lipopolysaccharide administration. A) Time course of the sickness and depressive-like behaviour. B) Molecular signatures that dissociate these two types of behaviour. Obtained from Dantzer et al. (2008).

3. Cannabidiol

Cannabis sativa has been cultivated for more than 6000 years and is widely used for recreational and therapeutic purposes. More than 565 compounds have been isolated from the plant, and more than 120 have been identified as natural cannabinoids or phytocannabinoids (Li et al., 2020a). Among them, the most abundant and the most studied by far are two lipophilic compounds: Δ^9-THC, the main psychotropic component of the plant, and cannabidiol (CBD), the main non-psychotropic component. Interestingly, the effects of CBD and Δ^9-THC are often the opposite, and CBD can prevent or counteract some of the effects induced by Δ^9-THC (Bhattacharyya et al., 2010; Niesink and van Laar, 2013; Gunasekera et al., 2020). CBD was first isolated in 1940 and its structure was elucidated in 1963. Since then, the scientific interest in this phytocannabinoid has increased exponentially, especially in the last 20 years (figure 12), and nowadays is seen as a promising pleiotropic therapeutic drug.

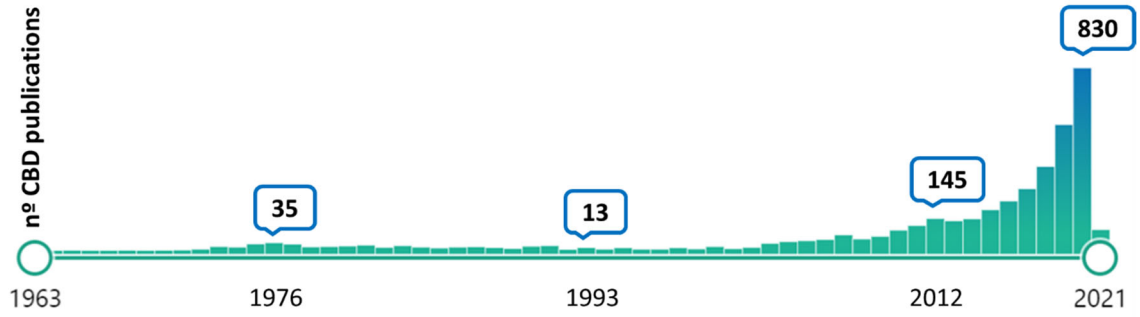


Figure 12. Number of peer-reviewed publications in Pubmed per year including the word "cannabidiol", from the year 1963 to present (search made in January 2021).

3.1 Pharmacokinetic aspects

3.1.1 Absorption

The two most common routes of CBD consumption are the inhalation route (smoked cannabis) and the oral route, as in the last years there has been a boom of commercialization of CBD dietary supplements in a variety of formats for various health complaints. The average bioavailability by the smoked route, as well as by aerosols and vaporization, is roughly 31% (Ohlsson et al., 1986; Devinsky et al., 2014). As CBD undergoes extensive first-pass liver metabolism, its oral bioavailability is low (around 6%) (Devinsky et al., 2014). However, plasma CBD levels are increased when CBD is administered with food. As CBD is a highly lipophilic molecule, it may dissolve in the fat content of food, increasing its absorption and bioavailability (Millar et al., 2018). The study of different routes of administration of CBD in humans has shown that peak plasma concentrations and the area under the curve are dose-dependent and show minimal accumulation. The time needed to reach the maximum concentration (Tmax) is not dose-dependent, and mostly occurs between 1 and 4 h (Millar et al., 2018).

3.1.2 Distribution

The distribution of CBD is determined by its high lipophilia and volume distribution, with a rapid distribution in the brain —as it readily crosses the BBB (Deiana et al., 2012)—, adipose tissue, and other organs. CBD presents high binding to plasma proteins, and approximately 10% is bound to red blood cells (Devinsky et al., 2014). Interestingly, the lipophilic nature of CBD allows its passive diffusion into the intracellular milieu (Ryan et al., 2009). The preferential distribution to fat increases the likelihood of accumulation in adipose tissue after its chronic administration, especially in patients with high adiposity (Devinsky et al., 2014). However, this was not reported in brain tissue (Deiana et al., 2012).

Therefore, CBD pharmacokinetics may respond to a tricompartmental model, and the adjustment of its dosage after prolonged exposure is fundamental.

The concentration of CBD in plasma and brain after oral and i.p. administration has been compared in rats and mice, the animals most used in psychiatric research. In mice, CBD i.p. administration led to a much higher brain and plasma concentration than oral dosing. In rats, the pharmacokinetic plasma profile was similar for both administration routes but, in contrast, brain levels were slightly higher after oral dosing. In both species, Tmax occurred between 1 and 2 h in plasma, and between 1 and 6 h in the brain, depending on the species and the route of administration (Deiana et al., 2012).

3.1.3 Metabolism and elimination

As most cannabinoids, CBD is metabolized extensively in the liver, where it is hydroxylated by P450 enzymes, predominantly by the CYP3A (2/4) and CYP2C (8/9/19) families of isozymes (Jiang et al., 2011). Its main metabolite is further metabolized in the liver, and the resulting molecules are excreted in the feces and, to a much lesser extent, in the urine (Devinsky et al., 2014). The mean half-life of CBD in humans was reported to be between 1 and 3 h following nebulizer, aerosol, and oral administration, between 1 and 11 h after oromucosal spray administration, 24 h after intravenous infusion, 31 h after smoking, and 2 - 5 days after chronic oral administration. Plasma apparent clearance has been reported to be 74.4 L/h following intravenous injection, and to range from 533 to 4741 L/h following an oromucosal spray administration, which depended on the fed/fasted state (Millar et al., 2018).

In rodents, the apparent elimination half-life after oral and i.p. CBD administration ranged from 240 to 600 min in plasma, and from 220 to 660 min in the brain, and differed according to administration routes and the species. Both in mice and rats, the elimination of CBD from plasma and brain appeared to be complete after 24 h (Deiana et al., 2012).

3.2 Pharmacodynamic aspects

3.2.1 Pharmacological effects

The reason for which CBD has attracted such a great interest may rely on its pleiotropic therapeutic potential in various disease states. Preclinical research has shown that CBD is anxiolytic, antidepressant, antipsychotic, anticonvulsant, neuroprotective, antinausea, antioxidant, anti-inflammatory, antiarthritic, antiapoptotic, analgesic, and antineoplastic (*reviewed in* Ligresti et al., 2016; Levinsohn and Hill, 2020).

Recently, two drugs containing CBD have been approved for therapeutic use. Sativex® (GW Pharmaceuticals, UK) was the first drug containing ingredients derived from Marijuana that was approved for its use in clinics. It is composed of Δ^9-THC and CBD in a 1:1 proportion and is indicated for the treatment of spasticity in adult multiple sclerosis (Syed et al., 2014; Giacoppo et al., 2017a). Apart from spasticity, Sativex® has been reported to be effective against multiple sclerosis neuropathic pain in a phase III study (Langford et al., 2013), and produced a strong relief of pain in patients with advanced cancer (Johnson et al., 2010). In 2018, FDA approved Epidiolex® (GW Pharmaceuticals, UK), the only CBD pure solution that has been approved in clinics, for the treatment of seizures associated with severe and treatment-resistant epilepsy syndromes (Sekar and Pack, 2019).

Preclinical and clinical studies point to possible future uses of CBD to treat psychiatric disorders, in special anxiety, depression, and schizophrenia (*reviewed in* Calapai et al., 2019; García-Gutiérrez et al., 2020). Of particular interest is the use of CBD to treat psychosis, as some clinical studies reported that CBD was effective in treating positive and negative symptoms of schizophrenia (Leweke et al., 2012; McGuire et al., 2018). Although the effectiveness of CBD in psychotic disorders has shown mixed results, this could be due to differences in dosing as well as in the stage of the disease (Mandolini et al., 2018). Interestingly, clinical data has shown that CBD counteracted the psychogenic effects of Δ^9-THC (Bhattacharyya et al., 2010), and normalized fMRI signatures in regions associated with psychosis in patients at high risk of psychosis (Bhattacharyya et al., 2018).

The anxiolytic properties of CBD have widely promoted its consumption and have been reported in clinical trials in patients with social phobia and social anxiety (Bergamaschi et al., 2011a; Crippa et al., 2011). In addition, CBD reverses the anxiogenic effects of Δ^9-THC (Karniol et al., 1974; Zuardi et al., 1982) and some case reports showed that CBD may be useful in the treatment of cannabis withdrawal (Mandolini et al., 2018). Evidence supporting the antidepressant effects of CBD is described above (*see 3.3 in the Introduction section*).

The neuroprotective, antioxidant, and anti-inflammatory properties of CBD have motivated the study of its effects in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease. CBD has induced beneficial effects in animal models of the aforementioned diseases (Esposito et al., 2007; García-Arencibia et al., 2007; Sagredo et al., 2007), and also in animal models of ischemia and stroke (Hayakawa et al., 2007; Castillo et al., 2010). The clinical evidence at this time is scarce and has shown that CBD may be useful for improving the overall well-being and comorbid psychiatric and motor symptoms in Parkinson's disease (Zuardi et al., 2009; Leehey et al.,

2020). However, CBD was not effective in reducing chorea severity in a clinical study of Huntington's disease (Consroe et al., 1991).

Due to the anti-inflammatory and immunomodulatory properties of CBD, its administration to animal models of various autoimmune conditions such as encephalomyelitis, rheumatoid arthritis, colitis, diabetes, and psoriasis resulted in an attenuation of symptoms (*reviewed in* Ligresti et al., 2016). In addition, CBD has shown promising effects in a phase II trial as an adjunctive medication to prevent graft-*versus*-host disease (Yeshurun et al., 2015).

Another prominent therapeutic effect of CBD is the antitumoral effect. It has been described that CBD exerted antiproliferative effects in several human tumour cell lines such as breast, prostate, and colorectal carcinoma, and gastric adenocarcinoma. Furthermore, CBD was able to inhibit cancer cell invasion and metastasis, as well as angiogenesis (*reviewed in* Ligresti et al., 2016).

3.2.2 Mechanisms of action

In contrast to what could be expected for a cannabinoid, most of the effects of CBD are not directly mediated by the cannabinoid receptors, but by its action in other systems. In addition, the antioxidant properties of CBD seem not to be mediated by any receptor or target, but to come from its chemical structure, which includes two hydroxyl groups (Mechoulam et al., 2002; Fernández-Ruiz et al., 2013; Dos-Santos-Pereira et al., 2020).

A total of 65 targets have been described for CBD, 49% of which are enzymes, 20% transporters, 15% ion channels, and 15% receptors (*reviewed in* Ibeas Bih et al., 2015). Its wide-ranging therapeutic effects may be due to this target pleiotropy, which in turn could make CBD more efficient than single-target drugs in the treatment of heterogeneous diseases such as depression (Mencher and Wang, 2005; Hopkins, 2008). It is important to mention that like other cannabinoids, CBD produces bell-shaped dose-response curves and can act by different mechanisms depending on its concentration (Zanelati et al., 2010; Campos et al., 2012; Fernández-Ruiz et al., 2013; Patricio et al., 2020). Indeed, it has been proposed that different CBD doses could be effective for the treatment of different behavioural manifestations (Campos et al., 2012; Shoval et al., 2016). The targets most described for CBD are depicted in figure 13 and are explained below.

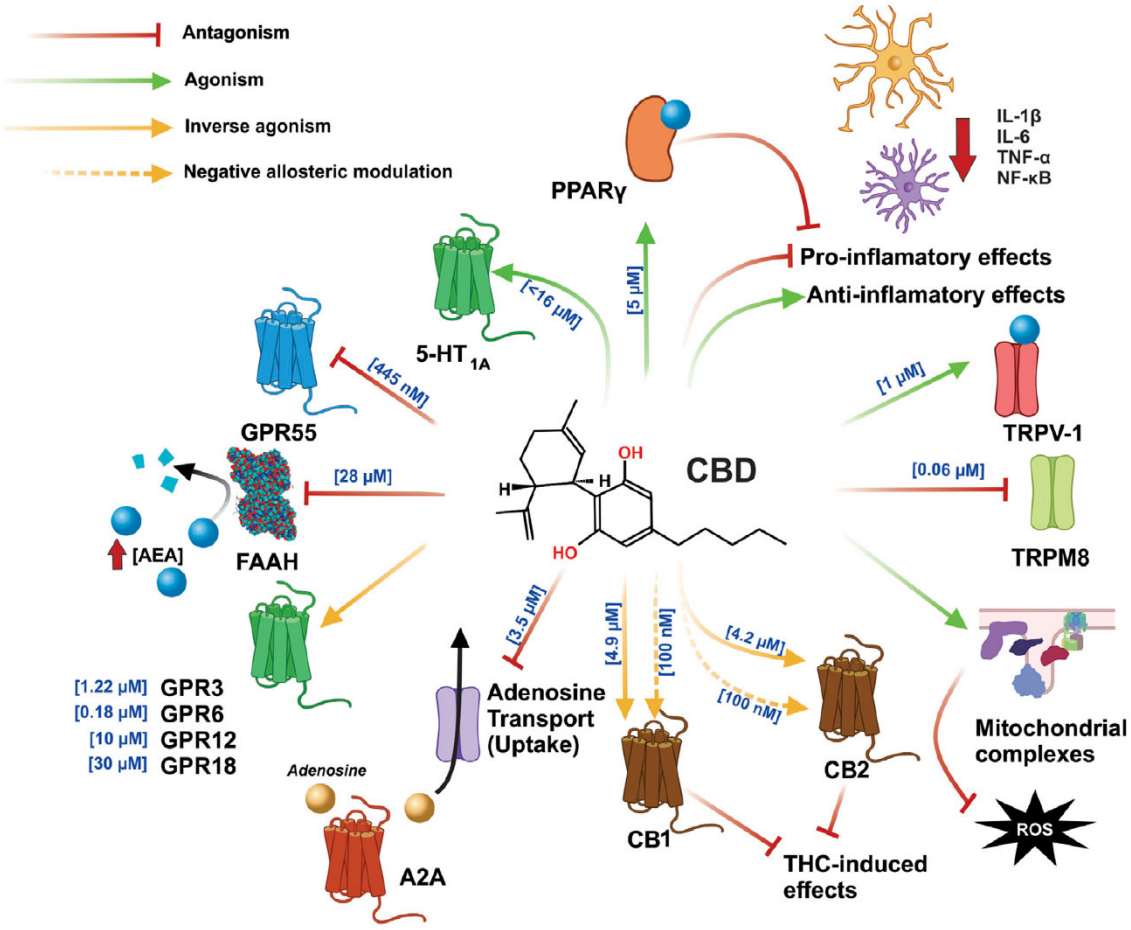


Figure 13. Principal targets described for cannabidiol. Obtained from Patricio et al. (2020).

Among the main mechanisms studied for CBD, its action on the ECS stands out in the first place. CBD is an antagonist/inverse agonist of the CB_1 and CB_2 receptors *in vitro*, although it presents a weak affinity for both of them (Thomas et al., 2007; Pertwee, 2008). This has been proposed as the principal reason for which CBD lacks the psychomimetic effects of Δ^9-THC and can antagonize some of the Δ^9-THC-induced effects. However, it has been reported that some therapeutic effects of CBD are mediated through the endocannabinoid receptors (Campos et al., 2013; Sartim et al., 2016), as CBD increases AEA levels by inhibiting its reuptake or by decreasing the activity of the FAAH enzyme (Bisogno et al., 2001; Leweke et al., 2012). Thus, CBD acts through the cannabinoid receptors indirectly. The effects of CBD on neurogenesis have been attributed to its action on the CB_1 and CB_2 receptors (Campos et al., 2013), and some of its anxiolytic- and antidepressant-like effects are mediated through the CB_1 receptor (Campos et al., 2013; Sartim et al., 2016). CBD also facilitates signaling through the transient receptor potential vanilloid (TRPV) ion channels 1 and 2, which are targets of AEA (Bisogno et al., 2001; De

Petrocellis et al., 2011). Related to this, it has been suggested that TRPV1 receptor activation contributes to the bell-shaped dose-response curve of the anxiolytic-like effects of CBD (Campos and Guimarães, 2009), a characteristic that has also been described in human studies (Zuardi et al., 2017; Linares et al., 2019). In addition, CBD acts as an antagonist of the orphan G protein-coupled receptor 55 (GPR55) (Ryberg et al., 2007), which has been suggested as a putative type 3 cannabinoid receptor because it shares numerous cannabinoid ligands with CB_1 and CB_2 receptors (Ryberg et al., 2007; Yang et al., 2016). The antagonism of GPR55 could take part in the neuromodulatory and anti-inflammatory effects of CBD (Sylantyev et al., 2013; Yang et al., 2016; Kaplan et al., 2017).

One of the most described targets of CBD is the 5-HT_{1A} receptor, acting as an agonist at high concentrations in *in vitro* studies (Russo et al., 2005), or as a positive allosteric modulator at lower doses CBD (Rock et al., 2012). The effects of CBD on anxiety (Campos and Guimarães, 2008), stress (Resstel et al., 2009), depression (Zanelati et al., 2010), panic (Soares et al., 2010), emesis (Rock et al., 2012), and neuroprotection (Pazos et al., 2013) have been attributed to its action on the 5-HT_{1A} receptor. For most of these effects, CBD produced a 5-HT_{1A} receptor-mediated bell-shaped dose-response curve (Rock et al., 2012).

The anti-inflammatory properties of CBD have been widely reported and rely on diverse mechanisms of action. CBD is a PPARγ receptor ligand (O'Sullivan et al., 2009) and can increase PPARγ levels (Giacoppo et al., 2017b). It has been reported that CBD reduced neuroinflammation and neurodegeneration and produced antitumoral effects by its action on PPARγ (Esposito et al., 2011; Ibeas et al., 2015; Vallée et al., 2017). Besides, CBD inhibited the NF-κB pathway and inducible nitric oxide synthase (iNOS) expression and activity (Esposito et al., 2006a), the activity of the IDO enzyme (Jenny et al., 2009), and modulated the activity of cyclooxygenase (COX) 1 and 2 enzymes (Wheal et al., 2014). Moreover, the modulation of the adenosine system has also been implicated in the anti-inflammatory and neuroprotective effects of CBD, as CBD facilitates signaling through the adenosine receptors A_{1A} and A_{2A} by inhibiting adenosine reuptake (Carrier et al., 2006; Castillo et al., 2010; Gonca and Darıcı, 2015). All these mechanisms could explain the beneficial effects of CBD on inflammatory and neurodegenerative diseases that have been described in preclinical studies.

Finally, another mechanism implicated in the neuroprotective actions of CBD may be the normalization of glutamatergic homeostasis. In *in vitro* studies, CBD prevented glutamatergic neurotoxicity (Hampson et al., 2000) and reduced the concentration of glutamate in brain slices after an ischemic insult (Castillo et al., 2010). Furthermore, CBD reverted the cocaine-induced increase in glutamate release in hippocampal

synaptosomes (Gobira et al., 2015), and the injury-induced increase in glutamate release in the mPFC (Belardo et al., 2019). The mechanism that could account for the modulatory effects of CBD on glutamatergic neurotransmission could be its action on CB_1 receptors located in glutamatergic/GABAergic terminals and astrocytes (Navarrete and Araque, 2008; Rey et al., 2012), and on TRPV1 receptors (Xing and Li, 2007).

The wide variety of mechanisms of action described for CBD, as well as its bell-shaped dose-response curves, depict both the high therapeutic potential of this compound and the high complexity of its pharmacology. Therefore, more research is needed to better understand the effects of CBD and to eventually increase its use in the clinic to improve the treatment of various diseases.

3.3 Evidence of the antidepressant effect of cannabidiol

3.3.1 Preclinical studies

Most of the evidence supporting the potential antidepressant effects of CBD comes from preclinical studies (*reviewed in* Silote et al., 2019). First, CBD was effective in animal models of anxiety and attenuated the stress-induced emotional and cardiovascular consequences of restraint stress by facilitating 5-HT_{1A} receptor neurotransmission (Campos and Guimarães, 2008; Resstel et al., 2009). As conventional antidepressants and 5-HT_{1A} receptor agonists can also attenuate the emotional outcomes induced by restraint stress (Guimarães et al., 1993), these pieces of evidence motivated the study of the effects of CBD in other psychiatric diseases with impaired stress-coping mechanisms, such as depression (Resstel et al., 2009).

The first study on this issue assessed the effects of an acute systemic CBD administration at different doses (3 - 100 mg/kg) to mice submitted to the forced swimming test (FST). The results revealed that CBD induced dose-dependent antidepressant-like effects that followed a bell-shaped dose-response curve, being the effective dose 30 mg/kg. These effects were mediated by the 5-HT_{1A} receptor, as the prior administration of the 5-HT_{1A} receptor antagonist WAY100635 blocked the antidepressant-like effects of CBD (Zanelati et al., 2010). A subsequent study corroborated the antidepressant-like effects of the acute systemic administration of CBD in the FST although at much higher doses (200 mg/kg) (El-Alfy et al., 2010). However, such a high dose has not been used again to study its antidepressant effects.

The antidepressant-like effects of CBD were also observed both after single and chronic (15 days) CBD administration (3 - 30 mg/kg) to mice subjected to the tail suspension test

(TST) (Schiavon et al., 2016). Moreover, these effects are not species-specific, as similar results were observed in Wistar rats submitted to the FST, where both acute and chronic (14 days) 30 mg/kg CBD administration were effective (Réus et al., 2011). This study also showed a bell-shaped dose-response curve of the antidepressant-like effects of CBD, being the doses of 15 and 60 mg/kg ineffective. In addition, CBD selectively increased the time spent swimming in the FST, a behaviour that has been associated with an increase in serotonergic signalling (Detke et al., 1995; Cryan et al., 2002, 2005). Further confirming the involvement of the serotonergic system in the antidepressant-like effects of CBD, the co-administration of CBD and the SSRI fluoxetine at sub-effective doses produced synergistic effects in the FST, and the pre-treatment with an inhibitor of 5-HT synthesis abolished the behavioural effects of CBD (Sales et al., 2018). This study also suggests that CBD may be useful as adjunctive therapy to the serotonergic antidepressant drugs currently used in the clinic.

The investigation on the site of action for the antidepressant-like effects of CBD has started in the last years. The two studies addressing this issue found that the direct infusion of CBD (45 - 60 nmol) into the PL and IL cortices of the mPFC and 10 nmol CBD into the dorsal hippocampus produced acute antidepressant-like effects in the FST. The 5-HT_{1A} and CB_1 receptors were involved in the effects of the intra-mPFC CBD infusion (Sartim et al., 2016), while the effects of the intra-hippocampal CBD infusion were mediated by the activation of the neuroplastic signalling pathway BDNF-TrkB-mTOR (Sartim et al., 2018).

CBD also produces antidepressant-like effects in animal models of depression. A single administration of CBD was effective in the olfactory bulbectomy model in mice (Linge et al., 2016) and the learned helplessness model in rats (Sales et al., 2019), suggesting a fast-acting antidepressant effect. In the olfactory bulbectomy model, CBD enhanced serotonergic and glutamatergic neurotransmission in the vmPFC, and its effects were mediated by the 5-HT_{1A} receptor (Linge et al., 2016). Moreover, the antidepressant-like effects of a single CBD administration (10 - 30 mg/kg; i.p.) lasted for up to one week both in mice and rats, indicating a sustained effect (Sales et al., 2019). Altogether, these results suggest that the pharmacological profile of CBD is similar to the described for ketamine, with rapid and lasting effects after a single administration (Berman et al., 2000; Zarate et al., 2006; Zhang et al., 2015b). In addition, the antidepressant-like effects of a sub-chronic (7 days) 30 mg/kg CBD treatment were extended until 21 days post-treatment in rats (Bis-Humbert et al., 2020).

Chronic CBD administration (30 mg/kg for 14 days) induced stress-coping behaviour in the elevated plus maze and novelty suppressed feeding tests (NSF) in mice submitted to the

chronic unpredictable stress model (Campos et al., 2013; Fogaça et al., 2018). The effects of CBD in this animal model were mediated by the facilitation of endocannabinoid neurotransmission through CB_1 and CB_2 receptors, which induced neurogenesis and dendritic remodeling in the hippocampus (Campos et al., 2013; Fogaça et al., 2018). Besides, CBD (10 - 30 mg/kg) also reduced behavioural despair and reverted anhedonia in genetic models of depression based on selective breeding, such as Wistar-Kyoto (Shoval et al., 2016) and Flinders Sensitive Line (Sales et al., 2019) rats. In addition, repeated (3 doses in 24 h), but not acute, 30 mg/kg CBD administration produced mild antidepressant-like effects in a rat model of diabetes that presents a depressive-like phenotype in the FST (de Morais et al., 2018).

In all, preclinical studies have shown that CBD exerts antidepressant-like effects both after acute and chronic treatment in different animal models, rodent species, and strains. The range of CBD effective doses in depressive-like behaviour is 3 - 50 mg/kg in mice and 10 - 30 mg/kg in rats. A recent review about the antidepressant effects of CBD stressed that the involvement of additional targets in CBD antidepressant-like effects, other than 5-HT_{1A} and $CB_{1/2}$ receptors, needs to be explored (*reviewed in* Silote et al., 2019). In particular, they noticed a lack of studies regarding the participation of immunomodulatory mechanisms in the CBD-induced antidepressant-like effects, an issue that has been explored in this thesis and that we have just published (Florensa-Zanuy et al., 2021).

Another question that should be addressed is whether the effects of CBD may be influenced by gender and age, as the vast majority of the antidepressant-like effects of CBD have been tested in male and adult animals. New evidence suggests that gender may influence, as CBD was effective in male Flinders Sensitive Line rats subjected to the FST but not in females. However, only one dose of CBD was tested in this study (30 mg/kg), and it induced prohedonic and antidepressant-like effects in female Wistar-Kyoto rats (Shbiro et al., 2019). Finally, a recent study found that the antidepressant-like effects of CBD were influenced by age, as the effective dose, the duration of the effects in the FST, and the outcome in the sucrose preference test were different in adolescent rats compared to adult ones (Bis-Humbert et al., 2020).

3.3.2 Clinical studies

To date, there are no clinical studies that had evaluated the antidepressant effects of CBD in MDD patients. Nevertheless, some data obtained in clinical studies that evaluated depressive symptoms as a secondary outcome of other pathologies, suggest that CBD could exert antidepressant effects in humans (*reviewed in* Pinto et al., 2020). CBD has been administered in interventional human studies of different health conditions. Among the studies in which CBD was co-administered with Δ^9-THC, a reduction in depressive

symptoms was reported in a study of chronic pain (Notcutt et al., 2004) and cannabis use disorder (Allsop et al., 2014), whereas no significant differences in affective symptoms were observed in three other studies (*reviewed in* Pinto et al., 2020). When CBD was administered in monotherapy, it reduced depressive symptoms in three studies of cannabis use disorder (Crippa et al., 2013; Beale et al., 2018; Solowij et al., 2018) and improved mood symptoms in treatment-resistant epilepsy patients (Gaston et al., 2019); however, three other studies did not find changes in affective symptoms (*reviewed in* Pinto et al., 2020). These conflicting results are probably due to important methodological differences among the studies in several aspects, such as the evaluation of patients with different health conditions, the dose of CBD administered, and the use of different rating scales to measure depression.

Some cannabis users, including patients suffering from mood disorders (Ashton et al., 2005), have revealed that they use cannabis as self-medication for mood symptoms. Interestingly, some observational clinical studies performed on cannabis users showed a reduction in depressive symptoms when the cannabis consumed had a high CBD content (Schubart et al., 2011; Morgan et al., 2012; Cuttler et al., 2018). In addition, in a cross-sectional study of CBD users including 2409 participants, 62% reported using CBD for treating a medical condition, and 25% of them used CBD to treat depression. The 66% of those individuals reported that CBD worked from moderately to very well for depressive symptoms (Corroon and Phillips, 2018). However, the study did not discriminate the different sources and purity of CBD and was based on self-reports.

Currently, two randomized placebo-controlled trials assessing CBD administration for the treatment of depression are being carried out. In Brazil, the administration of 150 - 300 mg CBD per day for 12 weeks is being studied as adjunctive therapy in bipolar depression. The study has 100 participants, started in November 2017, and is estimated to end in April 2022 (NCT03310593). Although another similar study was being performed in Germany, it was prematurely ended in November 2019 and the results have not been revealed yet (EUCTR 2015-000465-31). Interestingly, a trial of the antidepressant efficacy of CBD in 10 patients with treatment-resistant depression has just started in the United States. CBD will be administered for 9 weeks; the dose will be gradually increased from 250 mg/day to 1000 mg/day and afterward it will be gradually decreased. The completion of the study is estimated in March 2024 (NCT04732169). The results of these studies will be of significant interest.

3.4 Safety of cannabidiol in humans

Numerous clinical studies have assessed the safety of CBD in open-label and controlled trials, concluding that CBD is generally well tolerated and has a good safety profile in a wide range of doses. No severe adverse effects have been found in the CNS, in vital signs, nor in psychological functions at doses up to 1500 mg/day (oral) or 30 mg/kg (i.v.), both after acute and chronic administration (*reviewed in* Bergamaschi et al., 2011b). In studies for the treatment of epilepsy and psychotic disorders, the most commonly reported side effects were fatigue, somnolence, diarrhea, vomiting, changes of appetite and weight, and hepatic abnormalities. Overall, CBD had a better side effect profile, as the incidence of these adverse effects was low when compared to the drugs currently used for the treatment of these diseases (*reviewed in* Iffland and Grotenhermen, 2017; Huestis et al., 2019). To notice, children were included in these studies, which suggests that CBD has also a good safety profile in the young population. Indeed, the use of Epidiolex® is approved in patients 1 year of age and older for the treatment of refractory epilepsy. In addition, studies examining the abuse potential of CBD have not found any evidence of tolerance, dependence, or abuse potential. Given these findings, the World Health Organization has recommended not to label CBD as a scheduled substance (WHO, 2018). Notwithstanding this, more research is needed in larger cohorts using CBD. The evaluation of the effects of CBD following long-term exposure and in the elder population is also required, as the existing evidence on these topics is more limited.

It must be highlighted that CBD may produce drug interactions, especially with drugs metabolized in the liver. It has been demonstrated that CBD is not only a substrate but also an inhibitor and inducer of certain cytochrome P450 isoforms (Jiang et al., 2011; Ujváry and Hanuš, 2016; *reviewed in* Iffland and Grotenhermen, 2017), which increases the risk of drug interactions. For instance, it has been reported that due to CBD inhibition of CYP2C19, its co-administration with clobazam increased plasma levels of the active metabolite of this drug in children with refractory epilepsy (Geffrey et al., 2015). In addition, preclinical evidence has shown that CBD interacts with the p-glycoprotein drug transporters, which suggests that other currently unknown drug interactions may exist (*reviewed in* Iffland and Grotenhermen, 2017).

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

Based on the previous preclinical studies we hypothesized that the antidepressant-like effect of cannabidiol could be mediated by a fast increase in synaptic plasticity markers and/or the modulation of various neurotransmission systems and/or neuroinflammation. We also postulate that the infralimbic cortex and the dorsal raphe nucleus would have an important role in this effect.

The main objective of this thesis was to provide further knowledge about the mechanisms of action by which cannabidiol exerts its antidepressant-like effect and its site of action in the brain.

To answer the aforementioned hypothesis, the following objectives were proposed:

1. Study the acute and sustained behavioural (anxious- and depressive-like phenotype), molecular (synaptic plasticity markers and neuronal activation), and neurochemical (neurotransmitter levels) outcomes induced by the local infusion of cannabidiol into the infralimbic cortex and dorsal raphe nucleus in male rats.

2. Evaluate the behavioural effects (anxious-, depressive-like, and anhedonic phenotype) of the systemic administration of cannabidiol to male and female mice subjected to a model of neuroinflammation induced by lipopolysaccharide injection, and the implication of different inflammatory pathways, neurotransmitter systems, and the 5-HT_{1A} receptor in these effects.

MATERIAL AND METHODS

1. Animals

Both mice and rats were used in this thesis. The animals were housed in a 12 h light-dark cycle, at a temperature of 22 ± 1 ºC and relative humidity of 60 - 70%. Unless otherwise specified, food and water were given *ad libitum*.

Male 2 - 3 months old Sprague Dawley rats (Harlan, Barcelona, Spain) weighing 280 - 350 grams were used in the experiments related to the first objective. They were housed in groups of 2 - 3 in Specific Pathogen Free (SPF) conditions.

Male and female NMRI mice (Envigo RMS Spain SL., Barcelona, Spain) (2 - 3 months old, 30 - 35 grams) were used for the experiments in the second objective. They were housed in groups of 4 - 5 animals.

For microglial and neuronal primary cell cultures, P1 - P3 (postnatal day 1 - 3) male C57BL/6JRj mice (Janvier Labs, Pays de la Loire, France) and male Sprague Dawley rats were used, respectively. They were housed in SPF conditions.

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, por el que se modifica el Real Decreto 53/2013) and the European Communities Council Directive on "Protection of Animals Used in Experimental and Other Scientific Purposes" (86/609/EEC).

2. Drugs and reagents

Table 1. Drugs and reagents.

REAGENT	SUPPLIER
2-Mercaptoethanol	Sigma-Aldrich, Missouri, USA
5-HT hydrochloride (serotonin)	Sigma-Aldrich, Missouri, USA
Acetonitrile	Sigma-Aldrich, Missouri, USA
40% Acrylamide/Bis solution, 37.5:1	Bio-Rad, California, USA
Antipain	Sigma-Aldrich, Missouri, USA
Aprotinin	Affymetrix - USB® Products, Ohio, USA
APS (ammonium persulfate)	Sigma-Aldrich, Missouri, USA

REAGENT	SUPPLIER
B27	Thermo Fischer Scientific S.L., Madrid, Spain
BSA (bovine serum albumin)	Sigma-Aldrich, Missouri, USA
(-) Cannabidiol	Tocris, Bristol, UK
CHAPS	Sigma-Aldrich, Missouri, USA
Chloroform	Sigma-Aldrich, Missouri, USA
Chymostatin	Sigma-Aldrich, Missouri, USA
Ciprofloxacin	Sigma-Aldrich, Missouri, USA
Citric acid	EMD Millipore, Massachusetts, USA
DAB Substrate Kit	Vector Laboratories, California, USA
DC™ Protein Assay	Bio-Rad, California, USA
Dispase II	Sigma-Aldrich, Missouri, USA
DMEM	Biowest, Pays de la Loire, France
DMSO (dimethyl sulfoxide)	Sigma-Aldrich, Missouri, USA
DNAse I	Roche Diagnostics GmbH, , Baden-Württemberg,
DPBS	Sigma-Aldrich, Missouri, USA
DPX	BDH Prolabo/VWR, Tingalpa, Australia
DTT (Dithiothreitol)	Sigma-Aldrich, Missouri, USA
EDTA	Sigma-Aldrich, Missouri, USA
EGTA	Sigma-Aldrich, Missouri, USA
Ethanol	Scharlab S.L., Barcelona, Spain
FBS (fetal bovine serum)	Biowest, Pays de la Loire, France
GABA	Sigma-Aldrich, Missouri, USA
Gentamycin	Calbiochem, California, USA
Glutamate	Sigma-Aldrich, Missouri, USA
GlutaMax™	Gibco, Madrid, Spain
Glycerol	Scharlab S.L., Barcelona, Spain
Glycine	Sigma-Aldrich, Missouri, USA
H_2O_2	Sigma-Aldrich, Missouri, USA
HBSS	Sigma-Aldrich, Missouri, USA
Hematoxylin	EMD Millipore, Massachusetts, USA
HEPES	Usb, Ohio, USA
Igepal	Sigma-Aldrich, Missouri, USA
Isoflurane	Piramal Healthcare, Northumberland, UK
Isopropanol	Sigma-Aldrich, Missouri, USA
KCl	Sigma-Aldrich, Missouri, USA
Kynurenine	Sigma-Aldrich, Missouri, USA
Laemmli buffer 2x	Bio-rad, California, USA

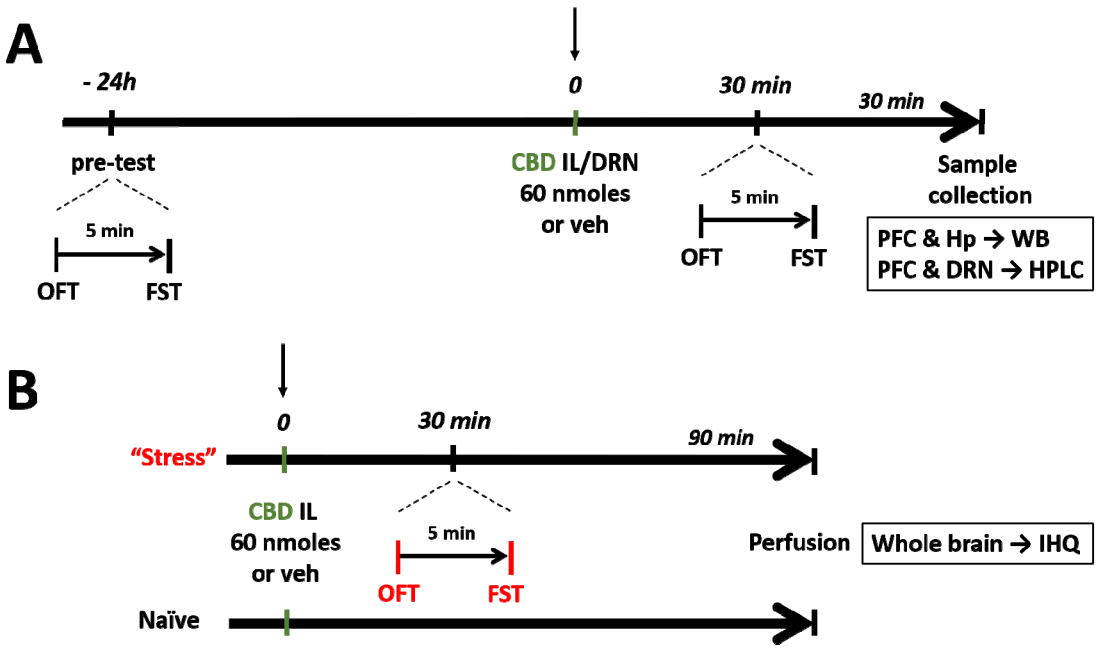
REAGENT	SUPPLIER
Leupeptin	Amersham, Buckinghamshire, UK
Lidocaine	Acros Organics, New Jersey, USA
Lipopolysaccharide (LPS)	Sigma-Aldrich, Missouri, USA
Methanol	Scharlab S.L., Barcelona, Spain
$MgCl_2$	Scharlab S.L., Barcelona, Spain
$MgSO_4$	Panreac, Barcelona, Spain
NA (noradrenaline)	Sigma-Aldrich, Missouri, USA
NaCl	BDH Prolabo/VWR, Tingalpa, Australia
NaF	Sigma-Aldrich, Missouri, USA
Na_3VO_4	Sigma-Aldrich, Missouri, USA
NDS (normal donkey serum)	EMD Millipore, Massachusetts, USA
Neurobasal A medium	Thermo Fischer Scientific S.L., Madrid, Spain
Nuclease free water	VWR International Ltd., Lutterworth, UK
OPA (o-phthalaldehyde)	Sigma-Aldrich, Missouri, USA
OSA (octanesulfonic acid)	EMD Millipore, Massachusetts, USA
PageRuler Plus Prestained Protein Ladder	Thermo Fischer Scientific S.L., Madrid, Spain
Papain	Sigma-Aldrich, Missouri, USA
Paraformaldehyde	Scharlab S.L., Barcelona, Spain
PDL (poly-D-lysine)	MP Biomedicals LLC, Ohio, USA
Penicillin/streptomycin	Sigma-Aldrich, Missouri, USA
Pepstatin	Sigma-Aldrich, Missouri, USA
Perchloric acid	Panreac, Barcelona, Spain
Phosphoric acid	EMD Millipore, Massachusetts, USA
PLL (poly-L-lysine)	Sigma-Aldrich, Missouri, USA
PMSF	Sigma-Aldrich, Missouri, USA
Powder skimmed milk	Central lechera asturiana, Asturias, Spain
Propilenglicol®	Panreac, Barcelona, Spain
Protease inhibitors cocktail	Sigma-Aldrich, Missouri, USA
SDS (sodium dodecyl sulfate)	Thermo Fischer Scientific S.L., Madrid, Spain
Sodium deoxycholate	Sigma-Aldrich, Missouri, USA
Sodium molybdate	Sigma-Aldrich, Missouri, USA
Sodium pentobarbital	Boeringher Ingelheim, Germany
Sodium pyruvate	Corning, Virginia, USA
Sodium sulfite	Sigma-Aldrich, Missouri, USA
Sucrose	Scharlab S.L., Barcelona, Spain
TEMED	Sigma-Aldrich, Missouri, USA
Tris-HCl	Sigma-Aldrich, Missouri, USA

REAGENT	SUPPLIER
Triton X-100	Sigma-Aldrich, Missouri, USA
TRI reagent®	Merck KGaA, Darmstadt, Germany
TRP (L-tryptophan)	Sigma-Aldrich, Missouri, USA
Trypsin/EDTA	Biochrom Ltd, Berlin, Germany
Tween 20	Sigma-Aldrich, Missouri, USA
Tween 80®	Scharlab S.L., Barcelona, Spain
Vectastain ABC kit	Vector Laboratories, California, USA
WAY100635 maleate	Tocris Bioscience, Bristol, UK
Xylene	Scharlab S.L., Barcelona, Spain

3. Experimental design

3.1 *In vivo* studies

The experimental design of the *in vivo* studies addressed to evaluate the behavioural, molecular and neurochemical effects induced by an infralimbic (IL) cortex and dorsal raphe nucleus (DRN) cannabidol (CBD) infusion (Objective 1) is presented in figure 14. The number of animals used in each experiment is detailed in the Results section.



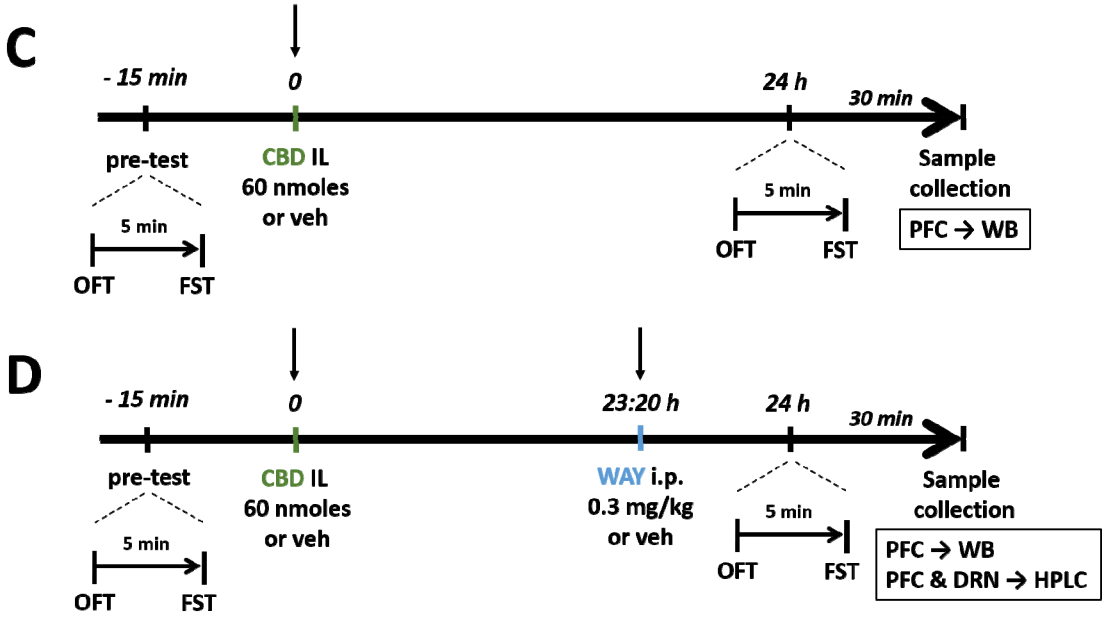


Figure 14. Experimental design of *in vivo* studies in Objective 1. A) Study of the behavioural and molecular effects of infralimbic cortex (IL) or dorsal raphe nucleus (DRN) CBD infusion after 30 min. B) Assessment of the effects of behavioural-related stress (open field and forced swimming tests) in neuronal activation evaluated in prefrontal cortex and DRN after the IL CBD infusion. Four experimental groups were compared: *naïve* (non-stressed), *naïve*-CBD, stress, and stress-CBD. C) Study of the behavioural and molecular effects of the IL CBD infusion after 24 h. D) Evaluation of the behavioural and molecular effects of the IL CBD infusion after 24 h and in the presence of a presynaptic 5-HT_{1A} receptor blockade (0.3 mg/kg WAY100635). Four experimental groups were used: vehicle, CBD, WAY, and CBD+WAY. CBD: cannabidiol, WAY: WAY100635, i.p.: intraperitoneal, FST: forced swimming test, OFT: open field test, DRN: dorsal raphe nucleus, Hp: hippocampus, IL: infralimbic cortex, PFC: prefrontal cortex, HPLC: high-performance liquid chromatography, IHQ: immunohistochemistry, WB: western blot.

The schedule of the *in vivo* experiments included in Objective 2 is shown in figure 15. The lipopolysaccharide (LPS) mouse model was induced by the administration of 0.83 mg/kg LPS and anxious/depressive-like behaviour was analyzed 12 h later. To evaluate the effect of CBD, this drug was administered 30 min before LPS injection.

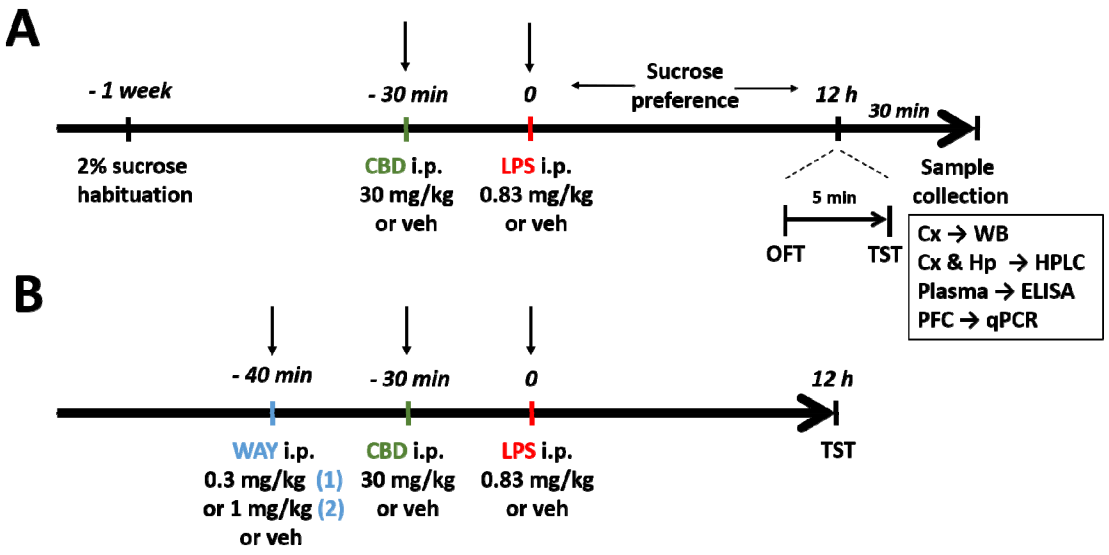


Figure 15. Experimental design of *in vivo* studies in Objective 2. A) Study of the effect of CBD in the LPS neuroinflammatory model. Four experimental groups were used: vehicle, LPS, CBD, and CBD+LPS. B) Evaluation of the effect of CBD in the LPS model in the presence of 0.3 mg/kg WAY100635 to block presynaptic 5-HT_{1A} receptors (1), or 1 mg/kg WAY100635 to block both, pre- and post-ynaptic 5-HT_{1A} receptors (2). The administration of 0.3 or 1 mg/kg WAY100635 was added to these four experimental groups: vehicle, LPS, CBD, and CBD+LPS. CBD: cannabidiol, LPS: lipopolysaccharide, WAY: WAY100635, OFT: open field test, TST: tail suspension test, Cx: cortex, Hp: hippocampus, PFC: prefrontal cortex, ELISA: enzyme-linked immunosorbent assay, HPLC: high-performance liquid chromatography, qPCR: quantitative polymerase chain reaction, WB: western blot.

3.2 *In vitro* studies

The experimental design of the *in vitro* studies is depicted in figure 16. Neuronal and microglial cell cultures were used to evaluate the effect of CBD on neuroplasticity and neuroinflammatory markers, respectively.

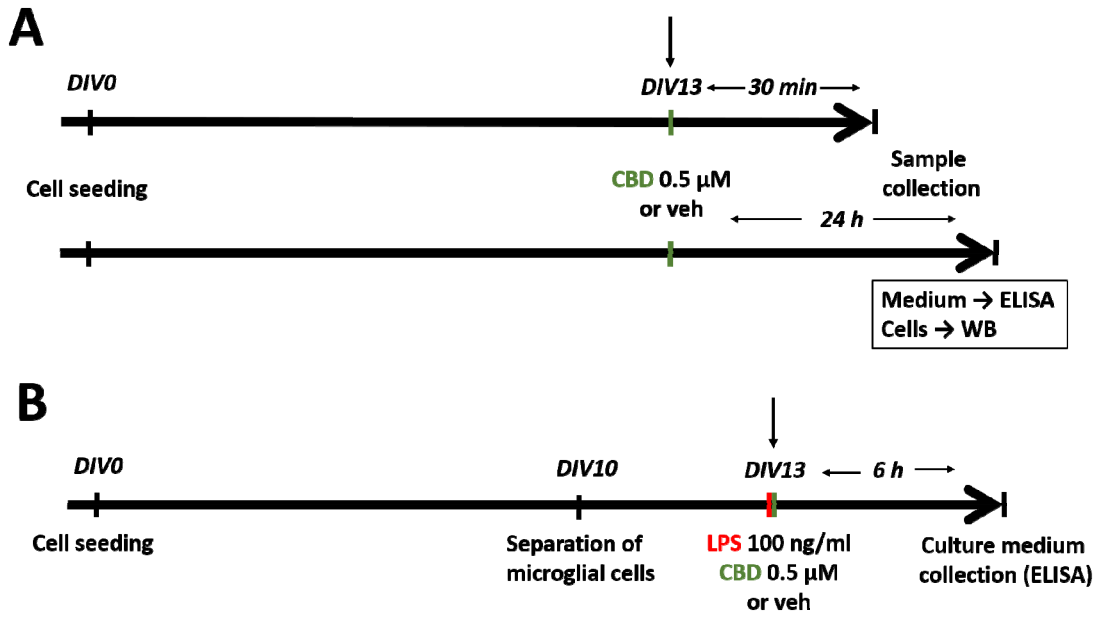


Figure 16. Experimental design of *in vitro* studies in Objectives 1 (A) and 2 (B). A) Study of the effects of CBD in neuroplasticity markers using primary neuronal cultures incubated for 30 min and 24 h. B) Evaluation of the effect of CBD incubation on inflamatory markers using primary microglial cell cultures activated with LPS. CBD: cannabidiol, LPS: lipopolysaccharide, DIV: days *in vitro*, ELISA: enzyme-linked immunosorbent assay, WB: western blot.

4. Pharmacological treatments

The main pharmacological treatment performed in this thesis was the administration of cannabidiol (CBD) for the study of its antidepressant-like effect. This was assessed using two approaches: (1) the local infusion of CBD to study the main brain areas implicated; (2) the systemic administration of CBD to mice subjected to a neuroinflammatory model induced by the injection of lipopolysaccharide (LPS) (O'Connor *et al.*, 2009). The other drug used was WAY100635, a selective antagonist of the 5-HT_{1A} receptor, to assess the role of this receptor in the effects of CBD.

All intraperitoneal (i.p.) injections (10 µl/g body weight) were performed with a 25 G needle. As CBD is an hydrophobic drug, it was dissolved in a solution containing 5% Propylenglycol®, 2% Tween 80®, in saline. The addition of the reactants following the stated order is crucial for the proper solubilization of CBD. The dose of CBD administered to mice was 30 mg/kg (Zanelati *et al.*, 2010). The selection of the i.p. route of administration for CBD was based on pharmacokinetic studies in mice (Deiana et al., 2012).

As WAY100635 maleate is a water-soluble drug, it was dissolved in water. Two doses of WAY100635 maleate were used: 0.3 mg/kg to mainly block the presynaptic 5-HT_{1A} receptors, and 1 mg/kg to block both the pre- and postsynaptic 5-HT_{1A} receptors (Serres et al., 2000; Ago et al., 2003; Carey et al., 2005). Rats received the dose of 0.3 mg/kg and mice received both doses.

For the intracerebral infusion, CBD was dissolved in a 60% DMSO and 40% saline solution, and administered during stereotaxic surgery using a 33 G needle (Hamilton®, Banat, Romania) with a flux of 0.2 µl/minute. One microliter containing 60 nmoles of CBD (Sartim *et al*., 2016) was infused into each brain hemisphere for the IL cortex administration, and a single infusion was performed for the DRN administration. After each infusion, the needle was left inside the tissue for 3 min to assure that the infusion was performed completely.

5. Stereotaxic surgery

Rats were administered CBD using a stereotaxic frame (David Kopf Instruments, California, USA) to target specific brain areas of interest. First, rats were anaesthetized with an O_2-isoflurane mixture (4% for induction and 2.5% for maintenance during all the procedure, with a flux of 2 L/min) and placed in an electric blanket to keep their corporal temperature. A sagittal cut was done to separate the skin above the cranium to visualize Bregma. The specific coordinates for drug infusion were calculated according to a rat brain atlas (Paxinos and Watson, 1987). The coordinates for the IL region of the prefrontal cortex (right and left hemisphere), expressed as mm from Bregma, were: anteroposterior (AP): + 3.2, mediolateral (ML): +/- 0.6, and dorsoventral (DV): - 5.2. For the DRN, the infusion was performed with a 30º angle to avoid the aqueduct. The coordinates were: AP: - 7.3, ML: + 1.7, and DV: - 5.9 (figure 17).

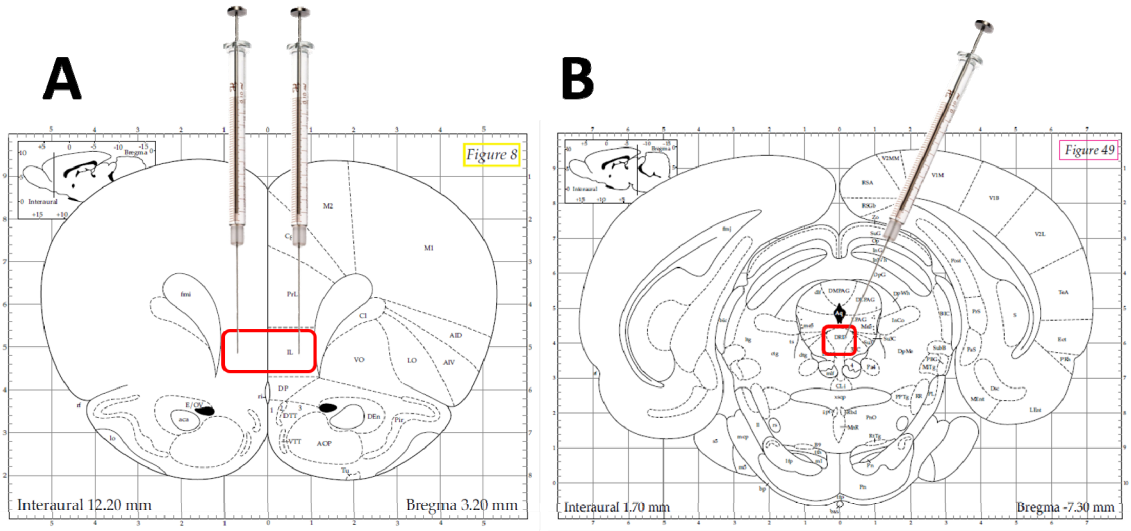


Figure 17. Brain areas in which CBD was directly infused. A) Bilateral infusion into the infralimbic cortex. B) Infusion into the dorsal raphe nucleus. Modified from Paxinos and Watson, 1987.

6. LPS-induced neuroinflammatory model

The lipopolysaccharide (LPS) used in this thesis was extracted from *Escherichia coli* serotype 0127:B8. The LPS dose administered to induce the neuroinflammatory model in mice was 0.83 mg/kg (O'Connor *et al*., 2009; Florensa-Zanuy et al., 2021). As LPS is water-soluble, it was dissolved in serum saline and administered i.p.. The behavioural effects were evaluated 12 h after LPS administration.

7. Behavioural tests

All tests were carried out during the light phase. Previous to the behavioral tests, animals were transported in their home cages from the animal room to the behavioral room and were given an hour to acclimatize to the new environment. When different tests were performed on consecutive days, the order of the behavioral battery was always from the least to the most stressful for the animals.

7.1 Open field test (OFT)

The open-field is a test used to evaluate the anxious-like behavior, which is characterized by reduced time spent in the center of the area. The open-field apparatus consists of a squared arena, the center of which is highly illuminated to create an aversive zone for

the animals. The dimensions were 100 x 100 cm width and 75 cm height for rats, and 40 x 40 cm width and 30 cm height for mice. The center was defined as a 50 x 50 cm area for rats and a 20 x 20 cm area for mice. The central illumination level was 350 lx for both rats and mice. The animals were placed in one corner of the apparatus and a computerized system (Any-maze Video-Tracking software, Stoelting Co., USA) was used to video track and analyze their behavior for 5 min. The parameters studied were the total distance traveled (indicative of the locomotor activity) and the time spent in the center (indicative of the anxious-like state of the animal).

7.2 Forced Swimming Test (FST)

The forced swimming test evaluates the depressive-like behavior and is used to assess both the behavior in diverse animal models of depression and the antidepressant-like effect of pharmacological treatments (Cryan et al., 2002). It consists in introducing the animal in a transparent cylinder (20 cm in diameter and 46 cm tall when used in rats) filled with water at 23 - 25ºC until the height of 30 cm, with the objective that the animal cannot touch the bottom of the cylinder with the tail. A pre-test of 15 min is performed 24 h before the test. Three different parameters are determined: time spent immobile, climbing, and swimming. The rat is considered immobile when it is floating passively, only performing the necessary movements to keep the head out of the water. The immobile state is considered a sign of behavioral despair and is decreased with the administration of antidepressant drugs. When the animal is doing horizontal movements along the cylinder is swimming, and when there are vertical energetic movements with the front paws, it is climbing (Slattery and Cryan, 2012).

Rats were recorded both from above and from the lateral for 6 min, and the time spent immobile, swimming and climbing was manually scored in the last 4 min of the test, by a blind observer to the experimental groups who was previously trained.

7.3 Tail Suspension Test (TST)

The tail suspension test is used to evaluate the depressive-like behavior, having high predictive validity for acute antidepressant effects. In this test, the animal is exposed to a brief stress episode without escape, as it is suspended by the tail, and after a short struggling episode will adopt an immobile state (Steru *et al.*, 1985). When a given drug decreases the time spent immobile in the TST, it is considered to have an antidepressant-like effect.

Mice were suspended using adhesive tape situated at 1 cm from the end of the tail and the TST sessions were videorecorded. The time spent immobile was manually scored during 5 min by a blind observer to the experimental groups. Immobility was considered when the mouse was “hanging passively and completely motionless”.

7.4 Sucrose Preference Test (SPT)

The sucrose preference test is used to evaluate anhedonia, which is described as the inability to experiment pleasure and is a cardinal symptom in depressive patients . Mice have a predilection for sweet, which is considered to produce a pleasant effect. A lower predilection for sweet solutions is considered to indicate an anhedonic state in mice.

Mice were individualized before the test. The test consists of the placement of two drinking bottles in the animal cage: one containing water, and the other a 2% sucrose solution (Sobrian et al., 2003; Goshen et al., 2008). Animals are given a free choice between these drinks, and the amount of liquid in each bottle is monitored. The animals were habituated to drink from the 2 bottles for 7 days before the test, to reach a sucrose preference around 80% before the start of the treatment. To avoid any possible position bias, the position of the bottles was switched every 12 h.

Sucrose preference was calculated as the percentage of the amount of sucrose solution consumed compared to the total liquid intake. This parameter was calculated weighing the bottles before drug administration and 12 hours after it. The preference before drug administration showed in the graphs is the mean of the sucrose preference of the 4 previous days.

8. Sample collection

Fresh tissue

Thirty minutes after behavioural testing, mice were sacrificed by cervical dislocation and rats by decapitation. Afterward, brains were extracted and dissected to obtain the prefrontal cortex (PFC), cortex (Cx), hippocampus (Hp), and dorsal raphe nucleus (DRN). Samples were stored at -80ºC until used.

Perfused tissue

For the immunohistochemical study, two hours after drug administration rats were deeply anaesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with cold saline solution (0.9% NaCl), followed by cold 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) using a perfusion bomb. Then, the brains were removed from the skull and post-fixed in PFA for 4 hours, and then submerged in a 30% sucrose solution (in PBS). Brains were stored at -20ºC until sectioned.

Plasma

For blood extraction, mice were deeply anaesthetized with sodium pentobarbital (40 mg/kg, i.p.) and the blood was collected directly from the heart with a syringe pre-coated with 0.25 M EDTA, pH 7.4 - 8. Then, the samples were centrifuged for 10 min at 2000xg and 4ºC to obtain the plasma. The supernatant was stored at -80ºC.

9. Primary neuronal cultures

Plate preparation

Before seeding the cells, 6-well polystyrene plates (9.5 cm^2/well) were pre-coated with 10 µg/ml of poly-L-lysine (PLL) dissolved in sterile water for 3 h at 37ºC. Before use, two washes with Dulbecco's phosphate-buffered saline (DPBS) were performed.

Brain dissection, cell extraction, and seeding

P1 - 3 rats were sacrificed by decapitation and the heads were rapidly cooled on ice. Then, the brains were extracted and placed in culture plates containing cold Hanks' Balanced Salt Solution (HBSS). A magnifying glass was used to microdissect the right and left cortices, after carefully removing the meninges. All the following steps were performed under a laminar flow hood in sterile conditions. The cortices were placed in a new plate with cold HBSS, cut into small pieces and transferred to a falcon tube. When the tissue pieces were deposited on the bottom of the tube, the HBSS was aspirated and 1 ml per rat of an enzymatic solution (0.01% Papain, 0.1 % Dispase II, 0.01% DNAse I and 14.4 mM $MgSO_4$ in HBSS without Ca^{2+} and Mg^{2+}) was added to disaggregate the cells. A mechanical cell dispersion was also carefully performed. Then, the cells were placed at 37ºC with agitation for 10 min. The last 2 steps (mechanical dispersion and 37ºC incubation) were repeated 2 more times. After that, cells were centrifuged 5 min at 300xg at RT, the supernatant was discarded and 2 ml per rat of Neurobasal A cell culture

medium at 37ºC were added to resuspend the cells. This step was repeated, and afterward, the cell suspension was filtered with a 40 µm cell strainer. Another centrifugation was performed, and cells were resuspended in 1 - 2 ml of complete Neurobasal A medium (2% B27, 1% penicillin/streptomycin, 0.12 mM ciprofloxacin and 1% GlutaMax™) to count cell density using an automated cell counter (Countess™ Invitrogen, California, USA). An adequate amount of complete Neurobasal A medium was added to seed the cells at a density of 10^5 cells/cm^2. Cells were maintained during all the cell culture in an incubator at 37ºC, 95% humidity, and 5% CO_2 conditions.

Primary neuronal culture maintenance

In day 1 of cell culture (DIV1), all the medium was replaced with complete Neurobasal A medium to eliminate dead cells and debris. At DIV7 all the medium was replaced with Neurobasal A medium containing 2% B27 and 1% GlutaMax™ to remove the antibiotics from the culture. At DIV10 half of the medium was removed and replaced with fresh medium.

Cell treatment

To establish the appropriate CBD concentration to treat the cells, the following CBD concentrations were used in a 24 h incubation: 0.05 µM, 0.1 µM, 0.25 µM, 0.5 µM, and 1 µM. Then, based on the highest increase of phospho-mTOR (p-mTOR) and BDNF levels determined by Western Blot that was reached with 0.5 µM CBD (figure 30), this concentration was selected for the following *in vitro* studies.

Cells were treated with 0.5 µM CBD at DIV13. CBD was first dissolved in DMSO and finally, a 1:2000 dilution was performed with Neurobasal A medium (supplemented with 2% B27 and 1% GlutaMax™). The vehicle group had the same dilution of DMSO. Cells were placed in the incubator during the treatment duration: 30 min and 24 h.

Sample collection

When the treatment was finished, the cell culture medium was collected in pyrogen/endotoxin-free tubes (Eppendorf, Hamburg, Germany), centrifuged 20 min at 1000xg at RT, and the supernatant was stored at -80ºC. To harvest the cells, plates were previously washed with PBS and then the cells were detached using a cell scraper in the presence of PBS. The cell suspension was collected and centrifuged for 10 min at 20800xg at 4ºC. Pellets were stored at -20ºC.

10. Primary microglial cultures

The protocol described by Bronstein et al. (2013) was followed with minor modifications.

Plate preparation

Cell culture polystyrene plates of 60 x 15 mm (19.5 cm^2) were pre-coated with 5 µg/ml of poly-D-lysine (PDL) diluted in DPBS for 3 h at 37ºC. Two washes with DPBS were performed before use.

Brain dissection, cell extraction, and seeding

P1 - 3 mice were sacrificed by decapitation and brains were rapidly placed on ice. The dissection of the cortices was performed as described in the previous section. After removing the HBSS, 1 ml per mouse of trypsin/EDTA was added and a mechanical cell dispersion was carefully performed. Cells were incubated in agitation for 15 min at 37ºC. To stop the enzymatic digestion, complete microglial medium [Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), 1% sodium pyruvate and 0.08% gentamycin] was added. The cell suspension was centrifuged for 5 min at 1000xg and RT. This washing step was repeated, and cells were resuspended in complete microglial medium and filtered through a 40 µm cell strainer. Then, cell density was determined using an automated cell counter and the appropriate amount of medium was added to seed the cells at a density of 10^5 cells/cm^2. The result of this first step is a mixed culture with different cell types. Cells were maintained during all the cell culture in an incubator at 37ºC, 95% humidity, and 5% CO_2 conditions. At DIV1 and 3, all the medium was changed with complete microglial medium.

Separation of microglial cells

Microglial cells were detached from the plate at DIV10 by adding lidocaine (first dissolved in ethanol and HBSS) to the culture medium to a final concentration of 4 mM. After 15 min at RT, the cell suspension was collected from the plate, together with an additional wash with HBSS to recover the remaining microglial cells. Then, EDTA (pH 8) was added to a final concentration of 50 µM, cells were centrifuged for 5 min at 1000xg and RT, and resuspended in 1 ml of DMEM with 1% FBS. The number of cells was quantified with an automated cell counter and cells were seeded at a density of 10^5 cells/cm^2 (12 well polystyrene plates, 3.8 cm^2/well, were pre-coated as explained in plate preparation). After 2 - 3 days the cells had fully returned to their ramified resting state.

Cell treatment

Cells were treated at DIV13. To trigger microglial activation, cells were incubated with 100 ng/ml LPS (first dissolved with water). CBD was first dissolved in DMSO, diluted 1:2000, and used at a final concentration of 0.5 µM. All the drugs were finally dissolved in 37ºC DMEM with 10% FBS. The vehicle groups had the same dilution of water/DMSO. Drug incubation was performed for 6 h at 37ºC.

Sample collection

When the incubation period was finished, the cell culture medium was collected in pyrogen/endotoxin-free tubes and stored at -80ºC.

11. Molecular techniques

11.1 Quantitative Polymerase Chain Reaction (qPCR)

The quantitative PCR is a variant of the PCR technique which permits the quantification of the DNA amplification product of the gene of interest at the end of each amplification cycle.

RNA extraction

Total RNA extraction was performed using TRI reagent® following the manufacturer instructions. PFC mouse samples (weighting 25 - 50 mg) were mechanically homogenized using a pellet pestle motor (DWK Life Sciences Kontes™, New Jersey, USA) in 500 µl of TRI reagent®. After 5 min at RT, 100 µl of chloroform were added and the tubes were shaken vigorously for 15 s and left for 15 min at RT. A centrifugation step was performed for 15 min at 4ºC and 13800xg to separate the samples in 3 phases: an aqueous upper phase containing the RNA, a lower pink phase containing the proteins, and a white phase in between containing the DNA. The upper aqueous phase was carefully collected in nuclease-free tubes. After this step, all the remaining procedures were performed under a laminar hood. Then, 250 µl of isopropanol were added, the samples were left for 10 min at RT and centrifuged for 10 min at 13800xg, 4ºC. The supernatant was discarded and after washing with 1 ml of 75% ethanol, samples were vortexed. This was followed by centrifugation of 5 min at 8600xg, 4ºC, after which the samples were air-dried for 20 min. Finally, the RNA was resuspended in 20 µl of nuclease-free water and stored at -80ºC. A NanoDrop 1000 (Thermo Fischer Scientific S.L., Madrid, Spain) was used to determine the quality and concentration of the RNA by

reading the absorbance at 230, 260, and 280 nm and calculating the ratios 260/280 and 260/230 to assess RNA purity.

Reverse transcription (RT)

A high capacity cDNA reverse transcriptase kit (Applied Biosystems, California, USA) was used for the reverse transcriptase reactions with 600 ng of total RNA per sample, following the manufacturer instructions. The cycles of the reaction, performed with a 2720 thermal cycler (Applied Biosystems, California, USA), were the following: i) 10 min at 25ºC, ii) 120 min at 37ºC, iii) 5 min at 85ºC, and iv) maintenance at 4ºC until use/storage at -80ºC.

Quantitative Polymerase Chain Reaction (qPCR)

Quantitative PCR was performed in a StepOne™ Real-Time PCR System (Applied Biosystems, California, USA) using TaqMan™ gene expression probes for the genes specified in table 2 and TaqMan™ Gene Expression Master Mix. Each reaction contained 125 ng of cDNA template and was performed in triplicate. Non-template controls were included in each plate. The reaction cycles were the following: i) hold step of 10 min at 95ºC, ii) 40 cycles composed by 2 steps: ii-a) 15 s at 95ºC and ii-b) 1 min at 60ºC. The expression levels of the house-keeping genes β-actin and GAPD were used to normalize mRNA expression levels of the genes of interest.

Table 2. Sequence and assay ID of the Taqman™ probes used for qPCR experiments (from Applied Biosystems, California, USA).

GENE	SEQUENCE	ASSAY ID
IL-6	5'-TGAGAAAAGAGTTGTGCAATGGCAA-3'	Mm00446190_m1
TNFα	5'-CCCAAAGGGATGAGAAGTTCCCAAA-3'	Mm00443258_m1
β-actin	N/A	Mm00607939_s1
GAPD	N/A	Mm99999915_g1

N/A: not available.

Determination of gene expression

In the amplification curve of the PCR, there is an exponential phase in which the increase in fluorescent signal is linear and proportional to the amplified product. An amplification

threshold is set above basal levels, and the threshold cycle (Ct) is the number of cycles in which the cDNA quantity of the studied gene reaches the set threshold. The Ct value is inversely proportional to the amount of cDNA present at the beginning of the reaction. Therefore, a lower Ct will correspond to a higher cDNA amount of the gene of interest.

The equation established by Livak and Schmittgen (2001) was used to calculate the relative changes of expression of the target genes based on the mRNA amount of the two house-keeping genes (the average of the 2 genes was calculated and used in the equation). The mentioned equation is $2^{-\Delta\Delta Ct}$, where ΔCt = (Ct target gene – Ct house-keeping genes average) and ΔΔCt = (ΔCt in the experimental condition – ΔCt in the control condition). Results were expressed as relative mRNA quantity ± S.E.M., in which a relative value of 1 is the average of the control group.

11.2 Western Blot (WB)

Protein extraction: total homogenate

The tissue was mechanically homogenized 1:15 w/v with buffer without detergents (10 mM HEPES, pH 7.9, 100 mM KCl, 1.5 mM $MgCl_2$, supplemented with the following protease inhibitors: 1 mM PMSF, 0.03 mM aprotinin, 0.02 mM leupeptin, 0.01 mM pepstatin, 0.02 mM antipain, 0.02 mM chymostatin, and phosphatase inhibitors: 1 mM Na_3VO_4 and 1 mM NaF) using a pellet pestle motor. Afterward, a buffer with detergents (buffer without detergents supplemented with 1% Igepal, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2.5 mM CHAPS) was added, and the lysis was performed for 30 min on ice. Then, samples underwent a 10 min centrifugation at 20800xg, 4ºC, and the supernatant containing the solubilized proteins was stored at -20ºC as total homogenate until needed.

Protein extraction: subcellular fractionation

To study the protein expression in the cytosol and the nucleus separately, subcellular fractionation was performed following the protocol used in García-Bueno et al. (2005) with minor modifications. In brief, the tissue was mechanically homogenized with 300 µl of a buffer containing 10 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol (DTT), 0.5 M sucrose, 10 mM sodium molybdate and supplemented with a protease inhibitors cocktail, using a pellet pestle motor. After 15 min on ice, 0.5% Igepal was added and the tubes were gently vortexed for 15 s.

For the subcellular fractionation, samples were centrifuged 5 min at 8000xg, 4ºC, and the supernatant was collected as the cytosolic fraction. Then, the pellet was resuspended with 100 µl of the previous buffer supplemented with 20% glycerol and 0.4 M KCl and gently shaken for 30 min at 4ºC. After a 5 min centrifugation at 13000xg and 4ºC, the supernatant was collected as the nuclear fraction. All the samples were stored at -80ºC.

Sample preparation

In order to use the same amount of protein of each sample, the Lowry method (Lowry *et al.*, 1951) was applied for protein quantification using the DC™ Protein Assay. In the total homogenate and cytosolic fractions 45 - 60 µg of protein per well were loaded, and 90 µg per well in the nuclear fractions. Laemmli buffer 2x supplemented with 2-mercapto ethanol was added to the protein samples, and they were boiled at 100ºC for 5 min, left 3 min on ice, and finally centrifuged during 5 min at 960xg, 4ºC. The supernatant was stored at -20ºC.

Electrophoresis and protein transfer

The acrylamide gels used in the Western Blot were 8.5%, 10%, 12.5%, and 15% of acrylamide, depending on the protein studied. For protein migration, the gels were placed in tanks with migration buffer (20 mM Tris, 0.2 M glycine, 1% SDS) and the samples were loaded into the wells in duplicate. A protein ladder (PageRuler Plus Prestained) was also loaded to identify the molecular weight of the bands. The electrophoresis was performed in two phases: 15 min at 100 V and 50 min at 160 V. When the protein migration was finished, the gels and nitrocellulose membranes (Bio-Rad, California, USA) were submerged separately in transference buffer (20 mM Tris, 0.2 M glycine and 20% methanol) in agitation for 30 min. After, a wet protein transfer to nitrocellulose membranes was performed at 4ºC with a transfer buffer at 100 V. The duration of the transfer was 90 min for proteins with a molecular weight higher than 40 kDa, and 45 min for proteins with a molecular weight lower than 40 kDa.

Membrane blocking, antibody incubation, and signal detection

Membranes underwent a blocking step of 1 h at room temperature (RT) in agitation to avoid the unspecific binding of the antibodies to the membrane. The blocking solution depends on the antibody. For most of the antibodies used, the blocking solution was 5% powder skimmed milk in TBS-T (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20). When the antibodies were against phosphorylated proteins, the blocking solution was 3% powder skimmed milk in TBS-T supplemented with phosphatase inhibitors (1

mM Na_3VO_4 and 1 mM NaF). For CREB, phospho-CREB, NF-κB, and PPARγ antibodies, membranes were blocked with 5% BSA in TBS-T 0.1 (with 0.1% Tween-20). For phospho-AKT, the blocking step was performed with 10% powder skimmed milk in TBS (50 mM Tris-HCl, pH 7.6, 150 mM NaCl) supplemented with 1 mM Na_3VO_4 and 1 mM NaF.

Table 3. Antibodies and dilutions used for protein detection by Western Blot and correspondent suppliers.

ANTIBODY	DILUTION	REFERENCE	SUPPLIER
AKT (m)	1:500	sc-5298	Santa Cruz Biotechnology
p-Ser473-AKT (Rb)	1:500	#9271	Cell signaling
β-catenin (m)	1:1000	sc-7963	Santa Cruz Biotechnology
β-tubulin III (Rb, m)	1:20000	T2200/T8660	Sigma
BDNF (Rb)	1:250	Ab108319	Abcam
CREB (m)	1:500	#9104	Cell signaling
p-Ser133-CREB (Rb)	1:1000	#9198	Cell signaling
ERK-2 (Rb)	1:2000	sc-154	Santa Cruz Biotechnology
p-Thr183/Tyr185-ERK-1/2 (m)	1:1000	M8159	Sigma
GAPDH (m)	1:2000	sc-32233	Santa Cruz Biotechnology
GSK3-β (m)	1:2000	#9832	Cell signaling
p-Ser9-GSK3-β (Rb)*	1:1000	#9323	Cell signaling
IκBα (Rb)	1:2000	AB3016	Chemicon
mTOR (m)	1:1000	#4517	Cell signaling
p-Ser2448-mTOR (Rb)	1:250	#2971	Cell signaling
NF-κB (m)	1:1000	#6956	Cell signaling
PCNA (m)	1:1000	sc-56	Santa Cruz Biotechnology
PPARγ (Rb)	1:1000	MA5-14889	Invitrogen
800CW anti-mouse	1:15000	926-32212	LI-COR®
700CW anti-mouse	1:15000	926-68072	LI-COR®
800CW anti-rabbit	1:15000	926-32213	LI-COR®
700CW anti-rabbit	1:15000	926-68073	LI-COR®
800CW anti-goat	1:15000	926-32214	LI-COR®

** All the phosphorylations targeted activate the proteins except for p-Ser9-GSK3-β, which inhibits the activity of the protein. m: mouse; Rb: rabbit.*

Afterward, membranes were incubated overnight at 4ºC with the primary antibody (table 3) diluted in a solution with the same composition as in the blocking step (except for phospho-AKT antibody, which was incubated with 5% powder skimmed milk in TBS-T 0.1 supplemented with 1 mM Na_3VO_4 and 1 mM NaF). In table 3, all the primary and secondary antibodies used are listed, specifying the dilution used and the supplier.

After primary antibody incubation, membranes underwent 3 washes (15 min with TBS-T in agitation) and were incubated with the correspondent secondary antibody (table 3) diluted in the blocking solution for 1 h at RT in the dark (phospho-AKT was again an exception, as the secondary antibody was incubated with 5% powder skimmed milk in TBS supplemented with 1 mM Na_3VO_4 and 1 mM NaF). After 3 more washes in the dark, 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 images obtained with Odyssey® CLx Imaging System were quantified using the Image Studio™ Software (LI-COR Biosciences, Nebraska, USA). To correct the possible deviations in the amount of protein loaded of each sample, the values of the protein of interest were normalized against the value obtained from a house-keeping gene. The house-keeping genes used were β-tubulin III and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for total homogenate and the cytosolic fraction, and proliferating cell nuclear antigen (PCNA) for the nuclear fraction (table 3). After performing this loading correction, the average of the replicates of each sample was calculated. Protein expression results were calculated in percentage *versus* the vehicle group (100%) of each experiment.

11.3 Enzyme-Linked Immunosorbent Assay (ELISA)

IL-6 and TNFα ELISA kits were purchased from Invitrogen (Life Technologies Corporation, California, USA), and BDNF kit from Cloud-Clone Corporation (Texas, USA). The procedure specified by each manufacturer was followed. The optical density (O.D.) was determined at 450 nm using a Mithras LB 940 (Berthold technologies, Baden-Württemberg, Germany). GraphPad Prism software (California, USA) was used to generate a standard curve and to extrapolate the amount of the protein of interest in each sample (pg of the target protein/ml). The average of two replicates of each sample

was calculated. For cell culture, data results were expressed in percentage *versus* the vehicle group.

11.4 Immunohistochemistry (IHQ)

For c-Fos immunolabeling, perfused brains were cut in coronal serial sections (40 µm thick) with a cryostat. After five 10 min washes in PBS, free-floating brain slices were incubated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 10 min, and after 5 more washes, they were blocked with 0.2% Triton X-100 and 3% normal donkey serum (NDS) in PBS at RT for 1h. Sections were incubated with a rabbit c-Fos primary antibody (sc-52, Santa Cruz Biotechnology, Texas, USA) at a 1:500 dilution in PBS containing 3% NDS, at 4ºC overnight. After five 10 min washes with 0.3% Triton X-100 in PBS, sections were incubated with a biotinylated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Europe Ltd., Cambridgeshire, UK) at a 1:500 dilution in 0.3% Triton X-100, 3% NDS in PBS for 2h at RT. Following five more washes, avidin-biotin complexes were formed using the Vectastain ABC kit (Vector, California, USA) for 30 min to amplify the antibody signal. Then, after 3 additional washes in PBS and 2 in 50 mM Tris-HCl, pH 7.4, c-Fos positive cells were labeled with diaminobenzidine (DAB) substrate kit (Vector, California, USA). The last 4 washes were performed with distilled water, and finally, brain slices were counterstained with hematoxylin and dehydrated in 70% ethanol, 95% ethanol, 100% ethanol (2 times), and xylene (2 times), 5 min each. DPX was used for mounting the slides.

Image acquisition and c-Fos counting

An AxioScanZ.1 (Carl Zeiss) scanner was used to digitalize the c-Fos IHQ images using a Plan-APO x20/NA 0.8 lens (0.220 mm /pixel) and a color camera HV-F201SCL (Hitachi) with a 1600x1200 pixel sensor, a size of 7.13 x 5.37 mm and an adapter of x1. The images were analyzed by a observer blind to the treatments and were processed using ZEN 2.3 SP1 (blue edition) software (version 14.0.0.201) (Carl Zeiss Microscopy GmbH, Germany). Before counting the number of c-Fos positive cells, the same brightness and contrast were applied to all the images obtained. The brain regions quantified were the prelimbic cortex (PL; from 4.70 mm to 2.20 mm relative to Bregma), the infralimbic cortex (IL; from 3.20 mm to 2.20 mm relative to Bregma), and the DRN (from -7.30 mm to -8.00 mm relative to Bregma). c-Fos quantification was expressed as c-Fos positive cells per area (mm^2).

11.5 High-Performance Liquid Chromatography (HPLC)

This technique was used to evaluate the levels of neurotransmitters and kynurenine pathway precursors and metabolites in *postmortem* brain samples.

Sample preparation

Samples were placed in nuclease-free tubes and mechanically homogenized in milli-Q water using a pellet pestle motor. The weight/volume ratio used ranged from 1:5 to 1:9, depending on the sample weight. Then, perchloric acid was added reaching a final concentration of 1.2% and samples were centrifuged for 15 min at 16000xg and 4ºC. The supernatant was collected and filtered through 0.45 µm filters. Samples were immediately used or stored at -80ºC.

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(s), LC 110S pump(s), a DECADE Elite electrochemical detector, Clarity chromatography software of DataApex (Prague, Czech Republic) and an AS 110 autosampler. In the following sections are described the columns and mobile phases that were used to detect the different neurotransmitters and metabolites.

Serotonin, noradrenaline, tryptophan, and kynurenine detection

The levels of 5-HT, NA, TRP, KYN were determined using an Acquity UPLC® BEH C18, 1.7 µm, 1 x 100 mm column for monoamines and their acidic metabolites detection (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 temperature was set at 42ºC for the separation and detection processes, the flow rate was 75 µl/min, and the oxidation potential used was 0.46 V for 5-HT and NA detection, and 0.7 V for TRP and KYN.

Glutamate,GABA, and 3-HK detection

The amount of glutamate, γ-aminobutyric acid (GABA), and 3-hydroxykynurenine (3-HK) was determined using an Acquity UPLC® HSS T3, 1.8 µm, 1 x 50 mm column (Waters, Massachusetts, USA). As these molecules are not directly detectable with electrochemistry, automatic pre-column derivatization with o-phthalaldehyde (OPA) and sodium sulfite was applied. In this case, two mobile phases were needed. The mobile phase used for separation consisted of 50 mM phosphoric acid, 50 mM citric acid, 0.1

mM EDTA, pH 3.5, and 2% acetonitrile. The mobile phase used in the post-separation step was made of 50 mM phosphoric acid, 50 mM citric acid, 0.1 mM EDTA, pH 3.5, and 50% acetonitrile. The temperature used for separation and detection was 40ºC, the flow rate was 200 µl/min, and the oxidation potential was set at 0.85 V.

HPLC quantification

To quantify the amount of the molecules of interest present in the samples, a standard curve was performed to calibrate each of them. The software integrated the peaks selected from the histogram and the results were obtained as pg/µl. The values were corrected for each sample volume and tissue weight and were finally presented as pg-ng/mg of tissue.

12. Statistical analysis

The statistical analysis of the data was performed using GraphPad Prism software, version 8.4.3 (GraphPad Software Inc., California, USA). All the results were expressed as mean ± standard error of mean (S.E.M.). The level of significance was set at $p<0.05$.

The statistical comparison between two normally distributed populations was made with the unpaired Student *t*-test. When more than two populations sharing the same variable were compared, the one-way analysis of variance (one-way ANOVA) was used. Additionally, when two factors could interact with each other, the two-way analysis of variance (two-way ANOVA) was used. For both types of analysis of variance, the statistical comparison between groups was complemented with the Newman-Keuls *posthoc* test. For the correlation studies, the Pearson's correlation coefficient (r) was used. All the statistical results obtained in this thesis are compiled in Annexe 1.

RESULTS

OBJECTIVE 1. CBD antidepressant-like effects: site and mechanism of action

1.1 Acute behavioural and molecular effects of the bilateral infralimbic cannabidiol infusion

1.1.1 Evaluation of the depressive-like, anxious-like and locomotor behaviour

The bilateral infralimbic (IL) CBD infusion in rats (*see figure 14A in Material and methods*) produced a significant decrease in the time spent immobile in the FST as assessed 30 min after the administration (94.38 ± 24.99 s in CBD *vs* 167.50 ± 14.59 s in the vehicle group, $p<0.05$), which is indicative of an acute antidepressant-like effect. CBD also induced an increase in the time spent swimming (120.60 ± 22.51 s in CBD *vs* 58.33 ± 13.08 s in the vehicle group, $p<0.05$), without changes in the climbing time (figure 18A).

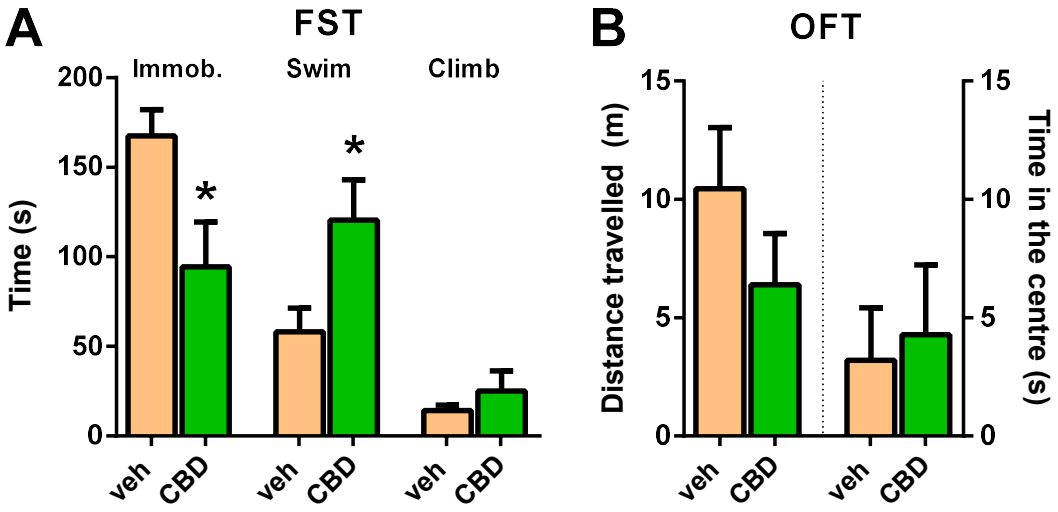


Figure 18. Acute effect of the bilateral IL CBD infusion in the depressive-like, anxious-like and locomotor behaviour. A) Time spent immobile, swimming, and climbing in the forced swimming test (FST). B) Distance travelled and time spent in the centre in the open field test (OFT). Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test *$p<0.05$ *vs* vehicle group. n= 6 - 8 animals per group.

No changes were observed in the distance travelled nor in the time spent in the centre in the OFT, indicating that the acute IL CBD infusion does not affect locomotion nor anxious-like behaviour (figure 18B) (Annexe 1, table 1).

1.1.2 Plasticity markers' expression in the prefrontal cortex and hippocampus

Following behavioural testing, rats were sacrificed and the expression of a battery of neuronal plasticity markers was studied in two crucial brain areas implicated in depression neurobiology and the antidepressant response: the prefrontal cortex and the hippocampus.

In the prefrontal cortex, the acute infusion of CBD into the IL cortex increased phospho-mTOR (142.70 ± 17.58% in CBD *vs* vehicle group, $p<0.05$), phospho-ERK (172.40 ± 28.20% in CBD *vs* vehicle group, $p<0.05$) and BDNF (749.00 ± 201.90% in CBD *vs* vehicle group, $p<0.01$) levels, whereas no changes were observed in the levels of phospho-GSK3β, β-catenin, phospho-AKT and phospho-CREB (figure 19A). Regarding the total (non-phosphorylated) protein levels, an increase in AKT levels in the CBD group was detected (120.20 ± 5.83% *vs* vehicle group, $p<0.05$), as well as a tendency to a decrease in CREB levels in the CBD group (88.49 ± 3.41% *vs* vehicle group, $p=0.065$) . The total amount of mTOR, GSK3β and ERK proteins were not modified.

Similar changes in the expression of plasticity markers were observed in the hippocampus. There was an increase in phospho-ERK (189.90 ± 35.48% in CBD *vs* vehicle group, $p<0.05$) and BDNF levels (126.80 ± 5.91% in CBD *vs* vehicle group, $p<0.01$), and a decrease in phospho-GSK3β levels (74.09 ± 8.40% in CBD *vs* vehicle group, $p<0.05$) (figure 19B). Regarding the total amount of mTOR, GSK3β, and ERK in the hippocampus, only a tendency to an increase in GSK3β levels was detected (80.22 ± 6.62% in CBD *vs* vehicle group, $p=0.066$) (Annexe 1, table 2).

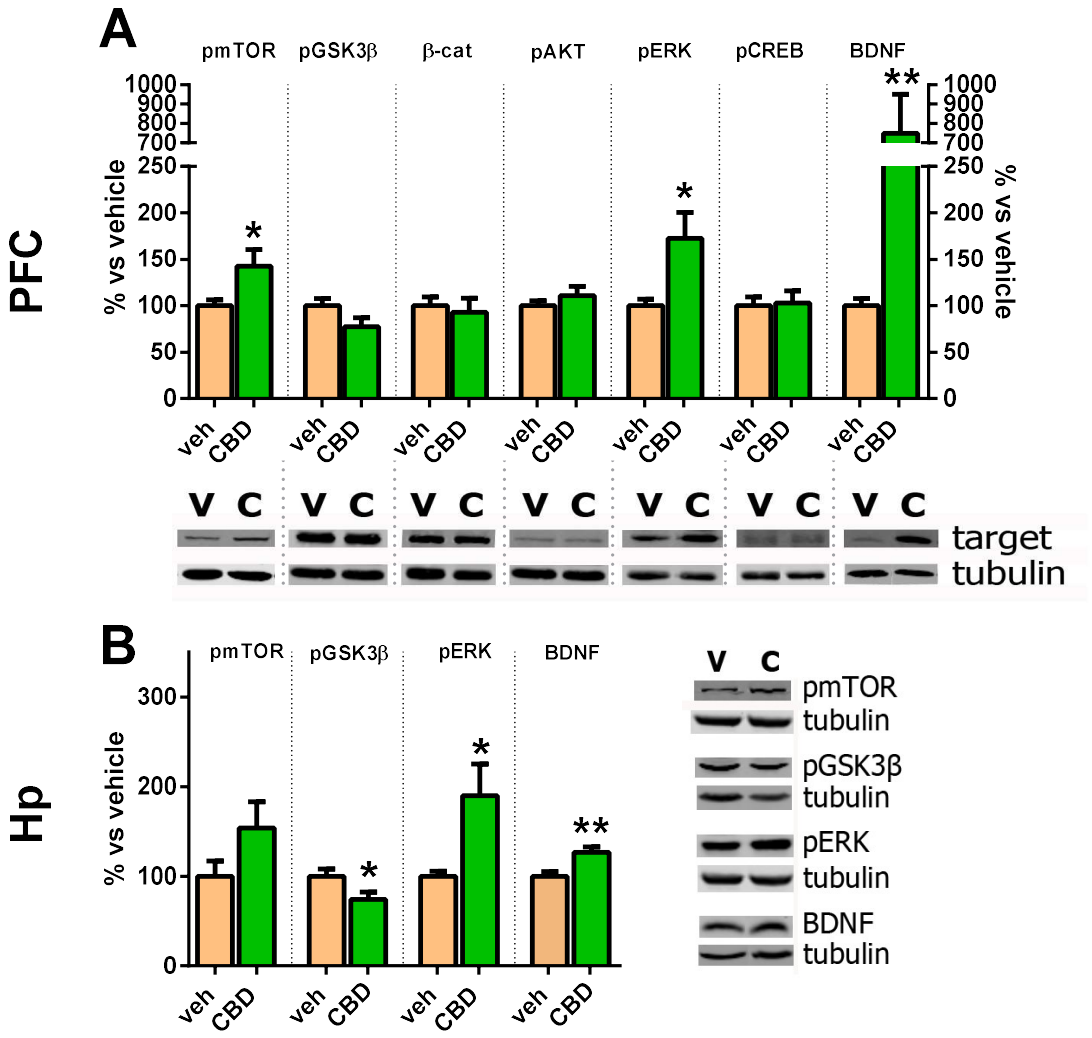


Figure 19. Plasticity markers' expression in the prefrontal cortex (A) and hippocampus (B) induced by the acute bilateral IL CBD infusion. Representative bands from the western blot of each marker and the corresponding housekeeping are shown. Results are expressed as percentage *versus* the vehicle group, and as mean ± S.E.M. Unpaired Student's *t*-test **p*<0.05, ***p*<0.01 *vs* vehicle group. n= 6 - 8 animals per group in (A) and 5 - 7 in (B).

1.1.3 Neurotransmitter levels in the prefrontal cortex and dorsal raphe nucleus

As the connectivity between the prefrontal cortex and the dorsal raphe nucleus plays an important role in depression, the levels of serotonin, noradrenaline, glutamate, and GABA were determined by HPLC in *postmortem* samples of these two brain areas obtained following behavioural testing. No significant changes were found in serotonin levels in none of the areas studied (figure 20A). CBD induced a decrease in noradrenaline

levels in the prefrontal cortex (176.4 ± 5.1 pg/mg in CBD *vs* 204.4 ± 4.9 pg/mg in the vehicle group, $p<0.01$) and no changes in the DRN (figure 20B). The infusion of CBD in the IL cortex induced a decrease of GABA in the DRN (347.4 ± 27.5 pg/mg in CBD *vs* 477.8 ± 50.6 pg/mg in the vehicle group, $p<0.05$) and no changes in the PFC (figure 20C). Regarding glutamate levels, CBD produced a significant decrease both in the PFC (7051 ± 387 pg/mg in CBD *vs* 9237 ± 906 pg/mg in the vehicle group, $p<0.05$) and the DRN (1367 ± 112 pg/mg in CBD *vs* 1816 ± 155 pg/mg in the vehicle group, $p<0.05$) (figure 20D) (Annexe 1, table 3).

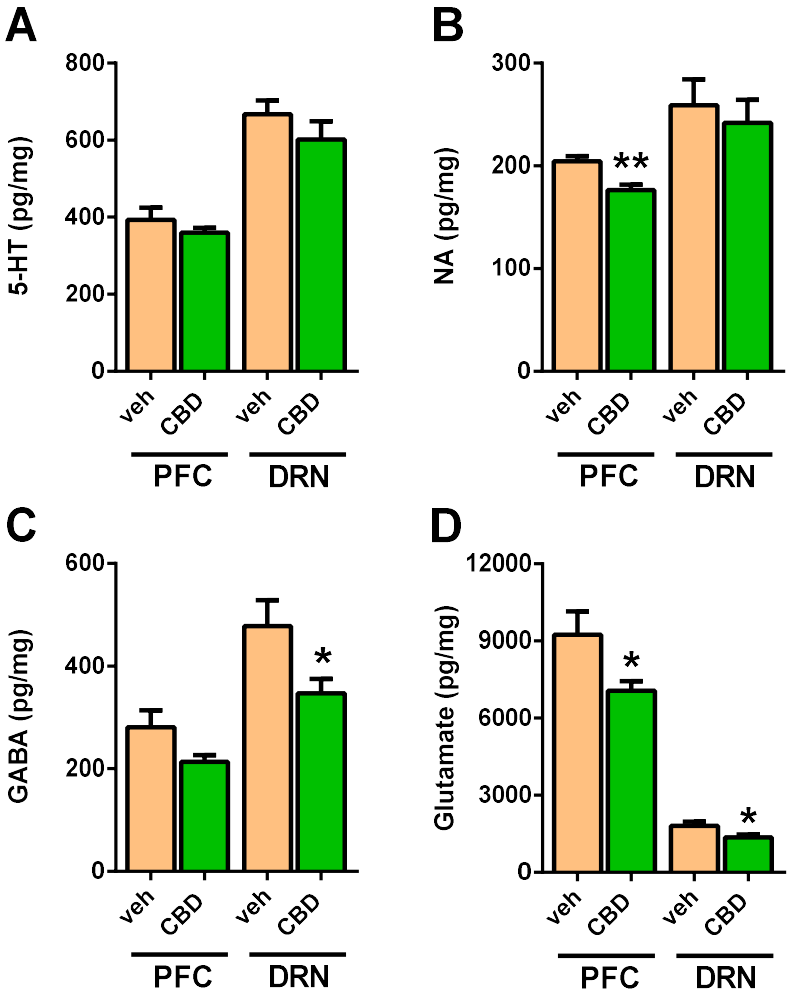


Figure 20. Neurotransmitters' levels in the prefrontal cortex and the dorsal raphe nucleus after the acute bilateral IL CBD infusion. A) serotonin. B) noradrenaline. C) GABA. D) glutamate. Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test *$p<0.05$, **$p<0.01$ *vs* vehicle group. n= 6 - 8 animals per group.

1.1.4 Neuronal activation in the prefrontal cortex and dorsal raphe nucleus: effect of CBD in *naïve* and stressed animals

The c-Fos marker was used to assess neuronal activation in the prelimbic and infralimbic cortex and the dorsal raphe nucleus following the acute bilateral IL CBD infusion and in the presence and absence of acute stress induced by behavioural testing (*see figure 14B in Material and methods*).

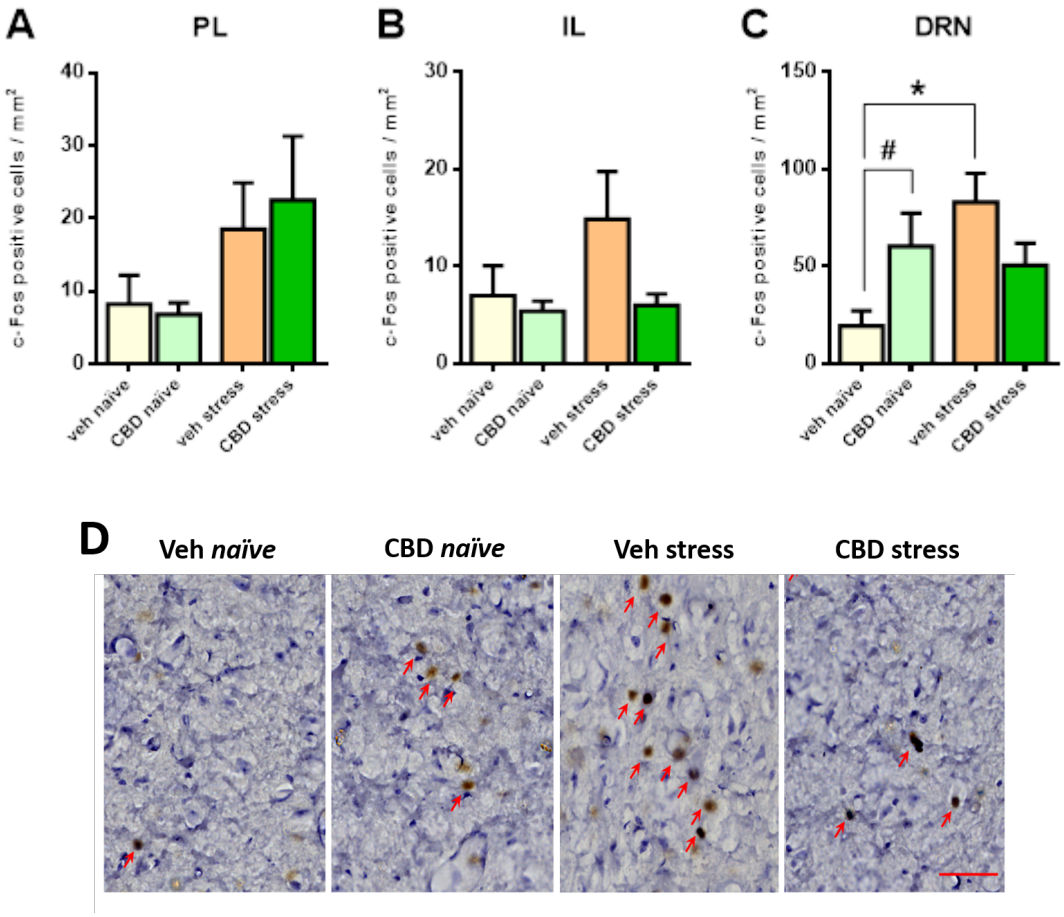


Figure 21. Immunohistochemical evaluation of c-Fos expression in the prelimbic (A) and infralimbic (B) cortex and the dorsal raphe nucleus (C) after the acute IL CBD infusion and acute stress exposure. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by a Newman Keuls *posthoc* test **p*<0.05 *vs* vehicle *naïve* group. Unpaired Student's *t*-test $^{\#}p$<0.05 *vs* vehicle *naïve* group. n= 4 - 6 animals per group. Representative images of the c-Fos immunolabeling in the dorsal raphe nucleus of vehicle naïve, vehicle stress, CBD naïve, and CBD stress conditions (D). Bar: 50 µm.

In the prelimbic cortex, acute stress produced a noticeable tendency to increase c-Fos expression in both vehicle and CBD groups (figure 21A). A two-way ANOVA showed a significant effect of the stress factor [$F_{(1,15)}$ = 4.881, p<0.05]. In contrast, in the infralimbic cortex, the stress produced a noteworthy tendency to increase c-Fos expression only in the vehicle group, and not when animals were infused with CBD in the IL cortex (figure 21B).

In the dorsal raphe nucleus, acute stress significantly increased c-Fos expression in the vehicle group (83.03 ± 14.89 c-Fos$^+$ cells/mm^2 in vehicle stress *vs* 19.41 ± 7.74 c-Fos$^+$ cells/mm^2 in vehicle *naïve* group, p<0.05). A two-way ANOVA *posthoc* test showed no statistical differences in the CBD-infused groups, although an unpaired Student's *t*-test showed that CBD infusion induced a significant increase in c-Fos levels under non-stress conditions (*naïve*) (60.45 ± 16.96 c-Fos$^+$ cells/mm^2 *vs* 19.41 ± 7.74 c-Fos$^+$ cells/mm^2 in the *naïve* vehicle group; t=2.374, df=7, p<0.05) (figures 21C and D). A two-way ANOVA revealed a significant effect of the interaction between treatment and stress factors [$F_{(1,15)}$ = 8.389, p<0.05] (Annexe 1, table 4).

1.2 Behavioural and molecular effects of the bilateral infralimbic cannabidiol infusion 24 hours after its administration

1.2.1 Evaluation of the depressive-like, anxious-like and locomotor behaviour

In order to evaluate the potential sustained behavioural and molecular effects induced by the bilateral IL CBD infusion, both the forced swimming test and the open field tests were performed 24 h after its administration (*see figure 14C in Material and methods*). No significant differences were found between rats treated with CBD and vehicle, in any of the parameters studied: time spent immobile, swimming and climbing in the FST (figure 22A) and time spent in the centre and total distance travelled in the OFT (figure 22B) (Annexe 1, table 5).

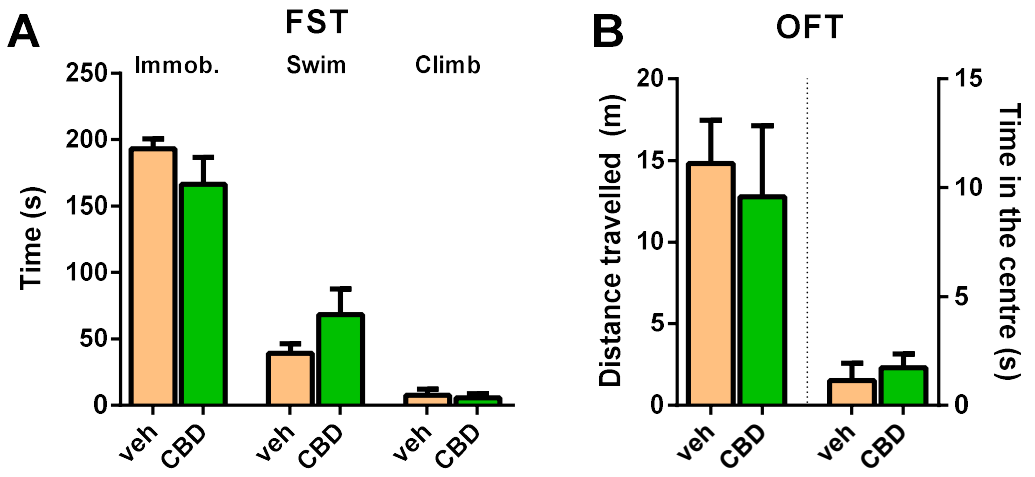


Figure 22. Effect of the IL CBD infusion in the depressive-like, anxious-like and locomotor behaviour assessed 24 h after its administration. A) Time spent immobile, swimming and climbing in the forced swimming test. B) Total distance travelled and time spent in the centre in the open field test. Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test. n= 6 - 8 animals per group.

1.2.2 Plasticity markers' expression in the prefrontal cortex

There was an increase in phospho-mTOR (165.60 ± 20.75% in CBD *vs* vehicle group, $p<0.05$) and BDNF levels (139.20 ± 13.42% in CBD *vs* vehicle group, $p<0.05$) and a decrease in phospho-GSK3β levels (61.93 ± 8.09% in CBD *vs* vehicle group, $p<0.05$) in the prefrontal cortex 24 h after the IL CBD infusion, compared to the vehicle group. No changes were observed in the levels of β-catenin, phospho-AKT, phospho-ERK and phospho-CREB (figure 23). The analysis of the total amount of GSK3β, AKT, ERK and CREB proteins showed no differences between both experimental groups, and only a tendency to increase was detected for mTOR levels in the CBD group (117.20 ± 7.14% in CBD *vs* vehicle, $p=0.069$) (Annexe 1, table 6).

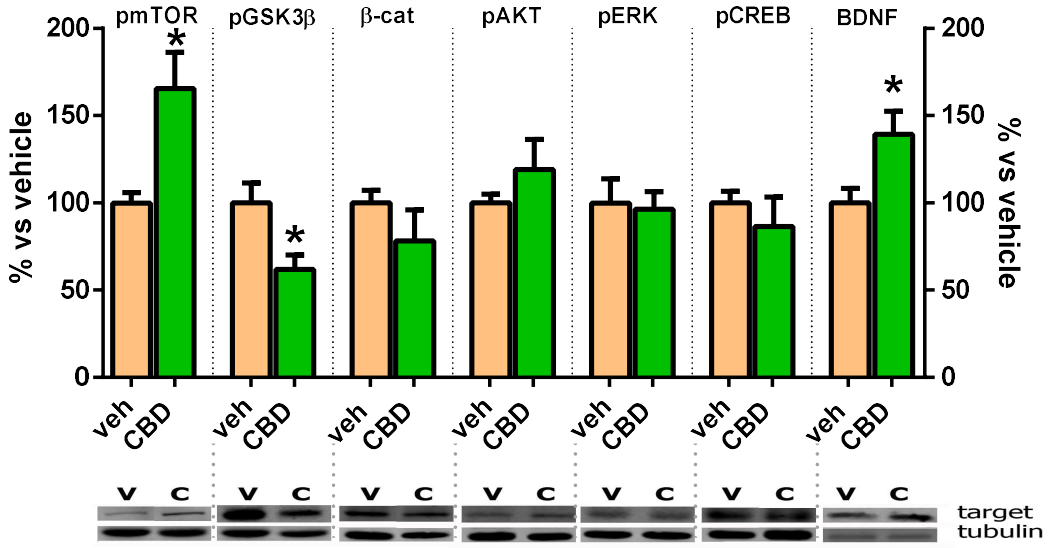


Figure 23. Plasticity markers' expression in the prefrontal cortex 24 h after the IL CBD or vehicle infusion. Results are expressed in percentage *versus* the vehicle group and as mean ± S.E.M. Unpaired Student's *t*-test **p*<0.05 *vs* vehicle group. n= 6 - 8 animals per group.

1.3 Behavioural and molecular effects of the blockade of presynaptic 5-HT$_{1A}$ receptors 24 hours after the bilateral infralimbic cannabidiol infusion

1.3.1 Evaluation of the depressive-like behaviour

The 5-HT$_{1A}$ receptor antagonist WAY100635, at a dose that preferentially blocks the 5-HT$_{1A}$ pre-synaptic receptors (0.3 mg/kg, i.p.), was administered to animals that had received an IL CBD infusion 24 h before (*see figure 14D in Material and methods*). This drug combination reduced the time spent immobile in the FST compared to the CBD group (59.0 s ± 21.5 s in CBD+WAY *vs* 155.0 s ± 15.1 s in the CBD group, $p<0.05$), and increased the time spent climbing compared to the vehicle and CBD groups (84.0 ± 6.5 s in CBD+WAY *vs* 36.5 ± 9.3 s in the vehicle group, $p<0.05$; 24.2 ± 8.3 s in the CBD group, $p<0.05$). A two-way ANOVA *posthoc* test showed no statistical differences in the time spent swimming, although an unpaired Student's *t*-test showed that WAY100635 administration to CBD-infused rats increased the swimming time compared to the CBD group (217.0 ± 25.2 s in CBD+WAY *vs* 106.9 ± 19.4 s in the CBD group; t=3.13, df=16, $p<0.01$) (figure 24). A two-way ANOVA showed a significant effect of the interaction between CBD and WAY100635 administration [$F_{(1,32)} = 4.38$, $p<0.05$] and a tendency to an effect of WAY100635 administration [$F_{(1,32)} = 3.69$, $p=0.064$] in the immobility time;

an effect of WAY100635 administration [$F_{(1,32)}$ = 5.89, p<0.05] in the swimming time; and an effect of WAY100635 administration [$F_{(1,32)}$ = 9.44, p=0.01] and of the interaction between CBD and WAY100635 administration [$F_{(1,32)}$ = 4.34, p<0.05] in the climbing time (Annexe 1, table 7).

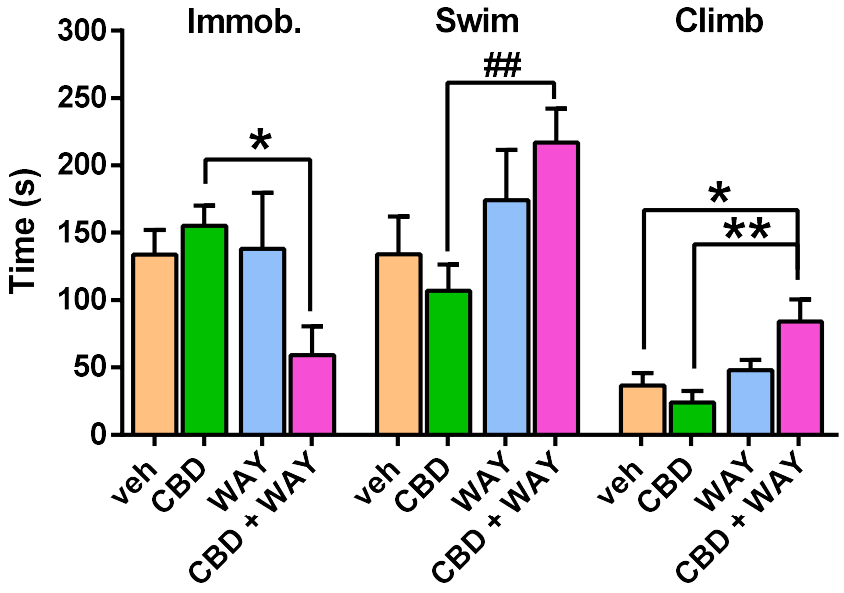


Figure 24. Effect of WAY100635 i.p. administration on the depressive-like behaviour evaluated in the forced swimming test 24 hours after the IL CBD infusion (time spent immobile, swimming and climbing). Results are expressed as mean ± S.E.M. Two-way ANOVA followed by a Newman Keuls *posthoc* test *p<0.05, **p<0.01. Unpaired Student's *t*-test $^{\#\#}p$<0.01. n= 8 - 10 animals per group.

1.3.2 Plasticity markers' expression in the prefrontal cortex

The results obtained in the CBD group were similar to the ones described above (24 h after IL CBD infusion). An increase in phospho-mTOR (135.3 ± 7.7% in CBD *vs* vehicle group, p<0.01) and BDNF (136.3 ± 9.9% in CBD *vs* vehicle group, p<0.05) levels was observed in the CBD group, which was also observed in the CBD+WAY group (p-mTOR: 131.8 ± 12.6% in CBD+WAY *vs* vehicle group, p<0.05; BDNF: 129.7 ± 12.6% in CBD+WAY *vs* vehicle group, p<0.05). Differing from the CBD group, and resembling the results obtained with the acute IL CBD infusion, there was an increase in phospho-ERK levels in the CBD+WAY group (124.9 ± 9.8% in CBD+WAY *vs* vehicle group, p<0.05) (figure 25). A

two-way ANOVA showed an effect of CBD in phospho-mTOR [$F_{(1,32)}$ = 17.3, p<0.001] and BDNF levels [$F_{(1,32)}$ = 10.2, p<0.01], and an effect of WAY100635 in phospho-ERK levels [$F_{(1,32)}$ = 4.5, p<0.05]. No differences were detected in the total amount of mTOR and ERK proteins (Annexe 1, table 8).

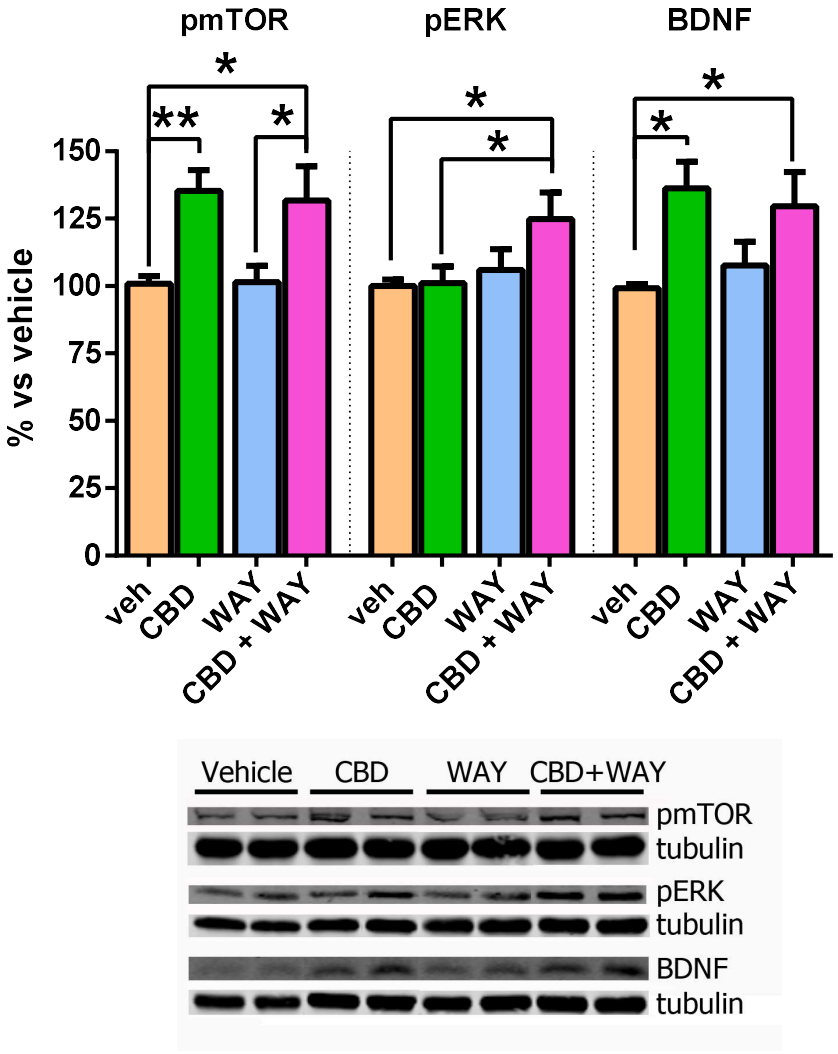


Figure 25. Plasticity markers' expression modulation in the prefrontal cortex following WAY100635 i.p. administration 24 hours after the IL CBD infusion. Representative bands from the western blot of each marker and the corresponding housekeeping are shown. Results are expressed in percentage *versus* the vehicle group and as mean ± S.E.M. Two-way ANOVA followed by a Newman Keuls *posthoc* test *p<0.05, **p<0.01. n= 8 - 10 animals per group.

1.3.3 Neurotransmitter levels in the prefrontal cortex and dorsal raphe nucleus

Serotonin levels were not affected by any of the drugs administered, neither in the PFC nor in the DRN (figure 26A), although a two-way ANOVA showed a significant effect of WAY100635 administration in the PFC [$F_{(1,20)}$ = 4.69, p<0.05] and a tendency in the DRN [$F_{(1,20)}$ = 4.21, p=0.054].

No differences between the different experimental groups were observed in PFC noradrenaline levels, but an increased amount was detected in the CBD+WAY group (480.3 ± 9.73 pg/mg) compared to the vehicle (390.7 ± 17.30 pg/mg, p<0.05), CBD (413.3 ± 14.88 pg/mg, p<0.05) and WAY (414.5 ± 35.08 pg/mg, p<0.05) groups in the DRN (figure 26B). In this brain area, a two-way ANOVA revealed a significant effect of WAY100635 [$F_{(1,20)}$ = 5.51, p<0.05] and of CBD [$F_{(1,20)}$ = 5.22, p<0.05].

GABA levels were increased in CBD (68.86 ± 12.87 pg/mg, p<0.05), WAY (49.79 ± 7.43 pg/mg, p<0.05) and CBD+WAY (58.11 ± 6.01 pg/mg, p<0.05) groups compared to the vehicle group (32.49 ± 4.59 pg/mg) in the PFC. GABA levels in the DRN were reduced in CBD (562.4 ± 65.1 pg/mg, p<0.05), WAY (402.8 ± 40.5 pg/mg, p<0.001) and CBD+WAY groups (492.9 ± 22.9 pg/mg, p<0.01) compared to the vehicle group (689.1 ± 37.3 pg/mg) (figure 26C). In PFC a two-way ANOVA showed an effect of CBD [$F_{(1,20)}$ = 8.54, p<0.01] and a tendency to produce an effect of the interaction between CBD and WAY factors [$F_{(1,20)}$ = 3.37, p=0.082]. The same statistical analysis performed in the DRN resulted in a significant effect of WAY [$F_{(1,20)}$ = 18.65, p<0.001] and of the interaction of CBD with WAY [$F_{(1,20)}$ = 6.92, p<0.05].

Glutamate levels were increased in the CBD (2746 ± 297.6 pg/mg, p<0.001), WAY (1540 ± 215.9 pg/mg, p<0.01), and CBD+WAY (3073 ± 100.2 pg/mg, p<0.001) groups compared to the vehicle group (747.2 ± 96.1 pg/mg) in the PFC. In the DRN, glutamate levels were also increased in CBD (602 ± 74.4 pg/mg, p<0.05), and CBD+WAY (834.4 ± 90.2 pg/mg, p<0.001) groups compared to the vehicle group (382.7 ± 46.1 pg/mg). Moreover, in the DRN there were significant differences between CBD and CBD+WAY groups (p<0.05) (figure 26D). In the PFC a two-way ANOVA showed an effect of CBD [$F_{(1,20)}$ = 103.6, p<0.001] and WAY [$F_{(1,20)}$ = 10.43, p<0.01] factors. In the DRN, there was a significant effect of CBD [$F_{(1,20)}$ = 32.36, p<0.001] and of the interaction between CBD and WAY factors [$F_{(1,20)}$ = 6.55, p<0.05] (Annexe 1, table 9).

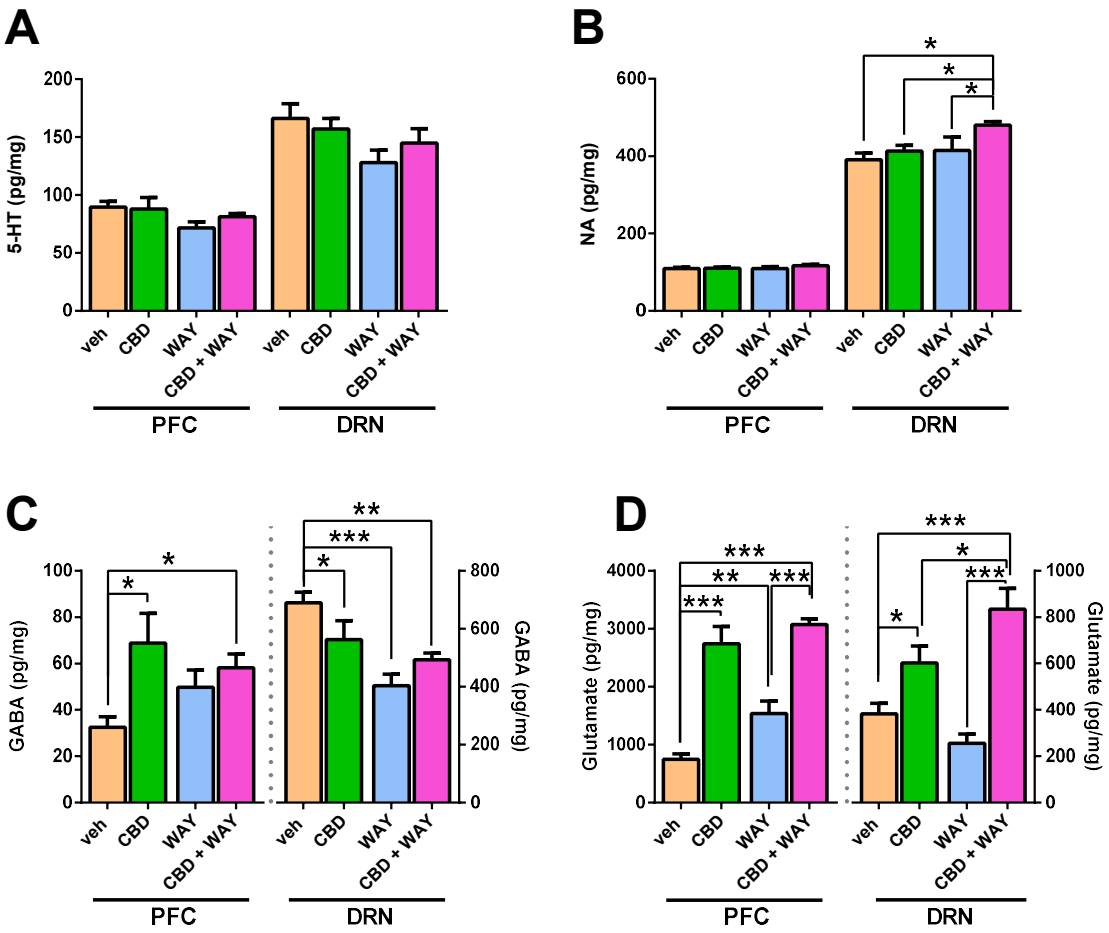


Figure 26. Neurotransmitter levels in the prefrontal cortex (PFC) and dorsal raphe nucleus (DRN) following WAY100635 i.p. administration 24 hours after the IL CBD infusion. A) Serotonin. B) Noradrenaline. C) GABA. D) Glutamate. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by a Newman Keuls *posthoc* test. **p*<0.05, ***p*<0.01, ****p*<0.001. n= 5 - 7 animals per group.

1.4 Acute behavioural and molecular effects of the dorsal raphe nucleus cannabidiol infusion

1.4.1 Evaluation of the depressive-like, anxious-like and locomotor behaviour

The behavioural effect of the CBD infusion into the DRN was assessed in rats 30 min after the administration (*see figure 14A in Material and methods*). In the forced swimming test, CBD increased the time spent swimming (222.90 ± 9.97 s in CBD *vs* 184.50 ± 10.99 s in the vehicle group, $p<0.05$) and decreased the time spent climbing (61.25 ± 7.84 s in CBD *vs* 124 ± 16.88 s in the vehicle group, $p<0.01$) compared to the

vehicle group, with no differences in the immobility time (figure 27A). No changes were detected in the parameters evaluated in the open field test (total distance travelled and time spent in the centre) (figure 27B) (Annexe 1, table 10).

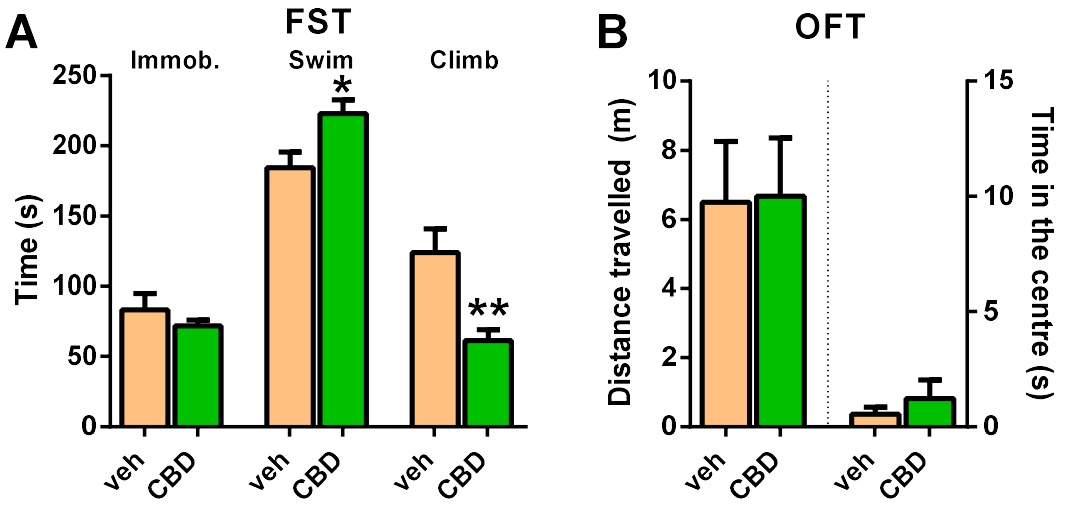


Figure 27. Effect of the DRN CBD infusion in the depressive-like, anxious-like and locomotor behaviour 30 min after the administration. Time spent immobile, swimming and climbing in the forced swimming test (A). Total distance travelled and time spent in the centre in the open field test (B). Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test *p<0.05, **p<0.01. n= 10 - 12 animals per group.

1.4.2 Plasticity markers' expression in the prefrontal cortex

Following behavioural testing, rats were sacrificed and the expression of a battery of neuronal plasticity markers was studied in the prefrontal cortex. The DRN CBD infusion produced an increase in BDNF levels in the prefrontal cortex after 30 min (262 ± 62.21% in CBD *vs* vehicle group, $p<0.05$), with no changes in the levels of phospho-mTOR, phospho-GSK3β, β-catenin, phospho-AKT, phospho-ERK and phospho-CREB compared to the control group (figure 28). None of the following non-phosphorylated proteins showed differences in their total amount between CBD and vehicle groups: mTOR, GSK3β, AKT, ERK and CREB (Annexe 1, table 11).

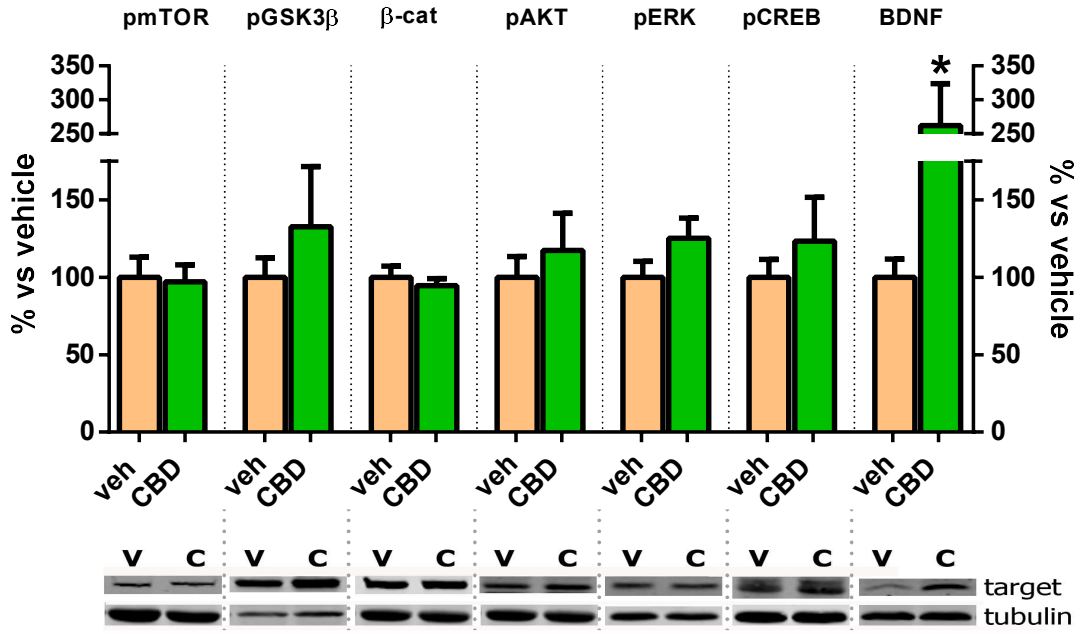


Figure 28. Plasticity markers' expression in the prefrontal cortex after the DRN CBD infusion. Representative western blot bands of each marker and the corresponding housekeeping are shown. Results are expressed in percentage *versus* the vehicle group and as mean ± S.E.M. Unpaired Student's *t*-test **p*<0.05 *vs* vehicle group. n= 6 - 8 animals per group.

1.4.3 Neurotransmitter levels in the prefrontal cortex and dorsal raphe nucleus

Rats infused with CBD in the DRN presented an increase in serotonin levels in the prefrontal cortex (444.7 ± 21.5 pg/mg in CBD *vs* 334.5 ± 34.7 pg/mg in the vehicle group, $p<0.05$), whereas no changes in serotonin levels were seen in the DRN at this time point (figure 29A). No changes were observed in noradrenaline, GABA and glutamate levels, in none of the brain areas studied (figure 29 B - D) (Annexe 1, table 12).

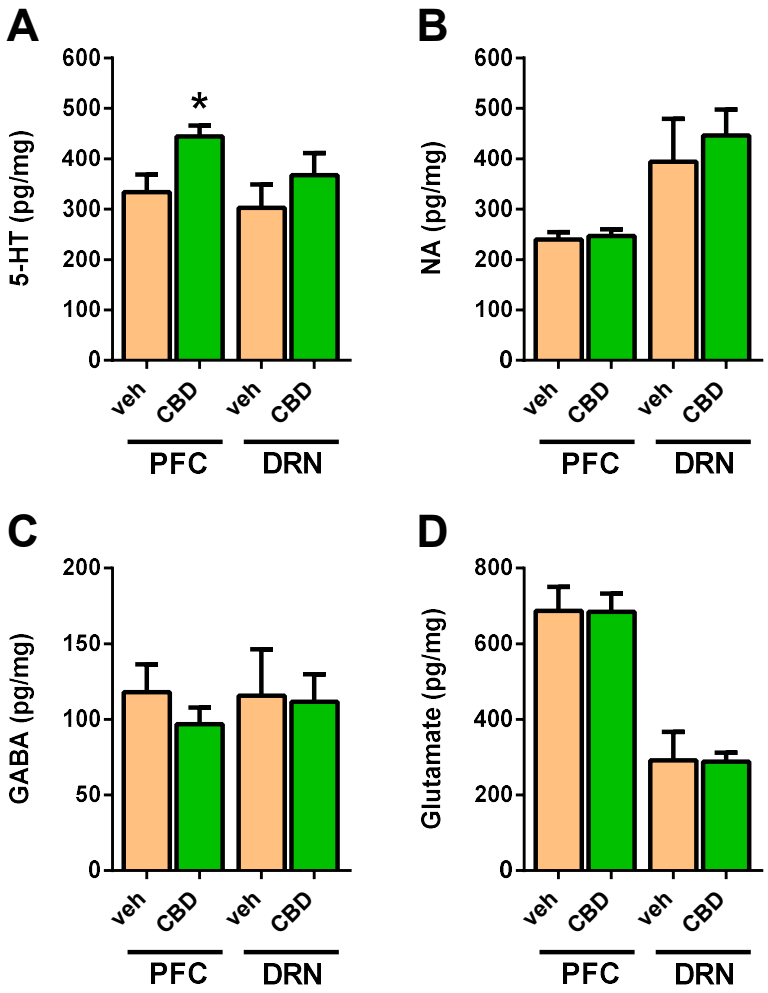


Figure 29. Neurotransmitter levels in the prefrontal cortex (PFC) and the dorsal raphe nucleus (DRN) after the DRN CBD infusion. A) serotonin. B) noradrenaline. C) GABA. D) glutamate. Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test *$p<0.05$ *vs* vehicle group. n= 6 - 9 animals per group in PFC and 5 - 8 in DRN.

1.5 Study of the effects of cannabidiol in rat primary neuronal cultures

1.5.1 Determination of the optimal CBD concentration for cell cultures: dose-response assays

To establish the adequate concentration of CBD, primary neuronal cultures were incubated for 24 hours with different CBD concentrations (0.05, 0.1, 0.25, 0.5 and 1 µM). In order to evaluate the neuronal response, the levels of two plasticity markers were determined: phospho-mTOR and BDNF. In figure 30 it is shown that 0.5 µM CBD was the

most effective concentration for both markers, being therefore selected for the following studies in cell cultures.

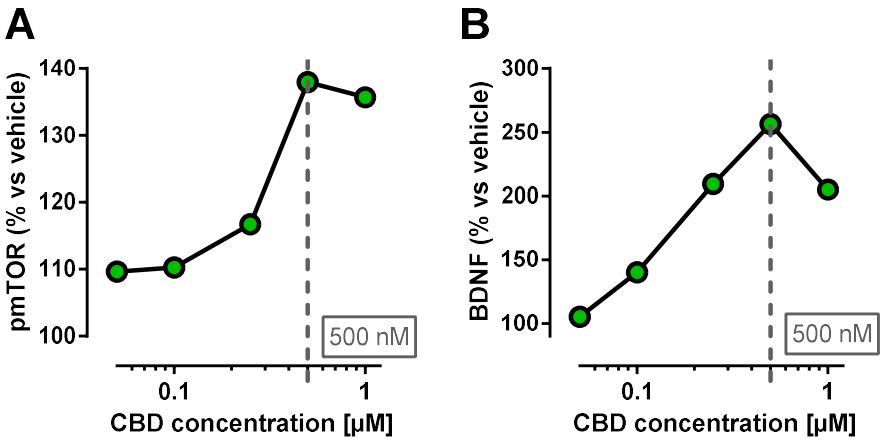


Figure 30. Phospho-mTOR (A) and BDNF (B) levels determined by Western Blot in rat primary neuronal cultures treated with different CBD concentrations for 24 hours. Results are expressed in percentage *versus* the vehicle group.

1.5.2 Effect of cannabidiol on plasticity markers in primary neuronal cultures from rat cortex

As an approach to understanding more in deep the fast and maintained increase of BDNF levels in the brain after an IL CBD infusion (*see 1.1.2 and 1.2.2 in the Results section*), primary neuronal cultures were treated with CBD for 30 min and 24 h, and BDNF levels were determined both in the culture medium and the neuronal cells (*see figure 16A in Material and methods*). Results showed that after 30 min, CBD produced an increase in intracellular BDNF levels (219.3 ± 48.4% in CBD *vs* vehicle, $p<0.05$) (figure 31B) but not in the culture medium (figure 31A). Conversely, the 24 hours CBD treatment increased BDNF levels in the culture medium (147.3 ± 13.1% in CBD *vs* vehicle, $p<0.01$) (figure 31A), but not in the cells (figure 31B).

Regarding phospho-mTOR and phospho-ERK levels, both were increased by CBD after 30 min (130.6 ± 5.7% in CBD *vs* vehicle, for phospho-mTOR, $p<0.05$; and 185.0 ± 22.7% in CBD *vs* vehicle, for phospho-ERK, $p<0.05$) (figure 31 C and D). A tendency to increase in total levels of mTOR was observed in the CBD group (120.1 ± 7.1% in CBD *vs* vehicle, $p=0.061$), with no changes in ERK levels. Conversely, there were no changes in phospho-mTOR and phospho-ERK levels 24 h after CBD incubation (figure 31 C and D). A tendency

to increase in the total levels of ERK was observed in the CBD group (121.6 ± 7.7% in CBD *vs* vehicle, *p*=0.064), with no changes in mTOR levels (Annexe 1, table 13).

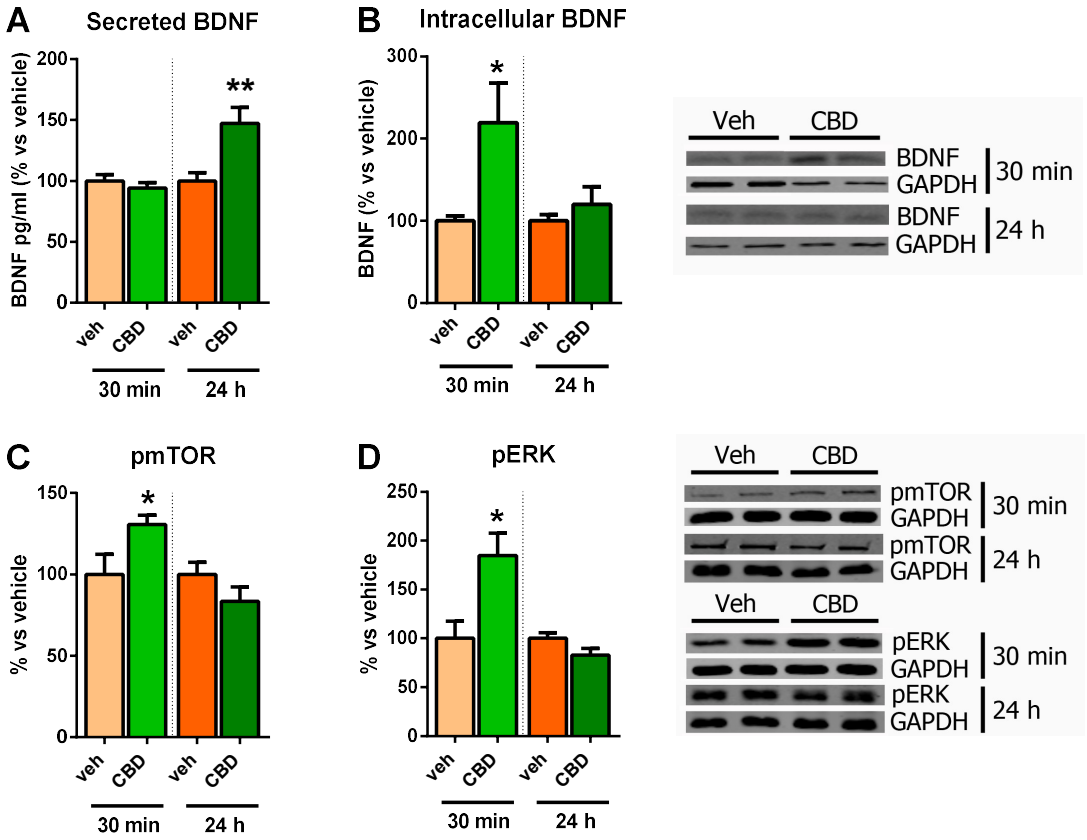


Figure 31. Plasticity markers' expression in primary neuronal cultures incubated with 0.5 µM CBD for 30 min and 24 h. A) Secreted BDNF levels determined by ELISA in the culture medium. B) Intracellular BDNF levels measured by western blot. C) Phospho-mTOR levels measured by western blot. D) Phospho-ERK levels measured by western blot. Representative bands from the western blot of each marker and the corresponding housekeeping are shown. Results are expressed in percentage *versus* the vehicle group, and as mean ± S.E.M. Unpaired Student's *t*-test **p*<0.05, ***p*<0.01 *vs* vehicle group. n= 6 - 11 samples per group.

Objective 2. Effects of CBD in experimental lipopolysaccharide models

2.1 Evaluation of the anti-inflammatory effect of cannabidiol in primary microglia cell cultures activated with lipopolysaccharide

As a confirmation of the anti-inflammatory effect of CBD, we incubated primary microglial cultures activated with 100 ng/ml LPS in the absence/presence of 0.5 µM CBD (*see figure 16B in Material and methods*). LPS incubation produced an increase in TNFα (227.1 ± 8.5% *vs* vehicle, $p<0.001$) and IL-6 (585.1 ± 106.5% *vs* vehicle, $p<0.01$) levels in the cell culture medium after 6 hours, and its coincubation with CBD attenuated the increase in TNFα levels (167.0 ± 7.3% in CBD+LPS *vs* 227.1 ± 8.5% in LPS, $p<0.001$), and prevented the increase in IL-6 levels (233.8 ± 40.7% in CBD+LPS *vs* 585.1 ± 106.5% in LPS, $p<0.01$) (figure 32). A two-way ANOVA of TNFα data showed an effect of the LPS [$F_{(1,6)}$ = 157.2, $p<0.001$], CBD [$F_{(1,6)}$ = 13.83, $p<0.01$], and of the interaction LPS x CBD [$F_{(1,6)}$ = 17.56, $p<0.01$]. In the case of IL-6, there was also an effect of the LPS [$F_{(1,6)}$ = 31.58, $p<0.01$], CBD [$F_{(1,6)}$ = 10.37, $p<0.05$], and of the interaction between both factors [$F_{(1,6)}$ = 9.49, $p<0.05$] (Annexe 1, table 14).

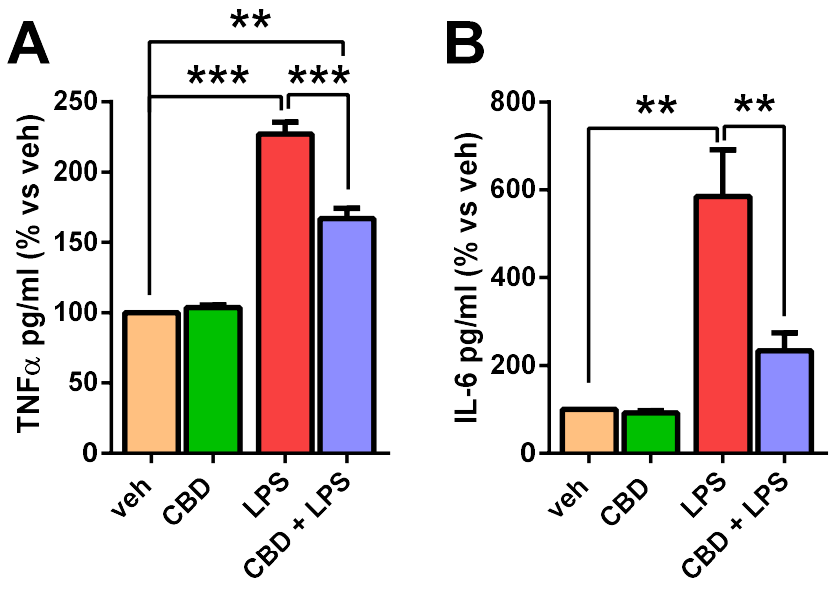


Figure 32. Pro-inflammatory cytokine release to the cell culture medium after microglial activation with LPS and incubation with CBD. A) Effect of CBD on LPS-induced TNFα release. B) Effect of CBD on LPS-induced IL-6 release. Results are expressed in percentage *versus* the vehicle group, and as mean ± S.E.M. Two-way ANOVA followed by a Newman Keuls *posthoc* test. **$p<0.01$, ***$p<0.001$. n= 2 - 4 samples per group.

As CBD significantly reverted the LPS-induced inflammatory effects in our preliminary *in vitro* study, we next studied its behavioral and molecular effects in a mouse model of neuroinflammation induced by an LPS injection.

2.2 Behavioural and molecular effects of cannabidiol in mice subjected to the neuro-inflammatory model induced by lipopolysaccharide: influence of gender

2.2.1 Optimization of the lipopolysaccharide model

In this study, we used NMRI mice since they present a lower basal immobility time in the TST compared to other mouse strains (Ripoll et al., 2003). Besides, we used both male and female mice to assess the gender-dependent effects of the model and the antidepressant response.

Diverse authors have reported depressive-like behaviour in mice 24 hours after a unique 0.83 mg/kg LPS injection (Frenois et al., 2007; O'connor et al., 2009). However, in our study, twenty-four hours after the LPS administration (0.83 mg/kg) there were no significant differences in the TST compared to the vehicle group, neither in males nor in females (figure 33 A and C). Thus, we performed the sucrose preference test at different time points after a unique 0.83 mg/kg LPS injection to evaluate the onset of a potential anhedonic state, as a behavioural manifestation of depression. There was a significant reduction in the sucrose preference 12 h after the LPS injection in male mice (49.4 ± 1.5% in LPS *vs* 80.7 ± 4.2% in vehicle, $p<0.001$), which was maintained up to 36 hours (figure 33B). The results obtained with females followed a similar pattern (figure 33D) (Annexe 1, table 15). In light of these results, the 12 h time point was selected for further experiments (*see figure 15A in Material and methods*).

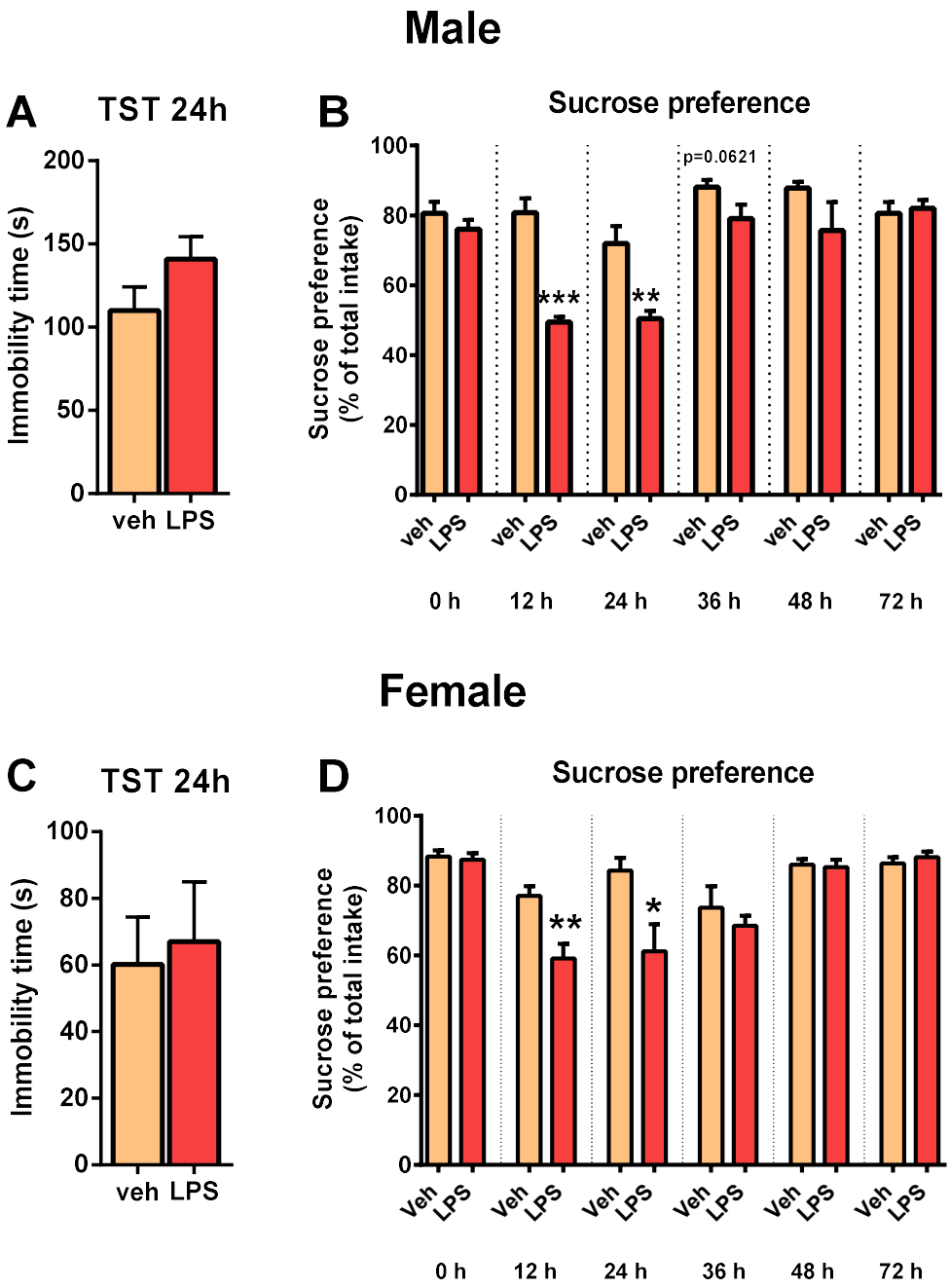


Figure 33. Optimization of the LPS model (0.83 mg/kg, unique administration) in NMRI mice. A) Tail suspension test after 24 h in males. B) Sucrose preference test in males. C) Tail suspension test after 24 h in females. D) Sucrose preference test in females. Results are expressed as mean ± S.E.M. Unpaired Student's *t*-test, **p*<0.05, ***p*<0.01, ****p*<0.001. n= 6 - 10 animals per group.

2.2.2 Evaluation of the influence of gender in the depressive- and anxious-like behaviour in the mouse LPS model

In the tail suspension test (TST), male mice receiving LPS presented an increase in the immobility time (126.8 ± 20.3 s in LPS *vs* 53.8 ± 10.7 s in vehicle, $p<0.05$), which was not shown in females. When comparing the basal immobility time of both genders, female mice had higher immobility (158.7 ± 19.68 s in the female vehicle group *vs* 53.8 ± 10.7 s in the male vehicle group, $p<0.01$) (figure 34A). A two-way ANOVA showed a significant effect of gender [$F_{(1,21)} = 20.32$, $p<0.001$] and of the LPS model [$F_{(1,21)} = 8.68$, $p<0.01$].

Males and females performed almost identically in the sucrose preference test (SPT), as LPS reduced sucrose preference in both cases (males: 49.9 ± 3.9% in LPS *vs* 81.4 ± 2.2% in vehicle, $p<0.001$; females: 51.73 ± 3.86% in LPS *vs* 78.9 ± 3.83% in vehicle, $p<0.01$) and no differences were observed between genders (figure 34B). A significant effect of the LPS model was obtained in the two-way ANOVA [$F_{(1,26)} = 72.85$, $p<0.001$].

Regarding weight variation, LPS administration caused weight loss in males and females (weight variation in males: -1.27 ± 0.28 g in LPS *vs* 0.87 ± 0.3 g in vehicle, $p<0.001$; females: -1.39 ± 0.07 g in LPS *vs* -0.30 ± 0.25 g in vehicle, $p<0.01$). Interestingly, the female vehicle group lost weight compared to the male vehicle group ($p<0.01$) (figure 34C). A two-way ANOVA resulted in an effect of gender [$F_{(1,21)} = 7.58$, $p<0.05$], of the LPS model [$F_{(1,21)} = 46.95$, $p<0.001$], and of the interaction between both factors [$F_{(1,21)} = 4.94$, $p<0.05$].

In the open field test (OFT), LPS administration induced a reduction in the time spent in the centre in males (2.4 ± 1.4 s in LPS *vs* 18.9 ± 4.7 s in vehicle, $p<0.01$), whereas no changes were seen in female mice, and between genders (figure 34D). A two-way ANOVA of this parameter showed an effect of the LPS model [$F_{(1,21)} = 17.55$, $p<0.001$]. The total distance travelled in the OFT was reduced by LPS in both genders (males: 7.41 ± 1.37 m in LPS *vs* 16.32 ± 2.46 m in vehicle, $p<0.05$; females: 9.85 ± 2.83 m in LPS *vs* 19.52 ± 1.48 m in vehicle, $p<0.05$), and no differences were observed between males and females (figure 34E). In the two-way ANOVA, there was an effect of the LPS model [$F_{(1,21)} = 16.97$, $p<0.001$] (Annexe 1, table 16).

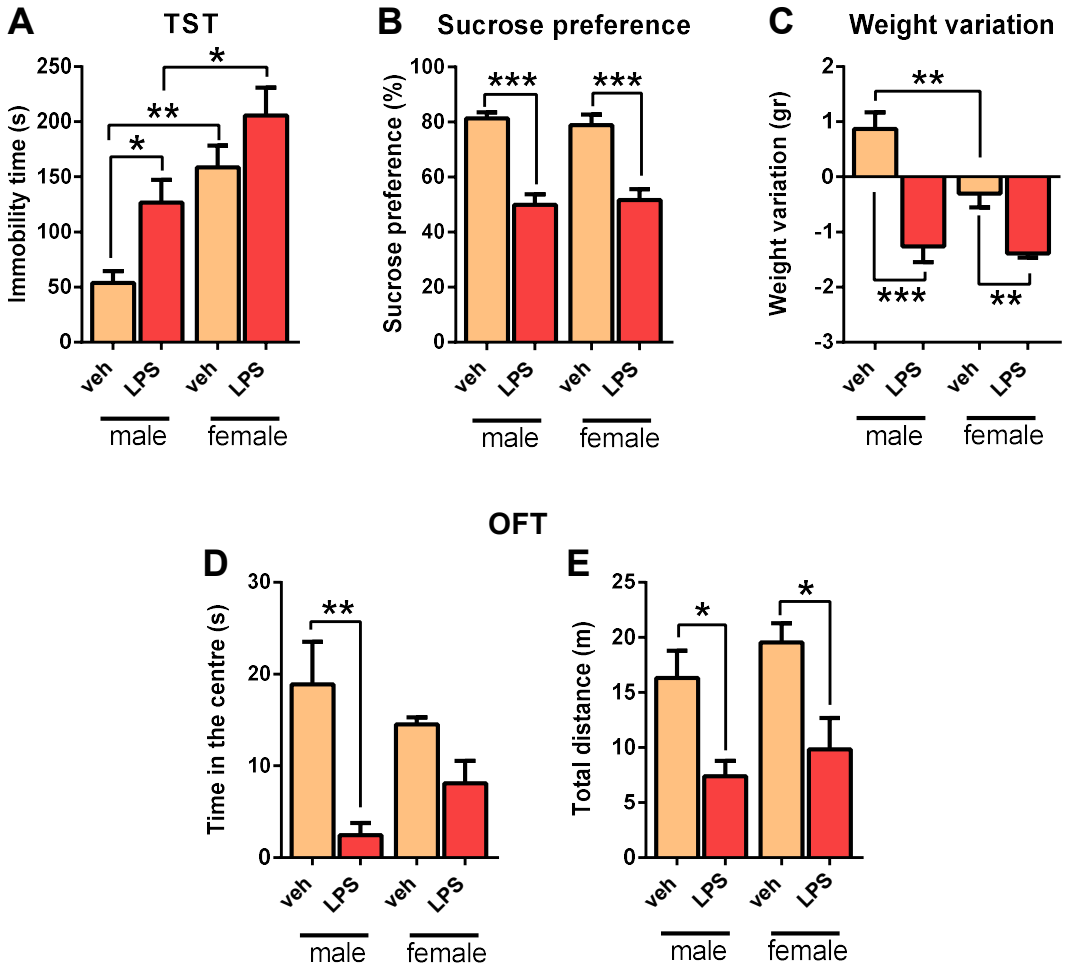


Figure 34. Evaluation of the depressive- and anxious-like behaviour in the LPS model performed in male and female NMRI mice. A) Tail suspension test. B) Sucrose preference test. C) Mouse weight variation. D-E) Open field test. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by a Newman Keuls *posthoc* test. *p<0.05, **p<0.01, ***p<0.001. n= 6 - 9 animals per group.

We analyzed the correlation of the different behavioural parameters with a locomotor component to check if the LPS-induced effects in locomotion could affect the interpretation of the tests. In both males and females, no correlation was found between the time spent immobile in the TST and the total distance travelled in the OFT (figure 35 A and B). Conversely, a significant positive correlation was observed between the time spent in the centre and the total distance travelled in the OFT (p<0.01 in males; p<0.001 in females, figure 35 C and D).

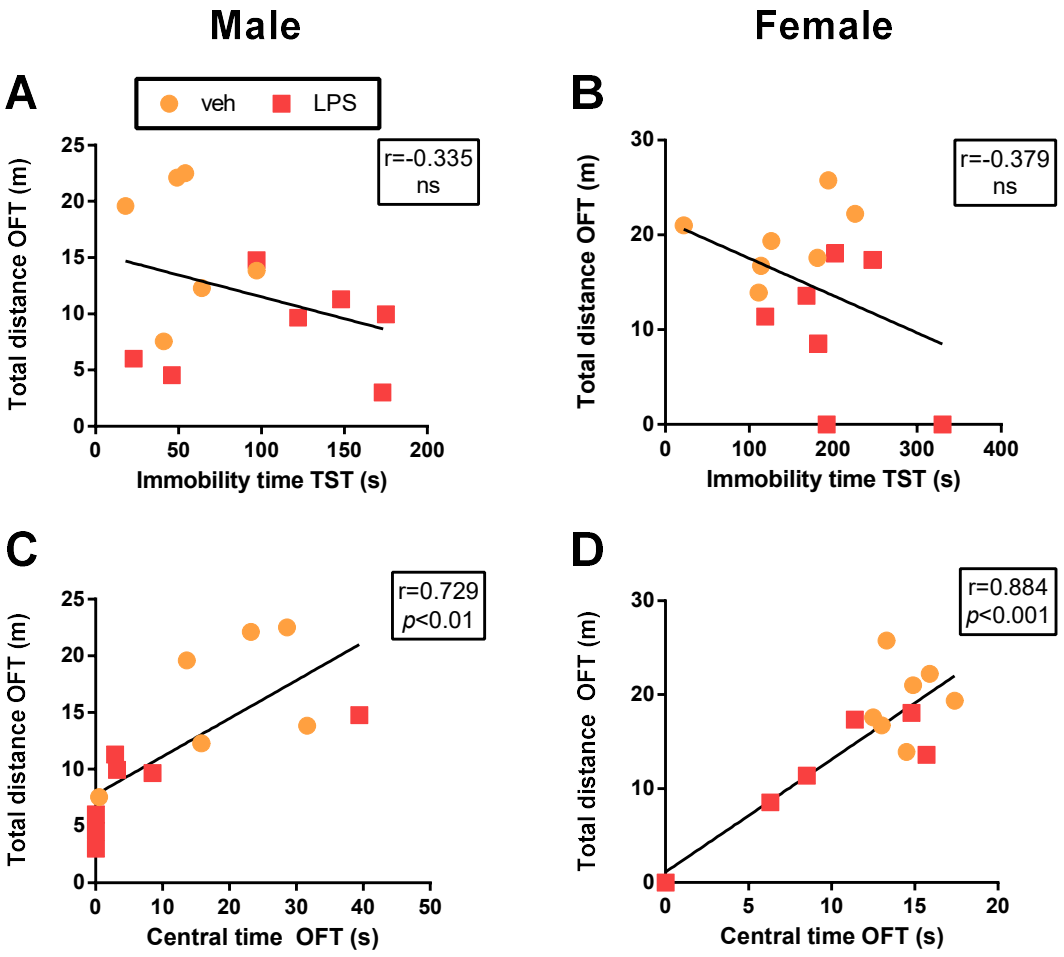


Figure 35. Correlation and linear regression between behavioural determinations in male (A and C) and female (B and D) mice injected with vehicle or LPS. A and B) Parameters from the open field test and the tail suspension test. C and D) Parameters evaluated in the open field test. n= 6 - 7 animals per group. r: Pearson's correlation coefficient. ns: not significant.

2.2.3 Evaluation of the depressive- and anxious-like behaviour following CBD administration to male mice subjected to the LPS model

In the tail suspension test (TST) the LPS administration induced an increase in the immobility time (126.8 ± 20.3 s in LPS *vs* 53.8 ± 10.7 s in vehicle, $p<0.01$), which was prevented by CBD administration (72.6 ± 10.3 s in CBD+LPS *vs* 126.8 ± 20.3 s in LPS, $p<0.01$) (figure 36A). A two-way ANOVA showed a significant effect of the LPS model [$F_{(1,22)}$ = 10.26, $p<0.01$], and of the interaction of model and treatment [$F_{(1,22)}$ = 5.54, $p<0.05$].

As a consequence of LPS injection, there was a decrease in the sucrose preference (49.9 ± 3.9% in LPS *vs* 81.4 ± 2.2% in vehicle, $p<0.001$). The administration of CBD before LPS administration in mice attenuated this decrease (63.3 ± 3.4% in CBD+LPS *vs* 49.9 ± 3.9% in the LPS group, $p<0.01$; 63.3 ± 3.4% in CBD+LPS *vs* 81.4 ± 2.2% in vehicle, $p<0.001$) (figure 36B). A two-way ANOVA revealed a significant effect of the LPS model [$F_{(1,28)}$ = 51.89, $p<0.001$] and of model and treatment interaction [$F_{(1,28)}$ = 7.00, $p<0.05$].

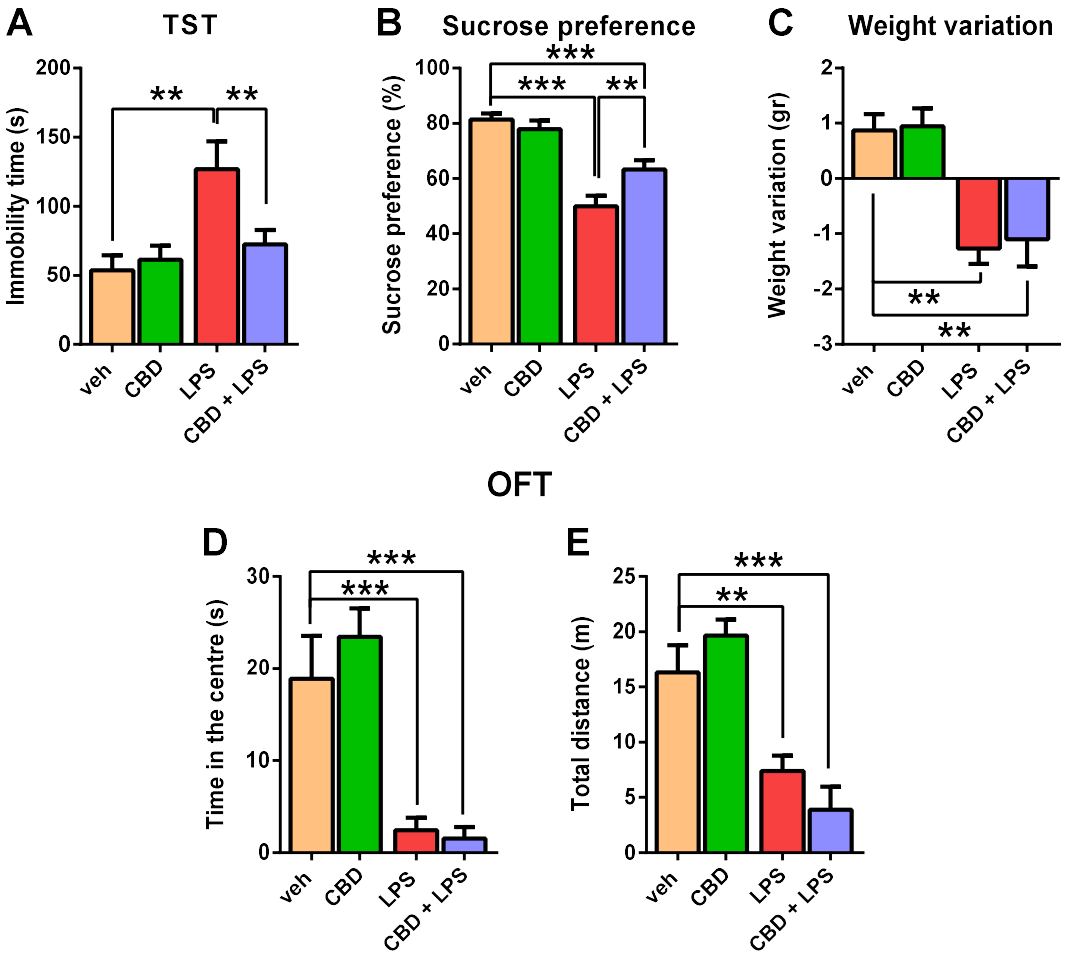


Figure 36. Evaluation of the depressive- and anxious-like behaviour following CBD administration in the LPS model in male NMRI mice. A) Tail suspension test. B) Sucrose preference test. C) Mouse weight variation. D-E) Open field test. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by a Newman Keuls *posthoc* test. **$p<0.01$, ***$p<0.001$. n= 6 - 9 animals per group.

Regarding the characteristic weight loss induced by LPS injection (weight variation of -1.27 ± 0.28 g in LPS *vs* 0.87 ± 0.3 g in vehicle, $p<0.01$), CBD did not have any effect on this parameter when administered to LPS mice (-1.1 ± 0.49 g in CBD+LPS) (figure 36C). A two-way ANOVA resulted in an effect of the LPS model [$F_{(1,22)} = 31.36$, $p<0.001$].

In the open field test (OFT), mice injected with LPS spent less time in the centre (2.4 ± 1.4 s in LPS *vs* 18.9 ± 4.7 s in vehicle, $p<0.001$) and travelled less distance compared to the vehicle group (7.41 ± 1.37 m in LPS *vs* 16.32 ± 2.46 m in vehicle, $p<0.001$). The administration of CBD to LPS mice did not produce an effect in these parameters (time in the centre 1.53 ± 1.27 s, and distance travelled 3.88 ± 2.1 m in CBD+LPS) (figure 36D and E). A two-way ANOVA of the time in the centre showed a significant effect of the LPS model [$F_{(1,22)} = 44.86$, $p<0.001$], and the same result was obtained for the distance travelled [$F_{(1,22)} = 42.31$, $p<0.001$]. In addition, there was a trend to produce an effect of the interaction of model and treatment in the total distance travelled [$F_{(1,22)} = 3.28$, $p=0.084$] (Annexe 1, table 17).

2.2.4 Evaluation of the depressive- and anxious-like behaviour following CBD administration to female mice subjected to the LPS model

Differing from the results obtained in male mice, LPS injection did not induce changes in the immobility time in the tail suspension test (TST) in females (figure 37A). A two-way ANOVA showed an effect of the CBD treatment [$F_{(1,23)} = 5.75$, $p<0.05$].

In the sucrose preference test, there was a reduction in the sucrose preference in the LPS group (51.73 ± 3.86% in LPS *vs* 78.9 ± 3.83% in vehicle, $p<0.01$), which was not prevented by CBD administration (47.73 ± 6.69% in CBD+LPS) (figure 37B). In the two-way ANOVA, an effect of the LPS model was obtained [$F_{(1,23)} = 18.28$, $p<0.001$].

Results in weight variation in females were parallel to males, as LPS produced weigh loss (-1.39 ± 0.07 g in LPS *vs* -0.30 ± 0.25 g in vehicle, $p<0.01$) and CBD did not counteract this effect when administered to mice injected with LPS (-1.12 ± 0.15 g in CBD+LPS) (figure 37C). A two-way ANOVA showed an effect of the LPS model [$F_{(1,23)} = 36.24$, $p<0.001$].

The open field test showed no significant differences between groups in the time spent in the centre (figure 37D). However, LPS induced a decrease in the total distance travelled (9.85 ± 2.83 m in LPS *vs* 19.52 ± 1.48 m in vehicle, $p<0.01$) that was not prevented by CBD administration to LPS mice (11.45 ± 1.47 m in CBD+LPS) (figure 37E). A

two-way ANOVA of the total distance indicated an effect of the LPS model [$F_{(1,23)}$ = 18.73, p<0.001] (Annexe 1, table 18).

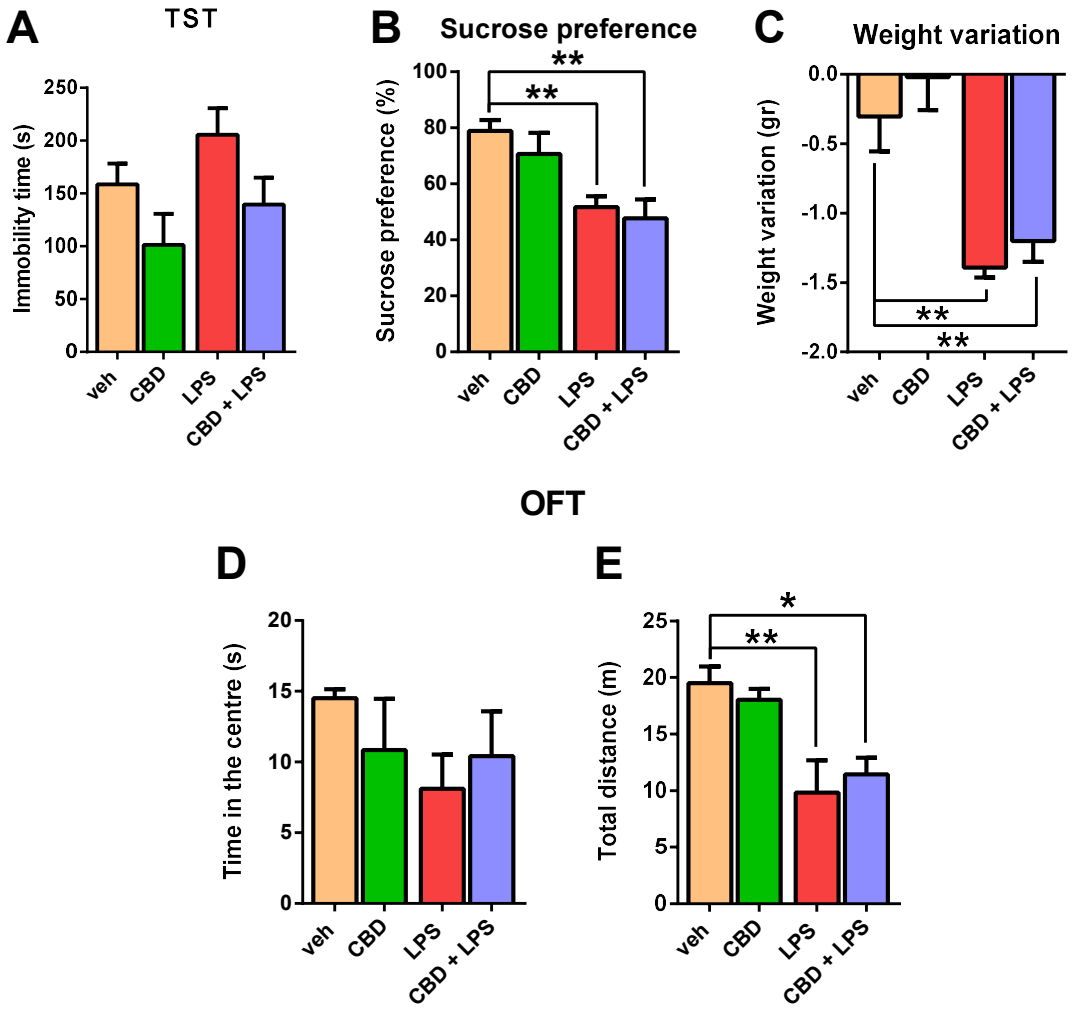


Figure 37. Evaluation of the depressive- and anxious-like behaviour following CBD administration in the LPS model in females. A) Tail suspension test. B) Sucrose preference test. C) Mouse weight variation. D-E) Open field test. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by a Newman Keuls *posthoc* test. *p<0.05, **p<0.01. n= 6 - 7 animals per group.

Taking into account the absence of LPS effects in some behavioural tests and the lack of preventive effects of CBD in female mice, the subsequent experiments were performed in male mice.

2.2.5 Role of 5-HT_{1A} receptors in the antidepressant-like effect of CBD in the LPS model

Since the 5-HT_{1A} receptors have been implicated in the behavioural effects of CBD (Zanelati et al., 2010; Linge et al., 2016; Sartim et al., 2016), we evaluated the effects of the pre- and postsynaptic 5-HT_{1A} receptor blockade with different doses of WAY100635 (*see figure 15B in Material and methods*).

As described above (*see 2.2.3 in the Results section*), CBD also prevented the LPS-induced immobility in the TST (50.4 ± 8.5 s in CBD+LPS *vs* 99.1 ± 9.1 s in LPS, $p<0.01$) in this set of experiments (figure 38, vehicle). A two-way ANOVA showed a significant effect of the interaction between model and treatment [$F_{(1,24)} = 13.35$, $p<0.01$].

The administration of 0.3 mg/kg of the selective 5-HT_{1A} receptor antagonist WAY100635 did not induce behavioural changes *versus* the groups not receiving WAY100635. Thus, CBD also prevented the increase in the immobility time produced by the LPS injection in the presence of 0.3 mg/kg WAY100635 (34.16 ± 10.54 s in CBD+LPS *vs* 97.50 ± 9.81 s in LPS, $p<0.001$) (figure 38, WAY 0.3 mg/g). A two-way ANOVA evidenced a significant effect of the model [$F_{(1,13)} = 11.21$, $p<0.01$], and of the interaction between model and treatment [$F_{(1,13)} = 28.57$, $p<0.001$].

Conversely, CBD failed to prevent the increase in the immobility time induced by LPS when it was co-administered with 1 mg/kg WAY100635 (83.9 ± 6.4 s in CBD+LPS *vs* 92.8 ± 15.5 s in LPS) (figure 38, WAY 1 mg/kg). A one-way ANOVA performed between the three CBD+LPS groups showed a significantly higher immobility time in the CBD+LPS group when 1 mg/kg WAY100635 was co-administered, compared to the same group in the presence of 0.3 mg/kg WAY100635 or vehicle [$F_{(2,14)} = 5.83$, $p<0.05$] (# in figure 38). In addition, a one-way ANOVA of the three vehicle groups showed an increase in the immobility time in the animals that received 1 mg/kg WAY100635 compared to the same group in the presence of 0.3 mg/kg WAY100635 or vehicle [$F_{(2,11)} = 5.648$, $p<0.05$] (& in figure 38) (Annexe 1, table 19).

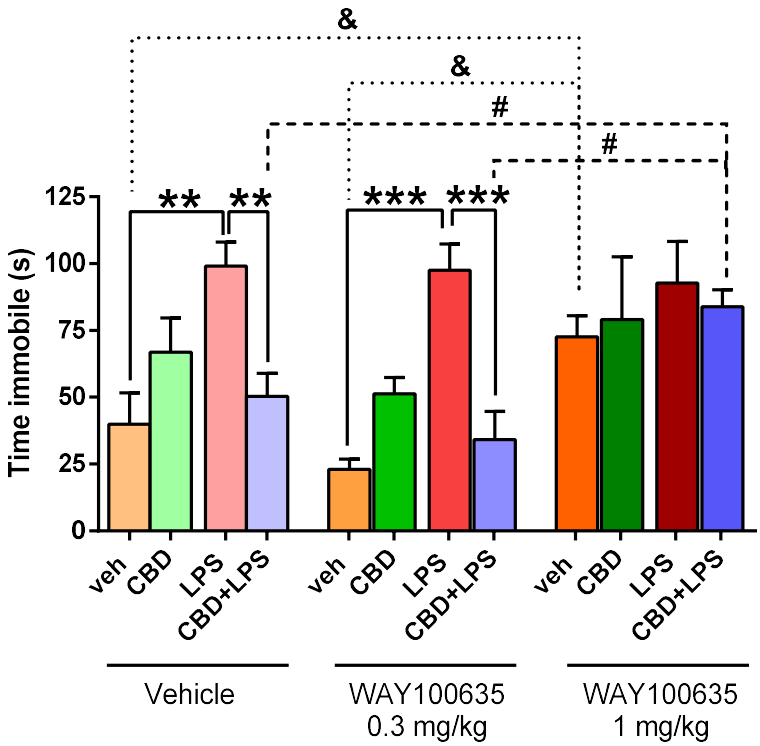


Figure 38. Evaluation of the depressive-like behaviour in the tail suspension test after 0.3 and 1 mg/kg WAY100635 administration to LPS mice pretreated with CBD. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by a Newman Keuls *posthoc* test. ***p*<0.01, ****p*<0.001. One-way ANOVA followed by a Newman Keuls *posthoc* test. $^{\&}p$<0.05 in the comparison of vehicle groups, $^{\#}p$<0.05 in the comparison of CBD+LPS groups. n= 6 - 8 animals per group in vehicle, 4 - 5 in WAY administration.

2.2.6 NF-κB, IκBα and PPARγ levels in nuclear and cytoplasmic fractions of cortical samples from LPS mice: effect of CBD

To study the NF-κB pathway and PPARγ activation, subcellular fractionation was performed in cortical samples in order to determine the protein levels of NF-κB, IκBα and PPARγ in the nucleus and the cytosol. After 12 h, LPS injection increased nuclear NF-κB levels in comparison to the vehicle group (197.9 ± 32.6% in LPS *vs* 100 ± 9.3% in vehicle, p<0.001), and CBD pretreatment significantly attenuated this changes (153 ± 37.7% in CBD+LPS *vs* 197.9 ± 32.6% in LPS, p<0.01) (figure 39A). An effect of the LPS model [$F_{(1,22)}$= 32.33, p<0.001] and of the LPS x CBD interaction [$F_{(1,22)}$ = 11.35, p<0.01] was observed in the two-way ANOVA.

Nuclear IκBα levels were also increased as a consequence of LPS injection (149.7 ± 31.9% in LPS *vs* 100 ± 6.8% in vehicle, $p<0.05$), but no changes were observed in the CBD+LPS group (135.3 ± 34.3%) (figure 39B). A two-way ANOVA revealed an effect of the LPS model [$F_{(1,22)} = 12.93$, $p<0.01$].

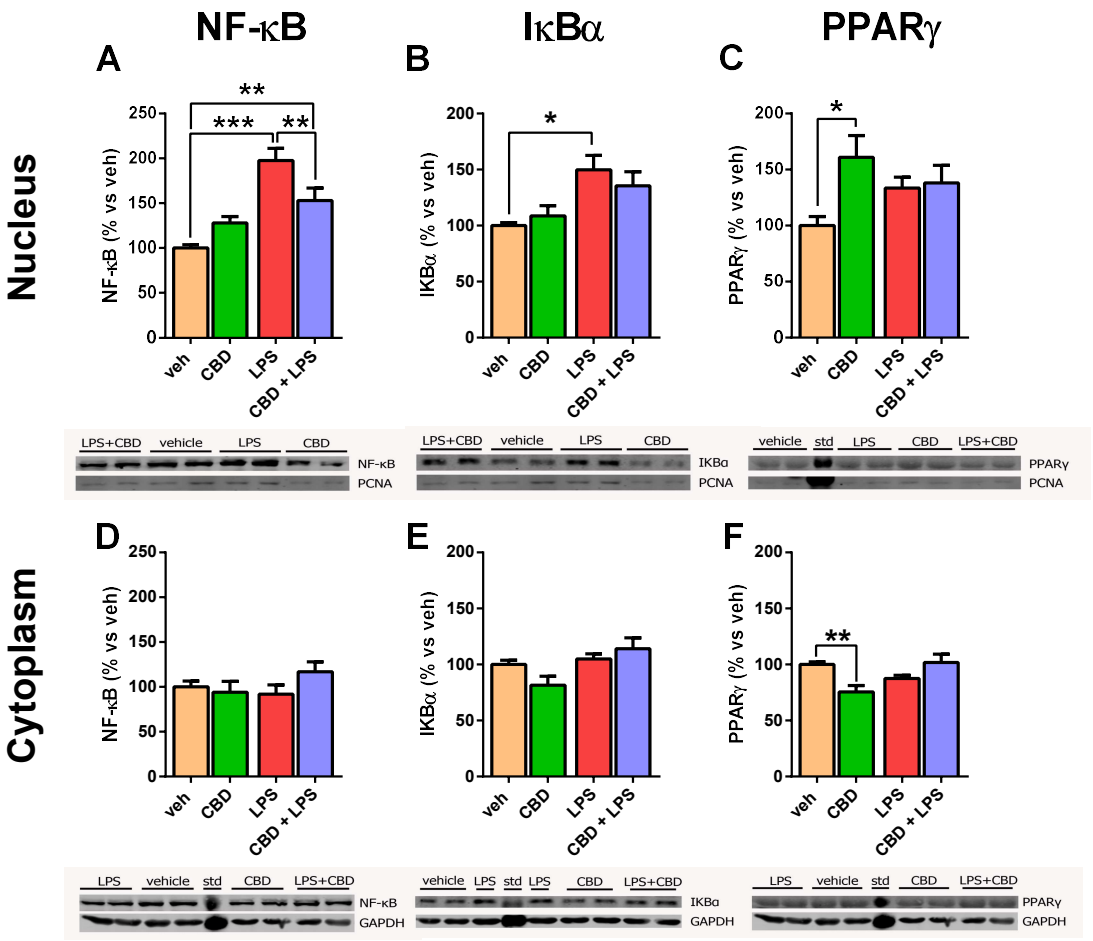


Figure 39. NF-κB (A and D), IκBα (B and E) and PPARγ (C and F) protein levels in nuclear (A-C) and cytosolic (D-F) fractions of cortices from LPS-injected mice and the effect of CBD preadministration. Representative bands from the western blot of each marker and the corresponding housekeeping are shown. Results are expressed as percentage *versus* the vehicle group and as mean ± S.E.M. Two-way ANOVA followed by a Newman Keuls *posthoc* test. **p*<0.05, ***p*<0.01, ****p*<0.001. n= 6 - 7 animals per group.

The cytosolic levels of NF-κB and IκBα (figures 39 D and E, respectively) remained unchanged in all experimental groups after the post-test analysis, although a two-way ANOVA showed an effect of the LPS model [$F_{(1,22)}$ = 6.56, p<0.05] on cytosolic IκBα levels.

Regarding PPARγ, the LPS injection did not affect its levels nor in the nucleus nor in the cytosol. Differently, CBD administration alone increased PPARγ levels in the nucleus (161 ± 50.9% in CBD *vs* 100 ± 19.8% in the vehicle group, p<0.05) (figure 39C), and decreased PPARγ levels in the cytosol (75.5 ± 14.4% in CBD *vs* 100 ± 6.1% in the vehicle group, p<0.01) (figure 39F). A two-way ANOVA resulted in an effect of CBD on PPARγ nuclear levels [$F_{(1,22)}$ = 5.01, p<0.05] and an effect of the interaction CBD x LPS on PPARγ cytosolic levels [$F_{(1,22)}$ = 13.54, p<0.01] (Annexe 1, table 20).

2.2.7 TNFα and IL-6 levels in plasma and prefrontal cortex of mice injected with LPS: effect of CBD

After 12 h, LPS injection produced a mild increase in TNFα plasma levels (9.3 ± 1.3 pg/ml in LPS *vs* 6.3 ± 0.2 pg/ml in vehicle, p<0.05). CBD preadministration to LPS injected mice did not produce any difference compared to the LPS group (figure 40A). A two-way ANOVA showed a significant effect of the LPS model [$F_{(1,25)}$ = 9.06, p<0.01]. Differently, IL-6 plasma levels were highly increased with LPS injection (1143 ± 191 pg/ml in LPS *vs* 27.6 ± 4.3 pg/ml in vehicle, p<0.001). CBD administration to LPS injected mice significantly reduced the levels of plasma IL-6 (573 ± 44 pg/ml in LPS+CBD group) compared to the LPS group (p<0.001), although this experimental group was still different from the vehicle group (p<0.001) (figure 40B). In the two-way ANOVA there was a significant effect of the LPS model [$F_{(1,25)}$ = 76.64, p<0.001], treatment [$F_{(1,25)}$ = 9.00, p<0.01], and the interaction of both factors [$F_{(1,25)}$ = 9.14, p<0.01].

In the prefrontal cortex, there was also an increase in TNFα mRNA levels after LPS injection (9.8 ± 1.8 in LPS *vs* 1.0 ± 0.1 in vehicle, p<0.001), that was not prevented by CBD (10.1 ± 1.6 in CBD+LPS *vs* vehicle, p<0.001) (figure 40C). A two-way ANOVA of TNFα mRNA levels in PFC revealed a significant effect of the LPS model [$F_{(1,25)}$ = 45.57, p<0.001]. Conversely, the LPS-induced increase in PFC IL-6 mRNA levels (3.6 ± 0.6 in LPS *vs* 1.0 ± 0.1 in vehicle, p<0.001) was prevented by CBD administration (2.2 ± 0.4 in CBD+LPS *vs* 3.6 ± 0.6 in LPS, p<0.05) (figure 40D). A two-way ANOVA showed a significant effect of the LPS model [$F_{(1,25)}$ = 22.83, p<0.001], and the model x treatment interaction [$F_{(1,25)}$ = 4.40, p<0.05] (Annexe 1, table 21).

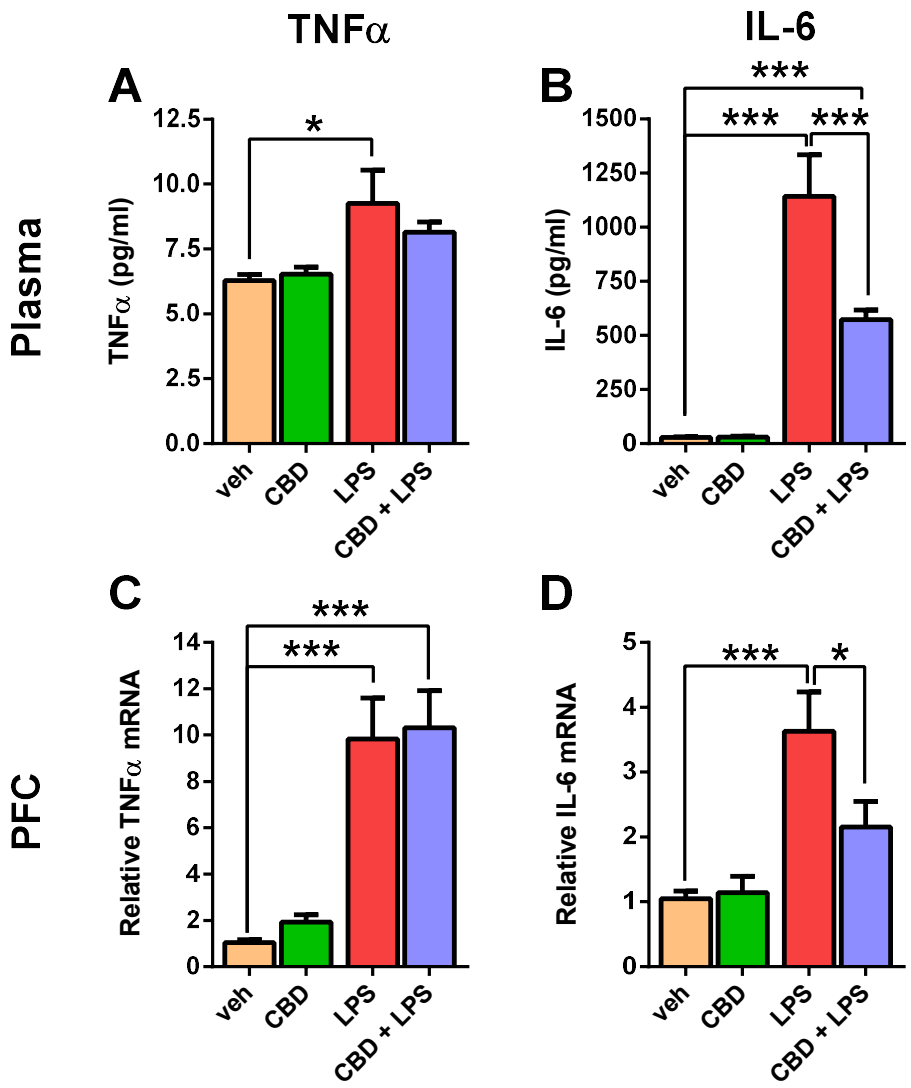


Figure 40. TNFα and IL-6 levels in plasma (protein) and prefrontal cortex (mRNA) of LPS injected mice, and the effects of CBD preadministration. TNFα levels (A and C), IL-6 levels (B and D), plasma samples (A and B), and prefrontal cortex samples (C and D). Results are expressed as mean ± S.E.M. Two-way ANOVA followed by a Newman Keuls *posthoc* test. *p<0.05, ***p<0.001. n= 6 - 7 animals per group.

2.2.8 Kynurenine pathway metabolites and neurotransmitters in hippocampus and cortex of LPS mice: effect of CBD

The kynurenine levels in hippocampus were increased in the LPS injected group compared to the vehicle group (p<0.001), which was prevented with CBD preadministration to LPS mice (CBD+LPS *vs* LPS, p<0.01) (table 4). A two-way ANOVA

showed an effect of the LPS model [$F_{(1,19)}$ = 15.50, p<0.001], CBD treatment [$F_{(1,19)}$ = 8.553, p<0.01] and the interaction of both factors [$F_{(1,19)}$ = 5.473, p<0.05]. Similar results were obtained in cortex, although in this brain region the prevention produced by CBD administration was partial, as there were still statistical differences between CBD+LPS and vehicle groups (p<0.001) (table 4). A two-way ANOVA of the kynurenine levels in cortex resulted in a significant effect of the model [$F_{(1,28)}$ = 72.33, p<0.001], CBD [$F_{(1,28)}$ = 5.200, p<0.05] and their interaction [$F_{(1,28)}$ = 7.718, p<0.01].

There were no differences in 3-hydroxy kynurenine (3-HK) levels in the hippocampus in the *posthoc* test (table 4), although a significant effect of the model was obtained in the two-way ANOVA [$F_{(1,19)}$ = 7.282, p<0.05]. In the cortex, the LPS injection produced an increase in 3-HK levels compared to the vehicle group (p<0.001), and CBD prevented this effect when administered to LPS mice (p<0.01) (table 4). A two-way ANOVA resulted in an effect of CBD [$F_{(1,28)}$ = 10.73, p<0.01] and of the model [$F_{(1,28)}$ = 31.68, p<0.001].

Table 4. Kynurenine (KYN), 3-hydroxy kynurenine (3-HK), serotonin (5-HT), and tryptophan (TRP) levels in hippocampus and cortex of LPS-injected mice and the effect of CBD preadministration. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by a Newman Keuls *posthoc* test. *p<0.05, **p<0.01, ***p<0.001 *versus* vehicle group; $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 *versus* LPS group. n= 5 - 6 animals per group in the hippocampus and 7 - 9 in the cortex.

	vehicle	CBD	LPS	CBD + LPS
ng/mg tissue	Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.
Hippocampus				
KYN	1.775 ± 0.442	1.595 ± 0.0689	3.712 ± 0.386***	2.088 ± 0.246$^{\#\#}$
3-HK	397 ± 136.8	268.2 ± 117.2	806.6 ± 153.5	660.3 ± 173.4
5-HT	2.761 ± 0.198	3.744 ± 0.074**	3.462 ± 0.128**	3.982 ± 0.207***
TRP	4.990 ± 0.969	3.782 ± 0.317	6.678 ± 0.524	5.784 ± 0.568
Cortex				
KYN	5.189 ± 0.165	5.540 ± 0.557	13.15 ± 1.039***	9.579 ± 0.792***/$^{\#\#}$
3-HK	922.9 ± 95.8	654.6 ± 209.8	2128 ± 182.8***	1312 ± 174.7$^{\#\#}$
5-HT	2.507 ± 0.174	2.941 ± 0.272	3.135 ± 0.133	3.436 ± 0.196**
TRP	2.496 ± 0.057	2.481 ± 0.209	3.796 ± 0.146***	3.258 ± 0.154***/$^{\#}$

***vs veh group**
#vs LPS group

Serotonin levels were affected differently in the hippocampus and cortex. The administration of LPS, CBD and CBD+LPS produced an increase in hippocampal serotonin

levels (p<0.01, p<0.01 and p<0.001, respectively) (table 4). In a two-way ANOVA there was an effect of the treatment [$F_{(1,19)}$ = 22.48, p<0.001] and of the model [$F_{(1,19)}$ = 8.769, p<0.01]. In the cortex, CBD administration to LPS-injected mice increased serotonin levels (p<0.01), whereas no changes were observed in the other experimental groups (table 4). A two-way ANOVA showed an effect of the model [$F_{(1,28)}$ = 8.335, p<0.01].

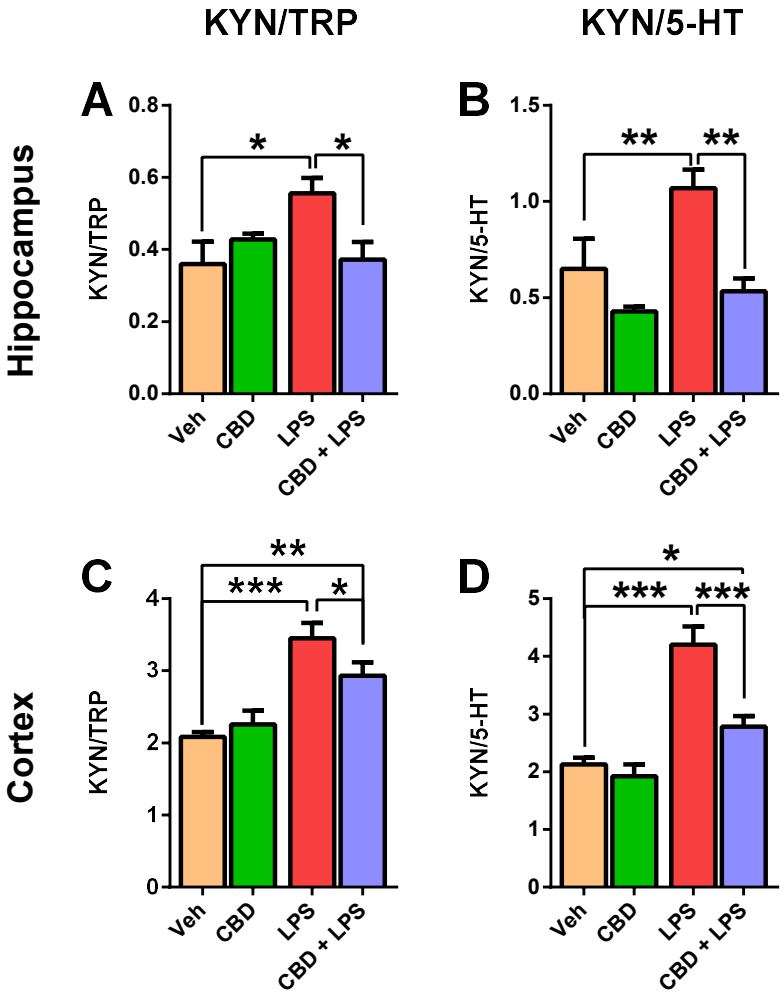


Figure 41. Kynurenine, tryptophan, and serotonin ratios in the hippocampus (A and B) and cortex (C and D) of LPS-injected mice and the effect of CBD preadministration. Kynurenine/tryptophan ratio (A and C), kynurenine/serotonin ratio (B and D). Results are expressed as mean ± S.E.M. Two-way ANOVA followed by a Newman Keuls *posthoc* test. *p<0.05, **p<0.01, ***p<0.001. n= 5 - 6 animals per group in the hippocampus and 7 - 9 in the cortex.

There were no statistically significant changes in the *posthoc* analysis of the hippocampal tryptophan levels (table 4), although a two-way ANOVA showed an effect of the model

[$F_{(1,19)}$ = 9.311, p<0.01]. In contrast, tryptophan levels were increased in the cortex by LPS injection (p<0.001). This effect was partially prevented by the CBD preadministration to LPS-injected mice (CBD+LPS *vs* LPS, p<0.05; CBD+LPS *vs* vehicle, p<0.001) (table 4). In the two-way ANOVA, there was an effect of the LPS model [$F_{(1,28)}$ = 52.05, p<0.001] (Annexe 1, table 22).

The evaluation of the kynurenine/tryptophan (KYN/TRP) ratio is used as a marker of the IDO enzyme activity (Fuchs et al., 1990; Widner et al., 2002; O'Connor et al., 2009). Mice injected with LPS had a higher KYN/TRP ratio in the hippocampus compared to the vehicle group (0.557 ± 0.041 in LPS *vs* 0.360 ± 0.062 in vehicle, p<0.05). CBD preadministration to LPS injected mice prevented the increase in the KYN/TRP ratio (0.373 ± 0.048 in CBD+LPS *vs* 0.557 ± 0.041 in LPS, p<0.05) (figure 41A). A two-way ANOVA of this data showed a significant effect of the interaction of model and treatment factors [$F_{(1,19)}$ = 8.389, p<0.01]. Analogous results were obtained in cortical samples, as the KYN/TRP ratio was also higher in the LPS group (3.449 ± 0.217 in LPS *vs* 2.084 ± 0.066 in vehicle, p<0.001) and this was attenuated by CBD preadministration to LPS injected mice (2.931 ± 0.189 in CBD+LPS *vs* 3.449 ± 0.217 in LPS, p<0.05; CBD+LPS *vs* vehicle, p<0.01) (figure 41C). A two-way ANOVA of the KYN/TRP ratio in cortex evidenced a significant effect of the LPS model [$F_{(1,28)}$ = 35.47, p<0.001], and a strong tendency of the interaction of model and treatment factors [$F_{(1,28)}$ = 4.060, p=0.054].

The kynurenine/serotonin (KYN/5-HT) ratio was used as a marker to evaluate the usage of tryptophan for the synthesis of either kynurenine or serotonin. There was an increase in this ratio in the hippocampus of mice injected with LPS (1.069 ± 0.097 in LPS *vs* 0.650 ± 0.156 in vehicle, p<0.01), and the preadministration of CBD prevented this effect (0.534 ± 0.066 in CBD+LPS *vs* 1.069 ± 0.097 in LPS, p<0.01) (figure 41B). A significant effect of the model [$F_{(1,19)}$ = 8.160, p<0.05], and of the treatment [$F_{(1,19)}$ = 17.04, p<0.001] was detected in a two-way ANOVA. There were parallel results in cortical samples, as a higher KYN/5-HT ratio was detected in LPS-injected mice (4.204 ± 0.315) compared to the vehicle group (2.127 ± 0.115, p<0.001), and CBD administration partially prevented this increase (2.786 ± 0.184 in CBD+LPS *vs* 4.204 ± 0.315 in LPS, p<0.001; CBD+LPS *vs* vehicle, p<0.05) (figure 41D). A two-way ANOVA of the KYN/5-HT ratio in cortical samples showed a significant effect of the model [$F_{(1,28)}$ = 47.30, p<0.001], CBD [$F_{(1,28)}$ = 14.39, p<0.001], and the interaction of both factors [$F_{(1,28)}$ = 8.062, p<0.01] (Annexe 1, table 23).

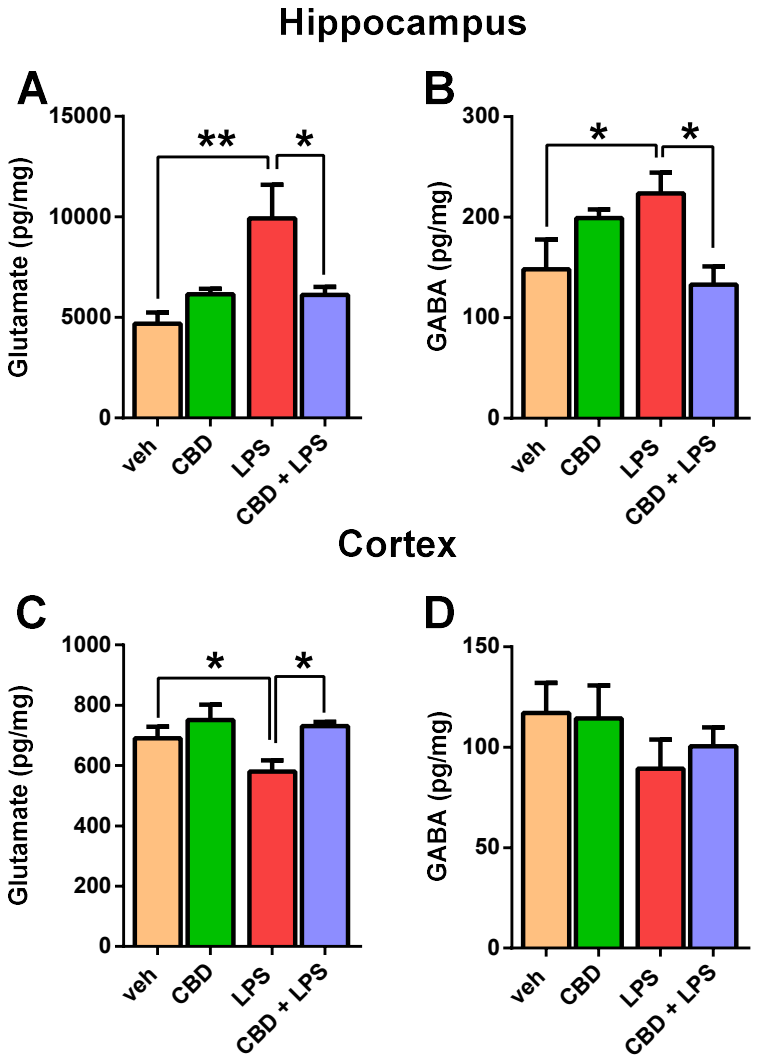


Figure 42. Glutamate (A and C) and GABA (B and D) levels in the hippocampus (A and B) and cortex (C and D) of LPS-injected mice and the effect of CBD preadministration. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by a Newman Keuls *posthoc* test. **p*<0.05, ***p*<0.01. n= 5 - 6 animals per group in hippocampus and 7 - 9 in cortex.

Regarding the brain levels of excitatory and inhibitory neurotransmitters, LPS injection increased glutamate and GABA levels in the hippocampus (glutamate: 9937 ± 1662 pg/mg in LPS *vs* 4688 ± 558 pg/mg in vehicle, *p*<0.01; GABA: 224 ± 20 pg/mg in LPS *vs* 148 ± 30 pg/mg in vehicle, *p*<0.05). CBD prevented the LPS-induced increase of both neurotransmitters in the hippocampus (glutamate: 6117 ± 406 pg/mg in CBD+LPS *vs* 9937 ± 1662 pg/mg in LPS, *p*<0.05; GABA: 133 ± 18 pg/mg in CBD+LPS *vs* 224 ± 20 pg/mg in LPS, *p*<0.05) (figure 42 A and B). A two-way ANOVA of glutamate levels showed an

effect of the model [$F_{(1,19)}$ = 7.679, p<0.05] and of the interaction between the model and CBD [$F_{(1,19)}$ = 7.892, p<0.05]. The same statistical analysis of GABA results showed an effect of the interaction of both factors [$F_{(1,19)}$ = 13.02, p<0.01].

In contrast, LPS injection decreased glutamate levels in the cortex (580 ± 36 pg/mg in LPS *vs* 690 ± 39 pg/mg in vehicle, p<0.05) and CBD prevented this decrease (732 ± 14 pg/mg in CBD+LPS *vs* 580 ± 36 pg/mg in LPS, p<0.05) (figure 42C). A two-way ANOVA of glutamate cortical levels showed a significant effect of CBD [$F_{(1,28)}$ = 8.837, p<0.01], and a trend towards significance of the LPS effect [$F_{(1,28)}$ = 3.289, p=0.081]. There were no changes in GABA cortical levels (figure 42D) (Annexe 1, table 24).

2.2.9 Correlation analysis between experimental findings

In the light of the results obtained in this section, the correlations that were most coherent with the data and most meaningful are presented.

Behaviour and molecular markers

The linear regressions between the sucrose preference test and TNFα and IL-6 levels are represented in figure 43. A strong negative correlation between sucrose preference and plasma IL-6 levels (r = -0.722, p<0.001) was observed, and the effect exerted by CBD in the LPS model could be clearly observed (figure 43B). A moderate negative correlation between sucrose preference and TNFα mRNA levels (r = -0.520, p<0.01, figure 43C), and IL-6 mRNA levels (r = -0.587, p<0.001, figure 43D) in the PFC was observed. Differently, the sucrose preference and TNFα plasma levels did not have a significant correlation (figure 43A).

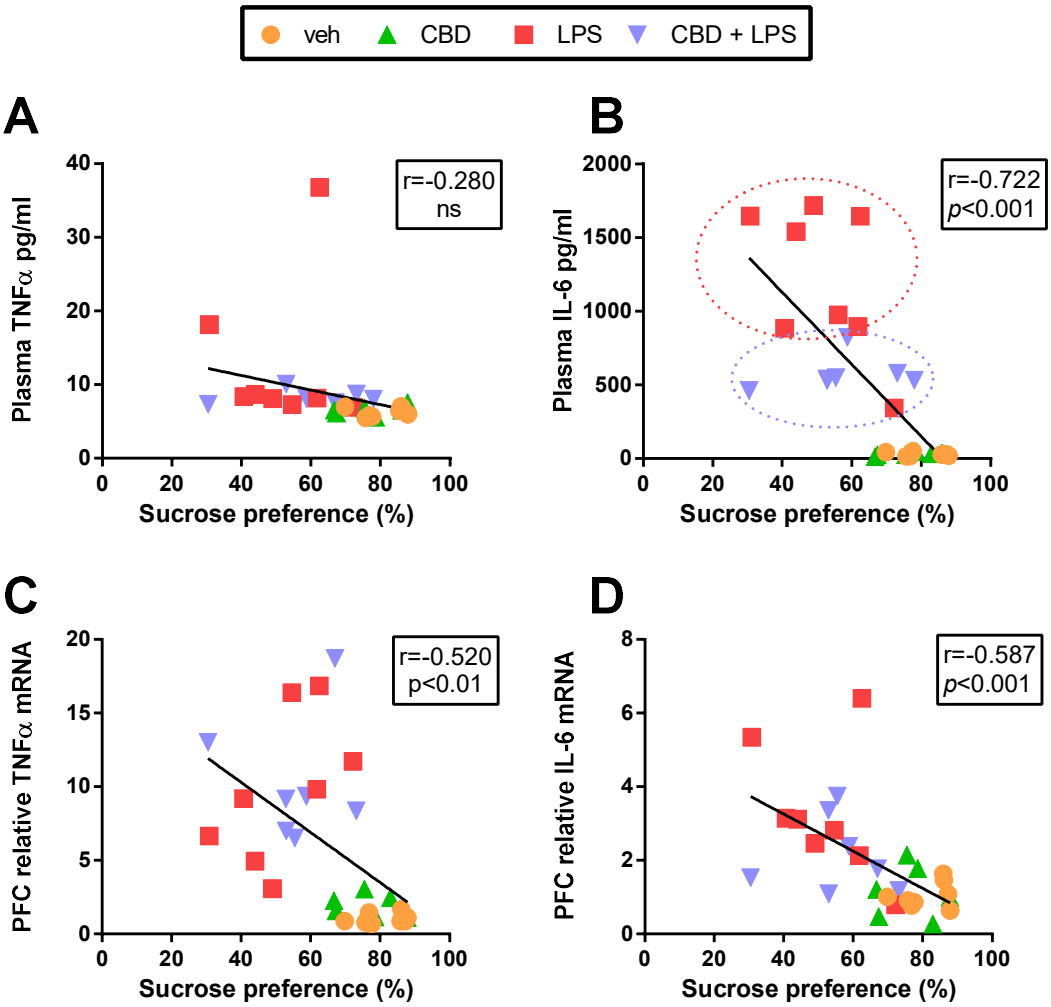


Figure 43. Correlation and linear regression between the sucrose preference and TNFα (A and C) and IL-6 (B and D) plasma protein levels (A and B) and prefrontal cortex mRNA levels (C and D) observed in the different experimental groups. n= 6 - 8 animals per group. r: Pearson's correlation coefficient. ns: not significant.

The correlations between the results obtained in the sucrose preference and the tail suspension tests and kynurenine brain levels are represented in figure 44. Linear regression showed a strong negative correlation between sucrose preference and cortical kynurenine levels (r = -0.671, $p<0.001$, figure 44A), and a moderate positive correlation between the immobility time and hippocampal kynurenine levels (r = 0.476, $p<0.05$, figure 44B). In both correlations, the effect exerted by CBD in the LPS model can be clearly observed.

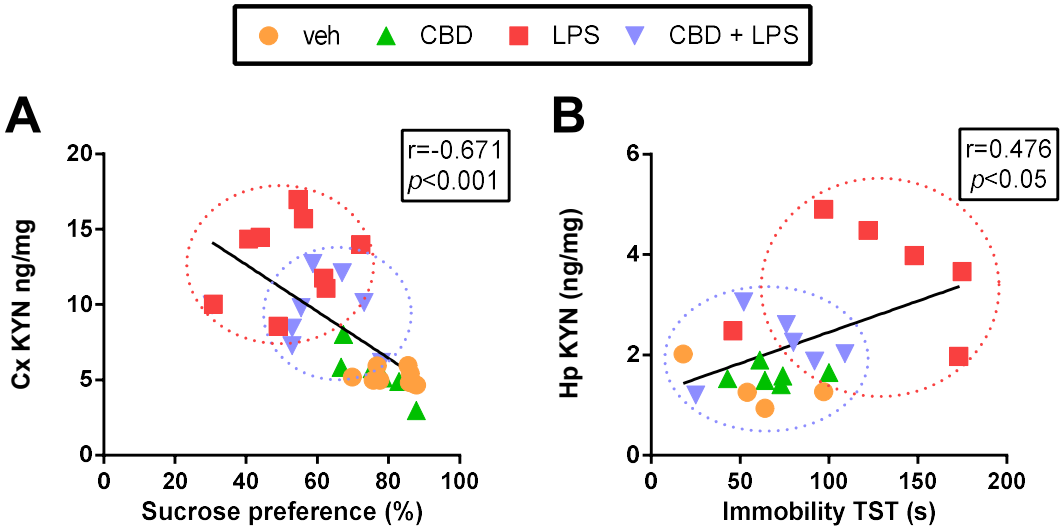


Figure 44. Correlation and linear regression between the sucrose preference (A) and the immobility time in the tail suspension test (TST) (B) and kynurenine (KYN) levels in the cortex (A) and hippocampus (B) of the different experimental groups. n= 7 - 9 animals per group in A, and 5 - 6 in B. r: Pearson's correlation coefficient.

Molecular markers

Plasma and prefrontal cortex IL-6 levels presented a strong positive correlation (r = 0.748, $p<0.001$, figure 45B), in which the LPS group is separated from the CBD+LPS group. Conversely, TNFα levels in plasma and prefrontal cortex did not show a significant correlation (figure 45A).

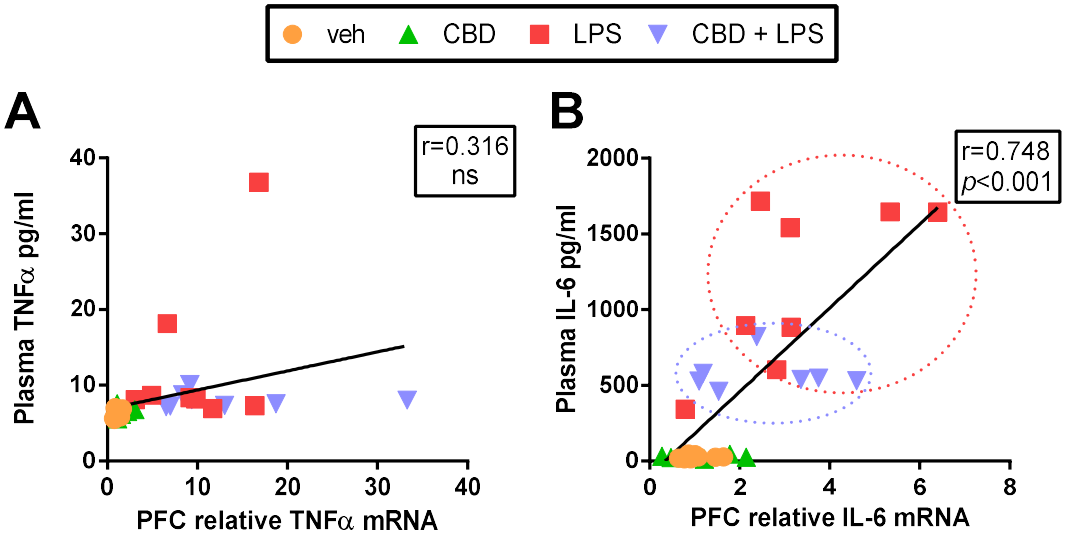


Figure 45. Correlation and linear regression between plasma protein levels and prefrontal cortex (PFC) mRNA levels of TNFα (A) and IL-6 (B) observed in the different experimental groups. n= 6 - 8 animals per group. r: Pearson's correlation coefficient. ns: not significant.

The correlations between plasma and PFC IL-6 levels and cortical kynurenine levels are presented in figure 46. Linear regression resulted in a strong positive correlation between plasma IL-6 levels and cortical kynurenine levels (r = 0.637, $p<0.001$, figure 46A), and in a weak positive correlation between IL-6 mRNA levels in PFC and kynurenine cortical levels (r = 0.400, $p<0.05$, figure 46B). In these correlations, a slight separation can be observed between the LPS and CBD+LPS groups.

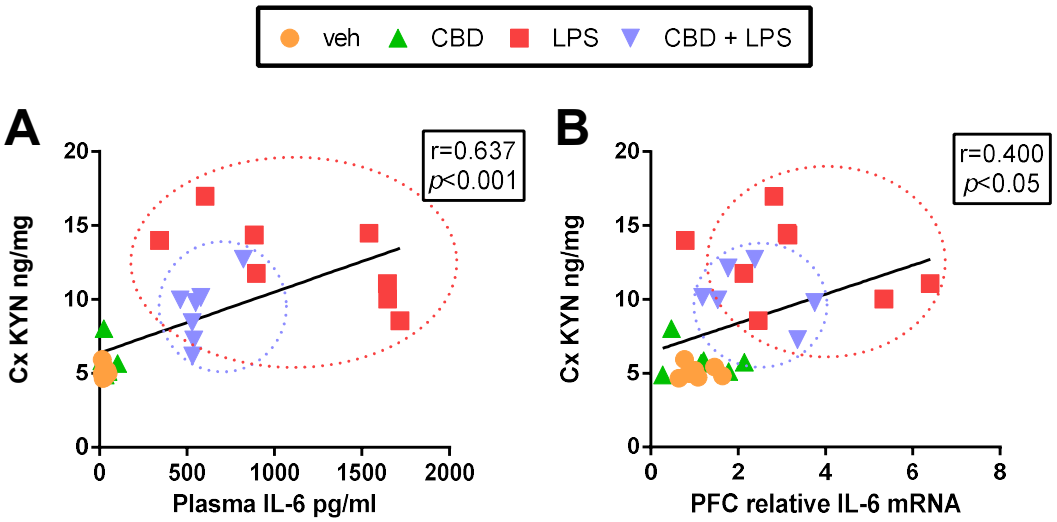


Figure 46. Correlation and linear regression between IL-6 plasma protein levels (A) and prefrontal cortex mRNA levels (B) and cortical kynurenine levels observed in the different experimental groups. n= 6 - 8 animals per group. r: Pearson's correlation coefficient.

DISCUSSION

1. CBD antidepressant-like effects: site and mechanism of action

There is growing preclinical evidence about the potential of cannabidiol as an antidepressant drug (Zanelati et al., 2010; Linge et al., 2016). In this thesis, we aimed to study the brain regions and the molecular and neurochemical actions underlying the antidepressant-like effect of CBD. Therefore, we performed intra-cerebral infusions of CBD into two key brain areas involved in mood regulation: the medial prefrontal cortex (mPFC) and the dorsal raphe nucleus (DRN) (Vertes, 2004; Berton and Nestler, 2006; Drevets et al., 2008; Pandya et al., 2012). As the infralímbic (IL) cortex has been described as a crucial area for the pathophysiology of depression (Garro Martínez, 2017; Fullana et al., 2019), for the effect of fast-acting antidepressants (Fuchikami et al., 2015; Jiménez-Sánchez et al., 2016; Gasull-Camós et al., 2017b), and for the 5-HT_{1A} receptor-dependent antidepressant-like effect of CBD (Sartim et al., 2016), we decided to focus on this region. On the other hand, we also selected the DRN, which is the location of most of the serotonergic neurones in the CNS, due to the importance of serotonin and the PFC-DRN connectivity in depression and the antidepressant treatment (Celada et al., 2001; Challis and Berton, 2015; Fullana et al., 2020). The behavioural, molecular, and neurochemical outcomes of these two experimental approaches are discussed in this section.

1.1 Bilateral cannabidiol infusion into the infralimbic cortex: acute effects

1.1.1 Depressive- and anxious-like behaviour

We have shown that CBD infusion into the IL cortex produced an acute antidepressant-like effect as evidenced by a decrease in behavioural despair (immobility time) in the FST, as previously described (Sartim et al., 2016), and without any detrimental effect on animals' locomotor activity, as evidenced in the open field test. This lack of acute locomotor effects of CBD has been also observed after its administration into the IL cortex (Lemos et al., 2010; Sartim et al., 2016), into other brain areas (Bitencourt et al., 2008; Gomes et al., 2011; Hsiao et al., 2012), and following systemic administration (Zanelati et al., 2010; Kasten et al., 2019; Sales et al., 2019).

It is worth to mention that with the data obtained from the modified FST (Detke et al., 1995) we demonstrated, for the first time, that the reduction in immobility elicited by the IL CBD infusion was due to an increase in the swimming behaviour, in line with the effects reported after the acute systemic administration of CBD (Réus et al., 2011; Shbiro et al.,

2019). In view of our results, and taking into account that most of the studies evaluating the effect of CBD in the FST only focus on the immobility time (Zanelati et al., 2010; Sartim et al., 2016, 2018; Sales et al., 2019), we believe that the use of the modified FST would provide more useful information in future studies, as it has been suggested that the active behavioural parameters are more sensitive to antidepressant drugs than immobility (Detke et al., 1995; Cryan et al., 2005; Kordestani-Moghadam et al., 2020). Therefore, we can postulate that the acute antidepressant-like effect of the IL CBD infusion is mediated by the activation of the serotonergic system, since it has been reported that an increased swimming behaviour in the FST is associated with a serotonergic-mediated response, while changes in the climbing activity are associated to a catecholaminergic-mediated response (Detke et al., 1995; Cryan et al., 2002, 2005).

Our findings are in good agreement with the acute effects of CBD after its infusion into the IL cortex (Sartim et al., 2016), the hippocampus (Sartim et al., 2018), and its intracerebroventricular administration (Sales et al., 2019). Moreover, the systemic administration of CBD also produces acute antidepressant-like effects (Zanelati et al., 2010; Réus et al., 2011; Sales et al., 2019; Shbiro et al., 2019). The pivotal role of the IL cortex in the fast antidepressant action is further evidenced by reports in which the IL infusion of drugs such as veratridine (a depolarizing agent) and dihydrokainic acid (DHK, a selective inhibitor of the glutamate transporter) (Gasull-Camós et al., 2017b), and other strategies as the deep brain stimulation in the IL cortex (Jimenez-Sánchez et al., 2016), produce a rapid antidepressant-like effect.

The acute anxiolytic-like properties of a systemic administration of CBD have been widely reported, but when the intra-cerebral CBD infusion approach is used, the IL cortex is not between the most targeted regions (*reviewed in* Blessing et al., 2015). Regarding the effect of the IL CBD infusion on anxiety-related behaviour, we did not observe any significative finding, in good agreement with a study using the same dose of CBD (60 nmol); in contrast, lower doses (15 and 30 nmol) produced an acute anxiolytic-like effect in the elevated plus maze (EPM) (Marinho et al., 2015). This bell-shaped dose-response curve of CBD on anxiety related responses has been also observed after systemic administration (*reviewed in* Blessing et al., 2015), and reinforces the notion that CBD exerts anxiolytic-like effects at low, but not at high doses (Guimaraes et al., 1990; Campos and Guimaraes, 2008; Lujan et al., 2018), at least in paradigms assessing ethological anxiety, such as the OFT and EPM tests, which are based on the exploratory behaviour of rodents and can be used to study generalized anxious disorders (Lister, 1990). In other study, the IL CBD infusion had effects on the anxious-like behaviour, but the authors explored other type of anxiety that is more related with fear (Lemos et al., 2010). Conversely, the tests based on fearful behaviour as the fear conditioning test used in that work are more indicated to study other anxiety

disorders, like phobias and post-traumatic stress disorder (Steimer, 2011). In fact, it has been proposed that anxiety and fear are distinct entities (Gray and McNaughton, 2000). Therefore, our results are not fully comparable with the work of Lemos et al. (2010) and with other studies using tests with a fearful component.

The infusion of CBD into other regions as the dorsolateral portion of periaqueductal gray (dlPAG) and the bed nucleus of the stria terminalis (BNST) are the most commonly studied regarding the anxiolytic-like effect. The administration of 60 nmol CBD into the BNST induced an acute anxiolytic-like effect in the EPM (Gomes et al., 2011). Other two studies reported an acute anxiolytic-like effect in the EPM with the infusion of 30 nmol CBD into the dlPAG, but no effect with the 60 nmol dose (Campos and Guimarães, 2008; Campos and Guimarães, 2009). These pieces of evidence indicate that the anxiolytic-like effects of locally administered CBD depend on the dose, the brain structure targeted, and the anxiety-related behaviour tested.

All in all, these findings confirm that the IL cortex is a key area for the acute antidepressant-like effect of CBD, but not the anxiolytic one.

1.1.2 Plasticity markers in the prefrontal cortex and hippocampus

To determine the acute effects of the IL CBD infusion on neuroplasticity, we studied the activation and expression of different neuroplasticity markers in two key brain areas for depression and the antidepressant effect: the prefrontal cortex and the hippocampus (Berton and Nestler, 2006; Drevets et al., 2008; Dusi et al., 2015; Liu et al., 2017a; Aleksandrova et al., 2019).

To our knowledge, this is the first work studying the acute effect of the IL CBD infusion on neuroplasticity signalling pathways. CBD infusion into the IL cortex increased the activation of MAPK/ERK and mTOR pathways and induced a huge increase in BDNF levels in the PFC, and increased MAPK/ERK activation and BDNF levels in the hippocampus. In support of these *in vivo* findings, our *in vitro* studies in cortical primary cultures show parallel results (*see 1.5 in the Discussion section*). Our results are in line with the effects reported after the intra-hippocampal CBD infusion (Sartim et al., 2018) and the acute systemic CBD administration on BDNF/TrkB/mTOR signaling in brain areas such as PFC and hippocampus (Sartim et al., 2018; Sales et al., 2019). Furthermore, the intracerebroventricular infusion of a TrkB receptor antagonist and the mTOR inhibitor rapamycin abolished the acute antidepressant-like effects of CBD (Sales et al., 2019), suggesting that BDNF and the mTOR pathway activation are mediating the acute

antidepressant-like effects of the drug. However, some reports showed a lack of changes in BDNF levels in areas as PFC and hippocampus after acute systemic CBD (30 mg/kg) administration (Zanelati et al., 2010; Réus et al., 2011). These discrepancies could be dependent on the dose of CBD administered. All in all, we propose that the main brain areas implicated in the acute antidepressant-like effects of the infusion of CBD into the IL cortex are the prefrontal cortex and the hippocampus, through a mechanism involving the activation of synaptic plasticity pathways.

The involvement of the mTOR pathway in the therapeutic effects of CBD has also been reported in animal models of seizures (Gobira et al., 2015; Lima et al., 2020). In this sense, the anticonvulsant effect of CBD is abolished in PI3Kγ knock-out mice, suggesting that this therapeutic effect of CBD is mediated by the activation of mTOR via PI3K/AKT pathway (Lima et al., 2020). The behavioural effects of CBD on the mTOR pathway have been evaluated in the presence of an mTOR inhibitor (Gobira et al., 2015; Sartim et al., 2018; Sales et al., 2019; Lima et al., 2020), but none of those studies demonstrated the activation of the mTOR pathway. Therefore, we report for the first time the increase in the phosphorylation of mTOR in brain after an acute administration of CBD.

Our findings are in line with the mechanism of action described for fast-acting antidepressants such as ketamine (Li et al., 2010; Duman et al., 2012; Yang et al., 2012; Zhou et al., 2014a; Aleksandrova et al., 2017), other drugs acting at NMDA receptors (Liu et al., 2017b), and scopolamine (Voleti et al., 2013). In addition, chronic treatment with classic antidepressants such as SSRIs increases BDNF levels (Nibuya et al., 1995; Martínez-Turrillas et al., 2005; Molteni et al., 2006; Björkholm and Monteggia, 2016), which highlight the potential use of BDNF as a biomarker for mood disorders (Sen et al., 2008; Hashimoto, 2010), and as a predictor of antidepressant efficacy (Sen et al., 2008). An initial study showed that classical antidepressants do not activate the mTOR pathway (Li et al., 2010), however, latter reports indicate that chronic treatment with SSRIs activates the mTOR pathway, especially in the hippocampus (Liu et al., 2015; Barone et al., 2018; Xu et al., 2018; 2020).

Regarding the Wnt/β-catenin pathway, the acute IL CBD infusion did not produce changes in the levels of β-catenin nor in the phosphorylation of GSK3β (Ser 9) and CREB in the PFC, suggesting that the acute effects of CBD are not mediated by this signalling pathway. These results are in agreement with the lack of changes in p-Akt levels, as activated Akt would phosphorylate and inactivate GSK3β (Grimes and Jope, 2001), which in turn could lead to the activation of the β-catenin pathway and increased CREB activity (*reviewed in* Pilar-Cuéllar et al., 2014). The Wnt/β-catenin pathway has been more related to cell proliferation and is a key regulator of adult neurogenesis (Lie et al., 2005; Wexler et al.,

2009), which suggests that the effects on this pathway would have more relevance in the hippocampus. Unexpectedly, CBD induced the reduction of p-Ser9-GSK3β levels in the hippocampus. As the phosphorylation of GSK3β serine 9 residue produces its inactivation (*reviewed in* Pilar-Cuéllar et al., 2014), our results suggest that CBD increased GSK3β activity in this area, which could lead to a decrease in β-catenin signalling. This result contrasts with the reported pro-neurogenic effects in the hippocampus after repeated CBD administration (Campos et al., 2013; Mori et al., 2017; Fogaça et al., 2018, reviewed in Silote et al., 2019). However, Schiavon et al. (2016) reported that, although chronic administration of CBD reduced hippocampal neurogenesis, the antidepressant-like effects were still present.

Besides, there seems to be a consensus in the literature pointing towards the increase in p-Ser9-GSK3β levels in prefrontal cortex, hippocampus, and striatum following the administration of classic antidepressant drugs (fluoxetine and imipramine), the mood stabilizer lithium, and the fast antidepressant ketamine (Beaulieu et al., 2004; Li et al., 2004; Beurel et al., 2011, Zhou et al., 2014b). The effects of CBD on GSK3β activity have been studied in an *in vitro* neurodegenerative model (Esposito et al., 2006b), and after subchronic CBD administration in an *in vivo* model of schizophrenia (Renard et al., 2016). However, this is the first *in vivo* study addressing GSK3β activity modulation after the acute administration of CBD. Still, as hippocampal neurogenesis is a process that takes some time to produce a noticeable effect –newborn neurones must differentiate and mature into functional neurones (Pittenger and Duman, 2008)– it is more likely to participate in the long-lasting antidepressant-like effects rather than in the acute effects. Therefore, it seems that these apparent contradictory effects of CBD on GSK3β activity in the hippocampus are not interfering with its acute antidepressant-like effects.

Regarding the mechanisms by which CBD could be modulating these signalling pathways, one possibility is through the agonistic or positive allosteric modulatory effect on the 5-HT_{1A} receptor (Russo et al., 2005; Rock et al., 2012). The activation of this receptor can induce synaptic plasticity pathways such as AKT, ERK, mTOR, and BDNF (Zhou et al., 2014c; Jiang et al., 2016; Fukumoto et al., 2018, 2020; *reviewed in* Masson et al., 2012; Rojas and Fielder, 2016; Albert and Vahid-Ansari, 2019). On the other hand, the antidepressant-like effects of CBD could also be mediated by the indirect activation of the endocannabinoid receptors CB_1 and CB_2, (Campos et al., 2013; Do Monte et al., 2013; Sartim et al., 2016; *reviewed in* Silote et al., 2019). Through the increase in endocannabinoid levels (Bisogno et al., 2001; De Petrocellis et al., 2011; Leweke et al., 2012), the IL CBD infusion may result in a higher activation of local CB_1 receptors, which promote the activation of BDNF, AKT, ERK and mTOR neuroplasticity signalling pathways (Puighermanal et al., 2009; Prenderville et al., 2015; Vilela et al., 2015; Mallipeddi et al., 2017; *reviewed in* Luján and

Valverde, 2020). Therefore, both 5-HT_{1A} and cannabinoid receptors might be contributing to the acute increase in phospho-ERK, phospho-mTOR, and BDNF levels observed after the IL CBD infusion.

The activation of CB_1 receptors in the vmPFC could activate the serotonergic neurotransmission, leading to an increase of serotonin release in projection areas such as the prefrontal cortex and hippocampus and therefore of postsynaptic 5-HT_{1A} receptor activation. This proposal is based on the experiments performed by McLaughlin et al. (2012), in which an intra-vmPFC infusion of a FAAH inhibitor produced acute antidepressant-like effects in the FST and increased the firing rate of DRN serotonergic neurones. Therefore, the effects observed in the hippocampus may be due to an increase of serotonin release in this brain area. The weaker effects observed in the hippocampus compared to the prefrontal cortex may be due to the indirect versus the direct effects of cannabidiol in these two areas.

All in all, our results suggest that the acute antidepressant-like effect of CBD is associated to the activation of the ERK and mTOR pathways and the increase in BDNF levels in the brain, without the contribution of PI3K/AKT and Wnt/β-catenin pathways.

1.1.3 Neurotransmittersin the prefrontal cortex and dorsal raphe nucleus

As an approximation to study the effects of CBD on the prefrontal cortex-DRN neurotransmission, we determined the levels of serotonin (5-HT), noradrenaline (NA), glutamate (GLUT), and γ-aminobutyric acid (GABA) in these two brain areas. The IL CBD infusion produced an acute decrease in GLUT and NA levels whithout changes in 5-HT and GABA levels in the PFC, while decreased GLUT and GABA levels without inducing changes in the levels of 5-HT and NA in the DRN.

In order to interpret these results correctly, it must be highlighted that: 1) the neurotransmitters levels have been determined in *postmortem* brain samples obtained 30 min after the behavioural tests, and therefore may not be reflecting what happened during the behavioural performance; 2) we are measuring tissular neurotransmitter levels that consist of the net balance between the neurotransmitters released into the synaptic cleft and the neurotransmitters kept in vesicles inside the cells. In addition, most of the studies in the literature were performed using *in vivo* microdialysis.

As suggested by the increase in the swimming behaviour in the FST, we were expecting that the IL CBD infusion would be associated to an increase in 5-HT levels in the PFC. However, this effect may be rapid and transient, leading to similar 5-HT levels in CBD and

control animals at the time point that were measured. In support of this assumption, some microdialysis studies report short duration increases in 5-HT levels in DRN projection areas after the administration of ketamine (Nishitani et al., 2014), CBD (Murillo-Rodríguez et al., 2006; Linge et al., 2016), and the blocker of glial glutamate transporter-1 (GLT-1) dihydrokainic acid (DHK) (Gasull-Camós et al., 2018). In line with the lack of changes on 5-HT levels in the DRN, it has been reported that other fast antidepressant estrategies, as ketamine administration and intra-IL cortex DBS, did not increase 5-HT levels in the DRN even if they were increased in the mPFC (Jiménez-Sánchez et al., 2016; Pham et al., 2017). Thus, the unmodified levels of 5-HT in the DRN may facilitate the rapid antidepressant-like effects of CBD by evading the auto-receptor mediated inhibition of the DRN firing that is produced by the acute administration of SSRIs (Gartside et al., 1995; Gobert et al., 1997; Sharp et al., 1997).

Regarding brain levels of glutamate, the neuroinflammatory hypothesis of depression postulates that brain inflammation can produce an increase of glutamate excitotoxicity (*reviewed in* Maes et al., 2011; Barone, 2019). Based on this, the brain surgery performed in our experiments to carry out the IL infusion of CBD may have induced an acute inflammatory response in the surrounding proximities of the infusion site (Travis et al., 2019), which may account for the higher glutamate levels observed in the PFC of vehicle-infused animals compared to the levels observed 24 h after the infusion or after the DRN infusion. These results could indicate that the inflammatory response had weakened over time. In this sense, the knock-down of astrocytic glutamate transporters in the rodent IL cortex produces a depressive-like phenotype (Fullana et al., 2019). As showed in the second objective of this thesis, CBD down-regulates inflammatory pathways (Florensa-Zanuy et al., 2021). Thus, CBD could have diminished the surgery-induced glutamate levels in the PFC, which in turn could contribute to its acute antidepressant-like effects, in line with the antidepressant-like effect induced by ketamine and citalopram administration to glutamate transporter knock-down mice that present a depressive-like phenotype (Fullana et al., 2019). The CBD-induced changes in PFC glutamate levels could influence glutamate (PFC-DRN direct pathway) and GABA (PFC-DRN indirect pathway) levels in the DRN (Celada et al., 2001).

In our study, a similar pattern in glutamate and GABA levels could be observed, which could be due to homeostatic mechanisms. Also, the CBD-mediated activation of 5-HT_{1A} heteroreceptors (Russo et al., 2005; Rock et al., 2012), could have contributed to the inhibition of local GABAergic neurones. Therefore, we cannot discard that a putative CBD-induced local decrease in GABA levels may be undetectable when analysing the whole PFC.

However, the antidepressant-like effects observed in the FST with the CBD infusion could also suggest that a higher neurotransmitter release from intracellular vesicles may have occurred, which would lead to its metabolization and the diminished levels of glutamate and GABA observed (Adell et al., 1988a, 1988b).

As the IL CBD infusion did not modify the climbing behaviour, we were not expecting to find reduced NA cortical levels. Indeed, it has been demonstrated that the depletion of NA does not block the acute antidepressant-like effects of CBD, suggesting that NA is not implicated on its mechanism of action (Sales et al., 2018). Even though, the reduction in PFC NA levels could be the consequence of 1) a putative lower glutamatergic neurotransmission towards the locus coeruleus (Kim and Lee, 2003), which would diminish noradrenergic neurotransmission; or 2) the release of NA from intracellular vesicles as previously proposed for glutamate and GABA. In any case, the fact that the three main monoaminergic brain nuclei (ventral tegmental area, locus coeruleus and DRN) closely regulate each other (Tritschler et al., 2018), and the locus coeruleus receives a great number of afferents and has a complex molecular heterogeneity (Schwarz and Luo, 2015), makes it difficult to interpret the CBD-induced changes in NA levels.

Notwithstanding all these possible explanations, future *in vivo* microdialysis studies would be needed to contrast our hypothesis regarding the changes in neurotransmitter levels in these two key brain areas, and its association to the acute antidepressant-like effect of the IL CBD infusion.

1.1.4 Cannabidiol effects on neuronal activation in prefrontal cortex and dorsal raphe nucleus

Different stimuli, such as drug administration and stress, increase c-Fos expression, which has been widely used as a marker of neuronal activation (Dragunow and Faull, 1989; Kovács, 1998). To study the acute effects of the IL CBD infusion on the modulation of neuronal activity in the PFC-DRN circuitry, we analyzed c-Fos immunolabeling in animals that had been exposed to acute stress (induced by the open field and forced swimming tests) in comparison with *naïve* animals. We determined c-Fos immunolabeling in 3 brain areas: 1) the infralimbic cortex, to study the local effects of the drug in this crucial brain area for the antidepressant-like effect; 2) the prelimbic cortex, to test if the effects of CBD would be the same in an adjacent area to the infusion site, or in contrast would be site-specific; 3) the DRN, to determine if the IL CBD infusion could modulate the PFC-DRN neuronal circuit.

The exposure to acute behavioural stress produced a significant increase in c-Fos levels in the DRN and a tendency in the IL and PL cortices, as previously described after different stressful stimuli, including the exposure to a novel environment and the FST (Kovács, 1998; Ons et al., 2004; Muigg et al., 2007; Silva et al., 2012; Pilar-Cuéllar et al., 2017; Pizzo et al., 2018). Other authors have described an increased neuronal activitation of the DRN GABAergic interneurones in knock-down mice of the astrocytic glutamate transporter, which present a depressive-like phenotype (Fullana et al., 2019). Based on this, it is tempting to especulate that the stress-induced increase in c-Fos levels observed in the DRN could be due to an increased activation of GABAergic neurones.

Regarding the acute effects of the IL CBD infusion, *naïve* animals showed an increase in c-Fos expression levels in the DRN. This agrees with previous reports after the intracerebroventricular (Murillo-Rodríguez et al., 2006) and DRN (Murillo-Rodríguez et al., 2008) infusion of CBD. In these two studies, CBD induced wakefulness, a state characterized by a higher firing rate of the DRN neurones (Wu et al., 2004; Urbain et al., 2006), which is accompanied by an increased release of 5-HT (Gartside et al., 1995). Thus, these pieces of evidence suggest that in our study, the IL CBD infusion may increase the activation of the serotonergic neurones in the DRN of *naïve* rats. Similarly, the infusion of fast antidepressant drugs as ketamine and the mGlu2/3 antagonist LY341495 into the mPFC increased c-Fos levels in DRN serotonergic neurones (Fukumoto et al., 2016). Interestingly, the infusion of a glutamate transporter blocker (dihydrokainic acid, DHK) and of S-AMPA into the IL cortex –which produced immediate antidepressant-like effects– increased c-Fos levels in the DRN serotonergic neurones (Gasull-Camós et al., 2018), but this was not observed when the drugs were infused into the PL cortex (Gasull-Camós et al., 2017b).

Differently, CBD did not change c-Fos levels in the PL and IL cortices of *naïve* rats. This is in accordance with the results reported after the acute systemic administration of CBD (Lemos et al., 2010), the PFC infusion of ketamine (Tarrés-Gatius et al., 2020), and chronic fluoxetine treatment (Lino-de-Oliveira et al., 2001). However, other authors reported an increase in c-Fos immunostaining in the mPFC after acute DBS into the mPFC (Veerakumar et al., 2014), and specifically in the PL and IL cortices both after an optogenetic stimulation of the IL cortex and after systemic ketamine administration (Fuchikami et al., 2015). Moreover, the NMDA glycine-site partial agonist GLYX-13 increased c-Fos levels in the IL but not in the PL cortex (Liu et al., 2017b). These differences could indicate that the effects elicited by CBD in *naïve* animals are different from the ones induced by other antidepressant treatments.

The CBD infusion into the IL cortex modulated differently the effects of stress depending on the brain area studied. CBD administration did not alter the stress-induced c-Fos expression in the PL cortex. In contrast, the results obtained in the IL cortex suggest that CBD infusion impeded the stress-induced neuronal activation in this brain area. The transient inactivation of the IL cortex has been described to produce antidepressant-like effects both in *naïve* rats (Scopinho et al., 2010; Slattery et al., 2011) and in a rat model of depression and anxiety (Slattery et al., 2011). Indeed, the results obtained in the IL cortex with chronic citalopram administration to rats subjected to chronic footshock stress were similar to the ones reported in this thesis (Kuipers et al., 2006). Similar results were obtained with the fear conditioning model, which increased c-Fos levels in the PL and IL cortices, and CBD pre-administration impeded those changes (Lemos et al., 2010). Also, other authors reported that the infusion of CBD into the PL cortex after fear conditioning reduced c-Fos levels in this brain area (Rossignoli et al., 2017). The different results obtained in the PL cortex could be possibly due to the systemic and the local CBD administration in that studies *vs* the IL CBD infusion performed in this thesis, which may not affect the PL cortex. In agreement with the results obtained in the IL cortex, it has also been described that CBD reduced c-Fos expression in other brain areas where c-Fos levels were increased as a consequence of a stressful stimulus (Hartmann et al., 2019). Conversely, the systemic administration of CBD did not modify PL and IL c-Fos expression after a low intensity stressful stimulus (Todd and Arnold., 2016).

In the DRN, both acute stress and the IL CBD infusion induced c-Fos expression. Regarding the statistical analysis, a two-way ANOVA showed a significant effect of the interaction between stress and CBD, indicating that CBD may oppositely modulate DRN activity under naïve or stress conditions, which could be due to a differential contribution of GABAergic and serotonergic neurones. Regarding the results obtained with clinically used antidepressant drugs, chronic paroxetine treatment reversed the effects induced by chronic mild stress in the DRN c-Fos levels (Elizalde et al., 2010), though chronic fluoxetine increased c-Fos expression in the DRN both in *naïve* and stressed animals (Lino-de-Oliveira et al., 2001). These pieces of evidence reinforce the possibility that the regulation of DRN neuronal activity participates in the antidepressant-like effects of the IL CBD infusion.

The study of c-Fos expression gives some valuable information, but it should be mentioned that this technique presents important limitations. For example, the high number of stimuli that lead to c-Fos expression makes it difficult to interpret its biological implications, and there are cases in which neuronal activation is not accompanied by c-Fos expression (Kovács, 2008; Appleyard, 2009). We have shown that an acute IL CBD infusion increased neuronal activity in the DRN in *naïve* animals, and that the stress-induced increase in neuronal activity in the IL cortex and the DRN appears to be

modulated by this CBD administration. In view of these results, it would be interesting to perform colocalization studies with c-Fos and glutamatergic and GABAergic neuronal markers to better understand the effects elicited by CBD. However, the complex outcomes observed in the DRN suggest that more modern techniques allowing the study of specific circuits and neuronal subpopulations, such as the recently developed optogenetic and chemogenetic tools (Hare and Duman, 2020), may be needed to clarify the effects of CBD on neuronal activation.

1.2 Bilateral cannabidiol infusion into the infralimbic cortex: effects after 24 h

1.2.1 Depressive- and anxious-like behaviour

The acute antidepressant-like effect of the IL CBD infusion was not observed after 24 h, as there were no differences in the immobility time in the FST. To our knowledge, this is the first time that the sustained effects of a single intra-cerebral CBD infusion is studied regarding its antidepressant-like effects. In line with our findings, other authors have observed the lack of sustained antidepressant-like effects 24 h after the infusion of drugs such as S-AMPA and DHK into the IL cortex (Gasull-Camós et al., 2017b; Gasull-Camós et al., 2018). However, the antidepressant-like effects were still present 24 h after the infusion of ketamine into the IL cortex and the optogenetic stimulation of this area (Fuchikami et al., 2015). Similarly, Hare et al. (2019) showed that the optogenetic stimulation of a specific type of neurones in the mPFC produced antidepressant-like effects at 24 h. As a single CBD infusion into the IL cortex is not enough to produce an antidepressant-like effect 24 h later, and the systemic administration of this drug has sustained antidepressant-like effects (Linge et al., 2016; Shoval et al., 2016; Sales et al., 2019), we can speculate that other brain areas may be implicated on its sustained effects.

It is important to note that we performed the IL CBD infusion in *naïve* rats, not in a rat model of depression. Therefore, there is still the possibility that, although the antidepressant-like effects of an IL CBD infusion were not sustained until 24 h in *naïve* rats, this could be different in an animal model of depression. In fact, we have demonstrated that CBD administration in the LPS model of neuroinflammation in mice (Objective 2 results; Florensa-Zanuy et al., 2021) and in the olfactory bulbectomy mouse model of depression (Linge et al., 2016) has different effects than in *naïve* animals. In line with this idea, ketamine administration produced acute antidepressant-like effects in both Flinders Resistant Line (FRL) and Flinders Sensitive Line (FSL) rats —a genetic model of depression—, but only exerted a sustained antidepressant-like effect in FSL rats (Sales et al., 2019). This distinct effect could be explained by the neurochemical differences that

the animal models of depression present in comparison to *naïve* animals (Wang et al., 2017).

There were no changes in the anxious-like behaviour assessed 24 h after the IL CBD infusion, as evidenced in the OFT. To our knowledge, there is only one study demonstrating the anxiolytic-like effect of CBD infusion into the IL cortex after 24 h, but it was assessed in a different behavioural paradigm (conditioned fear), with a longer duration of the treatment (3 days) and using a lower dose (Do Monte et al., 2013). Parallel results were obtained after i.c.v. infusions of a low dose of CBD (Bitencourt et al., 2008).

Regarding the anxious-like behavior, it is worth mentioning the implication of other brain areas in the acute and sustained anxiolytic-like effects that have been reported by other authors with CBD administration. For instance, the infusion of a low dose of CBD into the central amygdala produced anxiolytic-like effects in the OFT both acutely and after 24 h (Hsiao et al., 2012), and the intracisternal infusion of 30 nmol CBD reduced the anxious-like behaviour in the EPM induced by restraint stress after 24 h (Granjeiro et al., 2011). Moreover, the systemic administration of CBD also produces anxiolytic effects (Resstel et al., 2009; Linge et al., 2016). These pieces of evidence suggest that other brain areas and lower CBD doses may be more related to the anxiolytic-like properties of CBD.

Finally, the locomotor activity in the OFT was not modified 24 h after the IL CBD infusion, in line with the results obtained after its infusion into other brain areas (Hsiao et al., 2012) and after its systemic administration (Linge et al., 2016; Sales et al., 2019).

1.2.2 Plasticity markers in the prefrontal cortex

We have not found any *in vivo* study about the effects of a single CBD administration on neuroplasticity markers after 24 h. In our study, although phospho-mTOR and BDNF levels were still increased 24 h after the IL CBD infusion, the antidepressant-like effect was not maintained. It has been described that when BDNF binds to its receptor activates different mechanisms leading to more BDNF secretion, generating an autocrine feed-forward loop (Sadakata and Furuichi, 2010). The CBD-induced CB_1 and 5-HT_{1A} receptor activation together with the subsequent BDNF-dependent secretion of BDNF could have generated the huge acute increase in BDNF levels observed in the PFC. However, in view of the behavioural outcome, it is likely that these mechanisms would be gradually fading, which would have led to the still increased BDNF levels after 24 h but in a much lower magnitude, being therefore considered a subthreshold response (Gasull-Camós et al., 2018).

In contrast to the acute results, 24 h after CBD infusion the MAPK/ERK pathway activation had returned to control levels. BDNF binding to TrkB receptor is known to activate both PI3K/AKT and MAPK/ERK signalling pathways (*reviewed in* Mattson et al., 2004; Fenner, 2012; Pilar-Cuéllar et al., 2014; Duman et al., 2019b). However, this activation is transient, and fades away few hours after TrkB receptor stimulation (Proenca et al., 2016). Indeed, when TrkB receptor binds BDNF it becomes ubiquitinated and internalized, which could result in its degradation (Sommerfeld et al., 2000; Proenca et al., 2016). Therefore, we hypothesise that the continuous activation of TrkB receptors induced by the increased BDNF levels after CBD treatment could have reduced the amount of TrkB receptors in the cell surface, which could contribute to the fact that the MAPK/ERK pathway is no longer induced at 24 h. In addition, it has been reported that the agonism of the 5-HT_{1A} receptor induces a rapid but transient activation of ERK (Hsiung et al., 2005), and that the ketamine-induced increase on phospho-ERK and phospho-AKT levels in rat PFC is also transient, being detected at 30 min and having faded 2 h after drug administration (Li et al., 2010).

Surprisingly, phospho-mTOR levels were still increased 24 h after the IL CBD infusion, indicating that the activation of the mTOR pathway is less transient than the PI3K/AKT and MAPK/ERK pathways, and may be additionally regulated by other signalling pathways (*reviewed in* Pilar-Cuéllar et al., 2014). Still, our experiments suggest that the loss of the MAPK/ERK pathway activation may be an important factor underlying the lack of antidepressant-like effects of the IL CBD infusion after 24 h.

A CBD-induced decrease in phospho-Ser9-GSK3β levels in PFC was observed after 24 h, which is indicative of an increased activity of this kinase. As it is established that AKT inhibits GSK3β by phosphorylating its serine 9 residue (Grimes and Jope, 2001), it may seem that the lack of changes in AKT activity that we observed is incongruous. We have examined the activation of the PI3K/AKT pathway by determining the phosphorylation of AKT on the serine 473 residue, as most of the studies investigating the PI3K/AKT signalling pathway (Li et al., 2010; Renard et al., 2016; Liu et al., 2017b). However, it has been described that the phosphorylation of AKT on the threonine 308 residue is critical for the regulation of GSK3β (Beaulieu et al., 2004; Jacinto et al., 2006). Therefore, we cannot discard that CBD could have decreased p-Ser9-GSK3β levels by inducing a decrease on phospho-Thr308-AKT levels. Still, the regulation of Ser9-GSK3β phosphorylation by phospho-Ser473-AKT has also been suggested (Yung et al., 2011; Matsuo et al., 2018), which leaves unresolved the putative substrate specificity generated through AKT phosphorylation on Thr308 and Ser473. Alternatively, it is also possible that CBD regulates the activity of GSK3β independently of the PI3K/AKT pathway. Indeed, it has been described that chronic CBD treatment increased phospho-Ser9-GSK3β levels in the hippocampus of mice subjected to the chronic unpredictable stress (CUS) model but

produced no changes on phospho-Ser473-AKT and phospho-Thr308-AKT levels (Fogaça et al., 2018). It has been reported that MAPKs can also phosphorylate GSK3β (Gould et al., 2004). The acute IL CBD infusion increased phospho-ERK, with no changes on phospho-Ser9-GSK3β levels in the PFC. Since 24 h later the increase in ERK activity was lost, this could have contributed to the decrease in phospho-Ser9-GSK3β levels.

We hypothesise that the downregulation of phospho-Ser9-GSK3β levels observed 24 h after the IL CBD infusion could participate in the lack of antidepressant-like effects, since different antidepressant drugs increase phospho-Ser9-GSK3β levels (Beaulieu et al., 2004; Li et al., 2004; Beurel et al., 2011, Zhou et al., 2014b). When GSK3β is activated induces the degradation of β-catenin and this could reduce CREB activation (*reviewed in* Pilar-Cuéllar et al., 2014). However, we did not find changes in β-catenin nor phospho-CREB levels, which suggests that CBD is not affecting the Wnt/β-catenin pathway in the PFC. As GSK3β has multiple substrates (*reviewed in* Pilar-Cuéllar et al., 2014), more research is needed to elucidate the biological consequences of our finding.

Nevertheless, some authors have addressed this issue after chronic administration of CBD in different animal models. In line with our findings, the subchronic infusion of CBD into the nucleus accumbens shell produced antipsychotic-like effects in a model of schizophrenia, which was associated to reduced phospho-Ser9-GSK3β levels, with no changes on β-catenin levels (Renard et al., 2016). Moreover, CBD reduced phospho-Ser473-AKT levels but increased phospho-mTOR levels, suggesting that CBD induces the activation of the mTOR pathway trough another kinase than AKT (Renard et al., 2016, 2017). In contrast, other authors found that chronic CBD administration produced anxiolytic-like effects in mice subjected to the CUS model, together with a reduction on FAAH levels and an increase on phospho-Ser9-GSK3β levels in the hippocampus (Fogaça et al., 2018). Importantly, the CBD-induced increase on phospho-Ser9-GSK3β levels was only observed in the animal model, supporting that CBD molecular effects depend on the biological scenario in which it is administered. Therefore, these molecular effects may depend on the healthy/pathological state and could differ depending on the pathology studied. For instance, chronic CBD administration increased BDNF levels in the spinal cord and activated mTOR through the PI3K/AKT pathway in a mouse model of multiple sclerosis (Giacoppo et al., 2017b), and increased BDNF, phospho-ERK and phospho-CREB levels in the hippocampus in a mouse model of cocaine self-administration (Luján et al., 2018). Therefore, more research is needed concerning the neuroplastic effects of CBD in depression.

To sum up, the lack of ERK activation and the decrease on phospho-Ser9-GSK3β levels in the PFC of rats infused with CBD into the IL cortex could be involved in the absence of antidepressant-like effects at 24 h in *naïve* rats.

1.3 Effects of the bilateral cannabidiol infusion into the infralimbic cortex in the presence of a pre-synaptic 5-HT$_{1A}$ receptor blockade

1.3.1 Depressive-like behaviour

The IL CBD infusion produced acute antidepressant-like effects, but they were not maintained after 24 h. It is well stablished that the activation of 5-HT$_{1A}$ auto-receptors located in the DRN hinder the antidepressant effect (Rutter et al., 1995; Gartside et al., 1995; Hjorth et al., 2000; Gasull-Camós et al., 2018). A large number of basic research papers reported that the acute effect of SSRI administration was the reduction of 5-HT release in the forebrain due to the activation of 5-HT$_{1A}$ auto-receptors, whereas the coadministration of an SSRI plus WAY100635 (a selective 5-HT$_{1A}$ receptor antagonist) increased 5-HT release in DRN projection areas (Gartside et al., 1995; Gobert et al., 1997; Sharp et al., 1997). Therefore, we hypothesised that the blockade of those receptors could recover the antidepressant-like effects of CBD 24 h after its infusion into the IL cortex.

The administration of 0.3 mg/kg WAY100635 mainly blocks the 5-HT$_{1A}$ auto-receptors located in the DRN (Serres et al., 2000; Ago et al., 2003; Carey et al., 2005). The blockade of 5-HT$_{1A}$ presynaptic receptors short before the behavioural testing promoted the recovery of the antidepressant-like effect of the IL CBD infusion after 24 h, as evidenced in the three parameters assessed in the FST. It has been previously reported that the administration of this dose of WAY100635 does not alter the locomotor activity (Gasull-Camós et al., 2018). Our results may indicate that the inhibitory actions of the 5-HT$_{1A}$ auto-receptor on the serotonergic firing could be counteracting the prolongation of the antidepressant-like effect of CBD. In a similar experimental approach, Gasull-Camós et al. (2018) also demonstrated that the acute antidepressant-like effect of the IL infusion of dihydrokainic acid (DHK), a blocker of the glial glutamate transporter-1, was prolonged until 24 h in the presence of the antagonism of 5-HT$_{1A}$ auto-receptors.

There are also clinical studies supporting that the combination of SSRIs with pindolol (a mixed β-adrenergic and 5-HT$_{1A}$ receptor antagonist) could accelerate and enhance the antidepressant response in MDD patients (Artigas et al., 1994; Pérez et al., 1997, 2001; Portella et al., 2009). The potentiation effects of pindolol have not been consistent across clinical trials, but it is clear that the reduction of 5-HT$_{1A}$ auto-receptor signalling has the

potential to improve the antidepressant response to SSRIs (*reviewed in* Artigas et al., 2001; Albert and François, 2010). Regarding the benefitial effect observed on swimming behaviour, it may be associated to the potentiation of the serotonergic system by the blockade of the inhibitory effect of the 5-HT_{1A} presynaptic receptors. In this sense, an overactivation of 5-HT_{1A} auto-receptors in the DRN reduces the firing of serotonergic neurones (Gartside et al., 1995), impeding the antidepressant effect. On the other hand, the increase in the climbing behaviour in the FST may suggest an increased firing of noradrenergic neurones (Detke et al., 1995; Cryan et al., 2002, 2005). In line with these results, it has been reported that the activation of postsynaptic 5-HT_{1A} receptors increased noradrenaline release (Hajós-Korcsok et al., 1999).

Based on the results obtained, we hypothesise that the lack of antidepressant-like effects 24 h after the IL CBD infusion may be due to adaptative changes that result in a higher inhibitory serotonergic tone in the DRN mediated by presynaptic 5-HT_{1A} receptors.

1.3.2 Plasticity markers in the prefrontal cortex

Confirming our previous findings (*see 1.2.2 in the Discussion section*), 24 after the IL CBD infusion a significant increase in phospho-mTOR and BDNF levels, but not in phospho-ERK levels, was observed in the prefrontal cortex. Differently, in the presence of a presynaptic 5-HT_{1A} receptor blockade, we could observe a clear activation of phospho-ERK 24 h after the IL CBD infusion, resulting in a similar pattern of changes to the acute effects. This could be mediated by an increased serotonergic tone in postsynaptic areas, which could induce the MAPK/ERK pathway through the activation of 5-HT_{1A} heteroreceptors (Rojas and Fielder, 2016; Albert and Vahid-Ansari, 2019).

Therefore, the combination of the behavioural and molecular data obtained after the IL CBD infusion suggest that: 1) the activation of the synaptic plasticity signalling pathways mTOR and BDNF in the PFC may be important for the acute therapeutic effects of the IL CBD infusion but not sufficient to maintain them, at least in *naïve* rats; and that 2) the activation of ERK is important for the antidepressant-like effect of CBD. Our results are in line with the blockade of the antidepressant-like effects of ketamine through ERK inhibition (Li et al., 2010).

1.3.3 Neurotransmitters in the prefrontal cortex and dorsal raphe nucleus

Serotonin levels in PFC and DRN were not modified in any of the experimental conditions. In contrast, the IL infusion of DHK was associated to high 5-HT extracellular levels in the DRN 24 h after the infusion by using *in vivo* microdialysis, which was concomitant to a loss of the antidepressant-like effect. Those effects reappeared with the blockade of 5-HT_{1A} presynaptic receptors (Gasull-Camós et al., 2018). In our case, this strategy also recovered the antidepressant-like effects of CBD, but apparently without modifying serotonin levels in both areas. In this sense, it has been postulated that the release of glutamate in the DRN could increase the firing activity of a subset of serotonergic neurones without inducing the somatodendritic release of 5-HT in the nucleus (López-Gil et al., 2019). However, in our case, we cannot discard that the lack of changes observed could be due to the different technical approach used.

The higher NA levels observed in the DRN of animals treated with CBD and the 5-HT_{1A} antagonist WAY100635 may be explained by an increased excitatory noradrenergic input from the locus coeruleus (Tritschler et al., 2018), which agrees with the increased climbing behaviour observed (Detke et al., 1995; Cryan et al., 2002, 2005). Moreover, this increased noradrenergic firing leads to a subsequent increase in the firing of serotonergic neurones (Blier, 2001). However, although some authors report the increase in NA levels in the PFC following different antidepressant estrategies (Jimenez-Sánchez et al., 2016; López-Gil et al., 2019), we were not able to observe differences in this brain area.

Twenty-four hours after the IL CBD infusion glutamate levels were increased in both prefrontal cortex and DRN, and GABA levels were increased in PFC but decreased in DRN, with any behavioural outcome. The increase in GABA levels induced by CBD in PFC could be due to the higher excitatory signals from pyramidal neurones to GABAergic neurones (Pham and Gardier, 2019). In line with our neurochemichal results, both neurotransmitters were also increased 24 h after ketamine infusion into the mPFC (Pham et al., 2018, 2020). Interestingly, the systemic administration of WAY100635 in a dose that block the 5-HT_{1A} presynaptic receptors was able to recover the antidepressant-like effect of the IL CBD infusion, in parallel to a significant increase in glutamate levels in the DRN, but without modifying glutamate levels in the prefrontal cortex, or GABA levels in both structures, compared to the administration of CBD alone. Indeed, the antidepressant-like effect 24 h after ketamine (Pham et al., 2020) or (2R,6R)-hydroxynorketamine (HNK) (Pham et al., 2018) infusion into the mPFC has also been associated to increases in glutamate levels, and to the AMPA receptor activation in the DRN (Pham et al., 2020). Therefore, the increased levels of glutamate in DRN in the CBD group treated with WAY100635 together with the lower levels of GABA in this area (increased ratio

glutamate/GABA), would favour an antidepressant-like effect. Therefore, according to our experimental findings, we can postulate that the activation of the 5-HT_{1A} presynaptic receptors could be masking the beneficial effect of the IL CBD infusion on the glutamatergic neurotransmission in the PFC-DRN circuitry observed after 24 h.

Finally, WAY100635 administration increased glutamate levels in the prefrontal cortex and reduced GABA levels in the DRN. Although 5-HT_{1A} autoreceptors do not tonically inhibit 5-HT release, they act as sensors blocking an excessive serotonergic response (Adell et al., 2002). In this sense, we could speculate that an increased serotonin release induced by the exposure to the FST might underlie the effects observed after WAY100635 administration.

All these findings demonstrate that the infusion of a single dose of CBD into the IL cortex triggers changes in some neurotransmitter systems and molecular pathways associated to synaptic plasticity that are still present 24 h after its administration. However, the antidepressant-like effect is not observed at this time point. Importantly, the antidepressant, molecular, and neurochemical effects of CBD 24 h after its IL infusion can be recovered with a presynaptic 5-HT_{1A} receptor blockade.

1.4 Cannabidiol infusion into the dorsal raphe nucleus: acute effects

1.4.1 Depressive- and anxious-like behaviour

To our knowledge, this is the first time that the depressive and anxious behavioural outcomes of an intra-DRN CBD infusion are evaluated. The infusion of drugs into this brain area to test antidepressant-like effects are not common in the literature, perhaps because of the counter-productive effects that classical antidepressant drugs could produce in this area as a consequence of 5-HT_{1A} auto-receptor activation (Rutter et al., 1995; Gartside et al., 1995; Romero and Artigas, 1997). Despite this, as there are contradictory reports in the literature about the effects that CBD produces on the neuronal activation and the firing of serotonergic neurones in the DRN (Murillo-Rodríguez et al., 2006; Murillo-Rodríguez et al., 2008; Rock et al., 2012; De Gregorio et al., 2019), we decided to investigate the behavioural and molecular outcomes of a direct CBD administration into this brain area.

When we performed the modified FST after an acute intra-DRN CBD infusion, there were not changes in the immobility time, but a significant increase in the swimming behaviour together with a decreased climbing activity were observed. These behavioural outcomes are not dependent on changes in the locomotor activity induced by CBD, in good

agreement with a large number of papers that performed a systemic administration (Zanelati et al., 2010; Kasten et al., 2019; Sales et al., 2019), or local infusion into different brain areas such as the IL cortex, BNST and central amygdala (Lemos et al., 2010; Gomes et al., 2011; Hsiao et al., 2012; Sartim et al., 2016). Although not commonly reported in the literature, other authors have recently observed an opposite change in the active behaviours in the FST without modifications in the immobility time (Kordestani-Moghadam et al., 2020; Moreno-Santos et al., 2021).

As commented before, the increase in swimming has been associated to the activation of the serotonergic system, and the increase in climbing to a catecholaminergic response (Detke et al., 1995; Cryan et al., 2002, 2005). Therefore, at a first glance our behavioural results suggest that the infusion of CBD into the DRN rapidly activate the serotonergic system. However, the different behavioural outcomes observed compared to the IL CBD infusion (decrease in the immobility time with the IL CBD infusion *versus* decrease in the climbing time with the DRN CBD infusion) could indicate that, depending on the area of infusion, CBD might activate different serotonergic neurones within the DRN projecting to different brain areas. This could be possible because when infused into the IL cortex, CBD may activate the descending pathway that innervates specific serotonergic neurones in the DRN, whereas the intra-DRN CBD infusion would activate the DRN serotonin neurones in an unspecific manner. In line with this hypothesis, is has been suggested that the systemic administration of ketamine induces the activation of only a subpopulation of 5-HT neurones in the DRN (López-Gil et al., 2019), and the optogenetic stimulation of a particular subtype of pyramidal mPFC neurones produced antidepressant-like effects (Hare et al., 2019). Importantly, it has been reported that the specific activation of the mPFC neurones that project to the DRN induced an active behaviour in the FST, while the unspecific activation of mPFC neurones did not produce behavioural effects. Furthermore, dissimilar behavioural results were observed after a direct DRN stimulation and the specific simulation of the mPFC-DRN circuit (Warden et al., 2012). Moreover, the suggested general activation of 5-HT neurones with the DRN CBD infusion could be responsible for the decrease in the time spent climbing in the FST, as the DRN-Locus Coeruleus projections are inhibitory (Segal, 1979; Koyama and Kayama, 1993; Kim et al., 2004; Kishi et al., 2006; Dremencov et al., 2009; Tritschler et al., 2018).

Based on the mechanisms of action described for CBD, when infused into the DRN it could theoretically produce both the activation and the inhibition of the DRN firing. The regulation of the DRN firing could be produced by the particular agonistic properties of CBD on the 5-HT_{1A} receptor (Russo et al., 2005), for which it has a bell-shaped dose-response curve (Rock et al., 2012). In this sense, the intra-DRN infusion of low doses of CBD (32 nmol) induced the activation of 5-HT_{1A} auto-receptors in this brain area (Rock et

al., 2012), which would reduce the firing of the serotonergic neurones. In contrast, the intra-DRN infusion of 64 nmol CBD, similar to the dose used in our study, induced c-Fos activation in the DRN and wakefulness (Murillo-Rodríguez et al., 2008), a state characterized by an increased firing of DRN neurones (Wu et al., 2004; Urbain et al., 2006). Therefore, the increase in swimming behaviour that we observed in the FST is in line with previous findings (Murillo-Rodríguez et al., 2008), which suggests that in our experiment, the CBD infusion into the DRN increased the firing of the DRN neurones.

Additionally, the increase in endocannabinoids is among the described mechanisms of action of CBD (Watanabe et al., 1996; Bisogno et al., 2001), which in the DRN would lead to a decrease in GABA signalling facilitating the activation of serotonergic neurones (Geddes et al., 2016). In accordance, Murillo-Rodríguez et al. (2007) showed that the inhibition of the FAAH enzyme increased c-Fos activation in the DRN. Similarly, CB_1 receptor activation in the DRN has been reported to increase the DRN firing (Mendiguren and Pineda, 2009), a mechanism that could underlie the effect of CBD when infused in the DRN.

As observed after the acute IL CBD infusion, the DRN-CBD infusion did not modify anxious-like behaviour. Nevertheless, CBD has shown CB_1 and/or 5-HT_{1A} receptor-dependent anxiolytic-like effects when administered systemically or locally into different brain areas, but not into the DRN as in our study (*reviewed in* Blessing et al., 2015). Regarding the 5-HT_{1A} receptor agonist profile of CBD, it has been described that the intra-DRN infusion of a selective 5-HT_{1A} receptor agonist (8-OH-DPAT) produces anxiolytic-like effects (Graeff et al., 1996; Sena et al., 2003; Andrade et al., 2013), while the intra-DRN infusion of a 5-HT_{1A} receptor antagonist (WAY100635) induces an anxious-like behaviour (Pobbe and Zangrossi, 2005; Andrade et al., 2013). However, considering the differential effect of low *vs* high doses of CBD (*reviewed in* Blessing et al., 2015), we cannot discard a positive effect on anxiety of lower doses of CBD.

1.4.2 Plasticity markers in the prefrontal cortex

Due to the importance of the neural circuits between the PFC and the DRN in depression (Celada et al., 2001; Challis and Berton, 2015; Fullana et al., 2020) and the behavioural effects observed, we studied the impact of the acute intra-DRN CBD infusion on neuroplasticity markers in the PFC. We only detected an increase on BDNF levels, which could be due to an increase of 5-HT release, as we observed an increase in serotonin levels in this brain area. It is known that BDNF levels are strongly sensitive to serotonergic neurotransmission (Zetterström et al., 1999; Mattson et al., 2004; Martinowich and Lu,

2008), and that both molecules are influenced by the antidepressant treatment in a similar manner (Dranovsky and Hen, 2006; Mahar et al., 2014).

Although BDNF can induce signalling pathways as mTOR, we failed to observe any effect after the DRN CBD infusion. We could hypothesize that the activation of those pathways could be produced later, when the initial BDNF release would induce more BDNF release (Sadakata and Furuichi, 2010). Another possibility is that the magnitude of the increase in BDNF levels may not be enough to produce an effective activation of signalling pathways as mTOR or MAPK/ERK, which could also contribute to the lack of antidepressant-like effect, as previously reported for ketamine after the inhibition of mTOR or ERK pathways (Li et al., 2010). Therefore, CBD is more efficient inducing synaptic plasticity pathways when infused into the IL cortex compared to the DRN infusion, which may explain the different behavioural outcomes observed.

1.4.3 Neurotransmitters in the prefrontal cortex and dorsal raphe nucleus

The intra-DRN CBD infusion increased serotonin levels in the PFC, but not in DRN, without modifying noradrenaline, glutamate, and GABA levels in both areas. The increase in the serotonergic tone may explain the enhanced swimming behaviour observed in the FST, in good agreement with the behavioural outcome observed with pharmacological strategies that preferentially increase serotonergic neurotransmission (*reviewed in* Cryan et al., 2005). We previously proposed that the DRN CBD infusion would indiscriminately activate the serotonergic neurones, whereas the IL CBD infusion would activate only a subset of these neurones. Therefore, this might explain that we detected the increase in PFC serotonin levels induced by the DRN CBD infusion. In line with our results, the activation of the DRN firing increased the PFC serotonin levels without inducing changes in DRN (Jiménez-Sánchez et al., 2016).

The climbing activity in the FST has been associated to the administration of antidepressant drugs with a noradrenergic profile (Cryan et al., 2005). Moreover, the serotonergic projections from the DRN to the locus coeruleus are inhibitory (Segal, 1979; Koyama and Kayama, 1993; Kim et al., 2004; Kishi et al., 2006; Dremencov et al., 2009; Tritschler et al., 2018). However, although a decrease in the climbing behaviour was observed, we failed to detect changes in PFC and DRN noradrenaline levels. This could be due to transient changes in those neurotransmitter levels during the behavioural testing. Finally, the absence of changes in GABA and glutamate levels in the prefrontal cortex may be related to the lack of antidepressant-like effects observed after the CBD infusion into

the DRN, as different antidepressant strategies modulate the levels of those neurotransmitters (Jiménez-Sánchez et al., 2016; López-Gil et al., 2019; Pham et al., 2020).

1.5 Cannabidiol effects on plasticity markers in primary neuronal cultures

As an approximation to better understand how CBD induced synaptic plasticity *in vivo*, we incubated primary neuronal cultures with CBD for 30 min and 24 h to evaluate the molecular markers that resulted increased in the PFC after the acute IL CBD infusion: BDNF, phospho-ERK and phospho-mTOR.

It is known that, although with a different temporal pattern, BDNF levels are increased by classical and fast-acting antidepressant drugs (Molteni et al., 2006; Autry et al., 2011; Björkholm and Monteggia, 2016; Liu et al., 2016, *reviewed in* Duman et al., 2019b). In the last years, some studies have addressed whether these drugs may induce BDNF release. Diverse authors have shown that the rapid antidepressant-like effects of ketamine and other fast-acting drugs require the release of BDNF, not just an increase on its expression (Lepack et al., 2014; *reviewed in* Duman et al., 2019b). On the other hand, it has been reported that classical antidepressants increase BDNF expression but not its release (*reviewed in* Duman et al., 2019b). Therefore, this could be an important factor to consider, especially for the evaluation of putative fast antidepressant drugs. As the use of *in vivo* microdialysis is technically difficult to study large proteins such as BDNF, the quantification of BDNF in cell culture medium has been used to study BDNF release (Lepack et al., 2014, 2016; Ghosal et al., 2018; Kato et al., 2018; Fukumoto et al., 2019). Therefore, we used this approach to assess the effect of CBD in BDNF release and BDNF levels in cell lysates at different time points.

In primary neuronal cell cultures, we observed an increase on intracellular BDNF levels after 30 min that vanished after 24 h, while BDNF release was increased only after 24 h. This indicates that CBD rapidly increases BDNF levels inside the cell, that are gradually released later, which leads us to draw some conclusions. Firstly, the fast increase observed on intracellular BDNF levels could suggest that CBD rapidly stimulates BDNF translation instead of its transcription, as the whole process takes more than 30 min (Lanz et al., 2012). In line with this hypothesis, ketamine *in vivo* administration increased BDNF protein levels in the hippocampus but not BDNF mRNA levels after 30 min. Moreover, the acute antidepressant-like effects of ketamine were prevented by a protein synthesis inhibitor but not by an RNA polymerase inhibitor, demonstrating that the rapid translation of BDNF –but not its transcription– is required for the antidepressant-like effects of ketamine (Autry et al., 2011). Secondly, CBD induction of BDNF is transient in our *in vitro*

experiments, as has been also reported for ketamine (Autry et al., 2011). This strengthens the idea that the IL CBD infusion rapidly increased BDNF levels, and that the still increased BDNF levels in the PFC after 24 h may be due to the previous effects and not to a continued induction. Thirdly, CBD induced BDNF release from neurones after 24 hours, as it has been reported for fast-acting antidepressant drugs such as ketamine, hydroxynorketamine, scopolamine, and GLYX-13 after only 15 - 60 min (Lepack et al., 2014,2016; Ghosal et al., 2018; Kato et al., 2018; Fukumoto et al., 2019). The apparent delay in the CBD-induced BDNF release could be explained by methodological differences, as we have determined BDNF levels directly in the cell culture medium, whereas in the aforementioned studies an immunoprecipitation enrichment step was performed before the ELISA.

In primary neuronal cultures from cortical samples, we obtained an increase in phospho-ERK and phospho-mTOR levels after 30 min of incubation with CBD, which fits with the acute effects observed in the PFC after the IL CBD infusion. These results support that the increased BDNF release may have occurred at this time point, as the phosphorylation of ERK has been proposed as a consistent marker of BDNF-TrkB signaling (Lepack et al., 2016). In line with our results, other fast-acting antidepressant drugs increase phospho-ERK levels and the mTOR pathway activation after a short incubation time in primary neuronal cultures (Lepack et al., 2016; Ghosal et al., 2018; Kato et al., 2018; Fukumoto et al., 2019). Differently, there were no changes in phospho-ERK and phospho-mTOR levels after 24 h of incubation with CBD. This is in line with our results obtained in phospho-ERK levels 24 h after the IL CBD infusion, and with the transient activation of this pathway reported with other drugs both *in vitro* and *in vivo* (Hsiung et al., 2005; Li et al., 2010; Proenca et al., 2016). However, other rapid antidepressant drugs maintained the increase in phospho-ERK levels until 24 h (Lepack et al., 2016). The lack of in vitro changes in phosho-mTOR levels after 24 hours differs from the still increased phospho-mTOR levels in the PFC 24 h after the IL CBD infusion.

Some differences should be considered when comparing *in vivo* and *in vitro* findings and could underlie the different results obtained. First, in primary neuronal cultures from cortical samples there are glutamatergic and GABAergic neurones, and less than 1% of the cells are glia (Lepack et al., 2016). This scenario is distinct from the cell distribution in the brain, as glial cells represent a higher percentage of the total cell content in the rat cortex (Bass et al., 1971; Ren et al., 1992). These *in vitro* and *in vivo* differences are important because: a) CBD produces diverse effects on glial cells (Kozela et al., 2010; Esposito et al., 2011; di Giacomo et al., 2020; Lima et al., 2020), and this cell type has an important role in neuroplasticity related to mood disorders and the effect of antidepressant drugs (*reviewed in* Cotter et al., 2001; Sanacora and Banasr, 2013; Sild et al., 2017); and b) serotonin has been implicated in some effects of CBD (Sales et al., 2018), thus, the lack of

serotonergic neurones in cortical primary cell cultures could also be responsible for the differences observed. Second, the maintained presence of CBD in the culture medium could lead to receptor desensitization and explain the short duration of ERK activation (Daigle et al., 2008).

In view of these pieces of evidence, more research is needed to better understand the mechanism and the temporal pattern of the induction of plasticity signalling pathways elicited by CBD.

The most relevant results obtained in the Objective 1 are summarized in figure 47.

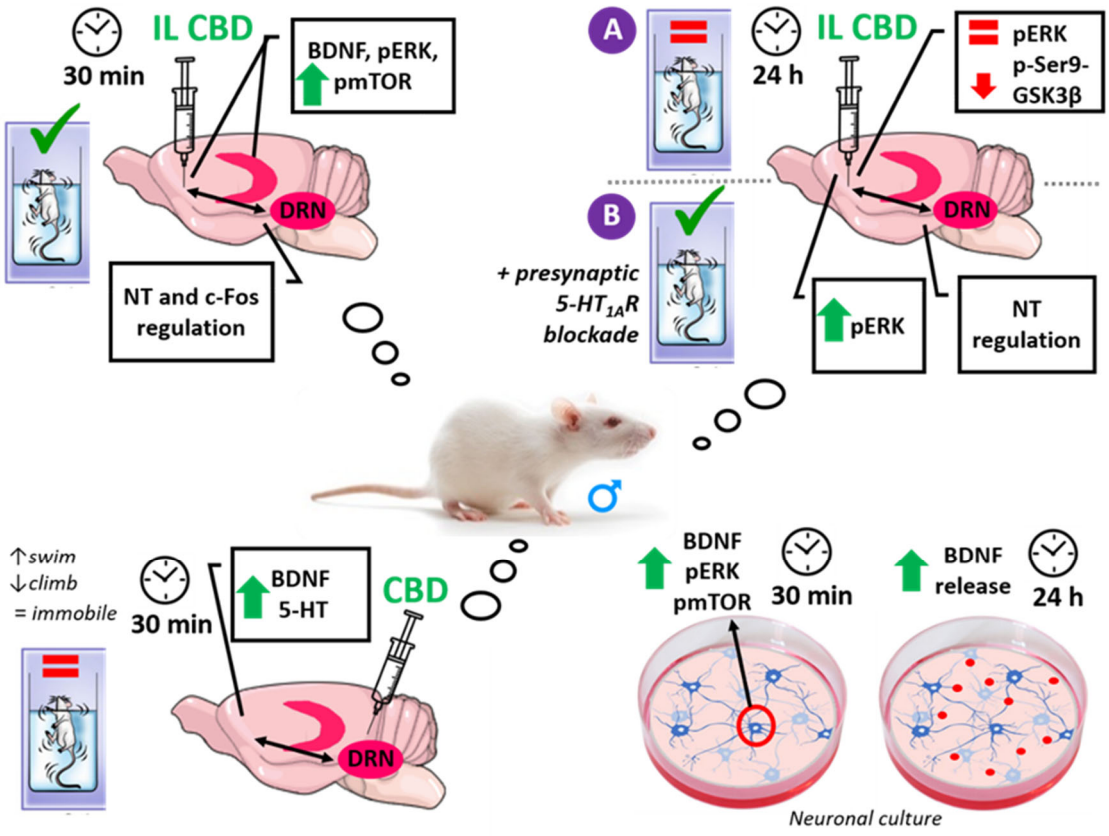


Figure 47. Scheme summarizing the behavioural and molecular effects of the intracerebral cannabidiol (CBD) infusion in rats, and the CBD-induced modulation of neuroplasticity markers in neuronal cultures. *In vivo*: the infusion of CBD into the infralimbic (IL) cortex decreased behavioural despair in the forced swimming test after 30 min, in parallel to an increase in phospho-ERK, phospho-mTOR, and BDNF levels in prefrontal cortex (PFC) and hippocampus, and the regulation of c-Fos and neurotransmitter (NT) levels in PFC and dorsal raphe nucleus (DRN). However, the IL CBD infusion did not modify behavioural despair after 24 h, which was concomitant to a lack of changes in phosho-ERK levels and a decrease in phosho-Ser9-GSK3β levels in PFC. Conversely, in the presence of a presynaptic 5-HT_{1A} receptor blockade (WAY100635 0.3 mg/kg), the IL CBD infusion decreased behavioural despair and increased phospho-ERK levels in PFC after 24 h. The infusion of CBD into the DRN did not modify the immobility time but increased the time spent swimming and decreased the time spent climbing in the forced swimming test after 30 min, which was accompanied by an increase in BDNF and serotonin (5-HT) levels in PFC. *In vitro*: CBD increased phospho-ERK, phospho-mTOR and BDNF intracellular levels in primary neuronal cultures after 30 min, and increased BDNF release after 24 h.

2. Effects of cannabidiol in experimental lipopolysaccharide models

To study the effect of CBD on neuroinflammatory-based depression we used an animal model that presents depressive-like behaviour induced by the acute LPS administration, which has been widely used due to its predictive validity (Frenois et al., 2007; O'Connor et al., 2009; Walker et al., 2013; Millett et al., 2019). We have included male and female mice to assess possible gender-dependent differences. The administration of CBD has been performed in the LPS model to study its effects in other pathologies, such as intestinal inflammation (De Filippis et al., 2011) and Alzheimer's disease (Martín-Moreno et al., 2011), but to our knowledge, this is the first time in which the antidepressant effect of CBD is evaluated in an *in vivo* neuroinflammatory model induced by LPS.

2.1 Effect of cannabidiol in LPS-activated microglial cells

In this set of experiments, we assessed the anti-inflammatory properties of CBD in microglial cultures activated by LPS. We evaluated the release of the pro-inflammatory cytokines TNFα and IL-6 to the culture medium. Our results demonstrate that LPS potently induced the release of both pro-inflammatory cytokines, while the co-incubation with CBD significantly reduced it, which confirmed its anti-inflammatory effects. The induction of inflammation by LPS incubation is a widely used method in *in vitro* studies and has been reported to induce the expression and release of pro-inflammatory cytokines (Juknat et al., 2013; Jia et al., 2017). Our results are in line with previous studies showing the anti-inflammatory effects of CBD in LPS-activated cells (Kozela et al., 2010; Dos-Santos-Pereira et al., 2020).

2.2 Behavioural effects of cannabidiol in the LPS model: influence of gender

Preliminary behavioural experiments were undertaken to set up the optimal experimental conditions to use the lipopolysaccharide (LPS)-induced neuroinflammatory model in our laboratory. It is reported that LPS injection produces systemic inflammation, animals rapidly exhibit sickness behaviour, which is characterized by reduced locomotion and exploratory behaviour, loss of body weight, and reduced food intake, among other

manifestations (Castanon et al., 2001). It has been proposed that this type of behaviour reaches its maximum expression 2 - 6 h after LPS injection (Pitychoutis et al., 2009; Mello et al., 2018), and it disappears completely after 24 h. Differently, the depressive-like behaviour can be detected after 6 h of LPS injection and it is still observed after 24 h (Dantzer et al., 2008). Most of the studies report the 24 h time point as an appropriate moment for the study of depressive-like behaviour without being mixed up with sickness behaviour, and the suitability of the 0.83 mg/kg dose to induce the model (Frenois et al., 2007; O'Connor et al., 2009; Walker et al., 2013; Millett et al., 2019). Based on these studies, we administered 0.83 mg/kg LPS to male and female NMRI mice to assess gender-dependent differences and checked the depressive-like behaviour after 24 h in the tail suspension test (TST), which measures behavioural despair as a feature of depression-related behaviour (Castagné et al., 2011). However, as we did not observe a clear depressive-like behaviour at 24 h in both genders, we searched for another experimental suitable time point to study depressive-like behaviour. Our experiments evaluating the onset and duration of the LPS-induced depressive-like behaviour confirmed the 12 h time point as the optimal one to evaluate the effects of CBD on behavioural and molecular studies. Some studies have evaluated depressive behavioural outcomes (Yu et al., 2016) and molecular effects (André et al., 2008) 12 h after LPS administration. Interestingly, Sens et al. (2017) reported that the LPS-induced reduction in sucrose preference in the SPT in both males and females could be observed as soon as 14 h after LPS injection, which is in line with our findings.

2.2.1 LPS-induced depressive-like behaviour: effect of cannabidiol and gender

In male mice, a single LPS injection increased the behavioural despair evaluated in the TST paradigm, as it has been widely described (Frenois et al., 2007; O'Connor et al., 2009; Yu et al., 2016). However, in female mice, the LPS injection did not induce significant behavioural despair. Other authors that have compared the depressive-like behaviour induced by an acute LPS injection in male and female mice reported similar results (Mello et al., 2018; Millett et al., 2019). Painsipp et al. (2011) described an LPS-induced reduction of behavioural despair in the FST in females, which is usually interpreted as an antidepressant-like effect. However, behavioural despair has also been reported after an acute LPS injection in female mice (Sens et al., 2017), evidencing the existence of controversy in this topic. A putative explanation for this discrepancy in female animals is the influence of the ovarian oestrous cycle in behavioural despair, as variations in swimming behaviour in the FST have been reported in female rats depending on the phase of the oestrus cycle (Consoli et al., 2005; Tonelli et al., 2008).

To determine if the lower locomotor activity, which is characteristic of the sickness behaviour, could be interfering with the immobility time observed in the TST, we studied if there was a correlation between these two parameters. The lack of correlation between the immobility time in the TST and the total distance travelled in the open field test (OFT) in both genders evidenced that the increased immobility time in the TST was indicative of depressive-like behaviour and was independent of the sickness behaviour. This dissociation between the effects elicited by LPS administration in depressive-like and sickness behaviours resembles the observations made by O'Connor et al. (2009).

In male mice, the administration of a single dose of cannabidiol before LPS completely prevented the LPS-induced behavioural despair, which is indicative of an antidepressant-like effect. This is in line with the effects induced by the acute administration of SSRIs as fluoxetine and paroxetine (Ohgi et al., 2013), the chronic administration of tricyclic antidepressants such as imipramine (Renault and Aubert, 2006), and drugs with fast antidepressant effects as ketamine (Walker et al., 2013) in this inflammatory model. Moreover, the fact that CBD administration to LPS mice reduced the immobility time in the TST, but did not affect the total distance travelled in the OFT, further supports our conclusion, and indicates that the behavioural effects of CBD were not due to an impact on sickness behaviour, but to a specific antidepressant-like effect in the TST paradigm. Regarding females, although LPS did not induce significant behavioural despair, a trend to an effect of cannabidiol in both naïve and LPS groups, could be observed. It is noteworthy to mention that females presented a higher basal immobility time in the TST indicating higher behavioural despair compared to males, as previously reported in NMRI mice (Liu and Gershenfeld, 2001) and other strains (Millett et al., 2019). This gender-dependent difference could explain the lack of the LPS pro-depressive effect and the effect of CBD on behavioural despair in *naïve* animals that we observed in females. Regarding the antidepressant-like effects of CBD in female models of depression, it has been recently reported that this effect depends on the animal model used (Shbiro et al., 2019). In view of the lack of the LPS-induced pro-depressive effect in females, this issue needs further investigation.

The sucrose preference test has been widely used to evaluate anhedonia in rodents as it is considered the most appropriate method to assess this core symptom of depression (Tao et al., 2019b). In our study, LPS induced an anhedonic state in male mice as previously described in this model of neuroinflammation (Frenois et al., 2007; Walker et al., 2013; Sens et al., 2017). The anhedonic state induced by LPS injection in female mice was comparable to male mice. This result is in line with other studies (Pitychoutis et al., 2009; Sens et al., 2017; Mello et al., 2018). It seems that the mentioned controversy about the effect of an acute LPS injection in female behavioural despair does not exist regarding its

anhedonic effect in females. Despite we observed similar anhedonic effects of LPS in both genders, some pieces of evidence are suggesting that female mice are more susceptible to suffer this behavioural manifestation than males. For instance, chronic LPS administration induced a reduction in sucrose preference in females but not in males (Kubera et al., 2013). In another study, Williams et al. (2020) showed that subchronic variable stress-induced anhedonia to female mice but not to males, and this was due to lower excitability of the nucleus accumbens neurones in males that was testosterone-dependent. However, the increased susceptibility of females to suffer anhedonia is controversial, as some knock-out approaches induced anhedonia in male but not in female mice (Georgiou et al., 2019; Lei et al., 2020).

In our study, cannabidiol pre-treatment attenuated the anhedonic state induced by LPS in male but not in female mice, which, together with the results obtained in behavioural despair, demonstrates the efficacy of cannabidiol in alleviating different depressive manifestations in male mice. The anti-anhedonic effect of CBD in this neuroinflammatory model is in line with the effect exerted by ketamine (Walker et al., 2013) and classical antidepressants (Renault and Aubert, 2006; Ohgi et al., 2013) in this model. This anti-anhedonic effect of CBD has been also described in other models of depression such as the olfactory bulbectomy in mice (Linge et al., 2016), and a genetic model of depression in rats (Shoval et al., 2016). Regarding the lack of anti-anhedonic effect of CBD on female mice, it has been suggested that the effective dose of an antidepressant drug could be different for males and females (Kokras et al., 2015), so we can not discard the positive effect of other CBD dose in the female LPS model. Indeed, an anti-anhedonic effect of CBD has been reported in a female rat model of depression (Shbiro et al., 2019). The gender-dependent discrepancies could be due to differences in many biological systems and/or molecular effects of LPS injection that are commented later. To our knowledge, this is the first time that CBD's effect on depressive-like behaviour is tested in the LPS model of neuroinflammation in females. Therefore, more studies about this issue are required.

2.2.2 LPS-induced anxiety-like behaviour: effect of cannabidiol and gender

In the LPS model, male mice showed reduced central time in the open field test, which is indicative of an anxious-like state. Although the anxiogenic effect of LPS has been reported before (Salazar et al., 2012; Savignac et al., 2016), other authors did not observe this behaviour (Mello et al., 2018; Millet et al., 2019). In females, LPS did not reduce significantly the time spent in the center in the OFT, as previously reported (Mello et al., 2018; Millet et al., 2019). However, Mello and colleagues (2018), performing another test to evaluate anxious-like behaviour, showed that female mice presented an anxiety-like

behaviour in the elevated plus maze and the grooming behaviour after LPS administration, which was not observed in males. These discrepancies observed within the battery of behavioural tests used in rodents for the evaluation of anxiety-like behaviours have been previously reported, as each test evaluates different aspects of anxiety (Van Gaalen and Steckler, 2000; Calabrese, 2008). Publications about this issue are scarce, so more studies regarding the effects on anxious-like behaviour of an acute LPS administration in female rodents are needed.

It is noteworthy to mention that the anxiogenic effect of LPS is often observed during the peak of the sickness behaviour together with reduced locomotion, and vanishes after 24 h (Custódio et al., 2013). Indeed, we demonstrate in both male and female mice, that the LPS-induced sickness behaviour was still present after 12 h, as there was a weight reduction and decreased ambulatory activity, in good agreement with previous studies (André et al., 2008; Yu et al., 2016). Some authors suggest the possibility that the reduced ambulatory activity might be interfering with the anxiety test (Custódio et al., 2013). Indeed, we demonstrate a significant positive correlation between the time spent in the center and the total distance travelled in the OFT, indicating the impossibility to draw any conclusions regarding the LPS-induced anxiety-like behaviour at this time point.

The pre-treatment with CBD did not prevent any of the mentioned parameters of the sickness behaviour observed in both genders. Regarding the behavioural effect of CBD on anxiety parameters, we could not detect any significant effect neither in *naïve* nor in LPS mice of both genders. The lack of effect on the latter groups could be due to the association of the anxious-like behaviour to the sickness behaviour at this time point. Despite this, there are discrepancies in the literature relative to the effect of CBD on anxiety in *naïve* and animal models (*reviewed in* Blessing et al., 2015) depending on the dose, the route of administration, the duration of the treatment, the animal model used, and the gender.

The anxiolytic-like effect of CBD is dose-dependent, as the intraperitoneal administration of CBD exerted anxiolytic-like effects when administered at low doses (*reviewed in* Blessing et al., 2015), but this effect was lost when CBD dose was increased (Guimaraes et al., 1990; Campos and Guimaraes, 2008). In addition, it has been proposed that different CBD doses could induce different behavioural manifestations (Campos et al., 2012; Shoval et al., 2016). Another factor to consider is the duration of the treatment (*reviewed in* Blessing et al., 2015), as an anxiolytic effect of CBD was reported after acute (Linge et al., 2016), or chronic administration (Campos et al., 2013).

The differences in the animal model used could also explain the aforementioned discrepancy, as the underlying mechanisms that produce anxiety in the LPS model and

other animal models are certainly distinct. For example, Linge et al. (2016) observed an anxiolytic-like effect of acute CBD administration in the olfactory bulbectomy model of depression, while in other animal models, as the chronic unpredictable stress (CUS), a long-term treatment was needed to observe those effects (Campos et al., 2013). In contrast, other authors have reported a lack of anxiolytic-like effect in Wistar-Kyoto rats (Shoval et al., 2016).

Regarding the gender-dependent effects of CBD, there are few studies on anxious-like behaviour. Kasten et al. (2019) reported an anxiolytic-like effect of acute CBD administration in adolescent females, while Campos et al. (2015) showed this effect in naïve females after 7 doses. Conversely, an anxiogenic effect in adult females after subchronic (2 - 8 days) (Kasten et al., 2019) and chronic (6 weeks) (Schleicher et al., 2019) CBD administration was reported. Therefore, CBD's effect on female anxious-like behaviour needs further investigation.

In all, more studies about the anxiolytic-like effects of CBD in the LPS model are required, and other tests of anxiety not interfered with the sickness behaviour could be useful to clarify the effect of CBD on this behaviour in LPS mice.

2.2.3 Gender differences in the behavioural effects

Apart from the putative explanations given for the gender dissimilitude in each type of behaviour studied, more general differences in the biological systems that take part in anhedonic, anxious- and depressive-like behaviours have been described between males and females, which could also underlie the dissimilarities observed in the behavioural tests performed.

In our study, females in the vehicle group lost weight compared to males, suggesting that NMRI female mice may be more sensitive to the stress induced by handling, intraperitoneal injection, and/or behavioural test performance. This higher sensitivity may underlie the higher behavioural despair showed by females compared to males, which is in line with previous reports (Bourke and Neigh, 2011; Goodwill et al., 2019). Supporting these gender-specific effects of stress, an extensive review by Brivio et al. (2020) presents a huge number of genes that are differentially regulated after stress in males and females. Research performed in rodents has still not reached a consensus about which gender is more susceptible to stress, but different studies point to females as the most stress-sensitive (LaPlant et al., 2009; Hodes et al., 2015; Bangasser and Wiersielis,

2018). It has also been reported that other stressors as individual housing increase the vulnerability to stress in female rodents (Baker and Bielajew, 2007; Takahashi et al., 2017).

There are also gender differences in the activity of the immune system that can result in behavioural and neurochemical dissimilarities between males and females after LPS administration (*reviewed in* Gaillard and Spinedi, 1998). In this review, the authors collected the gender differences that have been described in the immune system and the endocrine response, evidencing that there is sexual dimorphism in the communication between these two systems that are implicated in depression. Indeed, sex hormones modulate differently how the hypothalamic-pituitary-adrenal and immune axis respond to LPS injection. Other studies also showed that the LPS-induced changes in cytokine expression and the HPA axis activation differ between males and females (Tonelli et al., 2008; Pitychoutis et al., 2009; Cai et al., 2016). Interestingly, it has been reported that the expression of TLR4 presents a sexual dimorphism, as there are higher levels of this receptor in the cell surface of male macrophages (Marriott et al., 2006). Moreover, females have 17-β estradiol (E2), which is a sex hormone with broadly anti-inflammatory effects that can regulate cytokine release and NF-κB activation (Santos et al., 2017), two of the main processes mediating the effects of LPS.

Gender differences in oxidative stress regulation could also interfere in the outcome of LPS injection in males and females, as oxidative stress and inflammation are two processes closely related that can activate each other (Biswas, 2016). Mello et al. (2018) suggested that the different behavioural changes induced by LPS in males and females could be due to a sex-specific pattern of brain oxidative changes. In this line, Millet et al. (2019) postulated that differential modulation of antioxidant proteins in the hippocampus of female mice after LPS administration could be an intrinsic protective mechanism against oxidative stress and depressive-like behaviour in females. Again, estrogens can also participate in these differences, as they modulate the activity of various antioxidant systems (Strehlow et al., 2003; Bellanti et al., 2013).

In addition, gender-dependent differences in monoaminergic systems have been reported after LPS injection. Pitychoutis et al. (2009) described that 2 h after LPS females had an enhanced serotonergic activity in brain limbic areas involved in depression, such as the hippocampus, prefrontal cortex, and amygdala, in comparison to males. They also found that the dopaminergic system was generally more activated in females. Sens et al. (2017) reported similar findings, as there was sustained serotonergic hyperactivity 24 h after LPS injection in the female brain and gender differences in dopaminergic activation.

All in all, the literature shows a gender dimorphism in the different biological systems that are implicated in the depressive-like behaviour and the LPS-induced effects, which could

be the clue to understanding the different findings between males and females in the LPS model reported in this thesis.

2.2.4 Role of postsynaptic 5-HT$_{1A}$ receptors in the antidepressant-like effect of cannabidiol in the LPS model

As mentioned in the introduction, the consequences of 5-HT$_{1A}$ receptor activation profoundly depend on its location. The activation of auto-receptors reduces the firing of the serotonergic neurones in the DRN, which results in a decrease in serotonin release in projection areas (Hjorth and Sharp, 1991). Differently, the activation of hetero-receptors is part of the mechanism of action of most of the clinically used antidepressants, as SSRIs (Blier et al., 1997; Haddjeri et al., 1998; Béïque et al., 2000; Bambico et al., 2009; Celada et al., 2013; Kaufman et al., 2016).

As one of the described mechanisms of action of CBD is the activation of 5-HT$_{1A}$ receptors (Russo et al., 2005; Rock et al., 2012), we used the selective 5-HT$_{1A}$ receptor antagonist WAY100635 at two different doses: a low dose (0.3 mg/kg) that has been reported to block mainly the auto-receptors, and a higher dose (1 mg/kg) which acts on both auto- and hetero-receptors (Serres et al., 2000; Ago et al., 2003; Carey et al., 2005).

The persistence of the antidepressant-like effect elicited by CBD in mice subjected to the LPS model in the presence of a 5-HT$_{1A}$ auto-receptor blockade demonstrates that this auto-receptor is not implicated in its mechanism of action. Some authors describe that the blockade of 5-HT$_{1A}$ auto-receptors could potentiate the antidepressant-like effect, as it has been described for other antidepressant drugs whose mechanism of action implies postsynaptic 5-HT$_{1A}$ receptor activation (Artigas et al., 1996; Romero et al., 1996). However, we did not observe a significant behavioural effect with 30 mg/kg CBD i.p. and 0.3 mg/kg WAY100635 administration in both *naïve* and LPS-injected mice. In contrast, other authors reported that a similar dose of WAY100635 blocked the antidepressant-like effect elicited by CBD, although this study was performed in *naïve* animals, using a different mouse strain and evaluating a different time point (Zanelati et al., 2010).

Our results demonstrate the pivotal role of postsynaptic 5-HT$_{1A}$ receptors in the antidepressant-like effect of CBD in the LPS neuroinflammatory model since the administration of 1 mg/kg WAY100635 prevented this behavioural effect. In line with the importance of postsynaptic 5-HT$_{1A}$ receptors in the effects of antidepressant drugs (Celada et al., 2013), we found that the administration of 1 mg/kg WAY100635 had pro-depressive-like effects in vehicle animals, reinforcing the involvement of these receptors

in mood regulation and the importance of its activation for mood stabilization (Garcia-Garcia et al., 2014). The blockade of the antidepressant-like effect of CBD with WAY100635 has been reported in *naïve* animals (Zanelati et al., 2010) and in the olfactory bulbectomy model of depression (Linge et al., 2016), but to our knowledge this is the first time to be described in the LPS model of neuroinflammation. In addition, we demonstrated (Objective 1) that CBD exerts antidepressant-like effects when administered in brain areas rich in postsynaptic 5-HT_{1A} receptors as the infralimbic cortex, in agreement with others (Sartim et al., 2016). In contrast, CBD infusion in the DRN (Objective 1) did not produce antidepressant-like effects, which is in line with the results obtained after the presynaptic 5-HT_{1A} receptor blockade in mice.

2.3 Molecular effects of cannabidiol in the LPS model

2.3.1 NF-κB pathway

To investigate the mechanism of action of CBD responsible for the behavioral findings, we studied the NF-κB pathway, as it takes part in the pro-inflammatory mechanism of LPS (Kawai et al., 2001). The LPS model presented increased NF-κB nuclear levels as previously described (Perez-Nievas et al., 2010; MacDowell et al., 2013), evidencing the activation of the NF-κB pathway. CBD administration before LPS significantly attenuated the increase in nuclear NF-κB levels, in line with the inhibitor effect of CBD on this pathway reported in cell culture studies (Esposito et al., 2006a; Juknat et al., 2019; dos-Santos-Pereira et al., 2020) and in an animal model of Alzheimer's disease (Esposito et al., 2011). Interestingly, the widely used antidepressant fluoxetine also reversed the NF-κB-induced increase in a chronic LPS model in mice (Rodrigues et al., 2018). The direct inhibition of NF-κB also presents antidepressant-like effects in stress models (Koo et al., 2010).

The results obtained in NF-κB and IκBα nuclear and cytoplasmic levels fit with the time course of NF-κB pathway activation/deactivation described by Hobbs et al. (2018). They reported that LPS activation of TLR4 rapidly produced a decrease in IκBα cytoplasmic levels and induced NF-κB nuclear translocation. However, NF-κB deactivation was much slower, as this protein was still increased in the nucleus 10 h after LPS exposure when IκBα cytoplasmic levels had returned to their basal expression. It was not until 16 h after LPS when they reported the nuclear export of NF-κB (Hobbs et al., 2018). Our findings fit with this timeline, as 12 h after LPS we did not observe changes in IκBα cytosolic levels while NF-κB nuclear levels were still increased. Concerning the presence of IκBα in the nuclear compartment observed with LPS administration, it has been reported that after the activation of the pathway the re-synthesized IκBα translocates to the nucleus and

promotes NF-κB nuclear export (Arenzana-Seisdedos et al., 1997). This is one of the described mechanisms for the downregulation of the NF-κB pathway (Ruland, 2011).

Regarding PPARγ, it has been reported that LPS produces a reduction in its expression in BV2 cells (Juknat et al., 2013; Choi et al., 2017), and in the rat cortex (Perez-Nievas et al., 2010; MacDowell et al., 2013) in a few hours. Thus, the lack of changes observed in our experiments 12 hours after LPS exposure may be due to the recovery of PPARγ basal levels. In contrast, the administration of CBD, a PPARγ ligand (O'Sullivan et al., 2009; *reviewed in* O'Sullivan, 2016), induced an increase in PPARγ nuclear levels and a decrease in its cytosolic levels, in good agreement with the nuclear translocation of PPARγ mediated by other ligands (Khan and Abu-Amer, 2003; Kelly et al., 2004; Umemoto and Fujiki, 2012). In this regard, other authors have reported that chronic CBD treatment induced an increase in PPARγ levels in the spinal cord of an animal model of multiple sclerosis (Giacoppo et al., 2017b) and an increase in PPARγ coactivator 1-alpha (PGC-1α) mRNA levels in the striatum of an animal model of dyskinesia (Sonego et al., 2018). Thus, this is the first time, to our knowledge, that an increase in PPARγ nuclear levels is reported with a single CBD administration *in vivo*. Although PPARγ nuclear levels were increased in the CBD *naïve* group, this was not associated with changes in nuclear NF-κB levels. This could be explained by the lack of activation of the NF-κB pathway in this experimental group. The lack of changes in nuclear PPARγ levels after CBD administration to mice subjected to the LPS model could be due to the role of PPARγ in the downregulation of the pathway, as PPARγ can bind to NF-κB in the nucleus and induce the nuclear export of the PPARγ-NF-κB complex (Chung et al., 2000; Hou et al., 2012), promoting its translocation to the cytosol (Kelly et al., 2004). Nevertheless, we cannot exclude the possibility that CBD could produce its effects on the NF-κB pathway independently of PPARγ activation, as CBD has also shown anti-inflammatory effects that are not dependent on PPARγ (Alhamoruni et al., 2012; dos-Santos-Pereira et al., 2020).

In all, our results suggest that the reduction of the NF-κB pathway activation could participate in the antidepressant-like effect of cannabidiol in this neuroinflammatory model induced by LPS administration.

2.3.2 Pro-inflammatory cytokines

Our results in plasma determinations demonstrated that LPS increased TNFα protein levels to a lesser extent than IL-6 levels, as TNFα peaks earlier than IL-6 in response to LPS (Andreasen et al., 2008). In contrast, the increase in brain TNFα mRNA expression was higher than the one observed in IL-6 mRNA, as previously described (André et al., 2008).

This might be explained by a delayed beginning of the inflammatory response in the brain compared to the periphery. Herein, we observed a strong positive correlation between plasma and PFC IL-6 levels, which suggests the brain levels of this cytokine can be extrapolated from the plasma ones at this time point. In contrast, plasma and brain TNFα levels were not correlated, as previously reported (Qin et al., 2007; André et al., 2008). We have shown that the sucrose preference —a measure used to assess anhedonia in rodents— strongly correlated with IL-6 plasma levels, showing that the higher the plasma IL-6 levels, the higher the anhedonic behavior. These results are in line with the association between plasma IL-6 levels and anhedonia described in humans (Felger et al., 2016). The coexistence of depressive-like behavior and increased plasma IL-6 levels in this neuroinflammatory model agrees with the elevated IL-6 levels reported in stress-related animal models of depression (Sukoff Rizzo et al., 2012; Zhu et al., 2019), and with the depressive-like behaviour observed in animal models of peripheral inflammation (Sakić et al., 1997; 2001) and in mice with IL-6 overexpression (Sukoff Rizzo et al., 2012). Indeed, Zhang et al. (2016) proposed IL-6 as a susceptibility gene for MDD and as a putative biological marker for the disease, in agreement with meta-analyses of studies performed in MDD patients (Frommberger et al., 1997; Dowlati et al., 2010; Haapakoski et al., 2015). Moreover, a dose-response relationship between IL-6 plasma levels and the severity of depressive symptoms was reported (Howren et al., 2009), as observed in our study by the inverse correlation between IL-6 levels and sucrose preference. In addition, the dysregulation of both IL-6 production and serotonergic neurotransmission is associated with a greater vulnerability to suffering depression (Bull et al., 2009), as we observed in LPS mice by the increase in both IL-6 levels and the kynurenine/serotonin ratio.

In our study, CBD prevented the increase in IL-6 brain and plasma levels induced by LPS. This is in line with the reduction on LPS-induced pro-inflammatory cytokines reported with CBD administration in other inflammatory diseases (Malfait et al., 2000; Ruiz-Valdepeñas et al., 2011; Ribeiro et al., 2015), and is in accordance with its anti-inflammatory properties (*reviewed in* Nichols and Kaplan, 2020). Besides, it has been reported that IL-6 knockout animals exhibit reduced behavioral despair and resistance to stress-induced depression (Chourbaji et al., 2006). In parallel to our results, the increased IL-6 levels observed in acute depressed patients are reverted to control levels after classic antidepressant treatment (Frommberger et al., 1997), and after the administration of the fast-acting antidepressant drug ketamine (Verdonk et al., 2019). Moreover, antibodies against IL-6 reduced symptoms of depressed mood and anhedonia in inflammatory disease patients (Sun et al., 2017). The reduction of IL-6 could be of great importance, as high IL-6 levels are associated not only with depressive-like behavior but also with treatment resistance in animal (Sukoff Rizzo et al., 2012) and human (Haroon et al., 2018) studies, and with suicidal behaviour (Serafini et al., 2013).

Regarding TNFα, previous studies demonstrated that its administration induces sickness behavior and neuroinflammation, but not depressive symptoms as anhedonia (Biesmans et al., 2015). In addition, a cumulative meta-analysis of studies in MDD patients revealed that the association of TNFα with the disease is inconsistent, while other inflammatory markers as IL-6 are associated with MDD (Haapakoski et al., 2015). This is in accordance with the fact that, in our study, CBD exerted antidepressant-like effects in the LPS model without producing changes in TNFα levels. Moreover, this result is in line with the lack of effect on TNFα levels reported after ketamine (Verdonk et al., 2019) and an IDO inhibitor (O'Connor et al., 2009) administration to mice subjected to the LPS model, while both treatments induced antidepressant-like effects. However, we observed a reduction of TNFα protein levels in LPS-activated microglial cells coincubated with CBD, which could be due to difference in the time point studied (12 h in *in vivo* studies *versus* 6 h *in vitro*), in agreement with those *in vivo* studies which evaluated TNFα levels at shorter periods after CBD administration (90 min - 3 h) (Malfait et al., 2000; Ruiz-Valdepeñas et al., 2011).

In brief, our results suggest that the reduction of IL-6 levels in plasma and prefrontal cortex could be one mechanism mediating the antidepressant-like effects of CBD in the LPS model.

2.3.3 Kynurenine pathway

To further investigate the mechanism of action of CBD responsible for the findings presented here, we studied the kynurenine pathway. Our results show an increase in brain kynurenine levels, as well as in the kynurenine/tryptophan (KYN/TRP) ratio in the LPS model, which is indicative of an increased IDO activity (Fuchs et al., 1990; Widner et al., 2002; O'Connor et al., 2009). This IDO activation is further confirmed by the increased kynurenine/serotonin (KYN/5-HT) ratio in these animals, showing a tilted balance toward kynurenine synthesis. The increase in kynurenine levels and IDO activity has been widely described in the LPS neuroinflammatory model, both in the periphery and the brain (O'Connor et al., 2009; Walker et al., 2013; Zhang et al., 2019b). Interestingly, the activation of the kynurenine pathway has also been reported in animal models of depression such as the chronic unpredictable mild stress (CUMS) (Wang et al., 2018b; Li et al., 2020b) and the olfactory bulbectomy (OBX) (Bansal et al., 2018).

In fact, the correlation between behavioural despair and anhedonia and the increased activation of the brain kynurenine pathway obtained in LPS mice is in accordance with the behavioural despair (O'Connor et al., 2009) and anhedonia (Salazar et al., 2012) induced by the systemic administration of kynurenine. Similar parallelism between the activity of

the kynurenine pathway and depressive-like behaviour has been reported in the LPS model (Walker et al., 2019), in the CUMS model (Laugeray et al., 2011), and after chronic ethanol exposure (Giménez-Gómez et al., 2019) in mice. Importantly, the activation of the kynurenine pathway has also been correlated to anhedonia in adolescents suffering from MDD (Gabbay et al., 2012). Moreover, kynurenine levels and the KYN/TRP ratio in plasma were correlated to the severity of depressive symptoms in humans (Swardfager et al., 2009; Raison et al., 2010). Interestingly, this correlation was also found with the KYN/TRP ratio in the cerebrospinal fluid (CSF) (Raison et al., 2010).

We observed a correlation between plasma and brain IL-6 levels and kynurenine levels in the cortex. This is in accordance with the proposed sequence of events induced by LPS administration: 1) the activation of the IDO enzyme in the periphery, leading to an increase in kynurenine synthesis that crosses the blood brain barrier; and 2) the activation of the brain IDO enzyme and the local production of kynurenine in the brain (Leonard and Maes, 2012; Barone, 2019). Our experimental findings confirm the IL-6 induction of IDO activity and the production of kynurenine in the brain (Anderson et al., 2013). Even so, we cannot exclude the activation of the kynurenine pathway in the periphery, as a strong correlation between peripheral and brain kynurenine pathways has been reported in animal models with depressive-like behaviour (LPS model) (Verdonk et al., 2019), and MDD patients (Haroon et al., 2020).

As previously stated, the induction of brain IDO activity (increased KYN/TRP ratio) has been associated with the appearance of depressive-like behaviour and is not related to sickness behaviour (O'Connor et al., 2009). In this regard, Walker et al. (2013) reported that the LPS-induced increase in brain IDO enzyme activity was not detected 6 h after LPS injection but was increased after 28 h. In addition, other authors reported that the activity of this enzyme did not change in the lung 6 h after LPS-injection but was increased after 12 and 24 h (André et al., 2008). Our results are in accordance with these pieces of evidence, as the depressive-like behaviour that we observed in LPS mice after 12 h was concomitant to an increase in the brain KYN/TRP ratio.

CBD administration to LPS-injected mice produced antidepressant-like effects and a decrease in the KYN/TRP and KYN/5-HT ratios, which is in line with the antidepressant-like effect described following IDO enzyme inhibition (O'Connor et al., 2009; Dobos et al., 2012). Moreover, the inhibition of this enzyme is also associated with a lower anhedonic state, as observed in IDO knockout animals (Lawson et al., 2013). This body of evidence, together with the reversion of the LPS-induced behavioral and molecular changes within the kynurenine pathway promoted by CBD, indicate that the reduction of brain IDO activity is contributing to the antidepressant-like effects of CBD in the LPS model. This IDO

modulation could be produced by the direct action on the enzyme (Jenny et al., 2009), or indirectly through the decrease in pro-inflammatory cytokines (Lestage et al., 2002).

Within the kynurenine pathway, recent basic and clinical research highlight the involvement of the kynurenine metabolites in depression (*reviewed in* Maes et al., 2011; Barone, 2019). The neurotoxic branch of the KYN pathway [3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN)] is increased in animal models such as the neuroinflammatory LPS model and in MDD patients, which is in line with the increase in brain 3-HK levels reported here, while the neuroprotective branch [kynurenic acid (KYNA)] is decreased (Verdonk et al., 2019; *reviewed in* Barone, 2019). This imbalance between the two KYN branches has been related to depressive-like behavior (Parrott et al., 2016; Laumet et al., 2017). Brain kynurenine 3-monooxygenase (KMO) expression (the 3-HK enzyme of synthesis) and 3-HK levels are increased in animal models of depression such as the CUMS (Wang et al., 2018b; Li et al., 2020b). Moreover, the administration of 3-HK induces depressive-like behaviour in mice (Parrott et al., 2016). Conversely, KMO knockout mice are protected from the LPS-induced depressive-like phenotype (Parrott et al., 2016).

In our study, CBD administration prevented the LPS-induced increase in cortical 3-HK levels, as recently reported in *ex vivo* experiments (di Giacomo et al., 2020). This cortical 3-HK reduction, together with the antidepressant-like effects elicited by CBD, is in line with the effects of other antidepressant drugs as ketamine, which also reduced brain 3-HK levels in the LPS model (Verdonk et al., 2019). It has also been reported that the antidepressant fluoxetine reduced 3-HK levels in the periphery and some brain areas in the CUMS model of depression (Laugeray et al., 2016). Moreover, CBD also increases KYNA levels after a neurotoxic stimulus (di Giacomo et al., 2020).

We observed an increase in tryptophan and serotonin brain levels in the LPS model, which is in line with other reports using this neuroinflammatory model (Dunn and Welch, 1991; O'Connor et al., 2009; Walker et al., 2013). A factor that could contribute to the increase in tryptophan brain levels after LPS administration is the increased blood brain barrier permeability that has been described in this model (Ruiz-Valdepeñas et al., 2011), and in animal models of depression with increased pro-inflammatory cytokines (Menard et al., 2017; Cheng et al., 2018). The increased serotonin levels have been associated with an increase in brain 5-HT turnover after LPS administration (Dunn and Welch, 1991; O'Connor et al., 2009; Sens et al., 2017). This increased 5-HT metabolism has been interpreted as a stress-related response and is not specific to the LPS-induced depressive-like behaviour (Walker et al., 2013). In fact, an increased tryptophan concentration and 5-HT turnover have been described after IL-1, IL-6, LPS, and influenza virus administration, and after

commonly used stress-induction protocols as the electric shock and restraint (*reviewed in* Dunn et al., 1999).

CBD pre-treatment reduced tryptophan brain levels in LPS mice. This could be due to the attenuation of the inflammatory response elicited by CBD, as a similar effect was obtained with the administration of an antibody against IL-6 before LPS (Wang and Dunn, 1999). Another mechanism that could explain the reduction of tryptophan brain levels produced by CBD in LPS mice is the protection of the blood brain barrier reported in other inflammatory models (Ruiz-Valdepeñas et al., 2011; Mecha et al., 2013). In our experiments CBD tended to increase 5-HT brain levels in LPS mice, in line with its detrimental effect on IDO activity, tilting the balance of tryptophan metabolism towards serotonin synthesis. Moreover, CBD increased 5-HT hippocampal levels in *naïve* mice, in accordance with the CBD-induced increase in endocannabinoids and the subsequent facilitation of serotonin neurotransmission previously described (Bambico et al., 2007; Haj-Dahmane and Shen, 2011; Fernández-Ruiz et al., 2013).

In summary, we demonstrated that CBD prevents the LPS-enhanced kynurenine pathway and the production of its neurotoxic metabolite 3-HK in the brain, a mechanism that could underlie its antidepressant-like effects in the LPS model.

2.3.4 Glutamate and GABA

In the hippocampus, we observed an increase in glutamate levels in LPS mice, in line with previous studies reporting increased glutamatergic activity in this model (Gao et al., 2014; Chávez et al., 2019; Chen et al., 2020). These high glutamate levels may be a consequence of the activation of the kynurenine pathway, which leads to the dysregulation of the serotonergic and glutamatergic neurotransmission, among other alterations found in depression (*reviewed in* Maes et al., 2011). The dysregulation of the glutamatergic system would come from the effects of some kynurenine metabolites as the quinolinic acid, which induces glutamate release and excitotoxicity (*reviewed in* Barone, 2019). Therefore, the increased kynurenine and 3-HK levels found in this thesis suggest that the activation of the "neurotoxic branch" of the kynurenine pathway could be contributing to the high glutamate levels observed in the LPS model.

Other mechanisms that lead to the release of glutamate under neuroinflammatory conditions have been described. For instance, the oxidative stress produced by some kynurenine metabolites activates the cysteine/glutamate antiporter (x_c^- system), which is an endogenous antioxidant response that transports cystine into microglial cells in

exchange for glutamate (*reviewed in* Dantzer and Walker, 2014). Therefore, the increased activity of this system could also be responsible for the increase in glutamate after an LPS exposure, and it has been described both *in vitro* and *in vivo* following LPS administration (*reviewed in* Massie et al., 2015). Interestingly, Kitagawa et al. (2019) showed by microdialysis that a single systemic LPS injection increased glutamate release in the hippocampus as a consequence of an increase in the microglial x_c^- system, which fits with our results.

CBD pre-administration completely abolished the LPS-induced increase in hippocampal glutamate levels, which could be the neurochemical consequence of the direct inhibition of the IDO enzyme (Jenny et al., 2009), or the decrease in pro-inflammatory cytokines (Lestage et al., 2002), both leading to an inhibitory effect on the kynurenine pathway. Despite this, we cannot exclude the possibility that other mechanisms of action of CBD, such as the decrease of glutamate levels due to its intrinsic antioxidant effect as observed in LPS-induced microglial cultures (Dos-Santos-Pereira et al., 2020), could also participate in the effects observed.

In our study, the administration of LPS increased GABA levels in the hippocampus. The bibliography about the effects of neuroinflammation on GABA levels reveals a complex interplay between inflammation and the GABAergic system (*reviewed in* Crowley et al., 2016). In line with our results, other authors have described an increase in GABA neurotransmission or GABA hyperfunctionality after a unique dose of LPS (Kitamura et al., 2019; Tang et al., 2020), and increased GABAergic input in the hippocampus after chronic LPS exposure (Hellstrom et al., 2005). However, in the CUMS model of depression, which also presents neuroinflammation, a decrease in GABA levels has been reported (Zhang et al., 2019c). In our study, the pre-administration of CBD to mice subjected to the LPS model completely abolished the effects of LPS on hippocampal GABA levels. We could postulate that the LPS-induced changes in GABA levels could be a compensatory mechanism aiming to minimize glutamate-induced neurotoxicity. In line with this rationale, the blocking effect of CBD upon glutamate increase in the LPS model could make unnecessary to modify GABA levels. Supporting the suggested compensatory function of the increase in GABA levels in the LPS model, a review concluded that an increase in GABAergic signalling can have anti-inflammatory effects (*reviewed in* Crowley et al., 2016).

In the cortex of LPS-injected mice, we observed a decrease in glutamate levels that was completely prevented by CBD administration, in line with the effect of CBD in the olfactory bulbectomy model of depression (Linge et al., 2016). Although we did not find significant changes, a similar pattern was observed in cortical GABA levels.

Although LPS-induced neuroinflammation produced opposite effects on glutamate and GABA levels in the hippocampus and cortex, CBD prevented the LPS-induced dysregulation of these neurotransmitter levels in both brain structures. Therefore, CBD appears to produce a homeostatic effect on glutamate and GABA neurotransmission, which could be mediated by the upregulation of endocannabinoids (Watanabe et al., 1996; Bisogno et al., 2001), since these molecules modulate glutamate and GABA neurotransmitter systems (Castillo et al., 2012; Araque et al., 2017). Moreover, the agonistic profile of CBD on 5-HT_{1A} receptors (Russo et al., 2005; Rock et al., 2012), could lead to the hyperpolarization of glutamatergic neurones and the subsequent decrease in glutamate release (Mauk et al., 1988; Sprouse and Aghajanian, 1988; Mauler et al., 2001). Complementarily, we cannot discard that the effect of CBD on 5-HT_{1A} receptors situated in GABA hippocampal interneurones (Aznar et al., 2003; Russo et al., 2005; Rock et al., 2012), could have contributed to the effects observed.

All the previous findings reflect the complexity of the interaction between inflammation and the glutamatergic and GABAergic systems. Indeed, in MDD patients, glutamate levels have been found differently affected depending on the brain area studied (Sanacora et al., 2004; Yildiz-Yesiloglu and Ankerst, 2006; Hasler et al., 2007), while changes in the GABAergic system have been related to neuroinflammatory processes and point to reduced activity. However, some authors highlight that these changes may be region-specific, that the functional consequences of alterations on GABA synaptic availability are unclear, and that the increase of GABA signalling is not always beneficial regarding MDD symptoms (*reviewed in* Pehrson and Sanchez, 2015; Crowley et al., 2016). Consequently, the study of specific brain regions would help to better understand the biological implications of these findings.

The most relevant results obtained in the Objective 2 are summarized in figure 48.

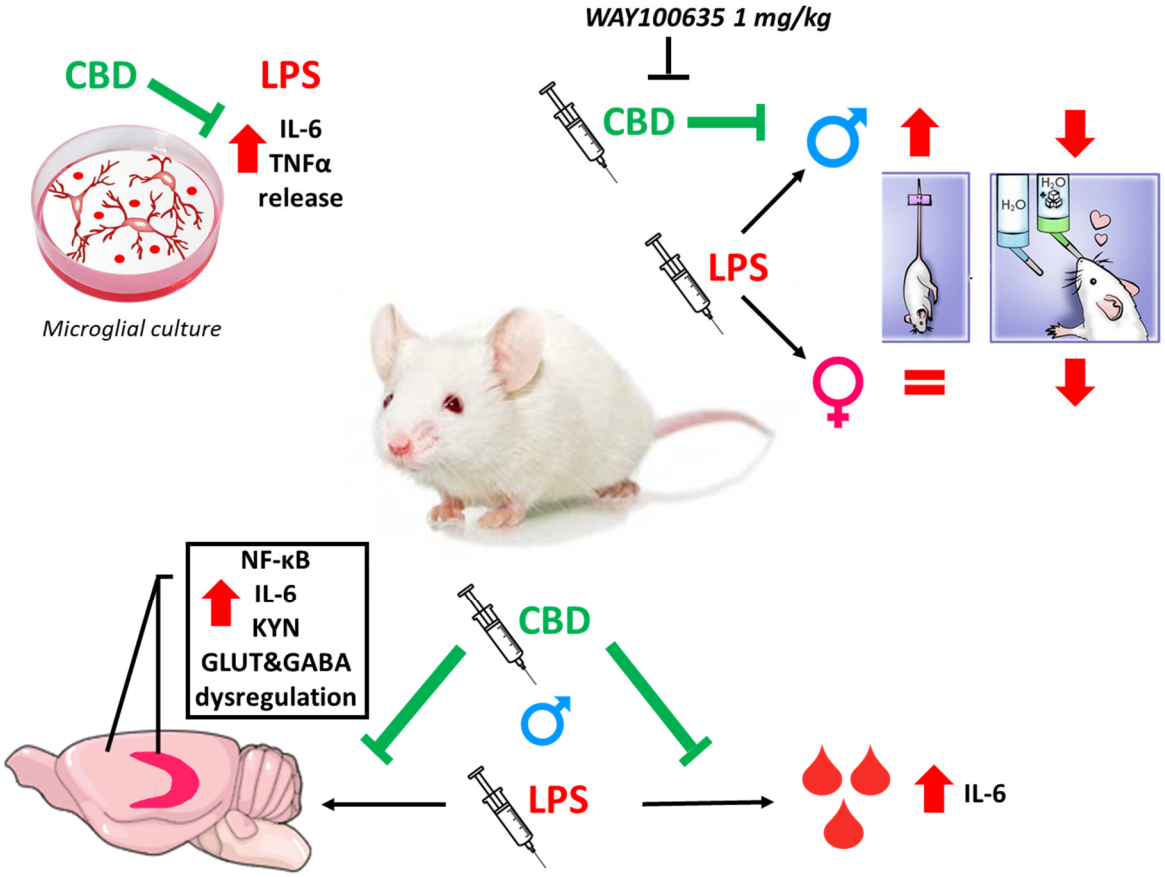


Figure 48. Scheme summarizing the effects of cannabidiol (CBD) in lipopolysaccharide (LPS) models. *In vitro*. The incubation of microglial cells with LPS increased IL-6 and TNFα release, an effect attenuated by CBD coincubation. *In vivo*. The systemic administration of LPS to male mice increased behavioural despair in the tail suspension test and anhedonia assessed in the sucrose preference test, while in female mice LPS injection increased anhedonia without altering behavioural despair. CBD pre-administration attenuated the LPS-induced behavioural manifestations in males, but not in females. Besides, the blockade of postsynaptic 5-HT_{1A} receptors with the selective antagonist WAY100635 (1 mg/kg) abolished the antidepressant-like effects of CBD observed in the tail suspension test in LPS male mice. LPS injection induced the activation of the NF-κB and kynurenine pathways, increased IL-6 levels, and dysregulated glutamate and GABA levels in cortex and hippocampus. Moreover, it increased IL-6 plasma levels. The pre-administration of CBD prevented all these LPS-induced changes.

CONCLUSIONS

1. The infralimbic cortex is a key brain area for the acute antidepressant-like effect of cannabidiol.

2. The acute antidepressant-like effect of cannabidiol infusion into the infralimbic cortex is associated with the activation of the ERK and mTOR pathways and the increase of BDNF in the prefrontal cortex and hippocampus.

3. Cannabidiol infusion into the infralimbic cortex increases neuronal activity in the dorsal raphe nucleus in *naïve* animals and modulates acute stress-induced neuronal activity in the infralimbic cortex and the dorsal raphe nucleus.

4. The antidepressant-like effect of cannabidiol infusion into the infralimbic cortex is not observed after 24 hours but is recovered after the blockade of presynaptic 5-HT_{1A} receptors.

5. This recovery of the antidepressant-like effect is associated with the activation of ERK in the prefrontal cortex, and with the increase in the levels of glutamate and noradrenaline in the dorsal raphe nucleus.

6. The infusion of cannabidiol in the dorsal raphe nucleus does not produce an acute antidepressant-like effect, although it increases serotonin levels in the prefrontal cortex, and is less efficient in inducing synaptic plasticity pathways in this area.

7. Gender differences were observed in the behavioural manifestations of the lippopolisaccharide model and the response to systemic cannabidiol administration. The systemic administration of cannabidiol produces an antidepressant-like effect in the lipopolysaccharide-induced neuroinflammatory model in male but not in female mice.

8. The activation of postsynaptic 5-HT_{1A} receptors plays a key role in the antidepressant-like effect of cannabidiol in this neuroinflammatory model.

9. The antidepressant-like effect of cannabidiol in the lipopolysaccharide model is associated with a decreased activation of the NF-κB pathway in the brain.

10. The antidepressant-like effect of cannabidiol in the lipopolysaccharide model is associated with a decrease in interleukin-6 levels in brain and plasma.

11. The antidepressant-like effect of cannabidiol in the lipopolysaccharide model is associated with a decreased activation of the kinurenine pathway in the brain.

12. Cannabidiol prevents the imbalance in the brain levels of glutamate and GABA induced by lipopolysaccharide administration.

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ANNEXES

ANNEXE 1: Statistical analyses

Objective 1

Table 1. Evaluation of the depressive and anxious-like behavior and locomotion 30 min after an IL CBD infusion (figure 18).			
Set of data	**Type of analysis**	**t, df**	**p**
A. Immobility, swimming and climbing behavior in the FST.	*t* -test		
Immobility		t = 2.306, df = 12	**p < 0.05**
Swimming		t = 2.183, df = 12	**p < 0.05**
Climbing		t = 0.793, df = 12	ns
B. Distance travelled (m) and time in the center (s) in the OFT.	*t* -test		
Distance		t = 1.213, df = 12	ns
Time in the center		t = 0.273, df = 12	ns

Table 2. Plasticity markers in PFC and Hp 1 h after an IL CBD infusion (figure 19).			
Set of data	**Type of analysis**	**t, df**	**p**
A. Prefrontal cortex.	*t* -test		
p-mTOR		t = 2.286, df = 12	**p < 0.05**
mTOR		t = 1.574, df = 12	ns
p-GSK3β		t = 1.821, df = 12	ns
GSK3β		t = 0.713, df = 12	ns
β-catenin		t = 0.383, df = 12	ns
p-Akt		t = 0.941, df = 12	ns
Akt		t = 3.026, df = 12	**p < 0.05**
p-Erk		t = 2.490, df = 12	**p < 0.05**
Erk		t = 1.205, df = 12	ns
p-CREB		t = 0.185, df = 12	ns
CREB		t = 2.033, df = 12	**p = 0.065**
BDNF		t = 3.212, df = 12	**p < 0.01**
B. Hippocampus.	*t* -test		
p-mTOR		t = 1.506, df = 11	ns
mTOR		t = 1.761, df = 11	ns
p-GSK3β		t = 2.216, df = 11	**p < 0.05**
GSK3β		t = 2.042, df = 11	**p = 0.066**
p-Erk		t = 2.711, df = 11	**p < 0.05**
Erk		t = 1.274, df = 11	ns
BDNF		t = 3.307, df = 9	**p < 0.01**

Table 3. Neurotransmitters in PFC and DRN 1 h after an IL CBD infusion (figure 20).			
Set of data	**Type of analysis**	**t, df**	**p**
A. 5-HT.	*t* -test		
PFC		t = 0.935, df = 12	ns
DRN		t = 1.090, df = 12	ns
B. NA.	*t* -test		
PFC		t = 3.940, df = 12	**p < 0.01**
DRN		t = 0.510, df = 12	ns
C. GABA.	*t* -test		
PFC		t = 1.843, df = 12	ns
DRN		t = 2.264, df = 12	**p < 0.05**
D. Glutamate.	*t* -test		
PFC		t = 2.220, df = 12	**p < 0.05**
DRN		t = 2.356, df = 12	**p < 0.05**

Table 4. Neuronal activation (c-Fos) 2 h after an IL CBD infusion (figure 21).			
Set of data	**Type of analysis**	**F, df**	**p**
A. Prelimbic (PL) cortex.	Two-way ANOVA		
Factor 1 CBD		F(1,15) = 0.047	ns
Factor 2 Stress		F(1,15) = 4.881	**p < 0.05**
Interaction (F1xF2)		F(1,15) = 0.215	ns
B. Infralimbic (IL) cortex.	Two-way ANOVA		
Factor 1 CBD		F(1,15) = 1.540	ns
Factor 2 Stress		F(1,15) = 1.007	ns
Interaction (F1xF2)		F(1,15) = 0.741	ns
C. Dorsal raphe nucleus.	Two-way ANOVA		
Factor 1 CBD		F(1,15) = 0.358	ns
Factor 2 Stress		F(1,15) = 2.605	ns
Interaction (F1xF2)		F(1,15) = 8.389	**p < 0.05**

Table 5. Study of the depressive and anxious-like behavior and locomotion 24 h after an IL CBD infusion (figure 22).			
Set of data	**Type of analysis**	**t, df**	**p**
A. Immobility, swimming and climbing behavior in the FST.	*t* -test		
Immobility		t = 1.092, df = 12	ns
Swimming		t = 1.228, df = 12	ns
Climbing		t = 0.332, df = 12	ns
B. Distance travelled (m) and time in the center (s) in the OFT.	*t* -test		
Distance		t = 0.367, df = 12	ns
Time in the center		t = 0.590, df = 12	ns

Table 6. Plasticity markers in PFC 24 h after an IL CBD infusion (figure 23).			
Set of data	**Type of analysis**	**t, df**	**p**
	t -test		
p-mTOR		t = 3.039, df = 12	**p < 0.05**
mTOR		t = 2.000, df = 12	**p = 0.069**
p-GSK3β		t = 2.734, df = 12	**p < 0.05**
GSK3β		t = 0.933, df = 12	ns
β-catenin		t = 1.003, df = 12	ns
p-Akt		t = 0.917, df = 12	ns
Akt		t = 0.413, df = 12	ns
p-Erk		t = 0.217, df = 12	ns
Erk		t = 0.608, df = 12	ns
p-CREB		t = 0.659, df = 12	ns
CREB		t = 0.263, df = 12	ns
BDNF		t = 2.490, df = 12	**p < 0.05**

Table 7. Evaluation of the depressive-like behaviour 24 h after an IL CBD infusion and i.p. WAY100635 administration (figure 24).			
Set of data	**Type of analysis**	**F, df**	**p**
Immobility.	Two-way ANOVA		
Factor 1 CBD		F(1,32) = 1.462	ns
Factor 2 WAY		F(1,32) = 3.687	**p = 0.064**
Interaction (F1xF2)		F(1,32) = 4.384	**p < 0.05**
Swimming.	Two-way ANOVA		
Factor 1 CBD		F(1,32) = 0.065	ns
Factor 2 WAY		F(1,32) = 5.890	**p < 0.05**
Interaction (F1xF2)		F(1,32) = 1.297	ns
Climbing.	Two-way ANOVA		
Factor 1 CBD		F(1,32) = 1.044	ns
Factor 2 WAY		F(1,32) = 9.438	**p < 0.01**
Interaction (F1xF2)		F(1,32) = 4.341	**p < 0.05**

Table 8. Plasticity markers in PFC 24 h after an IL CBD infusion and i.p. WAY100635 administration (figure 25).			
Set of data	**Type of analysis**	**F, df**	**p**
p-mTOR	Two-way ANOVA		
Factor 1 CBD		F(1,32) = 17.30	**p < 0.001**
Factor 2 WAY		F(1,32) = 0.035	ns
Interaction (F1xF2)		F(1,32) = 0.070	ns
mTOR	Two-way ANOVA		
Factor 1 CBD		F(1,32) = 0.201	ns
Factor 2 WAY		F(1,32) = 0.673	ns
Interaction (F1xF2)		F(1,32) = 1.428	ns
p-ERK	Two-way ANOVA		
Factor 1 CBD		F(1,32) = 2.029	ns
Factor 2 WAY		F(1,32) = 4.475	**p < 0.05**
Interaction (F1xF2)		F(1,32) = 1.631	ns
ERK	Two-way ANOVA		
Factor 1 CBD		F(1,32) = 1.793	ns
Factor 2 WAY		F(1,32) = 0.113	ns
Interaction (F1xF2)		F(1,32) = 2.111	ns
BDNF	Two-way ANOVA		
Factor 1 CBD		F(1,32) = 10.22	**p < 0.01**
Factor 2 WAY		F(1,32) = 0.010	ns
Interaction (F1xF2)		F(1,32) = 0.667	ns

Table 9. Neurotransmitters in PFC and DRN 24 h after an IL CBD infusion and i.p. WAY100635 administration (figure 26).			
	Type of analysis	**F, df**	**p**
A. 5-HT in PFC.	Two-way ANOVA		
Factor 1 CBD		F(1,20) = 0.470	ns
Factor 2 WAY		F(1,20) = 4.687	**p < 0.05**
Interaction (F1xF2)		F(1,20) = 0.979	ns
A. 5-HT in DRN.	Two-way ANOVA		
Factor 1 CBD		F(1,20) = 0.101	ns
Factor 2 WAY		F(1,20) = 4.210	**p = 0.054**
Interaction (F1xF2)		F(1,20) = 1.152	ns
B. NA in PFC.	Two-way ANOVA		
Factor 1 CBD		F(1,20) = 1.052	ns
Factor 2 WAY		F(1,20) = 0.819	ns
Interaction (F1xF2)		F(1,20) = 0.852	ns
B. NA in DRN.	Two-way ANOVA		
Factor 1 CBD		F(1,20) = 5.219	**p < 0.05**
Factor 2 WAY		F(1,20) = 5.512	**p < 0.05**
Interaction (F1xF2)		F(1,20) = 1.245	ns
C. GABA in PFC.	Two-way ANOVA		
Factor 1 CBD		F(1,20) = 8.544	**p < 0.01**
Factor 2 WAY		F(1,20) = 0.183	ns
Interaction (F1xF2)		F(1,20) = 3.365	**p = 0.082**
C. GABA in DRN.	Two-way ANOVA		
Factor 1 CBD		F(1,20) = 0.197	ns
Factor 2 WAY		F(1,20) = 18.65	**p < 0.001**
Interaction (F1xF2)		F(1,20) = 6.918	**p < 0.05**
D. Glutamate in PFC.	Two-way ANOVA		
Factor 1 CBD		F(1,20) = 103.6	**p < 0.001**
Factor 2 WAY		F(1,20) = 10.43	**p < 0.01**
Interaction (F1xF2)		F(1,20) = 1.799	ns
D. Glutamate in DRN.	Two-way ANOVA		
Factor 1 CBD		F(1,20) = 32.36	**p < 0.001**
Factor 2 WAY		F(1,20) = 0.568	ns
Interaction (F1xF2)		F(1,20) = 6.553	**p < 0.05**

Table 10. Assessment of the depressive and anxious-like behavior and locomotion 30 min after a DRN CBD infusion (figure 27).			
Set of data	**Type of analysis**	**t, df**	**p**
A. Immobility, swimming and climbing behavior in the FST.	*t* -test		
Immobility		t = 0.911, df = 20	ns
Swimming		t = 2.590, df = 20	**p < 0.05**
Climbing		t = 3.568, df = 20	**p < 0.01**
B. Distance travelled (m) and time in the center (s) in the OFT.	*t* -test		
Distance		t = 0.068, df = 20	ns
Time in the center		t = 0.793, df = 20	ns

Table 11. Plasticity markers in PFC 1 h after a DRN CBD infusion (figure 28).			
Set of data	**Type of analysis**	**t, df**	**p**
	t -test		
p-mTOR		t = 0.171, df = 12	ns
mTOR		t = 0.072, df = 12	ns
p-GSK3β		t =0.703, df = 12	ns
GSK3β		t = 1.085, df = 12	ns
β-catenin		t = 0.662, df = 12	ns
p-Akt		t = 0.570, df = 12	ns
Akt		t = 0.447, df = 12	ns
p-Erk		t = 1.437, df = 12	ns
Erk		t = 1.023, df = 12	ns
p-CREB		t = 0.669, df = 12	ns
CREB		t = 1.597, df = 12	ns
BDNF		t = 2.212, df = 12	**p < 0.05**

Table 12. Neurotransmitters in PFC and DRN 1 h after a DRN CBD infusion (figure 29).			
Set of data	**Type of analysis**	**t, df**	**p**
A. 5-HT.	*t* -test		
PFC		t = 2.867, df = 13	**p < 0.05**
DRN		t = 0.987, df = 11	ns
B. NA.	*t* -test		
PFC		t = 0.297, df = 13	ns
DRN		t = 0.566, df = 11	ns
C. GABA.	*t* -test		
PFC		t = 1.045, df = 13	ns
DRN		t = 0.114, df = 11	ns
B. Glutamate.	*t* -test		
PFC		t = 0.038, df = 13	ns
DRN		t = 0.053, df = 11	ns

Table 13. Plasticity markers in primary neuronal cultures treated with CBD for 30 min and 24 h (figure 31).			
Set of data	**Type of analysis**	**t, df**	**p**
A. Extracellular BDNF.	*t* -test		
30 min		t = 0.826, df = 18	ns
24 h		t = 3.215, df = 14	**p < 0.01**
B. Intracellular BDNF.	*t* -test		
30 min		t = 2.447, df = 18	**p < 0.05**
24 h		t = 0.870, df = 14	ns
C. p-mTOR and mTOR levels.	*t* -test		
p-mTOR 30 min		t = 2.250, df = 10	**p < 0.05**
mTOR 30 min		t = 2.115, df = 10	**p = 0.061**
p-mTOR 24 h		t = 1.439, df = 17	ns
mTOR 24 h		t = 0.816, df = 17	ns
D. p-ERK and ERK levels.	*t* -test		
p-ERK 30 min		t = 2.958, df = 10	**p < 0.05**
ERK 30 min		t = 1.632, df = 10	ns
p-ERK 24 h		t = 1.763, df = 17	ns
ERK 24 h		t = 1.978, df = 17	**p = 0.064**

Objective 2

Table 14. TNFα and IL-6 in microglial culture medium after activation with LPS and incubation with CBD (figure 32).			
Set of data	**Type of analysis**	**F, df**	**p**
A. CBD effect in TNFα.	Two-way ANOVA		
Factor 1 CBD		F(1,6) = 13.83	**p < 0.01**
Factor 2 Model		F(1,6) = 157.2	**p < 0.001**
Interaction (F1xF2)		F(1,6) = 17.56	**p < 0.01**
B. CBD efect in IL-6.	Two-way ANOVA		
Factor 1 CBD		F(1,6) = 10.37	**p < 0.05**
Factor 2 Model		F(1,6) = 31.58	**p < 0.01**
Interaction (F1xF2)		F(1,6) = 9.486	**p < 0.05**

Table 15. Optimization of the LPS model in NMRI mice (figure 33).			
Set of data	**Type of analysis**	**t, df**	**p**
A. TST: immobility time in males.	*t* -test		
		t = 1.574, df = 18	ns
B. Sucrose preference test in males.	*t* -test		
0h		t = 1.080, df = 16	ns
12h		t = 7.069, df = 16	**p < 0.001**
24h		t = 3.903, df = 16	**p < 0.01**
36h		t = 2.006, df = 16	**p = 0.062**
48h		t = 1.464, df = 16	ns
72h		t = 0.345, df = 16	ns
C. TST: immobility time in females.	*t* -test		
		t = 0.293, df = 11	ns
D. Sucrose preference test in females.	*t* -test		
0h		t = 0.358, df = 13	ns
12h		t = 3.467, df = 13	**p < 0.01**
24h		t = 2.556, df = 13	**p < 0.05**
36h		t = 0.799, df = 13	ns
48h		t = 0.259, df = 13	ns
72h		t = 0.794, df = 13	ns

Table 16. Evaluation of the effects of gender in the depressive and anxious-like behaviour in the mouse LPS model (figure 34).			
Set of data	**Type of analysis**	**F, df**	**p**
A. TST: immobility time.	Two-way ANOVA		
Factor 1 Sex		F(1,21) = 20.32	**p < 0.001**
Factor 2 Model		F(1,21) = 8.677	**p < 0.01**
Interaction (F1xF2)		F(1,21) = 0.405	ns
B. Sucrose preference test.	Two-way ANOVA		
Factor 1 Sex		F(1,26) = 0.011	ns
Factor 2 Model		F(1,26) = 72.85	**p < 0.001**
Interaction (F1xF2)		F(1,26) = 0.391	ns
C. Weight variation.	Two-way ANOVA		
Factor 1 Sex		F(1,21) = 7.585	**p < 0.05**
Factor 2 Model		F(1,21) = 46.95	**p < 0.001**
Interaction (F1xF2)		F(1,21) = 4.943	**p < 0.05**
D. OF test: time in the centre.	Two-way ANOVA		
Factor 1 Sex		F(1,21) = 0.055	ns
Factor 2 Model		F(1 21) = 17.55	**p < 0.001**
Interaction (F1xF2)		F(1,21) = 3.395	ns
E. OF test: total distance.	Two-way ANOVA		
Factor 1 Sex		F(1,21) = 1.572	ns
Factor 2 Model		F(1,21) = 16.97	**p < 0.001**
Interaction (F1xF2)		F(1,21) = 0.030	ns

Table 17. Evaluation of the depressive and anxious-like behaviour following CBD administration in the LPS model in males (figure 36).			
Set of data	**Type of analysis**	**F, df**	**p**
A. TST: immobility time.	Two-way ANOVA		
Factor 1 CBD		F(1,22) = 3.155	**p = 0.090**
Factor 2 Model		F(1,22) = 10.26	**p < 0.01**
Interaction (F1xF2)		F(1,22) = 5.544	**p < 0.05**
B. Sucrose preference test.	Two-way ANOVA		
Factor 1 CBD		F(1,28) = 2.363	ns
Factor 2 Model		F(1,28) = 51.89	**p < 0.001**
Interaction (F1xF2)		F(1,28) = 7.004	**p < 0.05**
C. Weight variation.	Two-way ANOVA		
Factor 1 CBD		F(1,22) = 0.106	ns
Factor 2 Model		F(1,22) = 31.36	**p < 0.001**
Interaction (F1xF2)		F(1,22) = 0.015	ns
D. OF test: time in the centre.	Two-way ANOVA		
Factor 1 CBD		F(1,22) = 0.413	ns
Factor 2 Model		F(1,22) = 44.86	**p < 0.001**
Interaction (F1xF2)		F(1,22) = 0.918	ns
E. OF test: total distance.	Two-way ANOVA		
Factor 1 CBD		F(1,22) = 0.003	ns
Factor 2 Model		F(1,22) = 42.31	**p < 0.001**
Interaction (F1xF2)		F(1,22) = 3.279	**p = 0.084**

Table 18. Evaluation of the depressive and anxious-like behaviour following CBD administration in the LPS model in females (figure 37).			
Set of data	**Type of analysis**	**F, df**	**p**
A. TST: immobility time.	Two-way ANOVA		
Factor 1 CBD		F(1,23) = 5.745	**p < 0.05**
Factor 2 Model		F(1,23) = 2.755	ns
Interaction (F1xF2)		F(1,23) = 0.029	ns
B. Sucrose preference test.	Two-way ANOVA		
Factor 1 CBD		F(1,23) = 1.089	ns
Factor 2 Model		F(1,23) = 18.28	**p < 0.001**
Interaction (F1xF2)		F(1,23) = 0.130	ns
C. Weight variation.	Two-way ANOVA		
Factor 1 CBD		F(1,23) = 1.599	ns
Factor 2 Model		F(1,23) = 36.24	**p < 0.001**
Interaction (F1xF2)		F(1,23) = 0.058	ns
D. OF test: time in the centre.	Two-way ANOVA		
Factor 1 CBD		F(1,23) = 0.062	ns
Factor 2 Model		F(1,23) = 1.660	ns
Interaction (F1xF2)		F(1,23) = 1.275	ns
E. OF test: total distance.	Two-way ANOVA		
Factor 1 CBD		F(1,23) = 0.001	ns
Factor 2 Model		F(1,23) = 18.73	**p < 0.001**
Interaction (F1xF2)		F(1,23) = 0.667	ns

Table 19. Evaluation of the depressive-like behaviour after 0.3 and 1 mg/kg WAY100635 admin. to LPS mice pretreated with CBD (figure 38).			
Set of data	**Type of analysis**	**F, df**	**p**
Vehicle groups.	Two-way ANOVA		
Factor 1 CBD		F(1,24) = 1.114	ns
Factor 2 Model		F(1,24) = 4.246	**p = 0.050**
Interaction (F1xF2)		F(1,24) = 13.35	**p < 0.01**
0.3 mg/kg WAY100635 groups.	Two-way ANOVA		
Factor 1 CBD		F(1,13) = 4.227	**p = 0.060**
Factor 2 Model		F(1,13) = 11.21	**p < 0.01**
Interaction (F1xF2)		F(1,13) = 28.57	**p < 0.001**
1 mg/kg WAY100635 groups.	Two-way ANOVA		
Factor 1 CBD		F(1,13) = 0.005	ns
Factor 2 Model		F(1,13) = 0.561	ns
Interaction (F1xF2)		F(1,13) = 0.207	ns
WAY100635 + Vehicle.	One-way ANOVA		
		F(2,11) = 5.648	**p < 0.05**
WAY100635 + LPS.	One-way ANOVA		
		F(2,13) = 0.080	ns
WAY100635 + CBD.	One-way ANOVA		
		F(2,12) = 0.619	ns
WAY100635 + LPS + CBD.	One-way ANOVA		
		F(2,14) = 5.832	**p < 0.05**

Table 20. NF-κB, IκBα and PPARγ levels in nuclear and cytoplasmic fractions from the cortex of LPS mice pretreated with CBD (figure 39).			
Set of data	**Type of analysis**	**F, df**	**p**
A. Nuclear NF-κB.	Two-way ANOVA		
Factor 1 CBD		F(1,22) = 0.609	ns
Factor 2 Model		F(1,22) = 32.33	**p < 0.001**
Interaction (F1xF2)		F(1,22) = 11.35	**p < 0.01**
B. Nuclear IκBα.	Two-way ANOVA		
Factor 1 CBD		F(1,22) = 0.070	ns
Factor 2 Model		F(1,22) = 12.93	**p < 0.01**
Interaction (F1xF2)		F(1,22) = 1.182	ns
C. Nuclear PPARγ.	Two-way ANOVA		
Factor 1 CBD		F(1,22) = 5.012	**p < 0.05**
Factor 2 Model		F(1,22) = 0.134	ns
Interaction (F1xF2)		F(1,22) = 3.723	**p = 0.067**
D. Cytoplasmic NF-κB.	Two-way ANOVA		
Factor 1 CBD		F(1,22) = 0.791	ns
Factor 2 Model		F(1,22) = 0.488	ns
Interaction (F1xF2)		F(1,22) = 2.161	ns
E. Cytoplasmic IκBα.	Two-way ANOVA		
Factor 1 CBD		F(1,22) = 0.414	ns
Factor 2 Model		F(1,22) = 6.563	**p < 0.05**
Interaction (F1xF2)		F(1,22) = 3.501	**p = 0.075**
F. Cytoplasmic PPARγ.	Two-way ANOVA		
Factor 1 CBD		F(1,22) = 0.863	ns
Factor 2 Model		F(1,22) = 1.676	ns
Interaction (F1xF2)		F(1,22) = 13.54	**p < 0.01**

Table 21. TNF and IL-6 levels in plasma (protein) and prefrontal cortex (mRNA) in mice treated with LPS and pretreated with CBD (figure 40).			
Set of data	**Type of analysis**	**F, df**	**p**
A. TNF-α in plasma.	Two-way ANOVA		
Factor 1 CBD		F(1,25) = 0.321	ns
Factor 2 Model		F(1,25) = 9.060	**p < 0.01**
Interaction (F1xF2)		F(1,25) = 0.774	ns
B. IL6 in plasma.	Two-way ANOVA		
Factor 1 CBD		F(1,25) = 8.996	**p < 0.01**
Factor 2 Model		F(1,25) = 76.64	**p < 0.001**
Interaction (F1xF2)		F(1,25) = 9.136	**p < 0.01**
C. TNF-α mRNA levels in prefrontal cortex.	Two-way ANOVA		
Factor 1 CBD		F(1,25) = 0.287	ns
Factor 2 Model		F(1,25) = 45.57	**p < 0.001**
Interaction (F1xF2)		F(1,25) = 0.025	ns
D. IL6 mRNA levels in prefrontal cortex.	Two-way ANOVA		
Factor 1 CBD		F(1,25) = 3.375	**p = 0.078**
Factor 2 Model		F(1,25) = 22.83	**p < 0.001**
Interaction (F1xF2)		F(1,25) = 4.401	**p < 0.05**

Table 22. Kynurenine, 3-hydroxy kynurenine, serotonin and tryptophan levels in Hp and Cx from LPS mice pretreated with CBD (table 4).			
Set of data	**Type of analysis**	**F, df**	**p**
Kynurenine in Hp.	Two-way ANOVA		
Factor 1 CBD		F(1,19) = 8.553	**p < 0.01**
Factor 2 Model		F(1,19) = 15.50	**p < 0.001**
Interaction (F1xF2)		F(1,19) = 5.473	**p < 0.05**
3-hydroxy kynurenine in Hp.	Two-way ANOVA		
Factor 1 CBD		F(1,19) = 0.857	ns
Factor 2 Model		F(1,19) = 7.282	**p < 0.05**
Interaction (F1xF2)		F(1,19) = 0.004	ns
Serotonine in Hp.	Two-way ANOVA		
Factor 1 CBD		F(1,19) = 22.48	**p < 0.001**
Factor 2 Model		F(1,19) = 8.769	**p < 0.01**
Interaction (F1xF2)		F(1,19) = 2.125	ns
Tryptophan in Hp.	Two-way ANOVA		
Factor 1 CBD		F(1,19) = 3.020	**p = 0.098**
Factor 2 Model		F(1,19) = 9.311	**p < 0.01**
Interaction (F1xF2)		F(1,19) = 0.067	ns
Kynurenine in Cx.	Two-way ANOVA		
Factor 1 CBD		F(1,28) = 5.200	**p < 0.05**
Factor 2 Model		F(1,28) = 72.33	**p < 0.001**
Interaction (F1xF2)		F(1,28) = 7.718	**p < 0.01**
3-hydroxy kynurenine in Cx.	Two-way ANOVA		
Factor 1 CBD		F(1,28) = 10.73	**p < 0.01**
Factor 2 Model		F(1,28) = 31.68	**p < 0.001**
Interaction (F1xF2)		F(1,28) = 2.738	ns
Serotonine in Cx.	Two-way ANOVA		
Factor 1 CBD		F(1,28) = 3.579	**p = 0.069**
Factor 2 Model		F(1,28) = 8.335	**p < 0.01**
Interaction (F1xF2)		F(1,28) = 0.118	ns
Tryptophan in Cx.	Two-way ANOVA		
Factor 1 CBD		F(1,28) = 3.689	**p = 0.065**
Factor 2 Model		F(1,28) = 52.05	**p < 0.001**
Interaction (F1xF2)		F(1,28) = 3.321	**p = 0.079**

Table 23. Ratio KYN/TRP and KYN/5-HT in hippocampus and cortex of LPS mice pretreated with CBD (figure 41).			
Set of data	**Type of analysis**	**F, df**	**p**
A. Ratio KYN/TRP in Hp.	Two-way ANOVA		
Factor 1 CBD		F(1,19) = 1.785	ns
Factor 2 Model		F(1,19) = 2.614	ns
Interaction (F1xF2)		F(1,19) = 8.389	**p < 0.01**
B. Ratio KYN/5-HT in Hp.	Two-way ANOVA		
Factor 1 CBD		F(1,19) = 17.04	**p < 0.001**
Factor 2 Model		F(1,19) = 8.160	**p < 0.05**
Interaction (F1xF2)		F(1,19) = 2.921	ns
C. Ratio KYN/TRP in Cx.	Two-way ANOVA		
Factor 1 CBD		F(1,28) = 1.020	ns
Factor 2 Model		F(1,28) = 35.47	**p < 0.001**
Interaction (F1xF2)		F(1,28) = 4.060	**p = 0.054**
D. Ratio KYN/5-HT in Cx.	Two-way ANOVA		
Factor 1 CBD		F(1,28) = 14.39	**p < 0.001**
Factor 2 Model		F(1,28) = 47.30	**p < 0.001**
Interaction (F1xF2)		F(1,28) = 8.062	**p < 0.01**

Table 24. Glutamate and GABA levels in hippocampus and cortex from mice treated with LPS and pretreated with CBD (figure 42).			
Set of data	**Type of analysis**	**F, df**	**p**
A. Glutamate in Hp.	Two-way ANOVA		
Factor 1 CBD		F(1,19) = 1.566	ns
Factor 2 Model		F(1,19) = 7.679	**p < 0.05**
Interaction (F1xF2)		F(1,19) = 7.892	**p < 0.05**
B. GABA in Hp.	Two-way ANOVA		
Factor 1 CBD		F(1,19) = 1.023	ns
Factor 2 Model		F(1,19) = 0.055	ns
Interaction (F1xF2)		F(1,19) = 13.02	**p < 0.01**
A. Glutamate in Cx.	Two-way ANOVA		
Factor 1 CBD		F(1,28) = 8.837	**p < 0.01**
Factor 2 Model		F(1,28) = 3.289	**p = 0.081**
Interaction (F1xF2)		F(1,28) = 1.610	ns
B. GABA in Cx.	Two-way ANOVA		
Factor 1 CBD		F(1,28) = 0.088	ns
Factor 2 Model		F(1,28) = 2.171	ns
Interaction (F1xF2)		F(1,28) = 0.250	ns

ANNEXE 2: Resumen

UNIVERSIDAD DE CANTABRIA

PROGRAMA DE DOCTORADO EN BIOLOGÍA
MOLECULAR Y BIOMEDICINA

TESIS DOCTORAL

Cannabidiol como antidepresivo de acción rápida: identificación de los mecanismos moleculares implicados en su efecto antidepresivo

(Resumen)

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Santander 2021

ÍNDICE

INTRODUCCIÓN 1

HIPÓTESIS Y OBJETIVOS 4

MATERIAL Y MÉTODOS 5

1. Animales 5
2. Diseño experimental 6
3. Tratamientos farmacológicos 8
4. Cirugía estereotáxica 8
5. Modelo neuroinflamatorio inducido por lipopolisacárido 8
6. Pruebas conductuales 9
7. Recolección de muestras 10
8. Técnicas moleculares 10
9. Análisis estadístico 14

RESULTADOS Y DISCUSIÓN 14

Objetivo 1: Efectos de tipo antidepresivo del cannabidiol: sitio y mecanismo de acción. 14

1. Infusión bilateral de cannabidiol en la corteza infralímbica: efectos agudos 14
2. Infusión bilateral de cannabidiol en la corteza infralímbica: efectos tras 24 h y en presencia de un bloqueo del receptor 5-HT_{1A} presináptico 22
3. Infusión de cannabidiol en el núcleo dorsal del rafe: efectos agudos 27

Objetivo 2: Efectos de la administración de cannabidiol a ratones sometidos al modelo de lipopolisacárido 31

1. Evaluación del comportamiento de tipo depresivo y ansioso 31
2. Papel de los receptores 5-HT_{1A} en los efectos de tipo antidepresivo del cannabidiol en el modelo de lipopolisacárido 34
3. Efectos moleculares del CBD en el modelo de LPS: vía NF-κB 36
4. Efectos moleculares del CBD en el modelo de LPS: citoquinas proinflamatorias 38
5. Efectos moleculares del CBD en el modelo de LPS: vía de la quinurenina 40
6. Efectos moleculares del CBD en el modelo de LPS: glutamato y GABA 42

CONCLUSIONES 45

BIBLIOGRAFÍA 46

INTRODUCCIÓN

La depresión mayor es una enfermedad mental común y severa caracterizada por síntomas como tristeza, anhedonia, agitación o enlentecimiento motor, sentimientos de culpa o inutilidad, dificultades de concentración, y pensamientos de muerte y suicidio, entre otros (American Psychiatric Association, 2013).

La etiología de la depresión es escasamente conocida, por lo que se han propuesto diferentes hipótesis. La hipótesis clásica de la depresión, *hipótesis monoaminérgica*, postula que hay una desregulación de la transmisión monoaminérgica (Schildkraut, 1965), y se complementa por el hecho de que la mayoría de los antidepresivos usados actualmente en la clínica tienen como diana los sistemas de neurotransmisión monoaminérgica (antidepresivos clásicos) (Gorman y Kent, 1999; Gautam et al., 2017). Entre los sistemas monoaminérgicos destaca el sistema serotonérgico por su implicación en la patología (*revisado en* Artigas, 2013). Las neuronas serotonérgicas están localizadas principalmente en el núcleo dorsal del rafe (DRN). De entre los 14 subtipos de receptores de serotonina descritos destacan los receptores 5-HT_{1A} por su importancia para el efecto antidepresivo, ya que la activación de los receptores 5-HT_{1A} somatodendríticos dificulta el efecto terapéutico, mientras que la activación de los receptores 5-HT_{1A} postsinápticos está implicada en dicho efecto (*revisado en* Artigas, 2013). Aún con numerosas evidencias que la respaldan, la hipótesis monoaminérgica no explica la totalidad de las alteraciones descritas en pacientes con depresión, por lo que se han propuesto otras hipótesis.

Una de las más relevantes es la *hipótesis neurotrófica/neuroplástica* de la depresión, la cual postula que la reducción de factores neurotróficos como el factor neurotrófico derivado de cerebro (BDNF) en áreas cerebrales tales como la corteza prefrontal (PFC) y el hipocampo, podría ser la causa de la atrofia neuronal asociada a la depresión mayor (Duman et al., 1997, 2016). Respaldando esta hipótesis, el incremento de BDNF se ha asociado a la respuesta antidepresiva, tal como se ha descrito en estudios con muestras humanas *postmortem* (Chen et al., 2001), y en estudios preclínicos después del tratamiento crónico con antidepresivos clásicos (Nibuya et al., 1995; Molteni et al., 2006; Björkholm y Monteggia, 2016) o de la administración aguda de antidepresivos de acción rápida como la ketamina, un antagonista del receptor de glutamato N-metil-D-aspartato (NMDA) (Li et al., 2010; Zhou et al., 2014a). El BDNF es esencial para los dos tipos de neuroplasticidad implicados en la fisiopatología de la depresión y el tratamiento antidepresivo: la neurogénesis y la plasticidad sináptica.

La neurogénesis es el proceso en el que las células progenitoras proliferan, maduran, y se integran en circuitos neuronales funcionales. En el cerebro adulto está limitada a la zona

subventricular y a la zona subgranular del giro dentado del hipocampo (Altman y Das, 1965). Algunos estímulos como el estrés, altamente relacionado con la depresión, reducen la neurogénesis en el hipocampo adulto, pudiendo contribuir a la atrofia neuronal observada en esta área en pacientes deprimidos (Schmidt y Duman, 2007). Por el contrario, el tratamiento crónico con antidepresivos incrementa la neurogénesis hipocampal (Warner-Schmidt y Duman, 2006). Las vías de señalización de la adenosina cíclica monofosfato (cAMP) - proteína de unión al elemento de respuesta al AMP cíclico (CREB), y Wnt/glucógeno sintasa quinasa-3β (GSK-3β)/β-catenina juegan un papel clave en la regulación de la neurogénesis en el cerebro adulto (Nakagawa et al., 2002; Adachi et al., 2007; Wexler et al., 2009). Las alteraciones de estas vías de señalización que se han descrito en pacientes deprimidos se revierten con el tratamiento antidepresivo (Thome et al., 2000; Wexler et al., 2008; Karege et al., 2012).

El estrés también reduce la plasticidad sináptica, reduciendo la longitud y el número de dendritas y la cantidad de espinas dendríticas en áreas como la corteza prefrontal y el hipocampo (Radley et al., 2004; Qiao et al., 2016). La vía de señalización de la diana de rapamicina en mamíferos (mTOR) juega un papel crucial en la plasticidad sináptica mediante la regulación de la traducción de proteínas (Hay y Sonenberg, 2004). Esta vía puede ser activada por varias proteínas, como la proteína quinasa B (AKT) y la quinasa regulada por señales extracelulares (ERK) (Swiech et al., 2008). Los niveles de mTOR y de sus proteínas diana se encuentran alterados tanto en pacientes deprimidos (Jernigan et al., 2011) como en modelos animales de depresión (Zhu et al., 2013; Yao et al., 2020). La vía de mTOR ha generado un gran interés estos últimos años, ya que su activación forma parte del mecanismo de acción de los antidepresivos de acción rápida (Li et al., 2010; Duman et al., 2012).

Por otro lado, en los últimos años se han incrementado exponencialmente las evidencias que apoyan la *hipótesis neuroinflamatoria* de la depresión, basada en que algunas enfermedades inflamatorias presentan depresión comórbida (Dregan et al., 2019), y en el incremento de los niveles de citoquinas proinflamatorias como el factor de necrosis tumoral alfa (TNFα) y la interleucina 6 (IL-6) en sangre descrito en pacientes con depresión mayor (Dowlati et al., 2010). Esta inflamación periférica, mediante diversas vías de comunicación con el sistema inmune del sistema nervioso central, puede inducir una respuesta proinflamatoria en el cerebro (Maier, 2003; Connor et al., 2008). Apoyando esto, se ha descrito la activación de la microglía y un incremento de citoquinas proinflamatorias en el líquido cefalorraquídeo y en muestras cerebrales *postmortem* de pacientes con depresión (Enache et al., 2019).

Un estímulo periférico capaz de activar la respuesta inmune es el lipopolisacárido (LPS), un componente de la pared bacteriana. La administración de LPS induce, además de síntomas generales de enfermedad (*sickness behaviour*), un comportamiento de tipo depresivo y ansioso en animales (Leonard y Maes, 2012; Maes et al., 2012), y síntomas depresivos en humanos (*revisado en* Lasselin et al., 2018). La administración de LPS activa el receptor de tipo toll 4 (TLR4) y éste activa la vía canónica del factor nuclear kappa B (NF-κB) (Kawai et al., 2001). NF-κB es un factor de transcripción que participa en la respuesta inmune. En condiciones basales, NF-κB se localiza en el citosol mediante su unión con el inhibidor del factor nuclear kappa B (IκBα). La activación del TLR4 por LPS induce la fosforilación de IκBα, modificación que provoca su degradación en la proteasoma. Esto permite la translocación de NF-κB al núcleo y la transcripción de sus genes diana, iniciando así la respuesta inflamatoria (Covert et al., 2005). Entre las proteínas inducidas por NF-κB se encuentran las citoquinas proinflamatorias IL-6 e interferón gamma (IFNγ) (Kawai et al., 2001). Además, se ha descrito que el LPS reduce los niveles del receptor gamma activado por el proliferador de peroxisoma (PPARγ) en células (Juknat et al., 2013, Choi et al., 2017) y en corteza de rata (Pérez-Nievas et al., 2010; MacDowell et al., 2013). De forma inversa, la activación de PPARγ antagoniza la actividad transcripcional de NF-κB, produciendo un efecto antiinflamatorio (Ricote et al., 1998).

El incremento en IL-6 y IFNγ inducido por LPS activa la enzima indolamina 2,3-dioxigenasa (IDO) (Lestage et al., 2002; Godbout et al., 2008). El incremento de la actividad de la IDO cerebral se ha asociado a la aparición del comportamiento de tipo depresivo en ratones (André et al., 2008; Dantzer et al., 2008). Además, la IDO metaboliza el triptófano a quinurenina, por lo que un incremento en su actividad lleva a un desequilibrio hacia la síntesis de quinurenina en lugar de serotonina (Cervenka et al., 2017). Es importante destacar que en los últimos años se ha descrito ampliamente la asociación de la quinurenina y sus metabolitos con la fisiopatología de la depresión, lo que ha llevado a proponer una nueva hipótesis de la depresión que integra el estrés, la inflamación, la vía de la quinurenina y la neurotransmisión serotonérgica y glutamatérgica (Maes et al., 2011; Barone, 2019).

Con relación a las áreas cerebrales implicadas en la depresión, la corteza cingulada anterior ventral (vACC, área de Brodman 25) ha generado un especial interés. Se ha detectado una hiperactividad de esta área en pacientes con depresión mayor (Mayberg et al., 2005) y varios estudios preclínicos han demostrado la importancia de la corteza infralímbica (IL, el equivalente en roedores del vACC humano) en los efectos antidepresivos de la ketamina (Fuchikami et al., 2015), la hidroxinorketamina (Fukumoto et al., 2019), la estimulación optogenética (Fuchikami et al., 2015), y la estimulación cerebral profunda (Jiménez-Sánchez et al., 2016). Además, la activación de la corteza IL

revierte los cambios observados en el DRN de animales sometidos al modelo de depresión de la derrota social (*social defeat*) (Veerakumar et al., 2014), confirmando la importancia de esta área cerebral y de sus proyecciones al DRN en la regulación de las emociones y la respuesta al estrés.

Debido a que los tratamientos actuales para la depresión poseen importantes limitaciones (Duman et al., 2016; Akil et al., 2018; Fornaro et al., 2019), es necesario encontrar otros fármacos que sean más rápidos y efectivos para tratar la enfermedad. El cannabidiol (CBD), el principal componente no psicoactivo de la planta del *Cannabis sativa* (Mechoulam et al., 2002), se postula como un posible candidato. Sus propiedades de tipo antidepresivo se han descrito en pruebas conductuales predictivas (El-Alfy et al., 2010; Zanelati et al., 2010), y en modelos animales de depresión tales como la bulbectomía olfatoria (Linge et al., 2016) y el estrés crónico impredecible (Campos et al., 2013), tras su administración aguda y crónica. Además, la duración del efecto de tipo antidepresivo tras una sola administración de CBD se extiende hasta una semana, indicando que el fármaco tiene un efecto sostenido (Sales et al., 2019).

Se han descrito muchos mecanismos de acción para el CBD, entre los que destacan la modulación alostérica positiva del receptor 5-HT_{1A} (Rock et al., 2012), el incremento de los niveles de endocannabinoides mediante la inhibición de la enzima amidohidrolasa de ácidos grasos (FAAH) (Bisogno et al., 2001; Leweke et al., 2012), y la consecuente activación de los receptores cannabinoides CB_1 y CB_2 (Campos et al., 2013; Sartim et al., 2016). Los efectos de tipo antidepresivo del CBD se han asociado a varios mecanismos de acción. Por ejemplo, el CBD produce cambios en marcadores de neuroplasticidad como el BDNF (Sales et al., 2019), en proliferación hipocampal en animales con estrés crónico (Campos et al., 2013), y regula la neurotransmisión serotonérgica y glutamatérgica (Linge et al., 2016). Este fármaco también posee propiedades antiinflamatorias e inmunomoduladoras, descritas en modelos animales de otras patologías como el Alzheimer, efectos que fueron mediados por la activación de PPARγ (Esposito et al., 2011). Sin embargo, la relación entre los efectos antiinflamatorios y antidepresivos del CBD en un modelo inflamatorio está aún por explorar (Silote et al., 2019).

HIPÓTESIS Y OBJETIVOS

En base a estas evidencias, postulamos que los efectos de tipo antidepresivo del cannabidiol (CBD) están mediados por un rápido incremento en marcadores de plasticidad sináptica, y por la modulación de varios sistemas de neurotransmisores y de procesos

neuroinflamatorios. También postulamos que la corteza infralímbica (IL) y el núcleo dorsal del rafe (DRN) tienen un papel importante en dichos efectos.

El objetivo general de esta tesis fue ampliar el conocimiento sobre los mecanismos de acción responsables del efecto de tipo antidepresivo del CBD y su lugar de acción en el cerebro. Para ello se propusieron dos **objetivos principales**:

1. Estudiar los efectos agudos y mantenidos de tipo conductual (fenotipo de tipo depresivo y ansioso), molecular (marcadores de neuroplasticidad y activación neuronal), y neuroquímico (niveles de neurotransmisores) inducidos por la infusión local de CBD en la corteza infralímbica y el DRN en ratas.

2. Evaluar los efectos conductuales (fenotipo de tipo depresivo y ansioso) de la administración sistémica de CBD a ratones macho y hembra sometidos a un modelo de neuroinflamación inducido por la inyección de lipopolisacárido, y la implicación de vías inflamatorias, neurotransmisores y del receptor 5-HT_{1A} en dichos efectos.

MATERIAL Y MÉTODOS

1. Animales

En esta tesis se usaron ratas y ratones de 2 - 3 meses de edad que fueron estabulados con un ciclo de luz-oscuridad de 12 h, temperatura de 22 ± 1 ºC, humedad relativa del 60 - 70%, y con libre acceso a la comida y a la bebida (*ab libitum*).

Para los experimentos del primer objetivo se usaron ratas Sprague Dawley macho (280 - 350 g) estabuladas en grupos de 2 - 3 animales en condiciones libres de patógenos específicos (SPF).

Para los experimentos del segundo objetivo se usaron ratones NMRI macho y hembra (30 - 35 g) estabulados en grupos de 4 - 5 animales.

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.

2. Diseño experimental

En la figura 1 se muestra el diseño experimental seguido para evaluar los efectos conductuales, moleculares, y neuroquímicos de la infusión de cannabidiol (CBD) en la corteza infralímbica (IL) y en el núcleo dorsal del rafe (DRN).

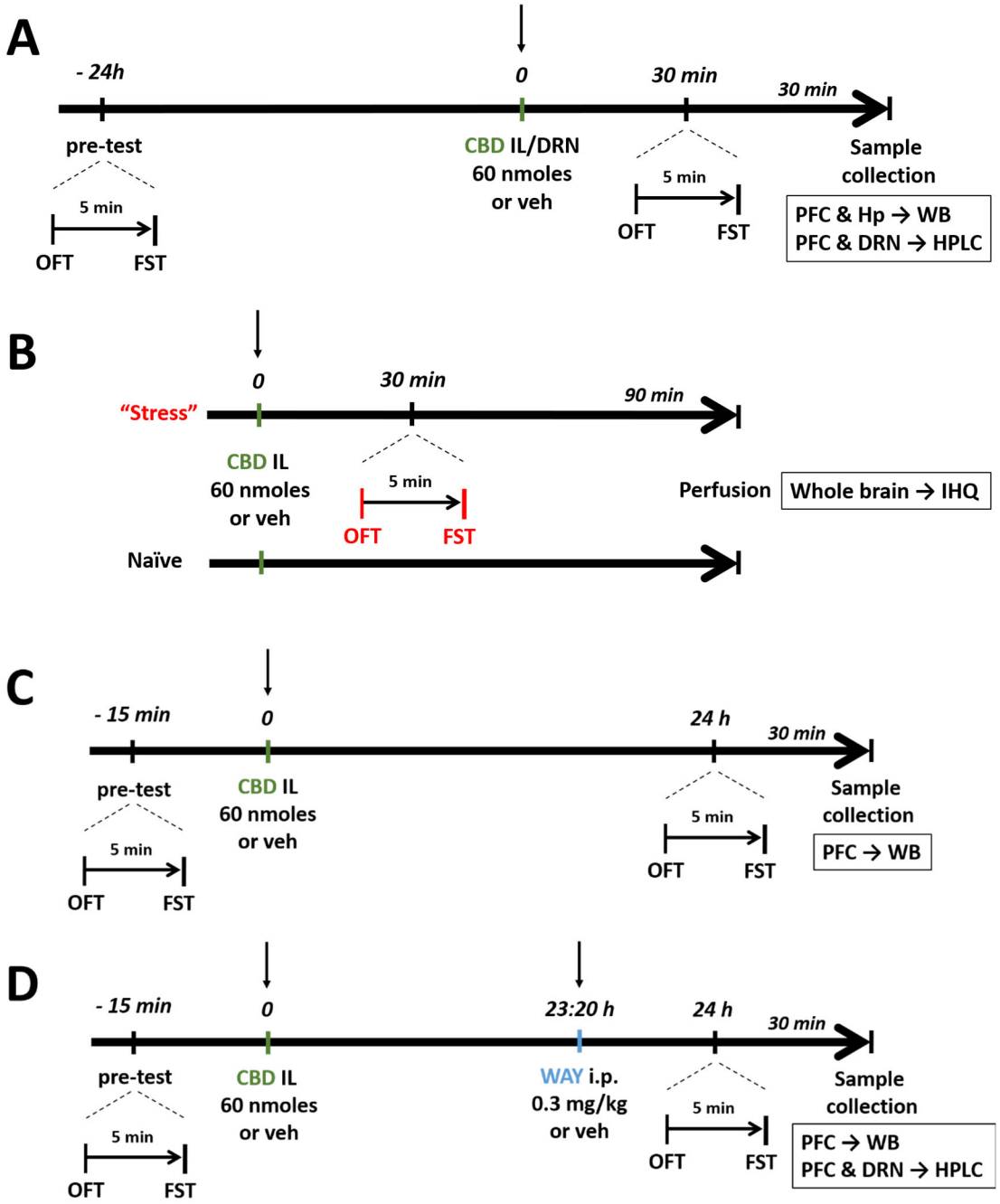

Figura 1. Cronograma de los experimentos del Objetivo 1. A) Estudios conductuales y moleculares de la infusión de cannabidiol (CBD) en la corteza infralímbica (IL) o en el núcleo dorsal del rafe (DRN) a los 30 min. B) Estudio de los efectos inducidos por el estrés (generado por las pruebas conductuales) en la activación neuronal de la corteza prefrontal y del DRN después de la infusión

IL de CBD. Se compararon cuatro grupos experimentales: *naïve* (sin estrés), *naïve*-CBD, estrés, y estrés-CBD. C) Evaluación de los efectos conductuales y moleculares de la infusión IL de CBD a las 24 h. D) Evaluación de los efectos conductuales y moleculares de la infusión IL de CBD a las 24 h en presencia de un bloqueo del receptor 5-HT_{1A} presináptico (WAY100635 0.3 mg/kg). Se usaron cuatro grupos experimentales: vehículo, CBD, WAY, y CBD+WAY. CBD: cannabidiol, WAY: WAY100635, i.p.: intraperitoneal, FST: prueba de la natación forzada, OFT: prueba del campo abierto, DRN: núcleo dorsal del rafe, Hp: hipocampo, IL: corteza infralímbica, PFC: corteza prefrontal, HPLC: cromatografía líquida de alto rendimiento, IHQ: inmunohistoquímica, WB: western blot.

En la figura 2 se muestra el diseño experimental seguido para evaluar los efectos conductuales y moleculares de la administración sistémica de CBD a ratones sometidos al modelo de neuroinflamación inducido por la inyección sistémica de lipopolisacárido (LPS).

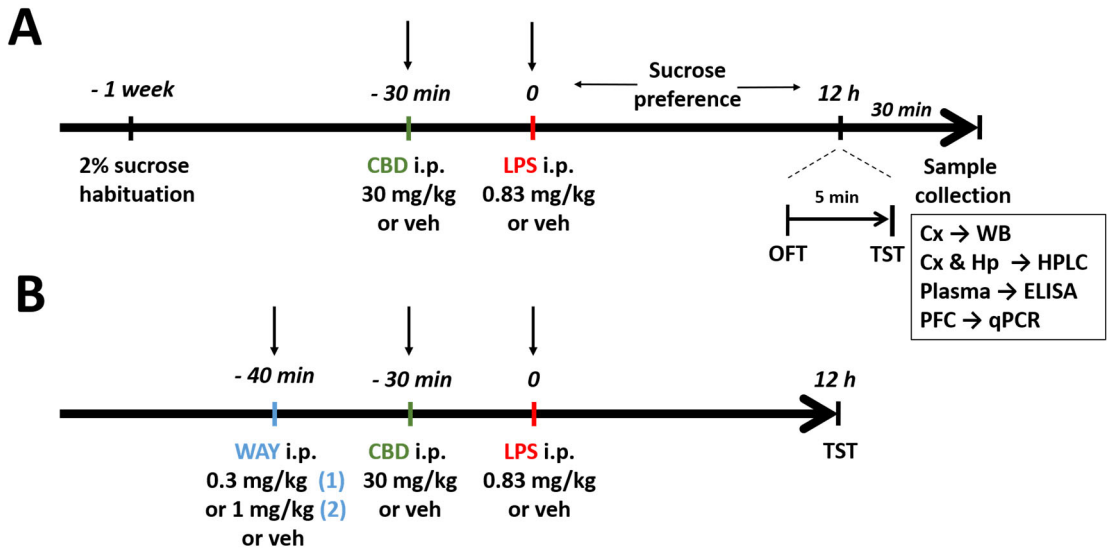

Figura 2. Cronograma de los experimentos del Objetivo 2. A) Estudio de los efectos conductuales y moleculares de la administración de cannabidiol (CBD) a ratones sometidos al modelo neuroinflamatorio inducido por la inyección de lipopolisacárido (LPS). El CBD se administró 30 min antes del LPS, y la conducta se evaluó a las 12 h. Se usaron cuatro grupos experimentales: vehículo, LPS, CBD, y CBD+LPS. B) Evaluación de los efectos del CBD en el modelo de LPS en presencia de un bloqueo de los receptores 5-HT_{1A} presinápticos (WAY100635 0.3 mg/kg) (1), o de los receptores 5-HT_{1A} pre- y postsinápticos (WAY100635 1 mg/kg) (2). CBD: cannabidiol, LPS: lipopolisacárido, WAY: WAY100635, OFT: prueba del campo abierto, TST: prueba de la suspensión por la cola, Cx: corteza, Hp: hipocampo, PFC: corteza prefrontal, ELISA: ensayo por inmunoabsorción ligado a enzimas, HPLC: cromatografía líquida de alto rendimiento, qPCR: reacción en cadena de la polimerasa cuantitativa, WB: western blot.

3. Tratamientos farmacológicos

Los tratamientos farmacológicos realizados en esta tesis fueron: (1) la infusión local en la corteza IL y en el DRN de CBD para estudiar las principales áreas cerebrales implicadas en sus efectos; (2) la administración sistémica de CBD en animales sometidos al modelo de neuroinflamación inducido por la inyección de lipopolisacárido (LPS) (O'Connor et al., 2009). Otro fármaco usado fue WAY100635, un antagonista selectivo del receptor 5-HT_{1A}, para evaluar el papel de dicho receptor en los efectos del CBD.

Para su infusión intracerebral, el CBD fue disuelto en una solución salina con 60% DMSO, y se administró durante la cirugía estereotáxica. Se infundió un microlitro conteniendo 60 nmoles de CBD (Sartim et al., 2016) en cada hemisferio en el caso de la infusión en la corteza infralímbica y se realizó una sola infusión en el núcleo dorsal del rafe.

Para su inyección intraperitoneal (i.p.) el CBD fue disuelto en solución salina con Propylenglycol® 5%, Tween 80® 2%. La dosis de CBD administrada en ratones fue 30 mg/kg (Zanelati et al., 2010).

Se usaron dos dosis diferentes de WAY100635: 0,3 mg/kg i.p. para bloquear principalmente los receptores 5-HT_{1A} presinápticos, y 1 mg/kg i.p. para bloquear los receptores 5-HT_{1A} pre- y postsinápticos (Serres et al., 2000; Ago et al., 2003; Carey et al., 2005). Las ratas recibieron la dosis de 0,3 mg/kg, y los ratones ambas dosis.

4. Cirugía estereotáxica

Las ratas se anestesiaron con una mezcla de O_2-isoflurano. Las coordenadas para la infusión bilateral en la corteza infralímbica fueron: anteroposterior (AP): + 3.2, mediolateral (ML): +/- 0.6, y dorsoventral (DV): - 5.2 (mm desde Bregma) (Paxinos y Watson, 1987). La infusión en el núcleo dorsal del rafe se realizó con un ángulo de 30º para evitar el acueducto, con las siguientes coordenadas: AP: - 7.3, ML: + 1.7, and DV: - 5.9 (Paxinos y Watson, 1987).

5. Modelo neuroinflamatorio inducido por lipopolisacárido

El lipopolisacárido (LPS) usado en esta tesis fue extraído del serotipo 0127:B8 de *Escherichia coli.* La dosis de LPS usada para inducir el modelo neuroinflamatorio en ratones fue de 0,83 mg/kg i.p. (disuelto en salino) (O'Connor et al., 2009; Florensa-Zanuy et al., 2021).

6. Pruebas conductuales

Todas las pruebas se realizaron durante la fase de luz. Los animales se trasladaron 1 h antes de las pruebas a la sala de conducta para habituarlos al nuevo entorno. El orden de realización de las pruebas fue de la menos a la más estresante.

Prueba del campo abierto (OFT)

El OFT se realizó en una caja gris con iluminación central (350 lx). Las dimensiones para rata fueron de 100 x 100 x 75 cm (centro 50 x 50 cm) y para ratón de 40 x 40 x 30 (centro 20 x 20 cm). Los animales se situaron en una esquina de la caja y su comportamiento se grabó mediante un sistema computarizado durante 5 min (Any-maze Video-Tracking software, Stoelting Co., USA). Se determinaron los parámetros de distancia total y tiempo en el centro para estudiar la locomoción y el comportamiento de tipo ansioso, respectivamente.

Prueba de la natación forzada (FST)

Se utilizó un cilindro transparente (20 cm de diámetro y 46 cm de alto) lleno de agua a 23 - 25 ºC hasta los 30 cm de altura. Se realizó una sesión de entrenamiento de 15 min 24 h antes de la prueba. El día del estudio, la conducta se grabó durante 6 min, y se evaluaron manualmente tres tipos de comportamiento durante los últimos 4 min: inmovilidad, natación y movimientos verticales (Slattery y Cryan, 2012). El evaluador desconocía los grupos experimentales. En esta prueba, la inmovilidad se considera un signo de desesperación conductual, y disminuye con la administración de antidepresivos (Cryan et al., 2002).

Prueba de la suspensión por la cola (TST)

Los ratones se suspendieron por la cola con cinta adhesiva y las sesiones se grabaron en vídeo. Se determinó el tiempo de inmovilidad manualmente durante 5 min. Un animal se consideró inmóvil cuando colgaba pasivamente sin realizar ningún movimiento, comportamiento que indica desesperación conductual. Esta prueba se usa para evaluar el comportamiento de tipo depresivo, y la administración de antidepresivos disminuye el tiempo de inmovilidad (Steru et al., 1985).

Prueba de la preferencia por sacarosa (SPT)

Los animales se individualizaron y se les dio libre elección entre agua o una solución azucarada al 2% (Sobrian et al., 2003; Goshen et al., 2008) durante 1 semana antes de la prueba para habituarse a disponibilidad de dos botellas. La posición de las botellas se fue

alternando cada 12 h para evitar una posible preferencia de posición. La preferencia por sacarosa se calculó 12 h después de la inyección de LPS como el porcentaje de sacarosa consumida con respecto al líquido total consumido durante ese periodo.

7. Recolección de muestras

Tejido fresco

Treinta minutos después de las pruebas conductuales, los ratones fueron sacrificados mediante dislocación cervical y las ratas mediante decapitación. Se extrajeron los cerebros y se diseccionaron la corteza prefrontal (PFC), la corteza (Cx), el hipocampo (Hp), y el núcleo dorsal del rafe (DRN). Las muestras se almacenaron a -80ºC.

Tejido perfundido

Para los estudios de inmunohistoquímica, las ratas se anestesiaron con pentobarbital sódico (100 mg/kg, i.p.) y se perfundieron transcardialmente con solución salina (0.9% NaCl), seguido de paraformaldehido (PFA) al 4% en solución salina tamponada con fosfato (PBS) con una bomba de perfusión. Se extrajeron los cerebros y se sumergieron en PFA durante 4 h y posteriormente en 30% de sacarosa en PBS. Los cerebros se almacenaron a -20ºC.

Plasma

Los ratones se anestesiaron con pentobarbital sódico (40 mg/kg, i.p.) para recoger sangre directamente del corazón con una jeringa pretratada con EDTA 0,25 M, pH 7,4 - 8. Las muestras se centrifugaron durante 10 min a 2000xg y a 4ºC para obtener el plasma. El sobrenadante se almacenó a -80ºC.

8. Técnicas moleculares

Reacción en cadena de la polimerasa cuantitativa (qPCR)

La extracción de RNA se realizó como se ha descrito previamente (Pilar-Cuéllar et al., 2017). El RNA se extrajo de muestras de corteza prefrontal usando TRI reagent (Merck KGaA, Darmstadt, Alemania) siguiendo las instrucciones del fabricante. La valoración de la pureza del RNA (ratios 260/280 y 260/230) y su cuantificación se realizaron con un NanoDrop 1000 (Thermo Fischer Scientific S.L., Madrid, España). La reacción de transcriptasa reversa se realizó utilizando 600 ng de RNA con el kit high capacity cDNA reverse transcriptase kit (Applied Biosystems, California, EEUU) siguiendo las instrucciones del fabricante.

La qPCR se realizó en un sistema de PCR a tiempo real StepOne™ (Applied Biosystems, California, EEUU) con sondas TaqMan para ensayos de expresión génica para TNFα (Mm00443258_m1), IL-6 (Mm00446190_m1), beta-actina (actb, Mm00607939_s1), y gliceraldehido 3-fosfato deshidrogenasa (GAPDH, Mm99999915_g1) comprados a Applied Biosystems (California, EEUU). Las reacciones se hicieron por triplicado con 125 ng cDNA por reacción. Como genes de control interno se usaron GAPDH y Actb. La cuantificación relativa de los genes diana se calculó mediante el método ΔΔCt (Livak y Schmittgen, 2001).

Western Blot (WB)

Extracción proteica: homogeneizado total

El tejido se homogeneizó mecánicamente 1:15 peso/volumen en una solución sin detergentes (10 mM HEPES, pH 7,9, 100 mM KCl, 1,5 mM $MgCl_2$, suplementado con los siguientes inhibidores de proteasa: 1 mM PMSF, 0,03 mM aprotinina, 0,02 mM leupeptina, 0,01 mM pepstatina, 0,02 mM antipain, 0,02 mM quimostatina, e inhibidores de fosfatasa: 1 mM Na_3VO_4 y 1 mM NaF). Posteriormente se añadió una solución con detergentes (solución sin detergentes suplementada con 1% Igepal, 0,5% desoxicolato sódico, 0,1% dodecilsulfato sódico (SDS), 2,5 mM CHAPS) y la lisis celular se realizó en hielo durante 30 min. Las muestras se centrifugaron 10 min a 20800xg, 4ºC y el sobrenadante se almacenó como homogeneizado total.

Extracción proteica: fraccionamiento subcelular

Se siguió el protocolo descrito en García-Bueno et al. (2005) con modificaciones menores. La homogeneización mecánica de las muestras se hizo en una solución con 10 mM HEPES, pH 7,9, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM ditiotreitol (DTT), 0,5 M sacarosa, 10 mM molibdato sódico, suplementado con un cóctel de inhibidores de proteasa. Después de 15 min en hielo se añadió 0,5% Igepal, se centrifugaron las muestras durante 5 min a 8000xg, 4ºC, y se recogió el sobrenadante como fracción citosólica. El pellet se resuspendió en la solución anterior suplementada con 20% glicerol y 0,4 M KCl, y se mantuvo 30 min en agitación a 4ºC. El sobrenadante resultante tras una centrifugación de 5 min a 13000xg, 4ºC se recogió como fracción nuclear.

Electroforesis, detección, y cuantificación proteica

Duplicados de cada muestra (45 - 60 µg de proteína del homogeneizado total y la fracción citosólica, y 90 µg de proteína de la fracción nuclear) se separaron por tamaño mediante electroforesis en geles de acrilamida (SDS-PAGE) y se transfirieron a una membrana de nitrocelulosa. Para la mayoría de los anticuerpos utilizados las membranas se bloquearon

con una solución de 5% leche desnatada en polvo en TBS-T (50 mM Tris-HCl, pH 7,6, 150 mM NaCl, 0,05% Tween-20). La solución de bloqueo para anticuerpos contra proteínas fosforiladas fue 3% leche desnatada en polvo en TBS-T suplementado con inhibidores de fosfatasas (1 mM Na_3VO_4 y 1 mM NaF). Para los anticuerpos contra CREB, fosfo-CREB, NF-κB y PPARγ las membranas se bloquearon con 5% albúmina fetal bovina (BSA) en TBS-T con 0,1% Tween-20. Para fosfo-AKT, el bloqueo se realizó con 10% leche desnatada en polvo en TBS (50 mM Tris-HCl, pH 7,6, 150 mM NaCl) suplementado con 1 mM Na_3VO_4 y 1 mM NaF.

Las membranas se incubaron durante toda la noche a 4ºC con el anticuerpo primario diluido en la solución de bloqueo correspondiente, excepto para fosfo-AKT, que se incubó en 5% leche desnatada en polvo en TBS-T con 0,1% Tween-20 suplementado con 1 mM Na_3VO_4 y 1 mM NaF. Posteriormente las membranas se incubaron con anticuerpos secundarios fluorescentes y la señal se detectó con un sistema de imagen Odyssey® CLx (LICOR Biosciences, Nebraska, EEUU).

La cuantificación proteica se llevó a cabo con el software Image Studio™ (LI-COR Biosciences, Nebraska, EEUU). Como proteínas de control interno se usaron la β-tubulina III y la gliceraldehido-3-fosfato deshidrogenasa (GAPDH) para el homogeneizado total y la fracción citosólica, y el antígeno nuclear de proliferación celular (PCNA) para la fracción nuclear. Se calculó la media de los duplicados de cada muestra y se representaron los resultados como porcentaje respecto al grupo vehículo.

Ensayo por inmunoabsorción ligado a enzimas (ELISA)

Se utilizaron kits de ELISA para la detección de TNFα y IL-6 (Life Technologies Corporation, California, EEUU) y BDNF (Cloud-Clone Corp., Texas, EEUU) siguiendo las instrucciones del proveedor. La densidad óptica se determinó a 450 nm con un Mithras LB 940 (Berthold technologies, Baden-Württemberg, Alemania). Las muestras se analizaron por duplicado, y los resultados se expresaron en pg de la proteína diana/ml.

Estudios de inmunohistoquímica (IHQ)

Para la detección de c-Fos, los cerebros perfundidos se cortaron en secciones coronales seriadas de 40 µm, las cuales se incubaron en *free-floating* con 0,3% peróxido de hidrogeno (H_2O_2) en PBS, y se bloquearon con 0,2% Triton X-100 y 3% suero normal de burro (NDS) en PBS. Las secciones se incubaron con un anticuerpo primario para c-Fos (sc-52, Santa Cruz Biotechnology, Texas, EEUU) a una dilución 1:500 en PBS con 3% NDS, a 4ºC durante toda la noche. Posteriormente las secciones se incubaron con un anticuerpo secundario biotinilado (Jackson ImmunoResearch Europe Ltd., Cambridgeshire, GB) a una

dilución de 1:500 en 0,3% Triton X-100, 3% NDS en PBS, y se formaron complejos de avidina-biotina con el kit Vectastain ABC (Vector, California, EEUU). Finalmente, las células positivas para c-Fos se marcaron con un kit de diaminobenzidina (DAB) (Vector, California, EEUU). Las secciones se contratiñeron con hematoxilina y se montaron en portaobjetos.

Las imágenes de la IHQ se digitalizaron con un escáner AxioScanZ.1 (Carl Zeiss) y se procesaron con el software ZEN 2.3 SP1 (Carl Zeiss Microscopy GmbH, Alemania). Las áreas cerebrales que se cuantificaron fueron la corteza prelímbica (PL; de 4,7 mm a 2,2 mm de Bregma), la corteza infralímbica (IL; de 3,2 mm a 2,2 mm de Bregma), y el núcleo dorsal del rafe (de -7,3 mm a -8,0 mm de Bregma) (Paxinos y Watson, 1987). Los resultados se expresaron como células c-Fos positivas por área (mm^2).

Cromatografía líquida de alto rendimiento (HPLC)

Las muestras se diluyeron 1:5 - 1:9 peso/volumen en agua milli-Q, se homogeneizaron mecánicamente, y se añadió ácido perclórico a una concentración final del 1,2%. Las muestras se centrifugaron a 16000xg durante 15 min a 4ºC, se recogió el sobrenadante, y se pasó por un filtro de 0,45 µm. El sistema de HPLC usado fue un ALEXYS® Neurotransmitter Analyzer (Antec Scientific, Leiden, Países Bajos).

Detección de serotonina, noradrenalina, triptófano, y quinurenina

Los niveles de dichas moléculas se determinaron con una columna Acquity UPLC® BEH C18, 1,7 µm, 1 × 100 mm (Waters, Massachusetts, EEUU). La fase móvil estaba compuesta por 100 mM ácido cítrico, 100 mM ácido fosfórico, pH 6, 0,1 mM EDTA, 950 mg/L ácido octanosulfónico, y 5% acetonitrilo. Se usó un potencial de oxidación de 0,46 V para serotonina y noradrenalina, y de 0,7 V para triptófano y quinurenina.

Detección de glutamato, GABA, y 3-hidroxiquinurenina (3-HK)

Los niveles de dichas moléculas se determinaron con una columna Acquity UPLC® HSS T3, 1,8 µm, 1 x 50 mm (Waters, Massachusetts, EEUU). Previo al paso por la columna, se realizó una derivatización automática con orto-ftalaldehído (OPA) y sulfito sódico. Se utilizaron dos fases móviles. La fase utilizada para la separación estaba compuesta por 50 mM ácido fosfórico, 50 mM ácido cítrico, 0,1 mM EDTA, pH 3,5, y 2% acetonitrilo. La fase utilizada para la post-separación estaba compuesta por 50 mM ácido fosfórico, 50 mM ácido cítrico, 0,1 mM EDTA, pH 3,5, y 50% acetonitrilo. Se usó un potencial de oxidación de 0,85 V.

Cuantificación

Los resultados se obtuvieron en pg/µl. Dichos valores se corrigieron por el volumen y el peso del tejido de cada muestra y se presentaron finalmente como pg-ng/mg de tejido.

9. Análisis estadístico

El análisis estadístico de los resultados se realizó con el software GraphPad Prism 8.4.3 (GraphPad Software Inc., California, EEUU). Los resultados se expresaron como media ± error estándar de la media (S.E.M.). El nivel de significación se fijó en $p<0,05$.

La comparación estadística de dos poblaciones con distribución normal se realizó con una prueba *t-Student* no pareada. Para comparar más de dos poblaciones compartiendo una misma variable se utilizó el análisis de la varianza (ANOVA) de una vía. Cuando dos variables podían interaccionar entre ellas, se usó el ANOVA de dos vías. En los ANOVA, la comparación estadística entre grupos se complementó con la prueba *posthoc* Newman-Keuls. Para los estudios de correlación se usó el coeficiente de correlación de Pearson (r).

RESULTADOS Y DISCUSIÓN

OBJETIVO 1: Efectos de tipo antidepresivo del cannabidiol: sitio y mecanismo de acción

1. Infusión bilateral de cannabidiol en la corteza infralímbica: efectos agudos

Conducta de tipo depresiva y ansiosa

La infusión bilateral de cannabidiol (CBD) en la corteza infralímbica (IL) redujo el tiempo de inmovilidad en la prueba de la natación forzada (FST) (figura 3A), produciendo por tanto un efecto agudo de tipo antidepresivo, como se había descrito previamente (Sartim et al., 2016). Este resultado está en línea con diversos estudios que han demostrado la importancia de la corteza IL para los efectos antidepresivos de distintos tratamientos (Fuchikami et al., 2015; Jiménez-Sánchez et al., 2016; Fukumoto et al., 2019). Es importante destacar que, debido a que utilizamos el FST modificado (estudio de los parámetros de natación y movimientos verticales además de la inmovilidad), demostramos por primera vez que **la disminución aguda de la desesperación conductual (*behavioural despair*) producida por la infusión IL de CBD fue debida a un incremento en el tiempo de natación** (figura 3A), concordando con los efectos agudos descritos tras su administración sistémica (Réus et al., 2011; Shbiro et al., 2019). En base a estos

resultados podemos postular que el efecto agudo de tipo antidepresivo inducido por la infusión IL de CBD está mediado por la activación del sistema serotonérgico, ya que un incremento en el tiempo de natación se ha asociado con una respuesta mediada por serotonina (Detke et al., 1995; Cryan et al., 2002, 2005).

Dicha infusión no afectó a la locomoción de los animales, ya que no hubo cambios en la distancia total recorrida en la prueba del campo abierto (OFT) (figura 3B). La ausencia de efectos agudos en la locomoción se había descrito anteriormente, tanto tras la infusión de CBD en la corteza IL (Lemos et al., 2010; Sartim et al., 2016), como tras su administración sistémica (Zanelati et al., 2010; Sales et al., 2019).

Por otra parte, la infusión IL de CBD no indujo cambios en el tiempo central en el OFT (figura 3B), por lo que no tuvo efectos en la conducta de tipo ansiosa. Esto concuerda con los resultados obtenidos tras la infusión IL de CBD a la misma dosis (60 nmoles) en la prueba del laberinto elevado (EPM) (Marinho et al., 2015). Sin embargo, estos autores describieron que dicha infusión produjo un efecto de tipo ansiolítico a dosis más bajas (15 y 30 nmoles). Esta curva de dosis-respuesta en forma de U invertida para los efectos de tipo ansiolítico del CBD también se han observado tras su administración sistémica (*revisado en* Blessing et al., 2015), apoyando la idea de que el CBD tiene efectos de tipo ansiolítico a dosis bajas, pero no a dosis altas (Campos y Guimaraes, 2008; Luján et al., 2018). Aun así, la corteza IL no está entre las regiones cerebrales comúnmente seleccionadas para estudiar el efecto ansiolítico del CBD, tales como la porción dorsolateral de la sustancia gris periacueductal y el núcleo del lecho de la estría terminal (*revisado en* Blessing et al., 2015). Estas evidencias sugieren que los efectos de tipo ansiolítico del CBD dependen de la dosis y de la estructura cerebral seleccionada.

En definitiva, **nuestros resultados confirman que la corteza IL es un área clave para los efectos agudos de tipo antidepresivo del CBD, pero no para sus efectos de tipo ansiolítico.**

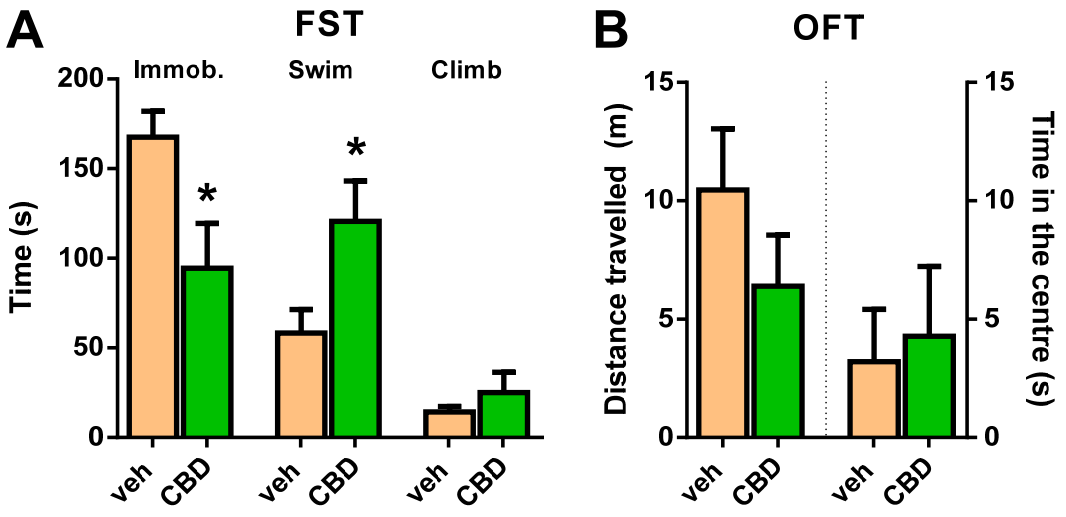

Figura 3. Efectos agudos de la infusión infralímbica bilateral de CBD en la conducta de tipo depresiva y ansiosa y en la locomoción. A) Tiempo de inmovilidad, natación, y movimientos verticales en la prueba de la natación forzada. B) Distancia recorrida y tiempo en el centro en la prueba del campo abierto. Los resultados están expresados como media ± S.E.M. Prueba *t-Student* no pareada *p<0,05 *vs* el grupo vehículo. n= 6 - 8 animales por grupo.

Marcadores de neuroplasticidad en la corteza prefrontal

La infusión IL de CBD indujo la activación de las proteínas ERK y mTOR e incrementó los niveles de BDNF en la corteza prefrontal de forma aguda (figura 4), siendo esta la primera vez que se estudia el efecto de dicha infusión en marcadores de neuroplasticidad. Nuestros resultados son similares a los descritos tras la infusión de CBD en el hipocampo (Sartim et al., 2018) y tras su infusión sistémica aguda (Sartim et al., 2018; Sales et al., 2019). Además, la infusión intracerebroventricular de un antagonista del receptor de BDNF y de un inhibidor de mTOR bloquearon el efecto antidepresivo agudo del CBD (Sales et al., 2019), sugiriendo que BDNF y mTOR median dichos efectos. Los resultados obtenidos están en línea con el mecanismo de acción descrito para fármacos antidepresivos de acción rápida como la ketamina (Li et al., 2010; Duman et al., 2012; Aleksandrova et al., 2017), y con los efectos observados tras la administración crónica de antidepresivos clásicos como los inhibidores selectivos de la recaptación de serotonina (ISRS) (Liu et al., 2015; Björkholm y Monteggia, 2016).

Por el contrario, la infusión IL de CBD no produjo cambios de forma aguda en los niveles de β-catenina, fosfo-Ser9-GSK-3β, ni fosfo-CREB en la corteza prefrontal (figura 4), sugiriendo que su efecto agudo de tipo antidepresivo no está mediado por la vía de

Wnt/β-catenina. Estos resultados concuerdan con la ausencia de cambios en la activación de AKT (figura 4), ya que la activación de dicha proteína fosforilaría e inactivaría a GSK-3β (Grimes y Jope, 2001), lo que a su vez llevaría a la activación de la vía de β-catenina e incrementaría la actividad de CREB (Pilar-Cuéllar et al., 2014). La vía de Wnt/β-catenina se ha relacionado más con la proliferación celular y ejerce una regulación clave sobre la neurogénesis adulta (Lie et al., 2005; Wexler et al., 2009), lo cual sugiere que los efectos en esta vía de señalización tendrían mayor relevancia en el hipocampo.

En relación con los mecanismos a través de los cuales el CBD podría estar modulando estas vías de señalización, se ha descrito que dos de sus dianas, el receptor 5-HT_{1A} (*revisado en* Masson et al., 2012; Zhou et al., 2014b; Jiang et al., 2016; Rojas y Fielder, 2016; Albert y Vahid-Ansari, 2019) y los receptores CB_1 y CB_2 (Mallipeddi et al., 2017; *revisado en* Luján y Valverde, 2020) promueven la activación de las vías de plasticidad sináptica AKT, ERK, mTOR y BDNF. Por consiguiente, ambos tipos de receptores podrían estar contribuyendo al incremento en los niveles de fosfo-ERK, fosfo-mTOR, y BDNF observado tras la infusión aguda de CBD en la corteza IL.

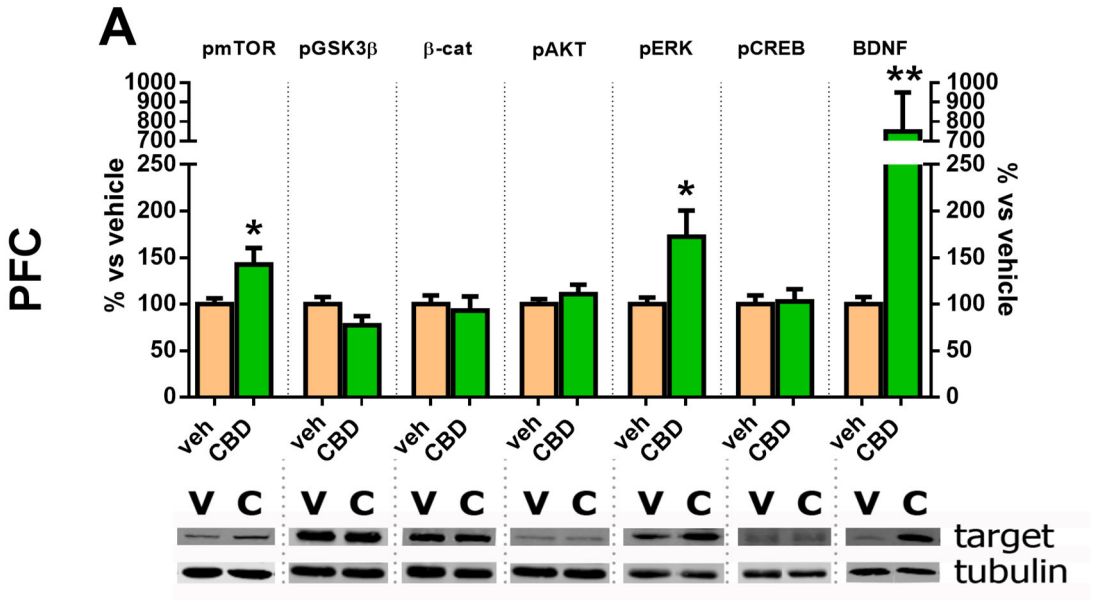

Figura 4. Expresión de diversos marcadores de neuroplasticidad en la corteza prefrontal tras la infusión bilateral aguda de cannabidiol en la corteza infralímbica. Se muestran bandas representativas del Western Blot de cada marcador y del correspondiente control interno. Los resultados están expresados en porcentaje respecto el grupo vehículo y como media ± S.E.M. Prueba *t-Student* no pareada *p<0,05, **p<0,01 *vs* el grupo vehículo. n= 6 - 8 animales por grupo.

En resumen, nuestros resultados muestran que **los efectos agudos de tipo antidepresivo del CBD están asociados a la activación de la vía de mTOR a través de ERK y al incremento de BDNF en la corteza prefrontal, sin la contribución de AKT ni de la vía Wnt/β-catenina.**

Neurotransmisores en la corteza prefrontal y en el núcleo dorsal del rafe

En primer lugar, cabe mencionar que para interpretar correctamente los resultados obtenidos en los niveles de neurotransmisores hay que tener en cuenta: 1) que han sido determinados en muestras cerebrales *postmortem* 30 min después de la conducta; y 2) que los valores obtenidos incluyen tanto los neurotransmisores liberados al espacio sináptico como los contenidos dentro de las vesículas neuronales.

La infusión IL de CBD produjo una disminución aguda de los niveles de glutamato (GLUT) y noradrenalina (NA), sin cambios en los niveles de ácido γ-aminobutírico (GABA) ni de serotonina (5-HT) en la corteza prefrontal (PFC), mientras que en el núcleo dorsal del rafe (DRN) produjo una disminución aguda de los niveles de glutamato y GABA, sin modificar los de 5-HT y NA (figura 5).

En relación con el glutamato, hemos observado que sus niveles en la corteza prefrontal aparecen elevados en el grupo experimental que recibió la infusión aguda de vehículo en la corteza IL (figura 5), en comparación con los resultados obtenidos después de 24 h de dicha administración (figura 9) o tras la infusión de vehículo en el DRN (figura 12). Esto podría deberse a una respuesta inflamatoria aguda provocada por la técnica de infusión (Travis et al., 2019), la cual induciría un incremento de excitotoxicidad por glutamato (Maes et al., 2011; Barone, 2019). Tal como se muestra en el segundo objetivo de esta tesis (Florensa-Zanuy et al., 2021), el CBD disminuye la activación de vías inflamatorias, pudiendo mediar así el descenso de los niveles de glutamato observado en PFC, que parece acompañarse de cambios homeostáticos en los niveles de GABA. Los cambios en los niveles corticales de glutamato podrían repercutir tanto en los niveles de glutamato (vía directa PFC-DRN) como de GABA (vía indirecta PFC-DRN) en el DRN (figura 5 C y D). Sin embargo, dado el efecto de tipo antidepresivo observado en el FST, también podría ser posible que haya habido más liberación de neurotransmisores de las vesículas intracelulares en estas áreas cerebrales, lo que conllevaría su metabolización y la disminución de sus niveles.

Debido al incremento de la natación observado en el FST, esperábamos encontrar un incremento de los niveles de 5-HT en PFC. Sin embargo, se ha descrito mediante estudios de microdiálisis que el incremento en dichos niveles tras la administración de CBD

(Murillo-Rodríguez et al., 2006; Linge et al., 2016), ketamina (Nishitani et al., 2014), y ácido dihidrocaínico (DHK) (Gasull-Camós et al., 2018) es de corta duración, por lo que especulamos que en el momento en que determinamos los niveles, éstos podrían haber vuelto a su estado basal. En línea con nuestros resultados obtenidos en DRN, otras estrategias con efecto antidepresivo rápido como la administración de ketamina y la estimulación cerebral profunda en la corteza IL, no incrementaron los niveles de 5-HT en esta área (Jiménez-Sánchez et al., 2016; Pham et al., 2017).

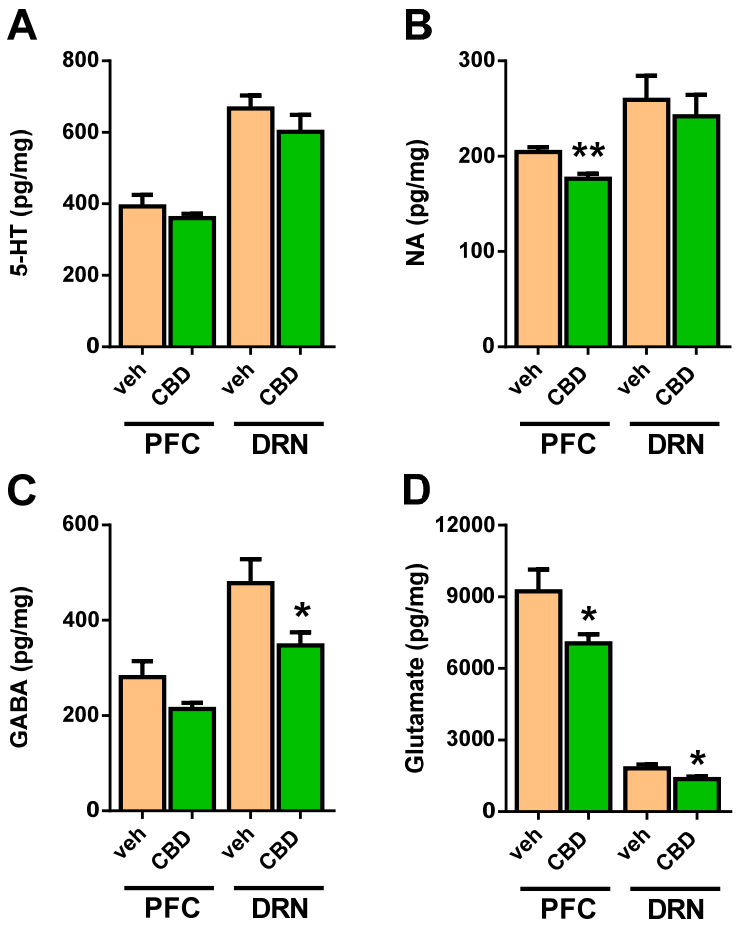

Figura 5. Niveles de neurotransmisores en la corteza prefrontal (PFC) y núcleo dorsal del rafe (DRN) tras la infusión aguda bilateral infralímbica de CBD. A) Serotonina. B) Noradrenalina. C) GABA. D) Glutamato. Los resultados están expresados como media ± S.E.M. Prueba *t-Student* no pareada **p*<0,05, ***p*<0,01 *vs* el grupo vehículo. n= 6 - 8 animales por grupo.

Dado que la infusión IL de CBD no modificó el tiempo de movimientos verticales en el FST, y este se ha asociado a la neurotransmisión noradrenérgica (Detke et al., 1995; Cryan et

al., 2002, 2005), no esperábamos encontrar cambios en los niveles de NA. Además, se ha postulado que el sistema noradrenérgico no estaría implicado en los efectos de tipo antidepresivo del CBD (Sales et al., 2018). Sin embargo, la reducción que observamos en los niveles de glutamato tras la infusión de CBD podría causar una menor activación de la conexión PFC-locus coeruleus excitatoria (Kim y Lee, 2003), conllevando una menor activación noradrenérgica. De cualquier manera, el hecho de que los tres núcleos monoaminérgicos (área tegmental ventral, locus coeruleus y DRN) se regulen mutuamente (Tritschler et al., 2018), y el locus coeruleus en particular reciba un gran número de aferencias y posea una compleja heterogeneidad molecular (Schwarz y Luo, 2015), dificulta la interpretación de los cambios en los niveles de NA inducidos por la infusión IL de CBD.

Activación neuronal en la corteza prefrontal y en el núcleo dorsal del rafe

La exposición a un estrés agudo conductual (OFT+FST) incrementó de forma significativa los niveles de c-Fos en el DRN y produjo una tendencia en las cortezas IL y prelímbica (PL) (figura 6), tal como se había descrito previamente tras diferentes estímulos estresantes (Kovács, 1998; Pilar-Cuéllar et al., 2017). En este sentido, en animales *knock-down* para el transportador astrocítico de glutamato, que presentan un fenotipo de tipo depresivo, se ha sugerido que el incremento en los niveles de c-Fos en DRN es debido a un mayor tono GABAérgico (Fullana et al., 2019). En base a esto, el incremento que de c-Fos que vemos en el DRN con la exposición a estrés podría deberse a un incremento de la actividad de las neuronas GABAérgicas.

La infusión IL de CBD incrementó los niveles de c-Fos en el DRN en animales *naïve* (figura 6C), de acuerdo con los efectos descritos tras su administración intracerebroventricular (Murillo-Rodríguez et al., 2006), y en el DRN (Murillo-Rodríguez et al., 2008). Los resultados de dichos estudios se relacionaron con un incremento del *firing* del DRN (Wu et al., 2004) y un incremento de la liberación de serotonina (Gartside et al., 1995), pudiendo esto indicar que la activación que observamos en el DRN tras la infusión IL de CBD en animales *naïve* podría deberse a la activación de las neuronas serotonérgicas. De acuerdo con esto, se ha descrito que la administración de fármacos con acción antidepresiva rápida en la PFC medial incrementó los niveles de c-Fos en las neuronas serotonérgicas del DRN (Fukumoto et al., 2016; Gasull-Camós et al., 2018). Por el contrario, la infusión IL de CBD no modificó los niveles de c-Fos en las cortezas IL y PL de animales *naïve* (figura 6 A y B), tal como se había descrito tras su administración sistémica (Lemos et al., 2010), la administración de ketamina en PFC (Tarrés-Gatius et al., 2020), y el tratamiento crónico con fluoxetina (Lino-de-Oliveira et al., 2001).

La infusión IL de CBD moduló de forma diferente los efectos del estrés dependiendo del área cerebral: no alteró los efectos inducidos por el estrés en la corteza PL (figura 6A) pero nuestros resultados apuntan a un bloqueo de los efectos del estrés en la corteza IL (figura 6B). En línea con los resultados obtenidos en la corteza IL, se ha descrito que la administración de CBD (Lemos et al., 2010; Hartmann et al., 2019) y de citalopram (Kuipers et al., 2006) redujo el incremento de c-Fos inducido por estrés en diversas áreas cerebrales, y que la inactivación transitoria de la corteza IL induce efectos de tipo antidepresivo (Scopinho et al., 2010; Slattery et al., 2011).

En el DRN, aunque tanto el estrés agudo como la infusión IL de CBD indujeron la expresión de c-Fos (figura 6C), el ANOVA de dos vías mostró una interacción significativa entre ambos factores. Esto podría indicar que el CBD modula de forma opuesta la actividad del DRN en condiciones basales o de estrés, lo que podría ser debido a una activación diferencial de las neuronas GABAérgicas o serotonérgicas. Esta modulación podría participar en el efecto de tipo antidepresivo del CBD, ya que la administración crónica de antidepresivos clásicos también modula la expresión de c-Fos en DRN tanto en animales *naïve* como estresados (Lino-de-Oliveira et al., 2001; Elizalde et al., 2010).

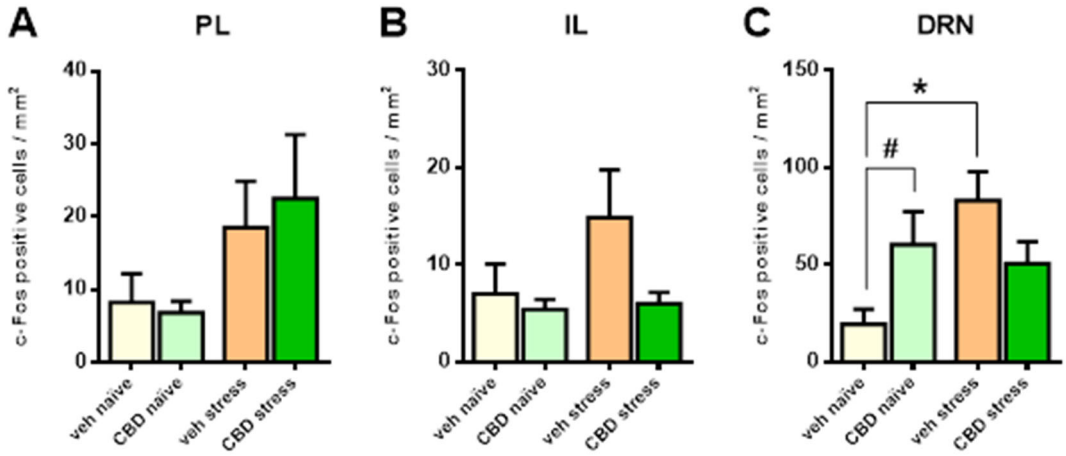

Figura 6. Evaluación de los niveles de c-Fos mediante inmunohistoquímica en las cortezas prelímbica (A) e infralímbica (B) y en el núcleo dorsal del rafe (C) tras la infusión aguda IL de CBD y la exposición a estrés agudo. Los resultados están expresados como media ± S.E.M. ANOVA de dos vías seguido de la prueba *posthoc* Newman Keuls **p*<0,05. Prueba *t-Student* no pareada $^{\#}p$<0,05. n= 4 - 6 animales por grupo.

Nuestros estudios de c-Fos muestran que **la infusión IL de CBD incrementa la actividad neuronal en el DRN en animales *naïve*, y sugieren que el CBD modula el incremento de actividad neuronal inducido por estrés agudo en la corteza infralímbica y el núcleo dorsal del rafe**. En futuros estudios, sería interesante estudiar el tipo de neurona que se activa en cada caso.

2. Infusión bilateral de cannabidiol en la corteza infralímbica: efectos tras 24 h y en presencia de un bloqueo del receptor 5-HT$_{1A}$ presináptico

Conducta de tipo depresiva

La infusión IL de CBD no produjo ningún efecto en el FST ni en el OFT 24 h después de la administración, habiendo perdido por tanto el efecto de tipo antidepresivo observado de forma aguda. Para estudiar si la activación de los receptores 5-HT$_{1A}$ presinápticos podría estar participando en la pérdida de efecto, administramos un antagonista selectivo de dicho receptor, WAY100635, a una dosis que bloquea mayoritariamente los receptores presinápticos del DRN (Serres et al., 2000; Ago et al., 2003; Carey et al., 2005). Dicha aproximación produjo un efecto de tipo antidepresivo 24 h después de la infusión IL de CBD, reduciendo el tiempo de inmovilidad e incrementando el de natación y el de movimientos verticales en el FST (figura 7). Estos resultados sugieren que **la acción inhibitoria sobre el *firing* del DRN que ejerce la activación de los receptores 5-HT$_{1A}$ presinápticos podría impedir la prolongación de los efectos antidepresivos de la infusión IL de CBD.** De forma similar, el antagonismo de los receptores 5-HT$_{1A}$ presinápticos también prolongó los efectos de tipo antidepresivo de la infusión IL de ácido dihidrocaínico (DHK) (Gasull-Camós et al., 2018). Además, varios estudios clínicos han demostrado que la inhibición de los receptores 5-HT$_{1A}$ presinápticos mejora el efecto terapéutico de los ISRSs (*revisado en* Artigas et al., 2001; Albert y François, 2010).

El efecto beneficioso sobre la natación podría ser debido a la potenciación serotonérgica producida por el antagonismo del autoreceptor de serotonina, ya que una sobre-activación de estos receptores reduce el *firing* de las neuronas serotonérgicas (Gartside et al., 1995), impidiendo el efecto antidepresivo. Por otro lado, el incremento del tiempo de movimientos verticales sugiere un incremento de la neurotransmisión noradrenérgica (Detke et al., 1995; Cryan et al., 2002; 2005), el cual podría estar mediado por la activación de los receptores 5-HT$_{1A}$ postsinápticos (Hajós-Korcsok et al., 1999).

En base a estos resultados, hipotetizamos que **la falta de efecto de tipo antidepresivo observado 24 h tras la infusión IL de CBD es debida a cambios adaptativos que resultan**

en un mayor tono inhibitorio serotonérgico mediado por el receptor 5-HT$_{1A}$ presináptico.

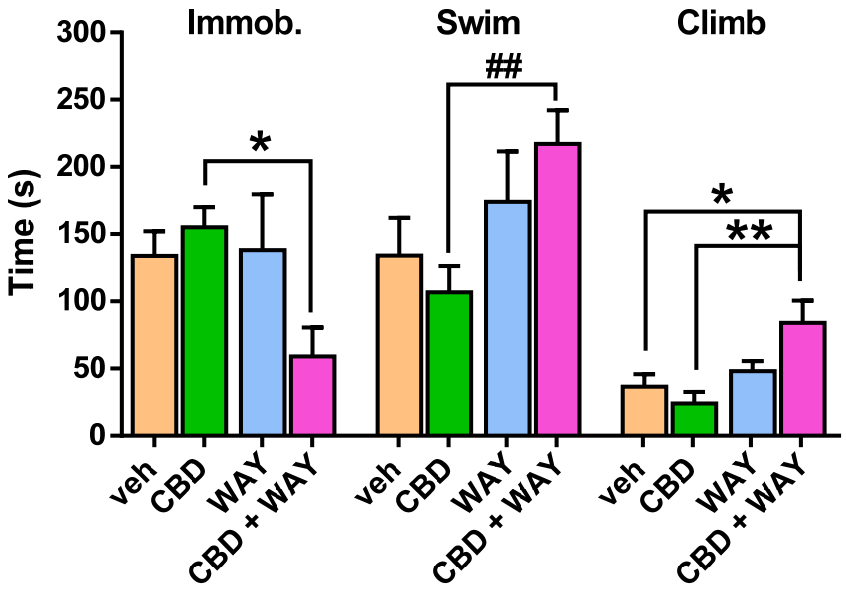

Figura 7. Efecto de la administración sistémica de WAY100635 (0,3 mg/kg) en el comportamiento de tipo depresivo evaluado en la prueba de la natación forzada 24 h después de la infusión IL de CBD (se muestra de izquierda a derecha el tiempo de inmovilidad, natación, y de movimientos verticales). Los resultados están expresados como media ± S.E.M. ANOVA de dos vías seguido de la prueba *posthoc* Newman Keuls **p*<0,05, ***p*<0,01. Prueba *t-Student* no pareada $^{\#\#}p$<0,01. n= 8 - 10 animales por grupo.

Marcadores de neuroplasticidad en la corteza prefrontal

El cambio más relevante que observamos al estudiar los marcadores de neuroplasticidad en PFC 24 h después de la infusión IL de CBD fue la pérdida de activación de ERK (figura 8) en comparación con los resultados agudos (figura 4). Sin embargo, tanto los niveles de **fosfo-mTOR** como los de **BDNF** seguían elevados al igual que tras la administración aguda de CBD, lo que **sugiere que la activación de dichas vías de señalización en la corteza prefrontal es importante para el efecto agudo de tipo antidepresivo del CBD, pero no suficiente para observarse un efecto mantenido**.

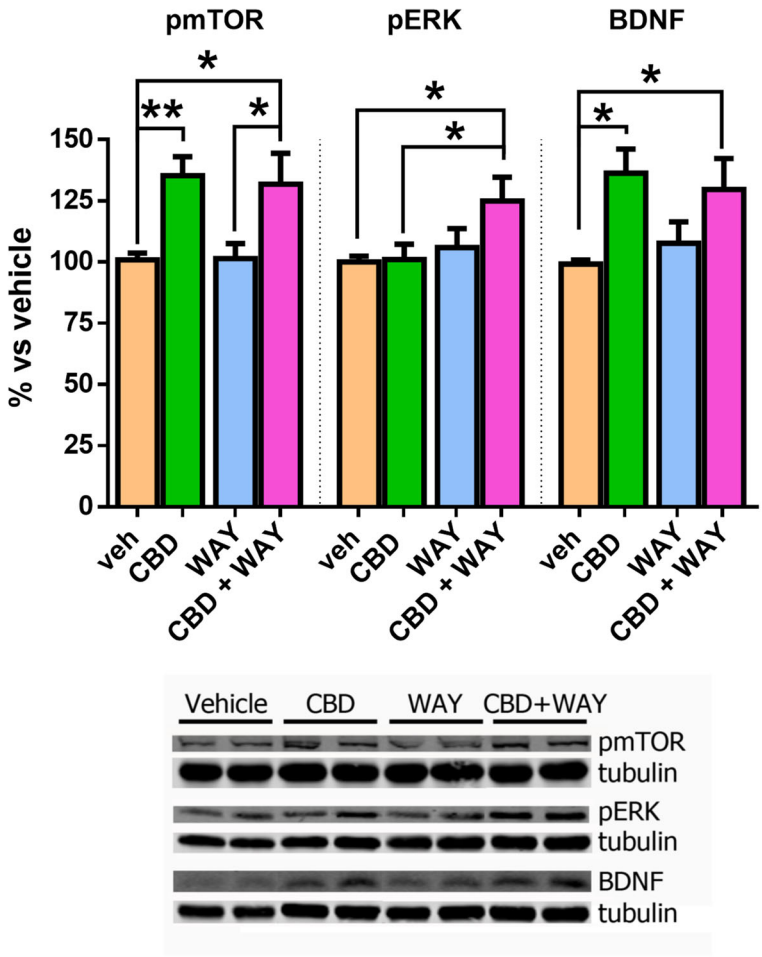

Figura 8. Expresión de marcadores de neuroplasticidad en la corteza prefrontal 24 h después de la infusión infralímbica de CBD y tras la administración sistémica de WAY100635 (0,3 mg/kg). Se muestran bandas representativas del Western Blot de cada marcador y del correspondiente control interno. Los resultados están expresados en porcentaje respecto el grupo vehículo y como media ± S.E.M. ANOVA de dos vías seguido de la prueba *posthoc* Newman Keuls **p*<0,05, ***p*<0,01. n= 8 - 10 animales por grupo.

Con el bloqueo de los receptores 5-HT_{1A} presinápticos observamos una recuperación del incremento de fosfo-ERK, sin cambios en los niveles ya elevados de fosfo-mTOR y BDNF en la corteza prefrontal (figura 8), un patrón similar a los resultados obtenidos tras la administración aguda de CBD en la corteza IL (figura 4). La combinación de los resultados conductuales y moleculares observados 24 h tras la infusión de CBD sugieren que **la activación de ERK es importante para el efecto de tipo antidepresivo del CBD**, y concuerdan con el bloqueo del efecto de tipo antidepresivo de la ketamina mediante la inhibición de ERK (Li et al., 2010). La activación de ERK inducida por la administración de CBD y WAY100635 podría producirse gracias a un incremento del tono serotonérgico en

la corteza prefrontal, que induciría la activación de dicha proteína a través de los receptores 5-HT_{1A} postsinápticos (Rojas y Fielder, 2016; Albert y Vahid-Ansari, 2019).

Neurotransmisores en la corteza prefrontal y en el núcleo dorsal del rafe

Veinticuatro horas después de la infusión de CBD en la corteza IL no se observaron cambios en los niveles de serotonina de la PFC ni del DRN (figura 9A), en paralelo a la falta de efecto de tipo antidepresivo. Sin embargo, mediante el uso de microdiálisis *in vivo* se han descrito mayores niveles extracelulares de serotonina en el DRN a las 24 h de la infusión de DHK en la corteza IL, lo que se asoció con la falta de efecto antidepresivo. Dicho efecto reapareció cuando se administró WAY100635 a una dosis que bloquea los receptores 5-HT_{1A} presinápticos (Gasull-Camós et al., 2018). En nuestro caso, dicha estrategia también recuperó el efecto de tipo antidepresivo del CBD, aunque sin modificar los niveles de serotonina en ambas áreas. En este sentido, se ha descrito que la activación de un subgrupo de neuronas serotonérgicas puede no traducirse en cambios en los niveles extracelulares de serotonina en el DRN (López-Gil et al., 2019), aunque no podemos descartar que la ausencia de cambios observada se deba al tipo de técnica utilizada.

En los animales tratados con CBD y el antagonista del receptor 5-HT_{1A} hubo un incremento de los niveles de noradrenalina en el DRN (figura 9B), lo que podría explicarse por un incremento del *firing* del locus coeruleus, el cual proyecta al DRN (Tritschler et al., 2018), y concuerda con el incremento en los movimientos verticales en el FST (figura 7). Otros autores detectaron un incremento de noradrenalina en la PFC con diferentes estrategias antidepresivas (Jiménez-Sánchez et al., 2016; López-Gil et al., 2019), aunque nosotros no observamos diferencias en esta área cerebral.

Después de 24 h de la infusión IL de CBD, los niveles de glutamato estaban incrementados en la PFC y el DRN, y los niveles de GABA estaban incrementados en la PFC y disminuidos en el DRN (figura 9 C y D), pero no observamos un efecto de tipo antidepresivo. La recuperación del efecto de tipo antidepresivo de la infusión IL de CBD tras 24 h con la administración de WAY100635 fue paralela a un incremento en los niveles de glutamato en el DRN, sin modificar los niveles de glutamato en la PFC, o los de GABA en ambas estructuras, comparado con el grupo que sólo recibió CBD (figura 9 C y D). Se ha descrito que el efecto de tipo antidepresivo 24 h tras la infusión de ketamina (Pham et al., 2020) o de (2R,6R)-hidroxinorketamina (Pham et al., 2018) en la PFC medial está asociado a un incremento de los niveles de glutamato, y a una activación del receptor AMPA de glutamato en el DRN (Pham et al., 2020). Por tanto, el incremento de la ratio glutamato/GABA en el DRN de los animales tratados con CBD y WAY100635 podría

favorecer el efecto de tipo antidepresivo. **Nuestros resultados sugieren que a las 24 h de la infusión de CBD en la corteza IL la transmisión glutamatérgica del circuito PFC-DRN estaría incrementada, aunque sin producir un efecto antidepresivo. El bloqueo de los receptores 5-HT$_{1A}$ presinápticos recuperaría la respuesta antidepresiva, en paralelo a una potenciación de los niveles de glutamato en el DRN.**

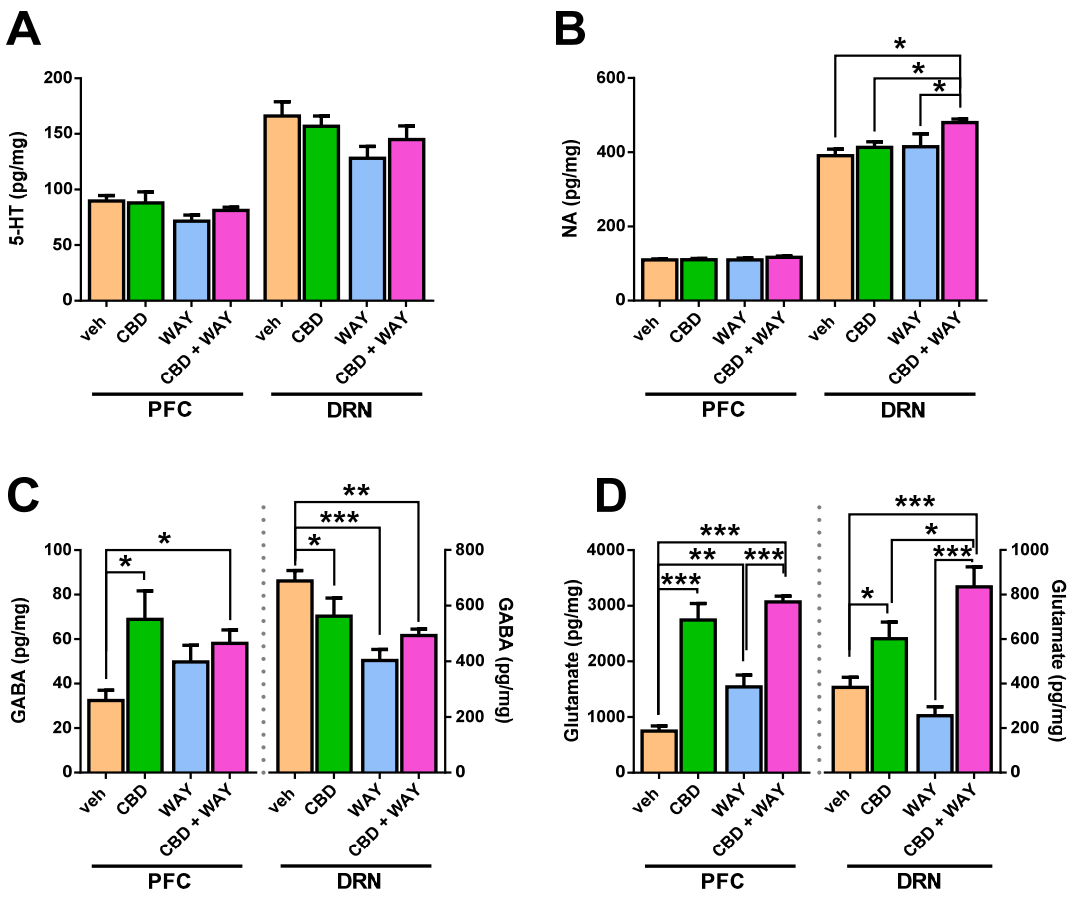

Figura 9. Niveles de neurotransmisores en la corteza prefrontal (PFC) y núcleo dorsal del rafe (DRN) 24 h tras la infusión bilateral infralímbica de CBD, y en presencia de un antagonismo de los receptores 5-HT$_{1A}$ presinápticos. A) Serotonina. B) Noradrenalina. C) GABA. D) Glutamato. Los resultados están expresados como media ± S.E.M. ANOVA de dos vías seguido de la prueba *posthoc* Newman Keuls *p<0,05, **p<0,01, ***p<0,001. n= 5 - 7 animales por grupo.

3. Infusión de cannabidiol en el núcleo dorsal del rafe: efectos agudos

Conducta de tipo depresiva y ansiosa

Hasta donde sabemos, esta es la primera vez que se estudia el efecto conductual de **la infusión de CBD en el DRN.** Esta administración **no indujo cambios en el tiempo de inmovilidad en el FST de forma aguda, pero provocó un incremento en el tiempo de natación y un descenso en el de movimientos verticales** (figura 10A). Estos efectos no fueron debidos a cambios en la locomoción, ya que no observamos cambios en la distancia total medida en el OFT (figura 10B), como se ha descrito tanto con la administración sistémica de CBD (Zanelati et al., 2010), como con su infusión en varias áreas cerebrales (Lemos et al., 2010; Gomes et al., 2011; Hsiao et al., 2012).

Como se ha comentado anteriormente, el incremento en el tiempo de natación que hemos observado tras la infusión de CBD en IL y en el DRN, sugiere una activación serotonérgica (Detke et al., 1995; Cryan et al., 2002; 2005). No obstante, las diferencias conductuales entre la infusión de CBD en la corteza IL (disminución de la inmovilidad) y en el DRN (disminución del tiempo de movimientos verticales) sugiere que, **dependiendo del área de infusión, el CBD podría activar diferentes poblaciones de neuronas serotonérgicas en el DRN** con proyección a áreas cerebrales distintas. Esto se podría explicar por una activación serotonérgica más selectiva con la infusión en IL, y una activación inespecífica con la infusión en el DRN. Apoyando esta hipótesis, se ha descrito que para inducir el efecto de tipo antidepresivo basta con la activación de sólo una subpoblación de neuronas en la PFC medial (Hare et al., 2019), en el DRN (López-Gil et al., 2019), o de neuronas de la PFC medial que proyectan al DRN (Warden et al., 2012), y que la estimulación directa del DRN tiene consecuencias conductuales diferentes a la estimulación específica del circuito PFC medial-DRN (Warden et al., 2012). Además, la activación serotonérgica inespecífica que sugerimos tras la infusión de CBD en el DRN, a través de la activación de las proyecciones del DRN al locus coeruleus, que son de caracter inhibitorio (Segal, 1979; Dremencov et al., 2009; Tritschler et al., 2018), podría contribuir a la disminución del tiempo de movimientos verticales observada.

Los resultados conductuales de la infusión de CBD en el DRN sugieren un incremento del *firing* serotonérgico del DRN, que podría ser consecuencia del incremento de endocannabinoides inducido por CBD (Bisogno et al., 2001), el cual podría reducir la señalización por GABA y facilitar la activación de las neuronas serotonérgicas (Geddes et al., 2016). Además, la activación de los receptores 5-HT_{1A} por CBD responde a una curva de dosis-respuesta en forma de U invertida (Rock et al., 2012). Se ha propuesto que la infusión de dosis más bajas de CBD (32 nmoles) en el DRN reduce el *firing* serotonérgico (Rock et al., 2012), mientras que una dosis similar a la administrada en esta tesis (64

nmoles) producen un incremento del *firing* serotonérgico (Murillo-Rodríguez et al., 2008), en línea con nuestros resultados.

Tal como observamos tras la infusión IL de CBD, **la infusión en el DRN no modificó parámetros de ansiedad** en el OFT de forma aguda (figura 10B). Dado que la administración de CBD, tanto sistémica como en otras áreas cerebrales, produce efectos de tipo ansiolítico, **nuestros resultados sugieren que el DRN tampoco sería el sitio de acción para dichos efectos del CBD**. Además, tal como se ha comentado anteriormente, el efecto de tipo ansiolítico del CBD se produce generalmente a dosis más bajas (*revisado en* Blessing et al., 2015).

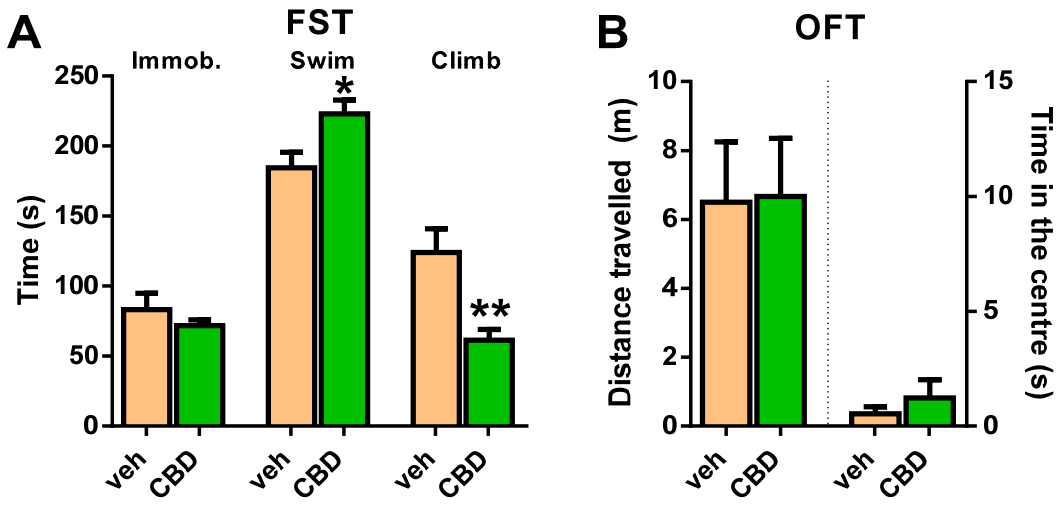

Figura 10. Efectos agudos de la infusión de CBD en el núcleo dorsal del rafe en la conducta de tipo depresiva y ansiosa y en la locomoción. A) Tiempo de inmovilidad, natación y movimientos verticales en la prueba de la natación forzada. B) Distancia total y tiempo en el centro en la prueba del campo abierto. Los resultados están expresados como media ± S.E.M. Prueba *t-Student* no pareada **p*<0,05, ***p*<0,01 *vs* el grupo vehículo. n= 10 - 12 animales por grupo.

Marcadores de neuroplasticidad en la corteza prefrontal

De entre la batería de marcadores de neuroplasticidad que estudiamos en la corteza prefrontal tras la administración aguda de CBD en el DRN, sólo detectamos un incremento en los niveles de BDNF (figura 11). Esto podría ser debido a un incremento en la liberación de serotonina en esta área –tal como sugieren los resultados conductuales y el incremento de los niveles de serotonina observado en la PFC (figura 12)– ya que los niveles de BDNF

son muy sensibles a la neurotransmisión serotonérgica (Zetterström et al., 1999; Mattson et al., 2004). Sin embargo, la magnitud de dicho incremento parece no ser suficiente para activar la vía de mTOR ni para producir un efecto de tipo antidepresivo, pudiendo tratarse de una respuesta por debajo del umbral necesario para producir un efecto (Gasull-Camós et al., 2018).

Por otro lado, el hecho de que no se activaran ERK y mTOR podría contribuir a la ausencia de cambios en el tiempo de inmovilidad en el FST (el parámetro comúnmente estudiado para determinar el efecto de tipo antidepresivo), tal como se ha descrito previamente por el bloqueo del efecto de tipo antidepresivo de la ketamina mediante la inhibición de ERK y mTOR (Li et al., 2010). Por tanto, podemos concluir **que el CBD es más eficiente induciendo vías de plasticidad sináptica cuando se infunde en la corteza infralímbica en comparación con su infusión en el núcleo dorsal del rafe, lo que podría explicar las diferencias conductuales observadas.**

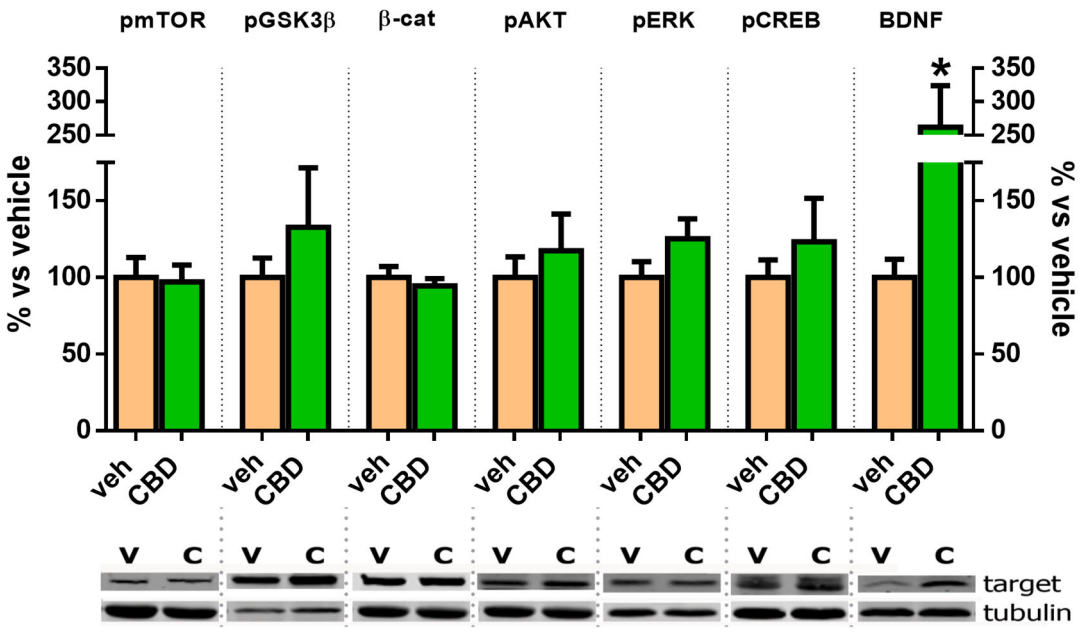

Figura 11. Expresión de varios marcadores de neuroplasticidad en la corteza prefrontal después de la infusión aguda de CBD en el núcleo dorsal del rafe. Se muestran bandas representativas del Western Blot de cada marcador y del correspondiente control interno. Los resultados están expresados en porcentaje respecto el grupo vehículo y como media ± S.E.M. Prueba *t-Student* no pareada *p<0,05 *vs* el grupo vehículo. n= 6 - 8 animales por grupo.

Neurotransmisores en la corteza prefrontal y en el núcleo dorsal del rafe

La **infusión de CBD en el DRN** incrementó los niveles de serotonina en la PFC, pero no en el DRN, sin modificar los niveles de noradrenalina, glutamato y GABA en ambas áreas (figura 12). De acuerdo con nuestros resultados, se ha descrito un incremento del tiempo de natación en el FST (Pham et al., 2017) y de los niveles serotonina en la PFC (Jiménez-Sánchez et al., 2016; Pham et al., 2017), sin cambios en los niveles de serotonina en el DRN. **Mayores niveles de serotonina en la PFC podrían explicar el incremento del tiempo de natación en el FST**, en concordancia con el efecto conductual observado tras la administración de fármacos que incrementan la neurotransmisión serotonérgica (*revisado en* Cryan et al., 2005). Tal como hemos propuesto anteriormente, la infusión de CBD en el DRN podría haber producido una activación indiscriminada de las neuronas serotonérgicas, mientras que la infusión en la corteza IL habría activado un subgrupo de neuronas serotonérgicas, lo que podría explicar que pudiéramos detectar el incremento de serotonina en la PFC tras su infusión en el DRN.

En relación con los niveles de noradrenalina no detectamos cambios en las áreas estudiadas, aunque en el FST observamos una reducción del tiempo de movimientos verticales, el cual se ha relacionado con la neurotransmisión noradrenérgica (Cryan et al., 2005). Esto podría deberse a cambios transitorios en dichos niveles durante la prueba conductual, tal como se ha propuesto para explicar la ausencia de cambios en los niveles de serotonina tras la infusión IL de CBD. Por otro lado, la ausencia de cambios en los niveles de glutamato y GABA podría explicar la falta de efectos de tipo antidepresivo tras la infusión de CBD en el DRN, ya que se ha descrito que varias estrategias antidepresivas modulan los niveles de estos neurotransmisores (Jiménez-Sánchez et al., 2016; López-Gil et al., 2019; Pham et al., 2020).

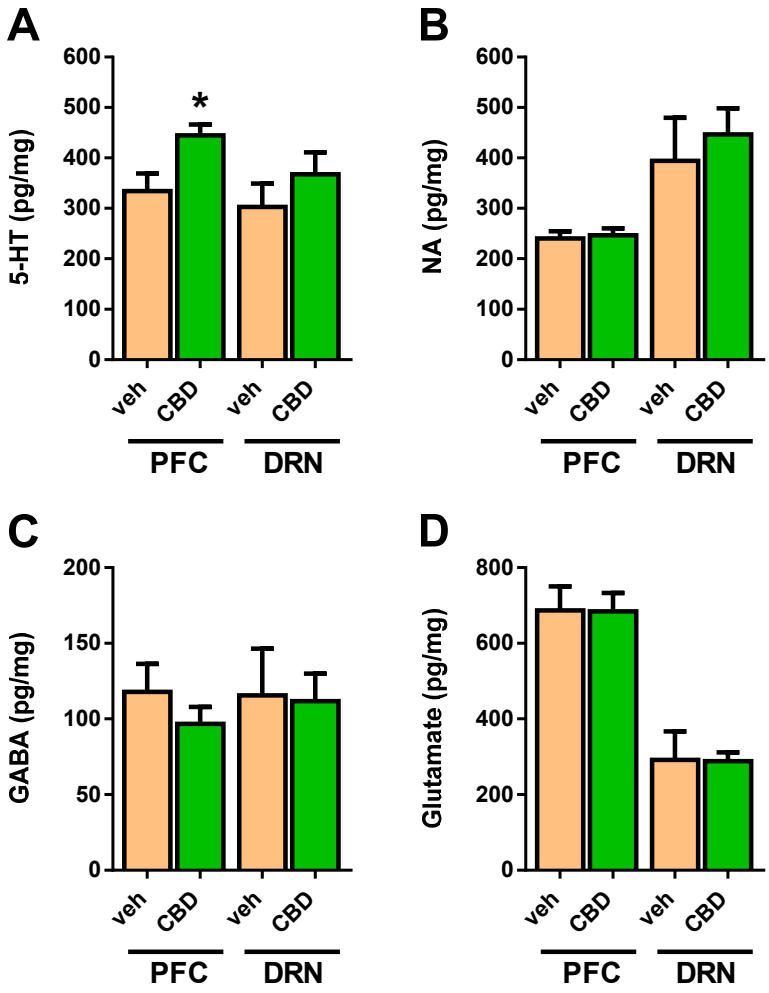

Figura 12. Niveles de neurotransmisores en la corteza prefrontal (PFC) y núcleo dorsal del rafe (DRN) tras la infusión aguda CBD en el DRN. A) Serotonina. B) Noradrenalina. C) GABA. D) Glutamato. Los resultados están expresados como media ± S.E.M. Prueba *t-Student* no pareada **p*<0,05 *vs* el grupo vehículo. n= 6 - 9 animales por grupo en la PFC y 5 - 8 en el DRN.

OBJETIVO 2: Efectos de la administración de cannabidiol a ratones sometidos al modelo de lipopolisacárido

1. Evaluación del comportamiento de tipo depresivo y ansioso

En el modelo inflamatorio por inyección de lipopolisacárido (LPS), la inflamación sistémica induce rápidamente síntomas generales de enfermedad (*sickness behaviour*), caracterizado por hipolocomoción, exploración reducida y pérdida de peso, entre otras manifestaciones (Castanon et al., 2001). Estas manifestaciones alcanzan su máximo en las 2 - 6 h posteriores a la inyección de LPS (Pitychoutis et al., 2009; Mello et al., 2018) y

desaparecen a las 24 h. Además, un comportamiento de tipo depresivo puede ser detectado a partir de las 6 h de la administración y sigue observándose a las 24 h (Dantzer et al., 2008).

Nuestros resultados demuestran que la administración sistémica de LPS indujo un **estado de tipo depresivo**, evidenciado por un incremento de la desesperanza conductual (*behavioural despair*) en la prueba de la suspensión por la cola (TST) (figura 13A), y la presencia de anhedonia, en la prueba de la preferencia de sacarosa (figura 13B). Las manifestaciones del *sickness behaviour* seguían presentes 12 h tras la administración de LPS, ya que observamos disminución de peso (figura 13C), e hipolocomoción en la prueba del campo abierto (OFT) (figura 13E). Para determinar si esta menor actividad locomotora podía interferir en el tiempo de inmovilidad observado en el TST, se analizó la correlación entre estos dos parámetros. La ausencia de correlación entre ambos (figura 14) indica que el comportamiento de tipo depresivo era independiente del *sickness behaviour* en este punto temporal.

Una única administración de cannabidiol (CBD) antes del LPS previno completamente el *behavioural despair* inducido por LPS (figura 13A). Esto concuerda con el efecto provocado por la administración aguda de inhibidores selectivos de la recaptación de serotonina (ISRS) como la fluoxetina y la paroxetina (Ohgi et al., 2013), fármacos con efecto antidepresivo rápido como la ketamina (Walker et al., 2013), y la administración crónica de antidepresivos tricíclicos como la imipramina (Renault y Aubert, 2006) en este modelo neuroinflamatorio. La reducción en el tiempo de inmovilidad que produjo el CBD en el TST contrasta con la ausencia de efectos en la locomoción estudiada en el OFT (figura 13E), respaldando el impacto del CBD sobre la conducta de tipo depresiva y no sobre el *sickness behaviour*. Además, **el CBD atenuó el estado anhedónico inducido por LPS, lo que junto con los resultados obtenidos en el *behavioural despair*, demuestra la eficacia del CBD en la remisión de diferentes manifestaciones de tipo depresivo presentes en el modelo neuroinflamatorio de LPS.** Estos resultados están en línea con los efectos del CBD descritos en otros modelos de depresión, tanto en ratón (Linge et al., 2016) como en rata (Shoval et al., 2016).

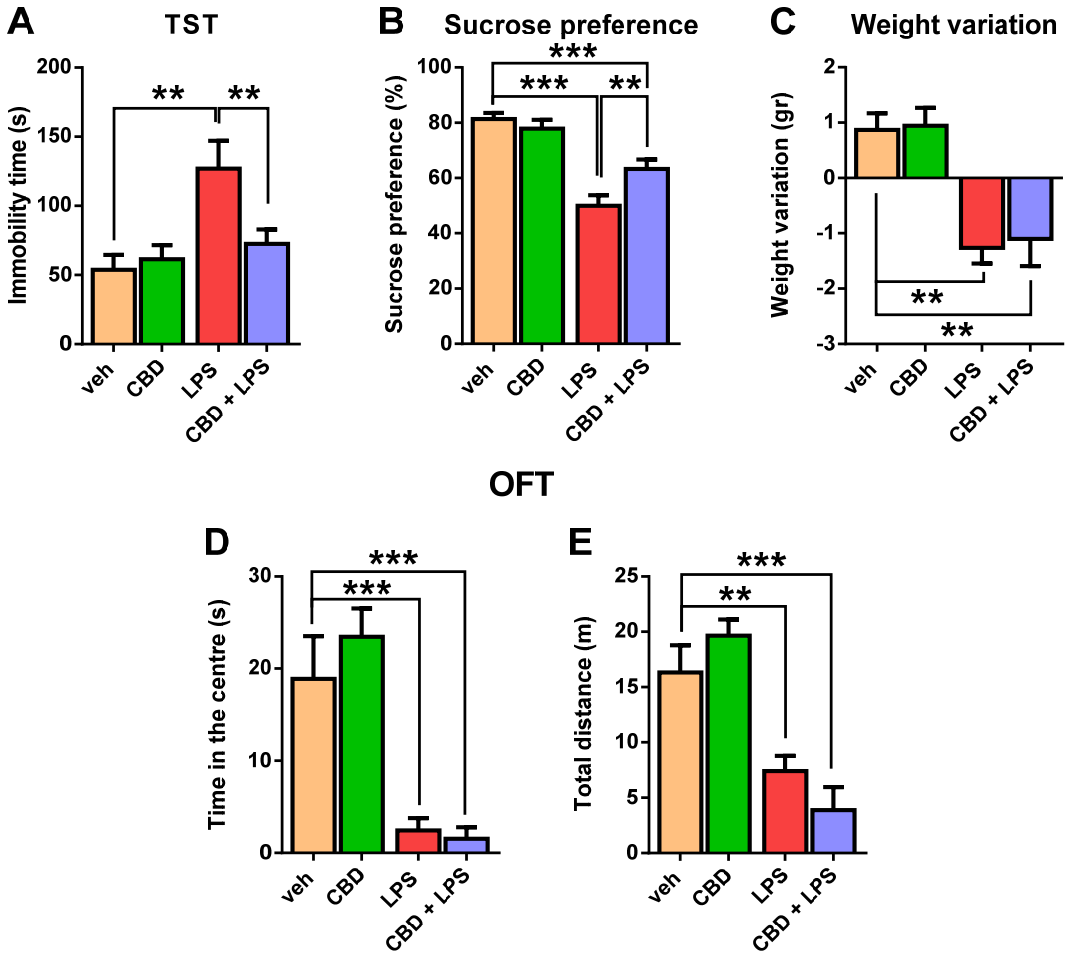

Figura 13. Evaluación de la conducta de tipo depresiva y ansiosa tras la administración de cannabidiol (CBD) a ratones macho sometidos al modelo de lipopolisacárido (LPS). A) Prueba de la suspensión por la cola (TST). B) Prueba de la preferencia por sacarosa. C) Variación de peso de los ratones. D-E) Prueba del campo abierto (OFT). Los resultados están expresados como media ± S.E.M. ANOVA de dos vías seguido de la prueba *posthoc* Newman Keuls. **p<0,01, ***p<0,001. n= 6 - 9 animales por grupo.

La administración de LPS disminuyó el tiempo central en el OFT (figura 13D), lo que indicaría un comportamiento de tipo ansioso. La administración de CBD no produjo modificaciones en el tiempo central en el OFT en el modelo de LPS (figura 13D), como se ha descrito también en el modelo genético de depresión de las ratas Wistar Kyoto (Shoval et al., 2016). Por el contrario, el CBD produce un efecto de tipo ansiolítico agudo en animales *naïve* (*revisado en* Blessing et al., 2015), y en el modelo de bulbectomía olfatoria (Linge et al., 2016). Sin embargo, obtuvimos una correlación significativa entre la distancia total y tiempo central en el OFT (figura 14), revelando la imposibilidad de separar la

conducta de tipo ansiosa del *sickness behaviour* en este punto temporal. Por tanto, no podemos sacar conclusiones sobre el efecto del CBD en ansiedad.

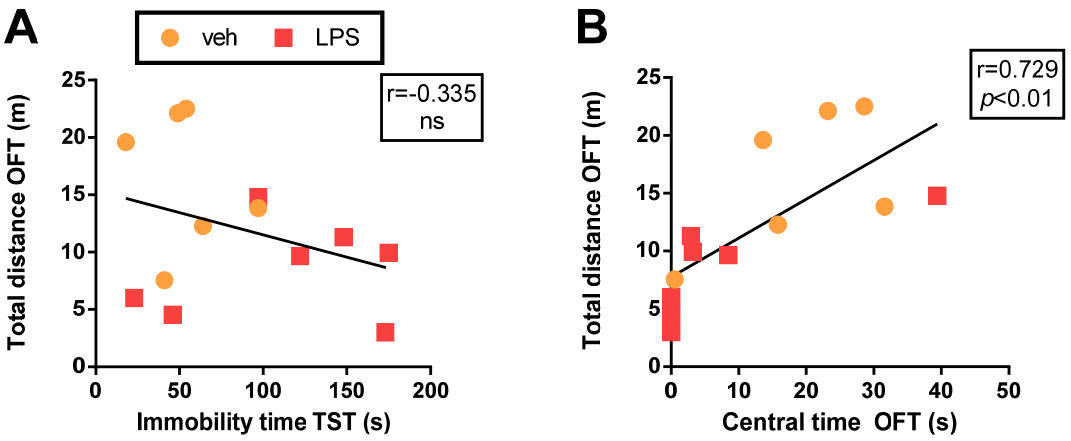

Figura 14. Correlación y regresión lineal entre los diferentes parámetros conductuales en ratones inyectados con vehículo o lipopolisacárido (LPS). A) Distancia total en la prueba del campo abierto (OFT) y tiempo de inmovilidad en la prueba de la suspensión por la cola (TST). B) Distancia total y tiempo en el centro determinados en el OFT. n= 6 - 7 animales por grupo. r: coeficiente de correlación de Pearson. ns: no significativo.

Debido a la existencia de diferencias de género tanto en el comportamiento de tipo depresivo como en la respuesta a fármacos antidepresivos en modelos animales de depresión (Kokras y Dalla, 2014), incluimos ratones hembra en nuestro estudio. Sin embargo, debido a que algunas de las manifestaciones conductuales inducidas por LPS en machos no se produjeron en hembras, y que el CBD no tuvo efectos preventivos en este género, los siguientes experimentos se realizaron con ratones macho.

2. Papel de los receptores 5-HT$_{1A}$ en los efectos de tipo antidepresivo del cannabidiol en el modelo de lipopolisacárido

Como la activación de los receptores 5-HT$_{1A}$ es uno de los mecanismos de acción descritos para el efecto de tipo antidepresivo del CBD (Zanelati et al., 2010; Linge et al., 2016; Sartim et al., 2016), usamos un antagonista selectivo de dicho receptor a dos dosis distintas: 0,3 mg/kg para bloquear mayoritariamente los receptores presinápticos del DRN, y 1 mg/kg

para bloquear tanto los receptores 5-HT_{1A} presinápticos como los postsinápticos (Serres et al., 2000; Ago et al., 2003; Carey et al., 2005).

En la prueba de la suspensión por la cola, **la persistencia de los efectos de tipo antidepresivo del CBD en ratones LPS aún en presencia de un bloqueo de los receptores 5-HT_{1A} presinápticos (WAY100635 0,3 mg/kg) demuestra que estos receptores no estarían implicados en su mecanismo de acción antidepresiva** (figura 15). Aunque otros autores han descrito que dicho bloqueo potencia el efecto de otros antidepresivos (Artigas et al., 1996; Romero et al., 1996), nosotros no observamos un efecto significativo de la combinación CBD+WAY 0,3 mg/kg ni en ratones *naïve,* ni en ratones que recibieron LPS (figura 15).

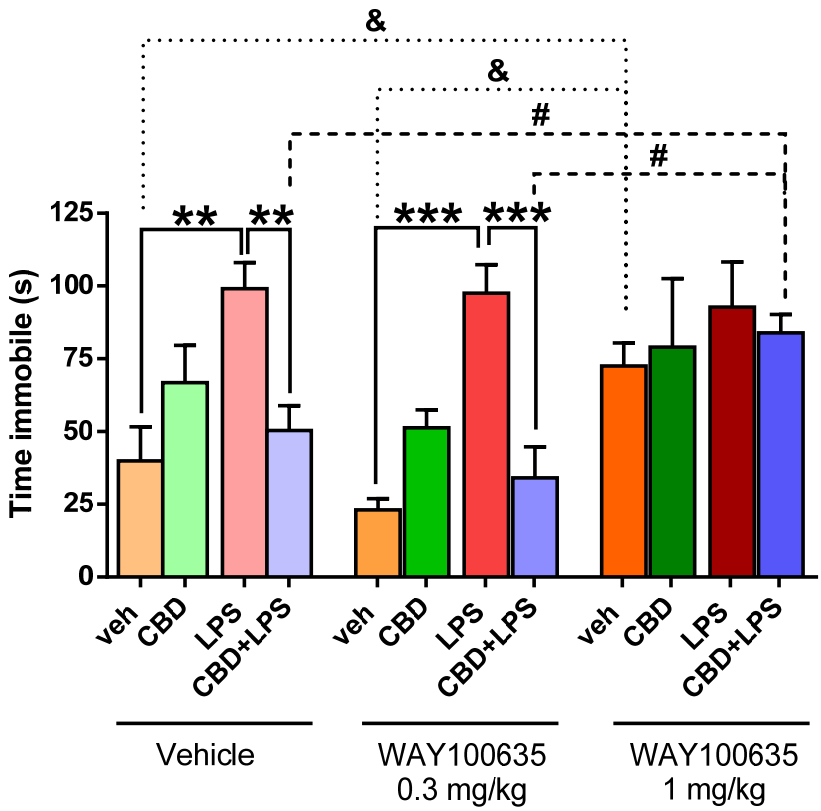

Figura 15. Evaluación de la conducta de tipo depresiva en la prueba de la suspensión por la cola tras la administración de WAY100635 0,3 y 1 mg/kg a ratones sometidos al modelo de LPS y pretratados con CBD. Los resultados están expresados como media ± S.E.M. ANOVA de dos vías seguido de la prueba *posthoc* Newman Keuls. **p<0,01, ***p<0,001. ANOVA de una vía seguido de la prueba *posthoc* Newman Keuls. $^{\&}p$<0.05 en la comparación de los grupos vehículo, $^{\#}p$<0.05 en la comparación de los grupos CBD+LPS. n= 6 - 8 animales por grupo en vehículo, 4 - 5 en la administración de WAY100635.

Por el contrario, el bloqueo de los receptores 5-HT_{1A} pre- y postsinápticos (WAY100635 1 mg/kg) evitó por completo el efecto de tipo antidepresivo del CBD en ratones sometidos al modelo de LPS (figura 15), demostrando un **papel clave del receptor 5-HT_{1A} postsináptico en el efecto de tipo antidepresivo del CBD en este modelo neuroinflamatorio.** Este bloqueo de los efectos de tipo antidepresivo del CBD con WAY100635 se ha descrito previamente en animales *naïve* (Zanelati et al., 2010; Sartim et al., 2016) y en el modelo de bulbectomía olfatoria (Linge et al., 2016), pero hasta donde sabemos, esta es la primera vez que se describe en el modelo neuroinflamatorio inducido por LPS.

3. Efectos moleculares del CBD en el modelo de LPS: vía NF-κB

Para investigar los mecanismos responsables de los efectos conductuales observados con la administración de CBD, estudiamos la vía de señalización NF-κB, ya que participa en el mecanismo proinflamatorio del LPS (Kawai et al., 2001). En los ratones a los que se administró LPS observamos un incremento de los niveles nucleares de NF-κB en corteza cerebral, sin cambios en los niveles citoplasmáticos (figura 16 A y D), lo que evidencia la activación de la vía NF-κB inducida por LPS, como se había descrito previamente (Pérez-Nievas et al., 2010; MacDowell et al., 2013). La administración de CBD previa a la inyección de LPS atenuó de forma significativa el incremento de los niveles nucleares de NF-κB (figura 16A), en línea con el efecto inhibitorio del CBD sobre esta vía que se ha descrito en cultivos celulares (Esposito et al., 2006; Juknat et al., 2019; Dos-Santos-Pereira et al., 2020) y en un modelo animal de Alzhéimer (Esposito et al., 2011). La fluoxetina, un antidepresivo ampliamente usado, también revierte el incremento de NF-κB en un modelo crónico de LPS en ratones (Rodrigues et al., 2018). Además, la inhibición directa de NF-κB produce efectos de tipo antidepresivo en modelos de estrés (Koo et al., 2010).

Con relación a IκBα, observamos un incremento de sus niveles nucleares en los ratones LPS, sin cambios en los niveles citoplasmáticos (figura 16 B y E). Tanto estos resultados como los obtenidos en NF-κB encajan con el curso temporal de activación/desactivación de la vía de NF-κB descrito por Hobbs et al. (2018). Describieron que la activación del TLR4 por LPS induce un rápido descenso en los niveles citoplasmáticos de IκBα y la translocación de NF-κB al núcleo. En cambio, la desactivación de la vía es más lenta, ya que el incremento de NF-κB en el núcleo aún se observa 10 h tras la exposición a LPS, cuando los niveles citoplasmáticos de IκBα ya han vuelto a su expresión basal (Hobbs et al., 2018). La presencia de IκBα en el compartimento nuclear que se observa tras la administración de LPS puede ser debida a que, tras la activación de la vía, el IκBα resintetizado transloca al núcleo y promueve la salida de NF-κB del mismo (Arenzana-Seisdedos et al., 1997). **Todo**

esto sugiere que la disminución de la activación de la vía NF-κB participa en los efectos de tipo antidepresivo del CBD en el modelo neuroinflamatorio inducido por lipopolisacárido.

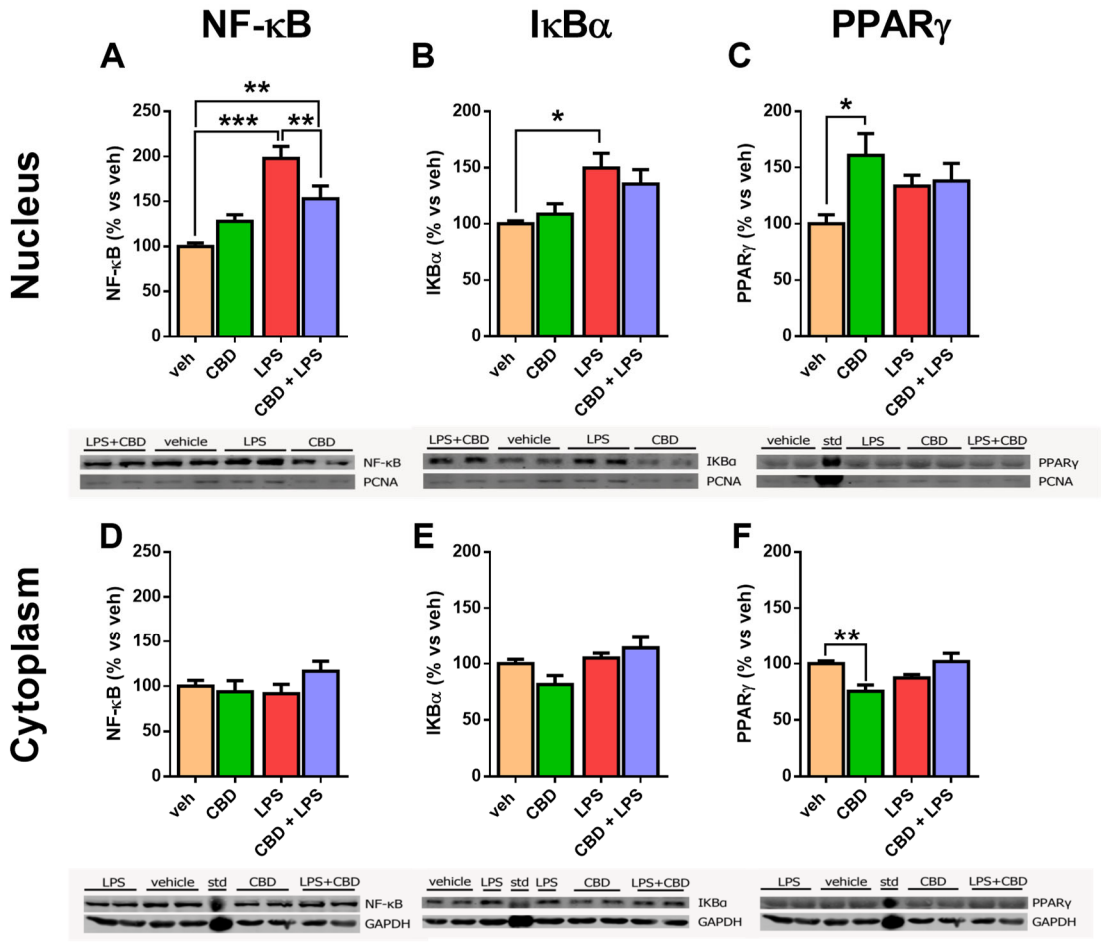

Figura 16. Niveles de las proteínas NF-κB (A y D), IκBα (B y E) y PPARγ (C y F) en la fracción nuclear (A-C) y citosólica (D-F) de la corteza cerebral de ratones LPS, y efecto de la pre-administración de CBD. Se muestran bandas representativas del Western Blot de cada marcador y del correspondiente control interno. Los resultados están expresados como media ± S.E.M. ANOVA de dos vías seguido de la prueba *posthoc* Newman Keuls. **p*<0.05, **p<0,01, ***p<0,001. n= 6 - 7 animales por grupo.

En cuanto a los niveles de PPARγ, no observamos cambios en los animales a los que se administró LPS (figura 16 C y F). Se ha descrito que el LPS reduce la expresión de PPARγ en células BV2 (Juknat et al., 2013; Choi et al., 2017) y en corteza de rata (Pérez-Nievas et al., 2010; MacDowell et al., 2013) en pocas horas. Por tanto, es probable que la ausencia

de cambios que observamos 12 h después de la administración de LPS sea debida a la recuperación de los niveles basales de PPARγ. Por el contrario, la administración de CBD produjo un incremento de los niveles nucleares de PPARγ y un descenso de los niveles citoplasmáticos en animales *naïve* (figura 16 C y F). Ya que el CBD es un ligando de PPARγ (O'Sullivan et al., 2009; *revisado en* O'Sullivan et al., 2016), podría activar a PPARγ y promover su traslocación al núcleo, tal como se ha descrito para otros ligandos (Khan y Abu-Amer, 2003; Kelly et al., 2004; Umemoto y Fujiki, 2012). En relación con esto, otros autores han descrito que el tratamiento crónico con CBD incrementó los niveles de PPARγ en la médula espinal en un modelo de esclerosis múltiple (Giacoppo et al., 2017), y los niveles de mRNA del coactivador de PPARγ 1-alfa (PGC-1α) en el estriado en un modelo de discinesia (Sonego et al., 2018). No obstante, esta es la primera vez que se observa el incremento de los niveles nucleares de PPARγ tras una única administración de CBD *in vivo*. La ausencia de cambios en los niveles nucleares de PPARγ en los animales sometidos al modelo de LPS y tratados con CBD podría deberse al papel de PPARγ en la inactivación de la vía, ya que PPARγ puede unirse a NF-κB en el núcleo e inducir su exportación nuclear (Chung et al., 2000; Hou et al., 2012), promoviendo su translocación al citosol (Kelly et al., 2004). Aun así, no podemos excluir la posibilidad de que los efectos inducidos por el CBD en la vía de NF-κB sean independientes de PPARγ, ya que se han descrito efectos antiinflamatorios del CBD que no dependen de dicha proteína (Haapakoski et al., 2015; Dos-Santos-Pereira et al., 2020).

4. Efectos moleculares del CBD en el modelo de LPS: citoquinas proinflamatorias

La administración de LPS incrementó los niveles plasmáticos de TNFα en menor medida que los de IL-6 (figura 17 A y B), ya que en respuesta al LPS el pico de TNFα se produce antes que el de IL-6 (Andreasen et al., 2008). Por el contrario, el incremento inducido por el LPS en los niveles de mRNA de TNFα en el cerebro fue mayor que el de los niveles de mRNA de IL-6 (figura 17 C y D), como se ha descrito anteriormente (André et al., 2008). La coexistencia de un comportamiento de tipo depresivo y un incremento en los niveles plasmáticos de IL-6 en este modelo neuroinflamatorio concuerda con los niveles elevados de IL-6 descritos en modelos animales de depresión (Sukoff Rizzo et al., 2012) y en metaanálisis de estudios con pacientes de depresión mayor (Dowlati et al.,2010; Haapakoski et al., 2015). Además, la desregulación de la producción de IL-6 y de la neurotransmisión serotonérgica –observado en los ratones sometidos al modelo de LPS por el incremento de la ratio quinurenina/serotonina (figura 18 B y D)– se ha asociado a una mayor vulnerabilidad a padecer depresión (Bull et al., 2009).

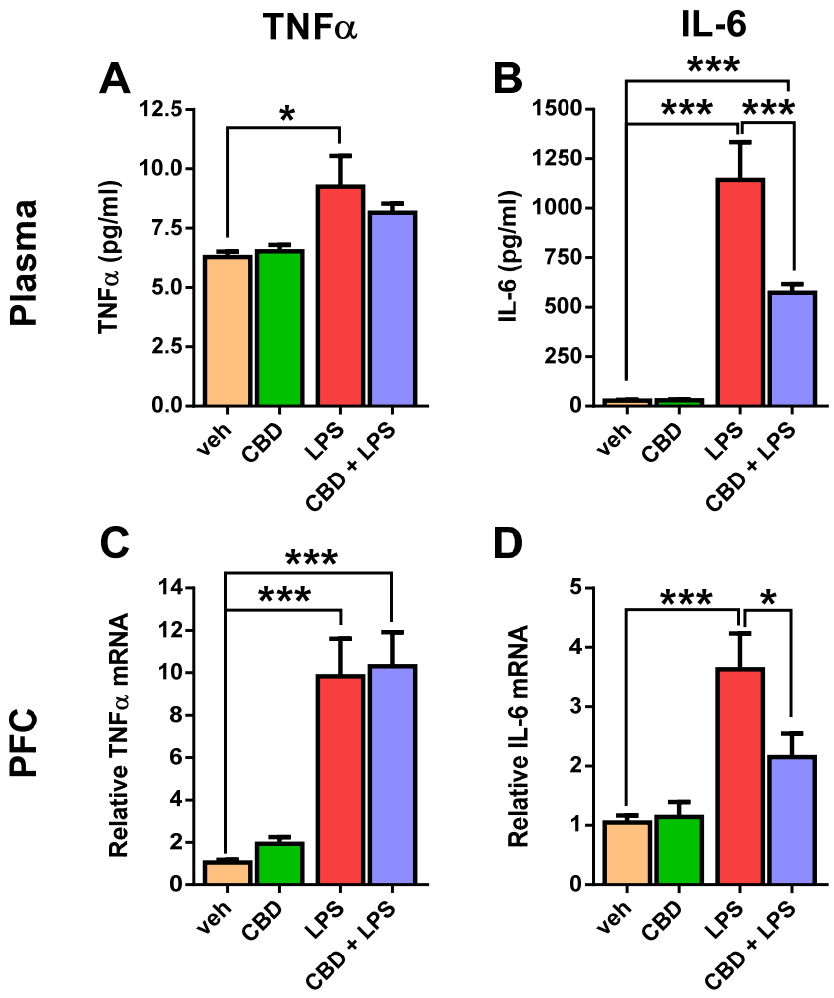

Figura 17. Niveles de TNFα e IL-6 en plasma (proteína) y corteza prefrontal (PFC) (mRNA) en ratones sometidos al modelo de LPS, y efectos de la administración de CBD. Niveles de TNFα (A y C), niveles de IL-6 (B y D), muestras de plasma (A y B), y muestras de corteza prefrontal (C y D). Los resultados están expresados como media ± S.E.M. ANOVA de dos vías seguido de la prueba *posthoc* Newman Keuls. **p*<0.05, ***p<0,001. n= 6 - 7 animales por grupo.

La administración de CBD atenuó el incremento de los niveles de IL-6 en plasma inducido por LPS (figura 17B), y previno totalmente dicho incremento en la corteza prefrontal (figura 17D). En línea con los efectos del CBD en la conducta de tipo depresiva y en los niveles de IL-6 descritos en esta tesis, los animales *knockout* para IL-6 presentan una reducción en el *behavioural despair* y son resistentes a la depresión inducida por estrés (Chourbaji et al., 2006). Además, los niveles elevados de IL-6 observados en pacientes con depresión aguda son revertidos a niveles control con el tratamiento antidepresivo (Frommberger et al., 1997). La disminución de IL-6 es de gran relevancia, dado que los

niveles altos de IL-6 se asocian no solamente con el comportamiento de tipo depresivo, sino también con la resistencia al tratamiento (Sukoff Rizzo et al., 2012).

Respecto a TNFα, se ha demostrado en estudios previos que su administración produce *sickness behaviour* y neuroinflamación, pero no síntomas de tipo depresivo y anhedonia (Biesmans et al., 2015). Además, un metaanálisis acumulativo de estudios en pacientes con depresión mayor reveló que la asociación de TNFα con la enfermedad es inconsistente, mientras que otros marcadores de inflamación como la IL-6 están claramente asociados con la depresión (Haapakoski et al., 2015). Esto va en línea con el hecho de que, en nuestro estudio, el CBD produjera efectos de tipo antidepresivo en el modelo de LPS sin producir cambios en los niveles de TNFα (figura17 A y C). **Estas evidencias sugieren que el efecto antidepresivo del CBD en el modelo de LPS puede estar mediado por sus efectos en los niveles de IL-6.**

5. Efectos moleculares del CBD en el modelo de LPS: vía de la quinurenina

Para seguir profundizando en el mecanismo responsable de los efectos de tipo antidepresivo observados con la administración de CBD, estudiamos la vía de la quinurenina. En el punto temporal estudiado aún había signos del *sickness behaviour* inducido por LPS. Sin embargo, este comportamiento se puede diferenciar del comportamiento de tipo depresivo (*behavioural despair* y anhedonia), ya que algunas moléculas como la quinurenina inducen una conducta de tipo depresiva pero no *sickness behaviour*, y el bloqueo de la indolamina 2,3-dioxigenasa (IDO) previene las manifestaciones de tipo depresivas sin alterar las de *sickness behaviour* inducidas por LPS (O'Connor et al., 2009).

Tras la administración de LPS observamos un incremento en la ratio quinurenina/triptófano tanto en hipocampo como en corteza (figura 18 A y C), lo cual es indicativo de un incremento en la actividad de la enzima IDO (Fuchs et al., 1990; Widner et al., 2002; O'Connor et al., 2009). Esta activación de la IDO se confirmó además mediante el incremento en la ratio quinurenina/serotonina en estos animales (figura 18 B y D), mostrando un desequilibro hacia la síntesis de quinurenina. La coexistencia de *behavioural despair* y anhedonia con niveles elevados de quinurenina observada en los animales sometidos al modelo de LPS, está en línea con el efecto de la administración de quinurenina en las conductas de *behavioural despair* (O'Connor et al., 2009) y anhedonia (Salazar et al., 2012).

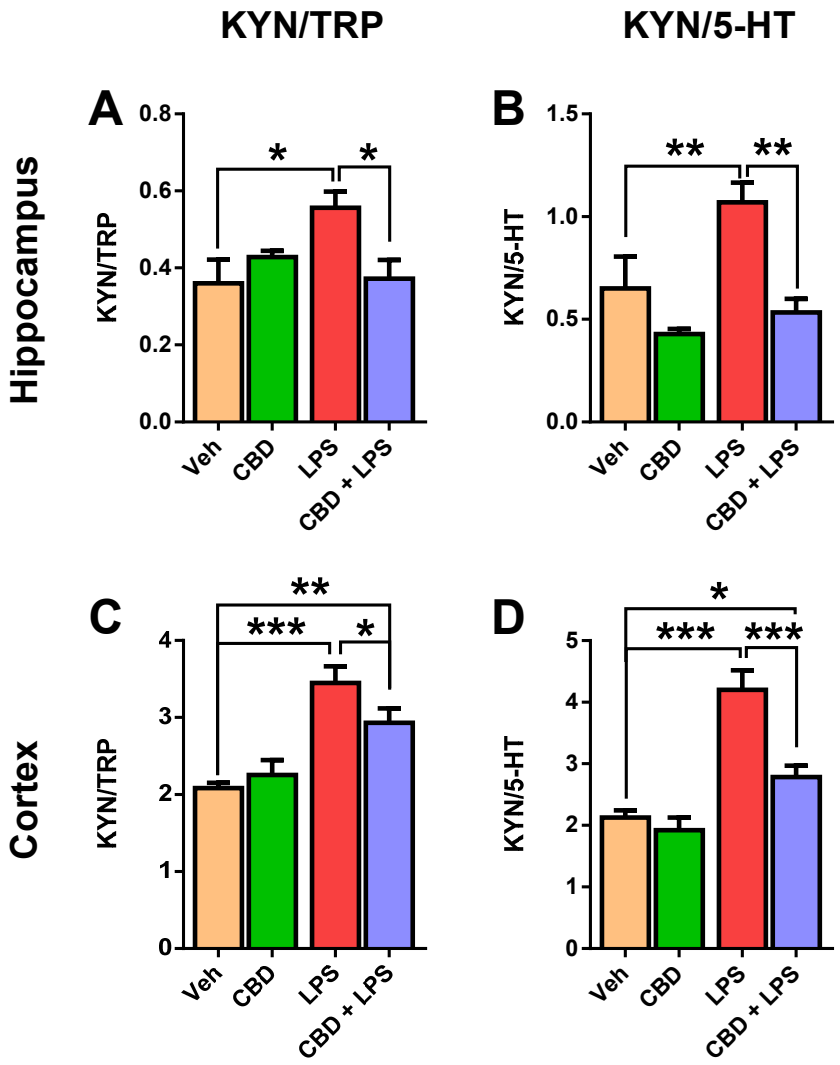

Figura 18. Ratios entre los niveles de quinurenina, triptófano y serotonina en el hipocampo (A y B) y la corteza (C y D) de ratones LPS, y efectos de la pre-administración de CBD. Ratio quinurenina/triptófano (A y C), y ratio quinurenina/serotonina (B y D). Los resultados están expresados como media ± S.E.M. ANOVA de dos vías seguido de la prueba *posthoc* Newman Keuls. **p*<0.05, ***p*<0.01, ***p<0,001. n= 5 - 6 animales por grupo en hipocampo y 7 - 9 en corteza.

Por el contrario, la administración de CBD produjo un efecto de tipo antidepresivo asociado a una reducción en las ratios quinurenina/triptófano y quinurenina/serotonina (figura 18), de acuerdo con los efectos de tipo antidepresivo observados tras la inhibición de la IDO (O'Connor et al., 2009; Dobos et al., 2012). Además, la inhibición de esta enzima también está asociada a menor anhedonia, como se ha descrito en animales *knockout* para la IDO (Lawson et al., 2013). Estas evidencias, junto con la reversión de los efectos conductuales y moleculares en la vía de la quinurenina promovidos por el CBD en el

modelo de LPS, indican que **los efectos de tipo antidepresivo que produce el CBD en este modelo podrían estar mediados por la reducción de la actividad de la IDO en el cerebro**. Esto podría ser consecuencia de la inhibición directa de la enzima (Jenny et al., 2009), o de un efecto indirecto a través de la disminución de los niveles de citoquinas proinflamatorias (Lestage et al., 2002).

Varios estudios recientes, tanto preclínicos como clínicos, resaltan la importancia de los metabolitos de la quinurenina en la depresión (*revisado en* Maes et al., 2011; Barone, 2019). En nuestro estudio observamos un incremento en los niveles de 3-hidroxiquinurenina (3-HK) en la corteza de los animales inyectados con LPS (2.128 ± 183 ng/mg en LPS *vs* 923 ± 96 ng/mg en vehículo, $p<0,001$). En concordancia, se ha descrito un incremento de la rama neurotóxica de la vía de la quinurenina (3-HK y ácido quinolínico, QUIN) y un descenso de la rama neuroprotectora (ácido quinurénico) en el modelo de LPS y en pacientes con depresión mayor (Verdonk et al., 2019; *revisado en* Barone, 2019). Este desequilibro entre las dos ramas de la vía de la quinurenina se ha relacionado con el comportamiento de tipo depresivo (Parrott et al., 2016; Laumet et al., 2017).

La administración de CBD en nuestro estudio produjo un efecto de tipo antidepresivo, en paralelo a la reversión del incremento de 3-HK inducido por el LPS (1.312 ± 175 ng/mg en CBD+LPS *vs* 2.128 ± 183 ng/mg en LPS, $p<0,01$). En línea con nuestros resultados, se ha descrito recientemente que el CBD reduce los niveles de 3-HK e incrementa los de ácido quinurénico, los cuales se habían visto alterados previamente por un estímulo neurotóxico en experimentos *ex vivo* (di Giacomo et al., 2020). Además, la administración de otros antidepresivos como la ketamina (Verdonk et al., 2019) y la fluoxetina (Laugeray et al., 2016) también redujo los niveles cerebrales de 3-HK en el modelo de LPS y del estrés leve impredecible crónico (CUMS), respectivamente. Este cúmulo de evidencias sugiere que **la restauración del equilibrio neurotóxico/neuroprotector en la vía de la quinurenina también podría participar en los efectos de tipo antidepresivo del CBD en el modelo de LPS.**

6. Efectos moleculares del CBD en el modelo de LPS: glutamato y GABA

La inyección de LPS incrementó los niveles de glutamato en el hipocampo (figura 19A), en línea con los resultados de un estudio de microdiálisis (Kitagawa et al., 2019), y con el incremento de la actividad glutamatérgica descrita en este modelo (Chávez et al., 2019; Chen et al., 2020). El incremento observado en los niveles de 3-hidroxiquinurenina, sugiere que en estos animales podría estar más activa la rama neurotóxica de la vía de la

quinurenina, produciéndose más ácido quinolínico. Este podría ser el responsable del incremento de glutamato observado, ya que induce la liberación de glutamato (*revisado en* Barone, 2019). Por el contrario, la administración de CBD evitó por completo dicho incremento (figura 19A), lo cual podría estar mediado por el efecto inhibitorio que tiene el CBD sobre la vía de la quinurenina (figura 18). Sin embargo, no podemos descartar otros mecanismos (Dos-Santos-Pereira et al., 2020).

También observamos un incremento de los niveles de GABA en el hipocampo tras la inyección de LPS (figura 19B), en concordancia con el incremento de la neurotransmisión GABAérgica descrita en este modelo (Kitamura et al., 2019; Tang et al., 2020). Este incremento podría tratarse de un mecanismo compensatorio, ya que se ha descrito que puede tener un efecto antiinflamatorio (*revisado en* Crowley et al., 2016). La pre-administración de CBD bloqueó por completo el efecto del LPS en los niveles de GABA en el hipocampo (figura 19B), apoyando el papel compensatorio propuesto para este neurotransmisor.

Por otro lado, la inyección de LPS redujo los niveles de glutamato en la corteza (figura 19C), efecto que fue completamente prevenido por el CBD, en línea con los efectos del CBD en el modelo de depresión de la bulbectomía olfatoria (Linge et al., 2016). Aunque no encontramos cambios significativos, observamos un patrón similar en los niveles de GABA en esta área (figura 19D).

Tanto en el hipocampo como en la corteza, **el CBD previno el desequilibro en los niveles de glutamato y GABA inducido por el LPS, sugiriendo que el CBD podría ejercer un efecto homeostático en estos sistemas de neurotransmisores**. Esto podría estar mediado por el incremento de endocannabinoides que produce (Bisogno et al., 2001), ya que estos modulan la neurotransmisión por glutamato y GABA (Castillo et al., 2012; Araque et al., 2017).

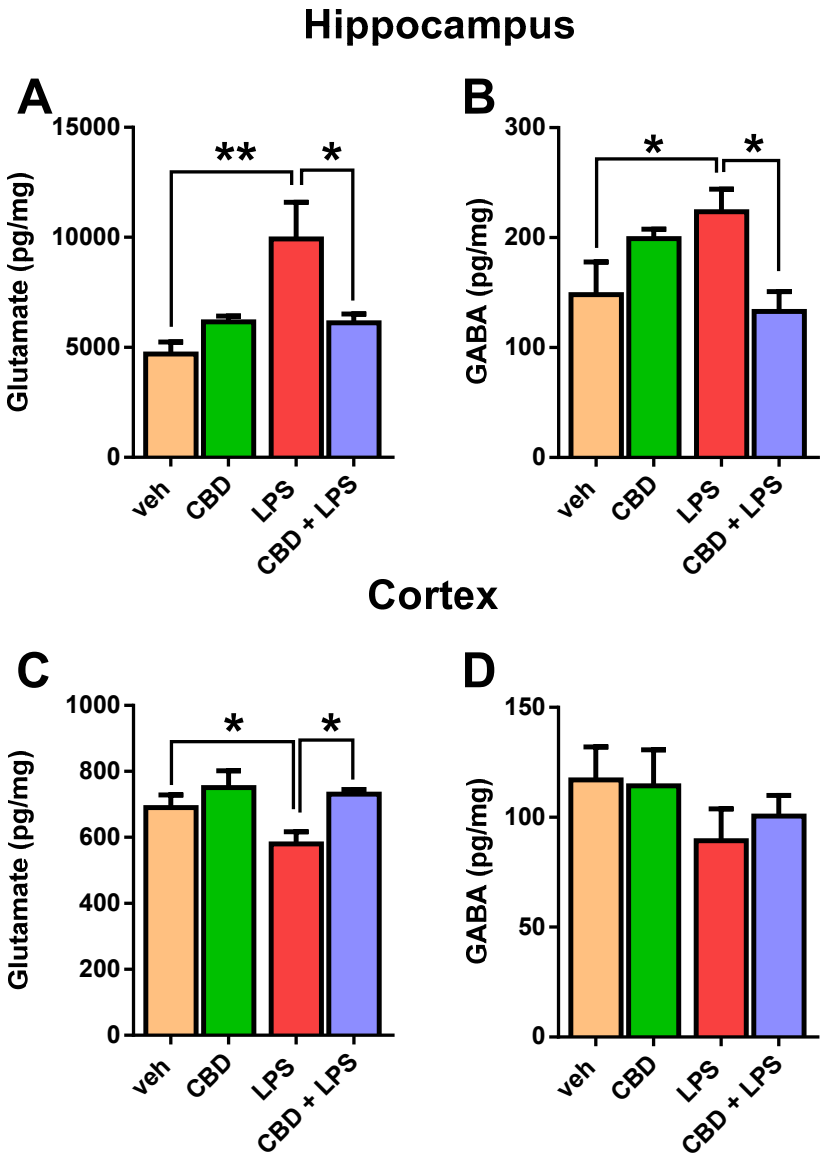

Figura 19. Niveles de glutamato (A y C) y de GABA (B y D) en el hipocampo (A y B) y la corteza prefrontal (C y D) de ratones inyectados con LPS, y efectos de la pre-administración de CBD. Los resultados están expresados como media ± S.E.M. ANOVA de dos vías seguido de la prueba *posthoc* Newman Keuls. **p*<0.05, ***p*<0.01. n= 5 - 6 animales por grupo en hipocampo y 7 - 9 en corteza.

CONCLUSIONES

1. La corteza infralímbica es un área clave para el efecto agudo de tipo antidepresivo del cannabidiol.

2. El efecto agudo de tipo antidepresivo de la infusión de cannabidiol en la corteza infralímbica está asociado a la activación de las vías de ERK y mTOR y al incremento de BDNF en la corteza prefrontal.

3. La infusión de cannabidiol en la corteza infralímbica incrementa la actividad neuronal en el núcleo dorsal del rafe en animales *naïve*, y modula la actividad neuronal inducida por estrés agudo en la corteza infralímbica y el núcleo dorsal del rafe.

4. El efecto de tipo antidepresivo de la infusión de cannabidiol en la corteza infralímbica no se observa a las 24 horas, pero se recupera tras el bloqueo de los receptores 5-HT_{1A} presinápticos.

5. Esta recuperación del efecto de tipo antidepresivo se asocia a la activación de ERK en la corteza prefrontal, y a un incremento de los niveles de glutamato y noradrenalina en el núcleo dorsal del rafe.

6. La infusión de cannabidiol en el núcleo dorsal del rafe no produce un efecto agudo de tipo antidepresivo, aunque incrementa los niveles de serotonina en la corteza prefrontal, y es menos eficiente induciendo vías de plasticidad sináptica en esta área.

7. La administración sistémica de cannabidiol produce un efecto de tipo antidepresivo en el modelo neuroinflamatorio inducido por lipopolisacárido.

8. La activación de los receptores 5-HT_{1A} postsinápticos juega un papel clave en el efecto de tipo antidepresivo del cannabidiol en este modelo neuroinflamatorio.

9. El efecto de tipo antidepresivo del cannabidiol en el modelo de lipopolisacárido está asociado a una disminución de la activación de la vía NF-κB en cerebro.

10. El efecto de tipo antidepresivo del cannabidiol en el modelo de lipopolisacárido está asociado a una disminución de los niveles de interleucina-6 en cerebro y plasma.

11. El efecto de tipo antidepresivo del cannabidiol en el modelo de lipopolisacárido está asociado a una disminución de la activación de la vía de la quinurenina en cerebro.

12. La administración de cannabidiol previene el desequilibro en los niveles de glutamato y GABA inducido por lipopolisacárido en cerebro.

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ANNEXE 3: Publications

Biochemical Pharmacology 185 (2021) 114433

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Cannabidiol antidepressant-like effect in the lipopolysaccharide model in mice: Modulation of inflammatory pathways

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ARTICLE INFO

Keywords:
Cannabidiol
Antidepressant
Kynurenine
IL-6
NF-κB

ABSTRACT

Major Depression is a severe psychiatric condition with a still poorly understood etiology. In the last years, evidence supporting the neuroinflammatory hypothesis of depression has increased. In the current clinical scenario, in which the available treatments for depression is far from optimal, there is an urgent need to develop fast-acting drugs with fewer side effects. In this regard, recent pieces of evidence suggest that cannabidiol (CBD), the major non-psychotropic component of *Cannabis sativa* with anti-inflammatory properties, appears as a drug with antidepressant properties. In this work, CBD 30 mg/kg was administered systemically to mice 30 min before lipopolysaccharide (LPS; 0.83 mg/kg) administration as a neuroinflammatory model, and behavioral tests for depressive-, anhedonic- and anxious-like behavior were performed. NF-κB, IκBα and PPARγ levels were analyzed by western blot in nuclear and cytosolic fractions of cortical samples. IL-6 and TNFα levels were determined in plasma and prefrontal cortex using ELISA and qPCR techniques, respectively. The precursor tryptophan (TRP), and its metabolites kynurenine (KYN) and serotonin (5-HT) were measured in hippocampus and cortex by HPLC. The ratios KYN/TRP and KYN/5-HT were used to estimate indoleamine 2,3-dioxygenase (IDO) activity and the balance of both metabolic pathways, respectively. CBD reduced the immobility time in the tail suspension test and increased sucrose preference in the LPS model, without affecting locomotion and central activity in the open-field test. CBD diminished cortical NF-κB activation, IL-6 levels in plasma and brain, and the increased KYN/TRP and KYN/5-HT ratios in hippocampus and cortex in the LPS model. Our results demonstrate that CBD produced antidepressant-like effects in the LPS neuroinflammatory model, associated to a reduction in the kynurenine pathway activation, IL-6 levels and NF-κB activation. As CBD stands out as a promising antidepressant drug, more research is needed to completely understand its mechanisms of action in depression linked to inflammation.

1. Introduction

Major Depressive Disorder (MDD) is a common and severe medical illness characterized by symptoms like sadness, anhedonia, psychomotor agitation or retardation, feeling worthless or guilty, difficulty concentrating, and thoughts of death or suicide, among others [1]. The etiology of this disease is poorly known, and different hypotheses have been proposed. The classical *monoaminergic hypothesis* of depression postulates that there is a deregulation in monoamine neurotransmission [2], which is complemented by the fact that most of the antidepressants currently used in clinic target these neurotransmitters systems (*rev. in* [3]). However, the *monoaminergic hypothesis* does not explain the totality of alterations present in depression and novel hypotheses have been proposed.

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https://doi.org/10.1016/j.bcp.2021.114433
Received 11 December 2020; Received in revised form 19 January 2021; Accepted 19 January 2021
Available online 26 January 2021

One of the hypotheses that has gained evidence in recent years is the *neuroinflammatory hypothesis*, as some inflammatory diseases present comorbid depression [4], and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) have been found increased in the blood of MDD patients [5]. This peripheral inflammation, via the cross-talk with the central immune system, can induce a pro-inflammatory response in the brain [6–8]. In this line, it has been reported that there is an activation of microglia and an increase of pro-inflammatory cytokines in cerebrospinal fluid and postmortem brain tissue of MDD patients [9].

One of the peripheral stimuli that can activate the immune response is lipopolysaccharide (LPS), a component of the bacterial wall. The administration of LPS induces not only sickness behavior, but also a depressive-like state, anhedonia, and anxiety-like behavior in animals [10,11], and depressive symptoms in humans [12,13]. LPS administration activates toll-like receptor 4 (TLR4), initiating the canonical nuclear factor kappa B (NF-κB) pathway [14]. NF-κB is a transcription factor that mediates the inflammatory response. Under basal conditions, NF-κB is located in the cytosol by its association with the inhibitor of nuclear factor kappa B (IκBα). When LPS activates TLR4, IκBα is phosphorylated and undergoes proteasome degradation. This allows NF-κB translocation to the nucleus and the transcription of its target genes, which initiate the inflammatory response [15]. Some of the proteins induced by NF-κB activation are pro-inflammatory cytokines like IL-6 and interferon-gamma (IFNγ) [14]. Moreover, LPS reduces the levels of the peroxisome proliferator-activated receptor gamma (PPARγ) in cells [16,17], and rat cortex [18,19]. Conversely, the activation of PPARγ can antagonize the transcription activity of NF-κB, consequently producing anti-inflammatory effects [20].

The increase in IL-6 and IFNγ induced by LPS activates the indoleamine 2,3-dioxygenase (IDO) enzyme [21,22]. In mice, the increase in brain IDO activity has been associated to the appearance of depressive-like behaviour [23,24]. In addition, the increased activity of the IDO enzyme, which transforms the precursor tryptophan into kynurenine, leads to a skewed pathway toward the formation of kynurenine instead of serotonin [25,26]. Importantly, in recent years kynurenine and its metabolites have been broadly associated with the pathophysiology of depression, leading to the proposal of a new hypothesis of depression that integrates stress, inflammation, the kynurenine pathway and serotonergic and glutamatergic neurotransmission [27–29].

In the last years, cannabidiol (CBD), the main non-psychotomimetic component of *Cannabis sativa* [30], has shown antidepressant-like properties in predictive behavioral tests [31,32], and in animal models of depression such as the olfactory bulbectomy [33], and the chronic unpredictable stress [34], following acute or chronic administration. CBD is known to produce changes in some neuroplasticity markers such as BDNF [35], and in hippocampal proliferation in chronically stressed animals [34], which has been associated to its antidepressant-like effect. This drug also presents anti-inflammatory and immunomodulatory effects, as reported in animal models of other pathologies such as Alzheimer's disease, which was mediated by PPARγ activation [36]. However, the link between the modulation of inflammatory pathways by cannabidiol and its antidepressant-like effects in an inflammatory model is yet to be explored [37].

Here we have studied the effect of acute cannabidiol administration in the depressive and anxious-like behavior in an animal model of inflammation induced by the systemic injection of lipopolysaccharide. We have also evaluated the effect of cannabidiol on different inflammatory markers as the NF-κB pathway, pro-inflammatory cytokines and the kynurenine pathway in order to figure out the mechanism of action of cannabidiol in this neuroinflammatory model.

2. Material and methods

2.1. Animals

Male NMRI mice (2–3 months old, 30–35 g) (Envigo, Indiana, USA) were housed in groups of 3–4 with a 12-h light–dark cycle, and food and water were provided *ad libitum*. All procedures were carried out with the previous approval of the Animal Care Committee of the University of Cantabria according to the Spanish legislation and the European Communities Council Directive on "Protection of Animals Used in Experimental and Other Scientific Purposes" (86/609/EEC).

2.2. Drugs

Cannabidiol (CBD, Tocris, Bristol, United Kingdom) was dissolved in 2% Tween 80®: 5% Propilenglycol®: saline, and used at a dose of 30 mg/kg [32]. Lipopolysaccharide (LPS from Escherichia coli O127:B8, Sigma-Aldrich, Darmstadt, Germany) was dissolved in saline and used at a dose of 0.83 mg/kg [38]. Both drugs were administered i.p. (10 μl/g body weight) just starting the dark period. Animals that did not receive CBD or LPS were injected with the corresponding vehicle.

2.3. Experimental design

A graphical representation of the experimental procedure is shown in Fig. 1. One week before the experiment, the animals were individually housed and habituated to a free choice of 2% sucrose solution and water. CBD was administered 30 min before the LPS injection. The open-field test (OFT) and the tail suspension test (TST) were performed 12 h after LPS administration. Sucrose preference was assessed during that 12 h period. Thirty min after the end of the behavioral assessment, a set of animals used for western blot and HPLC was sacrificed by cervical dislocation and brain samples were rapidly collected and stored at −80 °C until used. Another set of animals, used for cytokine determination in plasma (ELISA) and brain (qPCR), was sacrifized by pentobarbital administration (Fig. 1).

2.4. Behavioral assays

Tests were performed during the light phase. Animals were placed in the experimental room 1 h before the beginning of the procedures for habituation. Behavioral tests were performed during the light phase and were arranged from the least to most stressful ones (sucrose preference, open-field, and tail suspension test) (Fig. 1).

2.4.1. Sucrose preference test (SPT)

This test was used to evaluate anhedonia. Animals were individualized and given a free choice of water and a 2% sucrose solution during one week before the experiment in order to habituate to the availability of both bottles, as previously described [39]. The positions of the two bottles were switched every day to avoid any potential position bias. The sucrose preference was calculated 12 h after LPS injection as the percentage of the amount of sucrose solution consumed compared to the total amount of liquid intake during that period.

2.4.2. Open-Field test (OFT)

The open-field apparatus was a brightly lit (350 lx) grey wooden box (40 × 40 × 30 cm). The center was defined as a 20 × 20 cm area. Mice were placed in one corner of the apparatus, and the behavior was video tracked by a computerized system (Any-maze Video-Tracking software, Stoelting Co., USA) for 5 min [40]. The parameters 'total distance traveled' and 'time spent in the center' were obtained to study locomotion and anxiety-like behavior, respectively.

2.4.3. Tail suspension test (TST)

Five minutes after the OFT, mice were suspended by the tail and

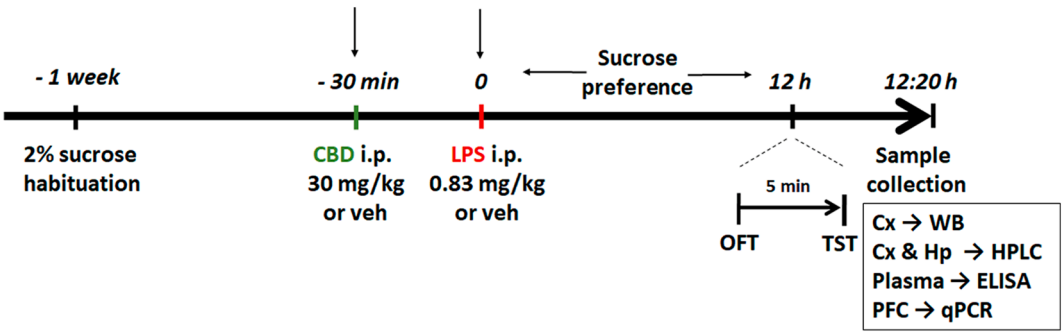

Fig. 1. Experimental design of drug administration and behavioral tests performed in this study, and sample collection for the different molecular techniques. CBD: cannabidiol, Cx: cortex, Hp: hippocampus, HPLC: high performance liquid chromatography, LPS: lipopolysaccharide, OFT: open-field test, PFC: prefrontal cortex, qPCR: quantitative PCR, TST: tail suspension test, veh: vehicle, WB: western blot.

video recorded for 6 min [41]. The time spent immobile was determined using an automated software (Biobserve GmbH, Bonn, Germany). The settings were previously adjusted by an experienced observer to count as immobility only when mice were hanging passively and completely motionless, which is indicative of behavioral despair.

2.5. Western blot (WB)

Cortical samples were processed for subcellular fractionation following the protocol described in [42] with minor modifications. A buffer containing 10 mM HEPES (USB, Ohio, USA), pH 7.9, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol (DTT) (Sigma Aldrich, Missouri, USA), 0.5 M sucrose (Scharlab S.L., Barcelona, Spain), 10 mM sodium molybdate and supplemented with a protease inhibitors cocktail (Sigma-Aldrich, Missouri, USA) was used for the homogenization of the samples. After 15 min on ice, 0.5% Igepal was added and samples were gently vortexed for 15 s. The cytosolic fraction was obtained by collecting the supernatant after a 5 min 8000×*g* centrifugation at 4 °C. The remaining pellet was resuspended with 100 µl of the previous buffer supplemented with 20% glycerol (Scharlab S.L., Barcelona, Spain) and 0.4 M KCl and gently shaken for 30 min at 4 °C. The nuclear fraction was obtained by collecting the supernatant after a 5 min 13000×*g* centrifugation at 4 °C.

Duplicates of 60–90 µg of cytosolic and nuclear protein, respectively, were size-separated with SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, California, USA). For IκBα detection, membranes were blocked with 5% powder skimmed milk in TBS-T (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20) (Sigma Aldrich, Missouri, USA), and for NF-κB and PPARγ in 5% BSA (Sigma Aldrich, Missouri, USA) in TBS-T 0.1 (0.1% Tween-20). The following primary antibodies were incubated with the corresponding blocking solution at 4 °C overnight: IκBα (1:2000, AB3016, Chemicon, Massachusetts, USA), NF- κB (1:1000, #6956, Cell signaling, Massachusetts, USA), PPARγ (1:1000, MA5-14889, Invitrogen, California, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2000, sc-32233, Santa Cruz Biotechnology, Texas, USA), and proliferating cell nuclear antigen (PCNA) (1:1000, sc-56, Santa Cruz Biotechnology, Texas, USA). LI-COR® fluorescent secondary antibodies (700 and 800CW) were used at 1:15000, and the signal was detected with an Odyssey® CLx Imaging System (LI-COR Biosciences, Nebraska, USA). Protein quantification was performed using Image Studio™ Software (LI-COR Biosciences, Nebraska, USA). GAPDH and PCNA were used as cytosolic and nuclear housekeeping proteins, respectively, and the average of the duplicates of each sample was calculated. Purity of subcellular fractions was assessed analyzing GAPDH and PCNA protein expression. All blots were performed at least 3 times in separate assays. Results are represented in percentage *versus* the vehicle group.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Mice were deeply anesthetized with a pentobarbital injection (40 mg/kg, i.p.) and the blood collected directly from the heart with an EDTA-treated syringe, centrifuged 10 min at 2000×*g*, 4 °C, and the supernatant collected and stored at −80 °C until used. TNFα and IL-6 ELISA kits were purchased from Invitrogen (Life Technologies Corporation, California, USA), and the procedure specified by the manufacturer was followed. The optical density was determined at 450 nm with a Mithras LB 940 (Berthold technologies, Baden-Württemberg, Germany). The data are represented as pg/ml.

2.7. RNA extraction and reverse transcription

RNA extraction was performed as previously described [43]. RNA was extracted from prefrontal cortex (PFC) samples using TRI reagent (Merck KGaA, Darmstadt, Germany) following manufacturer's instructions. RNA purity assessment (ratios 260/280 and 260/230) and quantification were performed with a NanoDrop 1000 (Thermo Fischer Scientific S.L., Madrid, Spain). 600 ng of total RNA were used in the reverse transcriptase reaction performed with a high capacity cDNA reverse transcriptase kit (Applied Biosystems, California, USA) following manufacturer's instructions.

2.8. Quantitative PCR (qPCR)

Quantitative PCR was performed in a StepOne™ Real-Time PCR System (Applied Biosystems, California, USA) using TaqMan gene expression assays for TNFα (category no. Mm00443258_m1; [38]), IL-6 (category no. Mm00446190_m1; [44]), beta-actin (actb, category no. Mm00607939_s1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, category no. Mm99999915_g1) that were purchased from Applied Biosystems (California, USA). Reactions were performed in triplicate using 125 ng cDNA template *per* reaction. GAPDH and Actb were used as endogenous housekeeping control genes. Non-template controls were included in the experiment. The average of the triplicates of each sample was calculated and the relative quantitative measurement of target gene levels was performed using the ΔΔCt method [45].

2.9. High performance liquid chromatography (HPLC)

Samples from PFC and hippocampus (Hp) were diluted 1:7 (weight/volume) in milli-Q water and homogenized with a pellet pestle motor. For each 100 µl of sample, 25 µl of 6% perchloric acid was added. Samples were centrifuged at 16,000 xg for 15 min at 4 °C, and the supernatants were collected and filtered with a 0.45 µm filter. Serotonin (5-HT), tryptophan (TRP), and kynurenine (KYN) levels were quantified by HPLC. The HPLC system used was an ALEXYS® Neurotransmitter

Analyzer (Antec Scientific, Leiden, The Netherlands) with an Acquity UPLC® BEH C18, 1.7 µm, 1 × 100 mm column (Waters, Massachusetts, USA). The oxidation potential was set at 0.7 V, and 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 ratios KYN/TRP and KYN/5-HT are represented.

2.10. Statistical analysis

Results are expressed as mean ± standard error of the mean (S.E.M.).

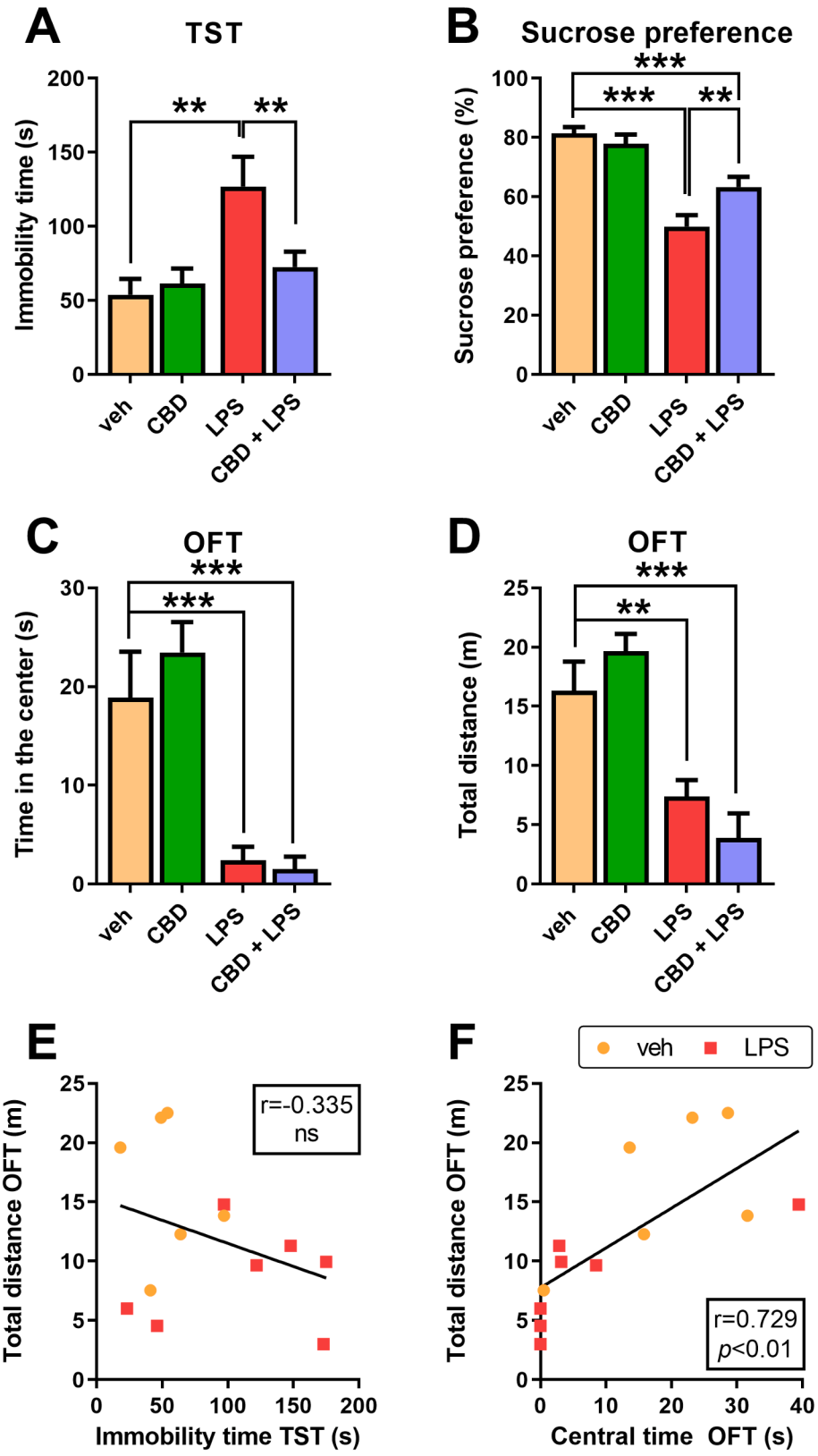

Fig. 2. Behavioral effect of cannabidiol (CBD) administration in the lipopolysaccharide (LPS) model. Immobility time in the tail suspension test (TST) (A), sucrose preference test (B), time in the center in the open-field test (OFT) (C), and total distance traveled in the OFT (D). Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Newman-Keuls *posthoc* test. $^{**}p < 0.01$, and $^{***}p < 0.001$. Correlation between the total distance traveled in the OFT and the immobility time in the TST (E). Correlation between the total distance traveled and the time spent in the center in the OFT (F). r: Pearson's correlation coefficient. ns: not significant. veh: vehicle group. n = 6–9 mice *per* group.

As CBD treatment was administered to both *naïve* and LPS mice, the statistical analysis was performed using two-way ANOVA followed by Newman-Keuls *posthoc* test. For the correlation studies, the Pearson's correlation coefficient (r) was used. Graphs and statistical analyses were made using the GraphPad Prism software, version 6.1 (GraphPad Software Inc., California, USA). The level of significance was set at $p < 0.05$. The number of animals used in each experimental group is indicated in the figure legends.

3. Results

3.1. Antidepressant-like effect of CBD in the LPS model.

In the tail suspension test (TST), the LPS group showed an increased immobility time (126.8 ± 20.3 s) compared to the vehicle group (53.8 ± 10.7 s, $p < 0.01$). The administration of CBD prevented the increase in the immobility time induced by LPS (72.6 ± 10.3 s, $p < 0.01$) (Fig. 2A). A two-way ANOVA showed a significant effect of the model [$F(1,22) = 10.26$, $p < 0.01$], and the interaction [$F(1,22) = 5.54$, $p < 0.05$].

In the sucrose preference test, the LPS group presented a decreased sucrose preference (49.9 ± 3.9%), compared to the vehicle group (81.4 ± 2.2%, $p < 0.001$). The administration of CBD to LPS mice (63.3 ± 3.4%) increased the sucrose preference compared to the LPS group (49.9 ± 3.9%, $p < 0.01$) (Fig. 2B). A two-way ANOVA showed a significant effect of the model [$F(1,28) = 51.89$, $p < 0.001$] and the interaction [$F(1,28) = 7.00$, $p < 0.05$].

In the open-field test (OFT), the LPS group showed a decrease in the time spent in the center (2.4 ± 1.4 s) compared to the vehicle group (18.9 ± 4.7 s, $p < 0.001$). The administration of CBD to LPS mice did not modify the central time compared to the LPS group (1.5 ± 1.3 s) (Fig. 2C). A two-way ANOVA showed a significant effect of the model [$F(1,22) = 44.86$, $p < 0.001$]. LPS mice also traveled less distance in this test (7.41 ± 1.37 s) compared to the vehicle group (16.32 ± 2.46 s, $p < 0.001$), and CBD administration to LPS mice did not alter the distance travelled (3.88 ± 2.1 s) (Fig. 2D). A two-way ANOVA showed a significant effect of the model [$F(1,22) = 42.31$, $p < 0.001$].

CBD administration to *naïve* mice did not modify any of the behavioral parameters studied.

Correlations were made to check if the reduced locomotion induced by LPS could interfere in the behavioral tests' interpretation. There was no correlation between the total distance traveled in the OFT and the

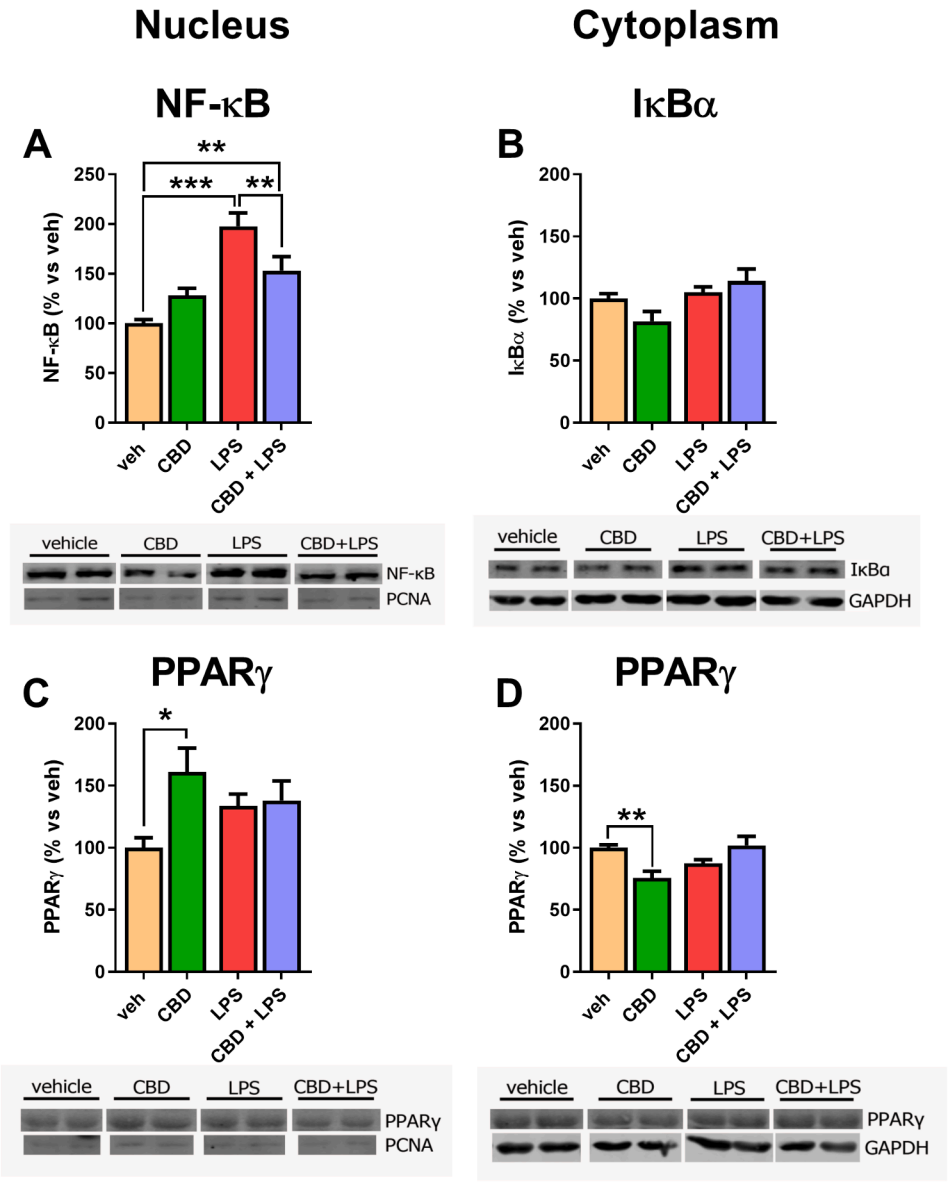

Fig. 3. NF-κB (A), IκBα (B) and PPARγ (C and D) levels in nuclear (A and C) and cytoplasmic (B and D) fractions of cortical samples from lipopolysaccharide (LPS) mice with cannabidiol (CBD) treatment. PCNA and GAPDH were used as nuclear and cytoplasmic housekeeping proteins, respectively. Representative bands from the western blot of each marker and the corresponding housekeeping proteins are shown. Results are expressed in percentage *vs* the vehicle group (veh) and as mean ± S.E.M. Two-way ANOVA followed by Newman-Keuls *posthoc* test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n = 6–7 animals *per* group.

time immobile in the TST (Fig. 2E). Conversely, a significant correlation was found between the total distance traveled and the time spent in the center in the OFT (Fig. 2F).

3.2. CBD-induced changes in the LPS model: NF-κB pathway activation

The activation of the NF-κB pathway and PPARγ was assessed by determining NF-κB, IκBα and PPARγ protein levels in the nuclear and cytosolic fractions from cortical samples. Twelve hours post-LPS injection, there was an increase of NF-κB nuclear levels (197.9 ± 32.6%) in comparison to the vehicle group (100 ± 9.3%, $p < 0.001$), and CBD pretreatment significantly attenuated this increase in LPS mice (153 ± 37.7%, $p < 0.01$) (Fig. 3A). A two-way ANOVA showed a significant effect of the LPS model [$F(1,22) = 32.33$, $p < 0.001$] and the interaction [$F(1,22) = 11.35$, $p < 0.01$].

No changes were observed in the cytosolic levels of IκBα in any of the experimental groups in the post-test analysis (Fig. 3B). A two-way ANOVA showed an effect of the LPS model [$F(1,22) = 6.56$, $p < 0.05$].

CBD administration in *naïve* animals produced an increase of PPARγ in the nucleus (161 ± 50.9%) compared to the vehicle group (100 ± 19.8%, $p < 0.05$) (Fig. 3C) and a decrease in the cytosolic fraction (75.5 ± 14.4% in CBD *vs* 100 ± 6.1% in the vehicle group, $p < 0.01$) (Fig. 3D). Neither the LPS nor the CBD + LPS groups presented differences in PPARγ protein levels compared to the vehicle group (Fig. 3C and D). A two-way ANOVA revealed a significant effect of CBD on PPARγ nuclear levels [$F(1,22) = 5.01$, $p < 0.05$] and a significant effect of the interaction on PPARγ cytosolic levels [$F(1,22) = 13.54$, $p < 0.01$].

3.3. CBD-induced changes in the LPS model: pro-inflammatory cytokines IL-6 and TNFα in plasma and brain

LPS administration induced a mild increase in plasma TNFα levels (9.3 ± 1.3 pg/ml) compared to the vehicle group (6.3 ± 0.2 pg/ml, $p < 0.05$). No changes were observed in the LPS mice treated with CBD (Fig. 4A). A two-way ANOVA showed a significant effect of the model [$F(1,25) = 9.06$, $p < 0.01$]. LPS injection produced a huge increase in plasma IL-6 levels in LPS (1143 ± 191 pg/ml) *versus* the vehicle group (27.6 ± 4.3 pg/ml, $p < 0.001$). The CBD + LPS group showed a significant reduction of IL-6 levels (573 ± 44 pg/ml) compared to the LPS group ($p < 0.001$) (Fig. 4B). A two-way ANOVA showed a significant effect of the model [$F(1,25) = 76.64$, $p < 0.001$], treatment [$F(1,25) = 9.00$, $p < 0.01$], and the interaction [$F(1,25) = 9.14$, $p < 0.01$].

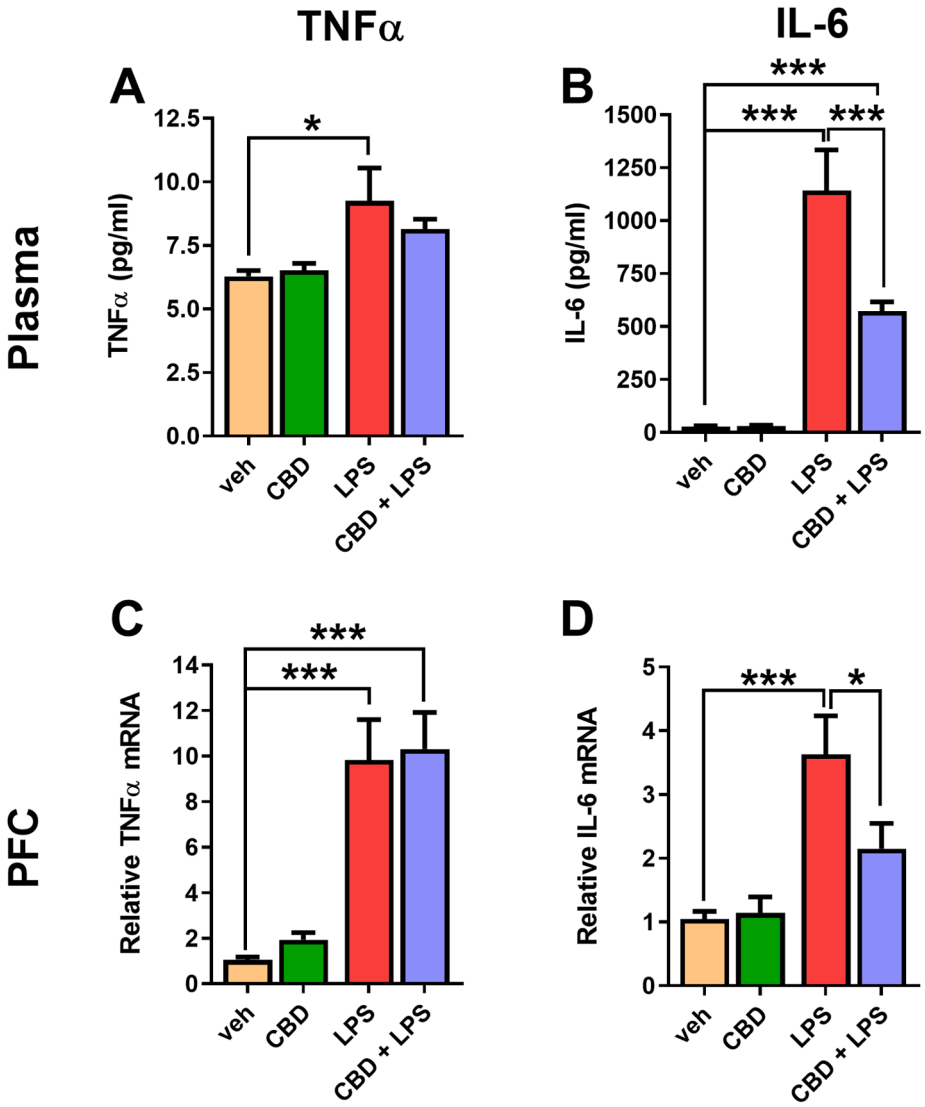

Fig. 4. TNFα (A) and IL-6 (B) levels in plasma, and TNFα (C) and IL-6 (D) mRNA expression levels in the prefrontal cortex (PFC) of lipopolysaccharide (LPS)-treated mice with cannabidiol (CBD) administration. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Newman-Keuls *posthoc* test, $^{*}p < 0.05$, $^{***}p < 0.001$. veh: vehicle group. n = 6–9 animals *per* group.

In PFC, the LPS administration increased TNFα mRNA levels (9.8 ± 1.8) compared to the vehicle group (1.0 ± 0.1, $p < 0.001$), and the previous CBD administration did not modify the TNFα expression (Fig. 4C). A two-way ANOVA showed a significant effect of the model [$F(1,25) = 45.57$, $p < 0.001$]. IL-6 mRNA levels in PFC were higher in the LPS group (3.6 ± 0.6) compared to the vehicle group (1.0 ± 0.1, $p < 0.001$), and CBD administration reduced LPS-induced IL-6 increase (2.2 ± 0.4, $p < 0.05$) (Fig. 4D). A two-way ANOVA showed a significant effect of the model [$F(1,25) = 22.83$, $p < 0.001$], and the interaction [$F(1,25) = 4.40$, $p < 0.05$]. CBD administration to *naïve* mice did not induce changes in the pro-inflammatory cytokines studied.

3.4. CBD-induced changes in the LPS model: Kynurenine pathway

The kynurenine/tryptophan (KYN/TRP) ratio is used as a marker of the IDO activity [38,46,47]. In the hippocampus of the LPS group a higher KYN/TRP ratio was found (0.557 ± 0.041) compared to the vehicle group (0.360 ± 0.062, $p < 0.05$). The administration of CBD prevented the increase in the KYN/TRP ratio in the LPS group (0.373 ± 0.048, $p < 0.05$) (Fig. 5A). A two-way ANOVA showed a significant effect of the interaction [$F(1,19) = 8.389$, $p < 0.01$]. Similar results were obtained in cortex, as the LPS group presented a higher KYN/TRP ratio (3.449 ± 0.217) *versus* the vehicle group (2.084 ± 0.066, $p < 0.001$). CBD attenuated this increase in the LPS group (2.931 ± 0.189, $p < 0.05$) (Fig. 5C). A two-way ANOVA showed a significant effect of the model [$F(1,28) = 35.47$, $p < 0.001$], and a strong tendency of the interaction [$F(1,28) = 4.060$, $p = 0.054$].

To examine the effects of LPS and CBD administration in the balance between the two major tryptophan metabolic pathways, we determined the kynurenine/serotonin (KYN/5-HT) ratio [48,49]. In the hippocampus of LPS mice, the KYN/5-HT ratio was higher (1.069 ± 0.097) than in the vehicle group (0.650 ± 0.156, $p < 0.01$), while the administration of CBD prevented this increase (0.534 ± 0.066, $p < 0.01$) (Fig. 5B). A two-way ANOVA showed a significant effect of the model [$F(1,19) = 8.160$, $p < 0.05$], and of CBD [$F(1,19) = 17.04$, $p < 0.001$]. In cortex, the KYN/5-HT ratio in the LPS group was higher (4.204 ± 0.315) than the vehicle group (2.127 ± 0.115, $p < 0.001$), and CBD attenuated that increase (2.786 ± 0.184, $p < 0.001$) (Fig. 5D). A two-way ANOVA showed a significant effect of the model [$F(1,28) = 47.30$, $p < 0.001$], CBD [$F(1,28) = 14.39$, $p < 0.001$], and the interaction [$F(1,28) = 8.062$,

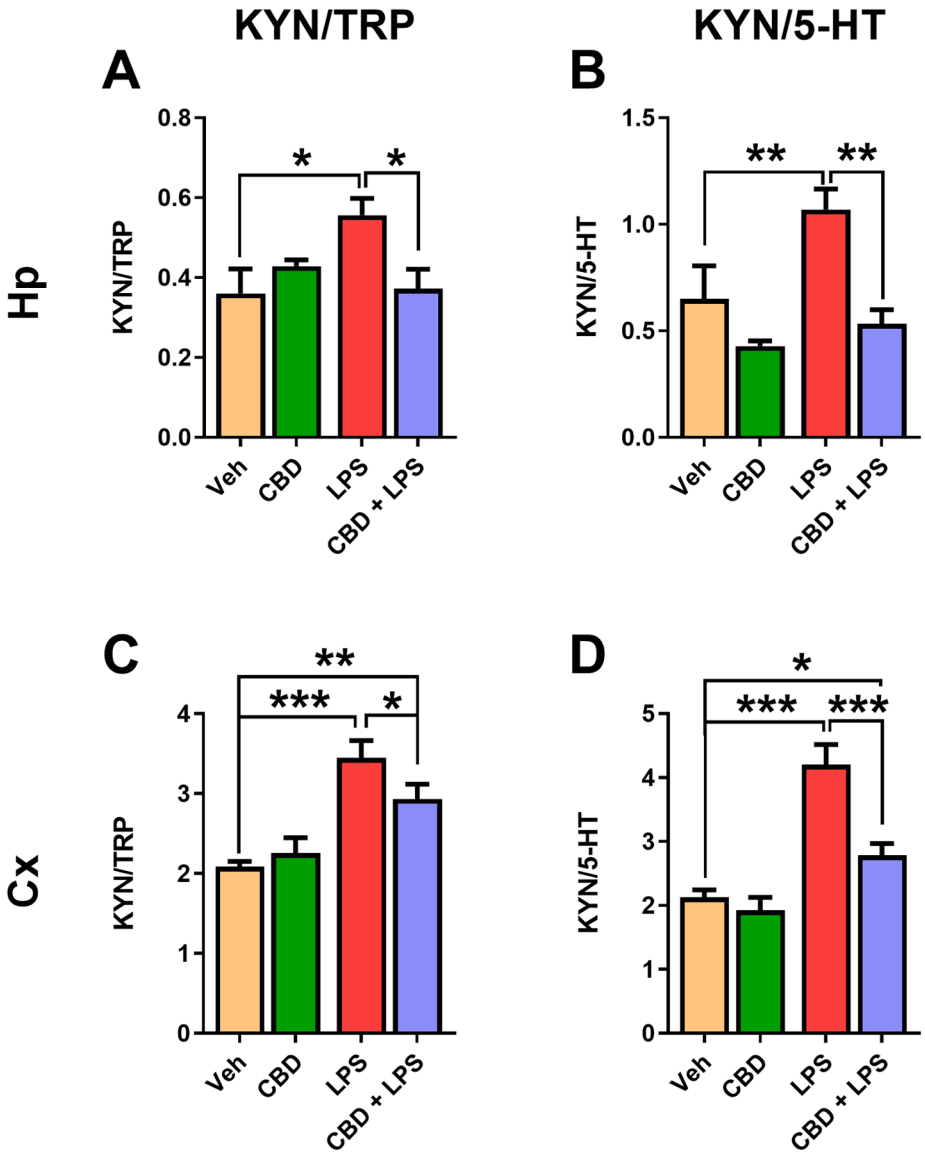

Fig. 5. Ratio kynurenine/tryptophan (KYN/TRP) (A and C) and kynurenine/serotonin (KYN/5-HT) (B and D) in hippocampus (Hp) (A and B) and cortex (Cx) (C and D) after cannabidiol (CBD) treatment in the lipopolysaccharide (LPS) neuroinflammatory model in mice. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Newman-Keuls *posthoc* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. veh: vehicle group. n = 5–9 mice *per* group.

$p < 0.01$]. CBD administration to *naïve* mice did not alter the kynurenine pathway.

4. Discussion

The present study shows the antidepressant-like effect elicited by an acute cannabidiol administration in a neuroinflammatory model induced by LPS administration, in parallel with the reduction of different inflammatory markers in the brain and periphery.

The systemic administration of LPS induces a depressive-like state, as evidenced by the increase in behavioral despair and anhedonia. Since the immobility time in the tail suspension test and the locomotion observed in the open field test did not show a statistically significant correlation in *naïve* and LPS animals, we can infer that the sickness behavior is not interfering with the depressive-like behavior at this time-point. The administration of a single dose of cannabidiol prior to LPS prevented the LPS-induced behavioral despair. This agrees with the effect elicited by the acute administration of SSRIs as fluoxetine and paroxetine [50], drugs with fast antidepressant effect as ketamine [51], and the chronic administration of tricyclic antidepressants such as imipramine [52] in this inflammatory model. The reduction in immobility elicited by cannabidiol in the tail suspension test contrasts with the lack of effect on locomotion observed in the open field test, supporting the impact of cannabidiol on the depressive-like behavior. Moreover, the anhedonic state induced by LPS is partially prevented by cannabidiol, supporting its role in the remission of the different depressive manifestations observed in this model, in line with the results obtained using CBD in other mouse [33] and rat [53] models of depression.

However, cannabidiol treatment did not show anxiolytic-like effects in the LPS model, as it has been described in Wistar Kyoto rats [53]. In contrast, there is an acute anxiolytic-like effect in naïve animals (*rev. in* [54]), and in the olfactory bulbectomy model [33]. Nevertheless, we obtained a significant correlation between locomotion and the anxious-like behavior in our inflammatory model, which reveals the impossibility to separate the anxious from the sickness behavior. Therefore, we cannot draw conclusions about the anxious-like behavior in this study.

The LPS model of neuroinflammation is characterized by an initial sickness behavior that resolves in less than 24 h, followed by a depressive-like behavior that overlaps with sickness in the initial phases, and lasts longer [24]. At the time-point studied in this work, there were still some manifestations characteristic of the sickness behavior (decreased locomotor activity, reduced body weight, etc.) [38,55]. However, this behavior can be differentiated from the depressive-like behavior (behavioral despair and anhedonia), as some molecules as kynurenine induce depressive-like but not sickness behavior, and the blockade of IDO prevents the manifestation of the depressive-like behavior, without altering the sickness behavior induced by LPS [38].

To investigate the mechanism of action of CBD responsible for the behavioral findings, we studied the NF-κB pathway, as it takes part in the pro-inflammatory mechanism of LPS [14]. The LPS model presented increased NF-κB nuclear levels as previously described [18,19], evidencing the activation of the NF-κB pathway. CBD administration prior to LPS significantly attenuated the increase in nuclear NF-κB levels, in line with the inhibitor effect of CBD on this pathway reported in cell culture studies [56–58] and in an animal model of Alzheimer's disease [36]. Interestingly, the widely used antidepressant fluoxetine also reversed the NF-κB-induced increase in a chronic LPS model in mice [59]. The direct inhibition of NF-kB also presents antidepressant-like effects in stress models [60]. The results obtained in NF-κB and IκBα levels fit with the time course of NF-κB pathway activation/deactivation described by Hobbs et al. [61]. They reported that LPS activation of TLR4 rapidly produces a decrease in IκBα cytoplasmic levels and NF-κB nuclear translocation. However, NF-κB deactivation is much slower, as this protein is still increased in the nucleus 10 h after LPS exposure, when IκBα cytoplasmic levels have returned to its basal expression [61]. All this suggests that the reduction of NF-κB pathway activation could participate in the antidepressant-like effect of cannabidiol in this neuroinflammatory model induced by LPS administration.

Regarding PPARγ, it has been reported that LPS produces a reduction in its expression in BV2 cells [16,17], and in rat cortex [18,19] in a few hours. Thus, the lack of changes observed in our experiments 12 h after LPS exposure may be due to the recovery of PPARγ basal levels. In contrast, the administration of CBD induced an increase in PPARγ nuclear levels and a decrease in its cytosolic levels. As CBD is a PPARγ ligand [62]; *rev. in* [63], the CBD-mediated activation of PPARγ in naïve animals would promote its translocation to the nucleus, as previously described [64–66]. In this regard, other authors have reported that chronic CBD treatment induced an increase in PPARγ levels in the spinal cord of an animal model of multiple sclerosis [67] and an increase in PPARγ coactivator 1-alpha (PGC-1α) mRNA levels in the striatum of an animal model of dyskinesia [68]. Thus, this is the first time, to our knowledge, that an increase in PPARγ nuclear levels is reported with a single CBD administration *in vivo*. Although PPARγ nuclear levels were increased in the CBD naïve group, this was not associated to changes in nuclear NF-κB levels. This could be explained by the lack of activation of the NF-κB pathway in this experimental group. The lack of changes in nuclear PPARγ levels in the CBD + LPS group could be due to the role of PPARγ in the downregulation of the pathway. PPARγ can bind to NF-κB in the nucleus and induce the nuclear export of PPARγ-NF-κB complex [69,70], promoting its translocation to the cytosol [65]. Nevertheless, we cannot exclude the possibility that CBD could produce its effects on the NF-κB pathway independently of PPARγ activation, as CBD has also shown anti-inflammatory effects that are not dependent on PPARγ [58,71].

In the evaluation of pro-inflammatory cytokines, LPS administration induced an elevation of TNFα plasma levels to a lesser extent than IL-6 levels, as TNFα peaks earlier than IL-6 in response to LPS [72]. In contrast, the increase in TNFα mRNA expression was higher than the one observed in IL-6 mRNA levels in the brain, as previously described [23]. The coexistence of depressive-like behavior and increased plasma IL-6 levels in this neuroinflammatory model, agrees with the elevated IL-6 levels observed in animal models of depression [73] and in meta-analyses of studies in MDD patients [5,74]. In addition, the dysregulation of both IL-6 production and serotonergic neurotransmission, as observed in our model by the increase in the ratio kynurenine/serotonin, is associated with a greater vulnerability to suffering depression [75]. In line with the effect of cannabidiol on the depressive-like behavior and IL-6 levels presented here, it has been reported that IL-6 knockout animals exhibit reduced behavioral despair and resistance to stress-induced depression [76]. In addition, the increased IL-6 levels observed in acute depressed patients are reverted to control levels after antidepressant treatment [77]. The reduction of IL-6 could be of great importance, as high IL-6 levels are associated not only with depressive-like behavior but also with treatment resistance [73]. Regarding TNFα, previous studies demonstrated that its administration induces sickness behavior and neuroinflammation, but not depressive symptoms as anhedonia [78]. In addition, a cumulative meta-analysis of studies in MDD patients revealed that the association of TNFα with the disease is inconsistent, while other inflammatory markers as IL-6 are clearly associated to MDD [74]. This is in accordance with the fact that, in this work, CBD exerted antidepressant-like effects in the LPS model without producing changes in TNFα levels. These pieces of evidence suggest that the antidepressant-like effect of cannabidiol in the LPS model may be partially due to its effect over IL-6 levels.

To further investigate the mechanism of action of CBD responsible for the findings presented here, we studied the kynurenine pathway. Our results show an increase in the kynurenine/tryptophan ratio in the LPS model, which is indicative of an increased IDO activity [38,46,47]. This IDO activation is further confirmed by the increased kynurenine/serotonin ratio in these animals, showing a tilted balance toward kynurenine synthesis. Moreover, the coexistence of behavioral despair and anhedonia with elevated kynurenine levels in our animal model is in line with

the behavioral despair [38] and anhedonia [79] induced by kynurenine administration. Conversely, CBD administration produced antidepressant-like effects and a decrease in kynurenine/tryptophan and kynurenine/serotonin ratios, which is in line with the antidepressant-like effect described following IDO enzyme inhibition [38,80]. Moreover, the inhibition of this enzyme is also associated to a lower anhedonic state, as observed in IDO knockout animals [81]. This body of evidence, together with the reversion of the behavioral and molecular changes within the kynurenine pathway promoted by cannabidiol in the LPS model, suggest that cannabidiol could be producing its antidepressant- and hedonic-like effects in this model by the reduction of brain IDO activity. This could be due to the direct inhibition of the enzyme [82], or to the decrease in pro-inflammatory cytokines [22]. Within the kynurenine pathway, recent basic and clinical research highlight the involvement of the kynurenine metabolites in depression [*rev. in* [27,29]]. In animal models such as LPS-induced neuroinflammation and in MDD patients, the neurotoxic branch of the KYN pathway [3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN)] is increased, while the neuroprotective branch [kynurenic acid (KYNA)] is decreased [83]; *rev. in* [29]. This imbalance between the two KYN branches has been related to a depressive-like behavior [84,85]. As it has been recently found that CBD can increase KYNA levels and decrease 3-HK and QUIN levels after a neurotoxic stimulus [86], all these pieces of evidence suggest that the restoration of this neurotoxic/neuroprotective balance could be also participating in the antidepressant-like effects elicited by CBD in the LPS model presented in this work.

In summary, our results show for the first time that cannabidiol exerts antidepressant-like effects in the LPS model of depression, together with a reduction of brain NF-κB pathway activation, plasma and brain IL-6 levels and IDO activity. However, we cannot exclude the possibility that other mechanisms of action proposed for cannabidiol could have also participated in the observed effects. In view of the anti-neuroinflammatory actions of CBD associated to its antidepressant-like effect, more research is needed to better characterize it as a potential drug in the treatment of depression.

5. Funding and acknowledgments

This research was supported by the Ministerio de Economía y Competitividad (SAF2015-67457-R MINECO/FEDER), the Ministerio de Ciencia, Innovación y Universidades (RTI2018-097534-B-I00), the Instituto de Salud Carlos III (PI19/00170), and Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM). E F-Z was supported by a predoctoral fellowship from the Universidad de Cantabria (Spain). We acknowledge the technical assistance of Annamaria Architravo and Deborah Vasturzo, and Dr Rebeca Vidal for her scientific advice.

CRediT authorship contribution statement

Eva Florensa-Zanuy: Investigation, Methodology, Validation, Visualization, Writing - original draft. **Emilio Garro-Martínez:** Investigation. **Albert Adell:** Methodology, Writing - review & editing. **Elena Castro:** Conceptualization, Writing - review & editing. **Álvaro Díaz:** Conceptualization, Writing - review & editing. **Ángel Pazos:** Funding acquisition. **Karina S. Mac-Dowell:** Methodology, Writing - review & editing. **David Martín-Hernández:** Methodology, Writing - review & editing. **Fuencisla Pilar-Cuéllar:** Conceptualization, Funding acquisition, Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Biochemical Pharmacology 157 (2018) 97–107

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Review

The endocannabinoid system in mental disorders: Evidence from human brain studies

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ARTICLE INFO

Keywords:
Human brain
Depression
Anxiety
Schizophrenia
Endocannabinoids
Cannabinoid receptors

ABSTRACT

Mental disorders have a high prevalence compared with many other health conditions and are the leading cause of disability worldwide. Several studies performed in the last years support the involvement of the endocannabinoid system in the etiopathogenesis of different mental disorders. The present review will summarize the latest information on the role of the endocannabinoid system in psychiatric disorders, specifically depression, anxiety, and schizophrenia. We will focus on the findings from human brain studies regarding alterations in endocannabinoid levels, cannabinoid receptors and endocannabinoid metabolizing enzymes in patients suffering mental disorders.

Studies carried out in humans have consistently demonstrated that the endocannabinoid system is fundamental for emotional homeostasis and cognitive function. Thus, deregulation of the different elements that are part of the endocannabinoid system may contribute to the pathophysiology of several mental disorders. However, the results reported are controversial. In this sense, different alterations in gene and/or protein expression of CB1 receptors have been shown depending on the technical approach used or the brain region studied. Despite the current discrepancies regarding cannabinoid receptors changes in depression and schizophrenia, present findings point to the endocannabinoid system as a pivotal neuromodulatory pathway relevant in the pathophysiology of mental disorders.

1. Introduction

Mental disorders are responsible for the largest proportion of the global burden of disease worldwide. It has been suggested that by 2030 depression will be the leading cause of disease burden globally. In this way, mood-related disorders contribute most of the non-fatal burden of mental illness followed by anxiety-related disorders, substance abuse and schizophrenia [1]. They present a major medical, societal and economic burden that has a large impact on individuals, families and communities.

Actual knowledge about the etiology and pathophysiology of mental disorders is mainly a result of an interaction between the development of new technology and the direct study of the brain tissue of patients. Thus, the description of morphological differences, functional deficits and molecular alterations is widely accepted today as existing in the brain of psychiatric patients due to the advance of *in vivo* neuroimaging techniques, genetic and genomic development, and the use of post-mortem brain tissue as a key substrate of the disease [2]. Nevertheless, despite the huge economic and scientific effort developed in the last decades, the pathophysiology of mental disorders remains elusive. In this context, many studies have focused in the possible involvement of alterations of the endocannabinoid system (ECS) in the pathophysiology of mental disorders such as depression or schizophrenia. The ECS participates, in part, in the control of emotional behavior and mood

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https://doi.org/10.1016/j.bcp.2018.07.009
Received 29 May 2018; Accepted 12 July 2018
Available online 17 July 2018
0006-2952/

through a functional coupling with monoaminergic systems in the brain [3]. These functional interactions have suggested a potential role for ECS signaling in the neurobiology of various psychiatric disorders [4–7]. The ECS is composed of two inhibitory G-protein coupled receptors (GPCRs), cannabinoid receptor 1 and 2 (CB1 and CB2, respectively), and two major endogenous ligands, N-arachidonoylethanolamine (anandamide/AEA) and 2-arachidonoylglycerol (2-AG). The ECS also includes two main metabolic enzymes, the fatty acid amide hydrolase (FAAH) and the monoacylglycerol lipase (MAGL) which hydrolyze AEA and 2-AG, respectively; and two main synthetizing enzymes, N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) and the diacylglycerol lipase (DAGL) which synthesize AEA and 2-AG, respectively. The correct interplay between all these ECS elements plays an important role in central nervous system (CNS) development, synaptic plasticity, and the homeostatic maintenance of cognitive, behavioral, emotional, developmental, and physiological processes [8,9]. In the brain, CB1 receptors are present in GABAergic and glutamatergic neurons, exerting a presynaptic inhibitory function when they are activated by the released endocannabinoids [10,11]. They are the most abundant G-protein coupled receptors and are widely expressed all throughout the brain, being located in cortical, subcortical, cerebellar and brainstem structures [8]. The CB2 receptors are less numerous and were initially thought to be located mainly in the immune system; however, currently they seem to be widely distributed in the CNS, taking part in immune-mediated responses and supporting a neuroprotective role against inflammation [12]. The two main endogenous ligands, AEA and 2-AG, are eicosanoid neuromodulatory lipids derived from membrane phospholipids, synthesized when and where they are required, and acting presynaptically on both type of cannabinoid receptors [8].

In the present review, we will summarize data obtained from human studies providing evidence about the role of the different ECS components (endocannabinoids, metabolizing/synthetizing enzymes and cannabinoid receptors) in the pathophysiology and treatment of several psychiatric disorders, with a focus on results from postmortem and living human brain studies. We will review findings from patients suffering a mood-related disorder (depression, anxiety, posttraumatic stress disorder (PTSD)) or schizophrenia compared to healthy subjects.

2. The endocannabinoid system and the emotional homeostasis

The ECS influences the activity of multiple brain areas involved in the regulation of the hypothalamic-pituitary-adrenal system (HPA), mood, anxiety and other related behaviors (i.e. extinction of fear learning, reward…). Indeed, the ECS enables the efficient interaction within and between brain regions that modulate cognitive and behavioral functioning.

A considerable number of studies suggest the relationship between changes in one or more components of the ECS and some of the symptoms that are present in depression and anxiety-related disorders. The ECS modulates fear and anxiety-related behaviors in both humans and rodents [13–15]. Augmented ECS signaling is usually followed by reduced conditioned fear and anxiety, whereas the opposite effect is observed when it is inhibited [16–19]. This is not surprising since the ECS is present in key structures within the brain such as prefrontal cortex (PFC), amygdala, and hippocampus [20–25].

There are also animal studies showing the correlation between CB1 receptor-deficiency and depressive/comorbid symptoms (anhedonia, anxiety, and heightened stress-response) [26–28]. In line with this relationship, chronic stress, as a pathogenic factor for depressive-behavior, has been associated with a dysfunctional endocannabinoid signaling in the brain [29]. Thus, strategies that are directed to the augmentation of the endocannabinoid signaling are reported to mitigate many of the adverse effects of chronic stress, such as anhedonia and anxiety [30–32]. The readers are directed to comprehensive reviews on this topic that is beyond the scope of this review [33–38].

3. The endocannabinoid system and depression

Depression is one of the most prevalent major neuropsychiatric diseases, affecting 20% of the population, being almost twice as common in females than males [39]. There are two main challenges to fight against depression. First, we still poorly understand its neurobiological and pathological bases. Second, there needs to be more effective antidepressant drugs overcoming the therapeutic lag between drug administration and the onset of clinical improvement, the lack of response in some patients and safety/tolerability issues [40,41].

The implication of the ECS in depression comes from observational findings regarding the mood-related effects of cannabis in humans, though contradictory results are reported. On one hand, the heavy use of cannabis is associated to a higher incidence of depressive disorders [42]. A recent meta-analysis showed a positive correlation between cannabis use and depression, being more evident among heavy cannabis users [43]. Moreover, the abuse of cannabis has been linked to a higher risk of suicide in patients with mood disorders [44]. On the other hand, other authors indicate that the use of marijuana, or its main psychoactive component Δ^9-tetrahidrocannabinol (THC), reduces the depressive behavior [45–48]. In addition, other authors describe that the administration of THC to patients with moderate to severe depression shows a lack of effect on mood [49], or on the suicidal ideation [50], but an increased anxiety [49].

Curiously, the pharmacological blockade of CB1 receptors using antagonists or inverse agonists was initially suggested as a potential novel target for antidepressant treatment, according to different evidences in preclinical studies [51]. However, further studies revealed that drugs as the CB1 receptor antagonist rimonabant (SR 141716A) that was marketed to treat obesity, induced depressed mood [52,53]. In animal studies, the pharmacological activation or blockade of CB1 receptors also give rise to contradictory results, since both approaches lead to an antidepressant-like effect [5,51,54–58].

Although these studies draw conflicting findings, they point to the involvement of the brain ECS in the modulation of mood and, especially, the contribution of CB1 receptors to major depression have received particular attention [59]. The activation of cannabinoid receptors produces the release of stress hormones as ACTH and cortisol in the HPA axis [60], which has been observed following acute marijuana administration [61]. However, this presents tolerance after chronic administration [62], as the THC-induced cortisol release is blunted in frequent marijuana users [63].

Neuroimaging studies in non-cannabis users show that the administration of THC produces a reduced activation of some brain areas in response to a negative content [64,65]. Conversely, there is an increased activation in response to a positive content, mediated by the activation of areas such as prefrontal and occipital cortices, amygdala, hippocampus and orbitofrontal gyrus [65], which is associated to a reduction in the negative attentional bias, and the potentiation of positive attentional bias [65]. This type of studies have also shown that the activation of cannabinoid receptors leads to morphological changes, such as the reduction in white matter (WM) observed in marijuana users that negatively correlates with the severity of the depressive disorder [66,67]. This decrease in WM volume has been associated with the presence of cannabinoid receptors in oligodendrocytes, the myelin-forming cells [68].

3.1. Cannabinoid receptors in depression

Human studies have corroborated the existence of an altered ECS activity associated to major depression [34,69]. The CB1 receptor density [70] and mRNA [71] is increased in the dorsolateral prefrontal cortex of patients with major depression, in parallel to CB1 receptor functionality (evaluated by [^{35}S]GTPγS binding studies) [70] (Mato et al., in this issue). However, no changes in CB1 immunoreactivity in the dorsolateral prefrontal cortex [72], or a reduction in CB1

Table 1
Studies about alterations of the different components of the ECS in the brain of patients with depression or anxiety-related disorders.

ECS element	Finding (% change)	Brain region	Cohort (n: disease-Ct)	Method/sample	References
CB1 (mRNA)	↑ (60%)	DLPC (BA46)	MDD:Ct 26:46	Gene expression microarray/PMBT	[71]
CB1 (protein)	≈	DLPC (BA46)	MDD:Ct 14:14	IHC/PMBT	[72]
	↑ (38%)	DLPC (BA9)	MDD with suicide:Ct 10:10	WB/PMBT	[70]
	↓ (22.1%)	Glial$^+$ cells in ACC GM	MDD:Ct 15:15	IHC/PMBT	[73]
	≈	Neurons$^+$ cells in ACC	MDD:Ct 15:15	IHC/PMBT	[73]
	↓ (~8.5–9.5%)	Neurons$^+$ cells in ACC	MDD + SSRI:MDD no-SSRI	IHC/PMBT	[73]
CB1 (density)	↑ (31%)	DLPC (BA9)	MDD with suicide:Ct 10:10	[^{3}H]CP-55,940 binding/PMBT	[70]
CB1 (functionality)	↑ (45%)	DLPC (BA9)	MDD with suicide:Ct 10:10	[^{3}H]CP-55,940 and [^{35}S]GTPγS binding/PMBT	[70]
CB1 (availability)	↑ (19.5%)	Brain-wide	PTSD:Ct 25:23	*In vivo* brain PET scan [^{11}C]OMAR	[100]
	↑ (14.5%)	Brain-wide	PTSD:TC 25:12	*In vivo* brain PET scan [^{11}C]OMAR	[100]
	↑	Amygdala	PTSD:TC:Ct 12:4:4	In vivo brain PET scan [^{11}C]OMAR	[101]
CNR1 gene rs1049353 (A)	↑ activity associated to emotional processing	Bilateral amygdala, putamen and pallidum	MDD(AG) MDD(GG) 13:20	Genetic association study with fMRI/peripheral cells	[80]
CB1-HINT1 (protein)	≈	PFC (BA9)	MDD:Ct 24:24	Co-IP/PMBT	[86]
CB1-NR1 C1 (protein)	≈	PFC (BA9)	MDD:Ct 24:24	Co-IP/PMBT	[86]
CB2 (mRNA)	≈	DLPC (BA46)	MDD:Ct 26:46	Gene expression microarray/PMBT	[71]

↑ Increase; ↓ decrease; ≈ no significant change. ACC: anterior cingulate cortex; BA: Brodmann's area; CB1, CNR1: cannabinoid receptor 1; CB2: cannabinoid receptor 2; Co-IP: co-immunoprecipitation; Ct: control; DLPC: dorsolateral prefrontal cortex; FAAH: fatty acid amide hydrolase; fMRI: functional magnetic resonance image; GM: grey matter; HINT-1: histidine triad nucleotide binding protein 1; IHC: immunohistochemistry; MDD: major depression disorder; PET: positron emission tomography; PFC: prefrontal cortex; PMBT: post-mortem brain tissue; PTSD: posttraumatic stress disorder; SSRI: serotonin selective reuptake inhibitor; TC: trauma controls; WB: western blot.

immunoreactivity in glial cells in the anterior cingulate cortex [73] have been reported (Table 1). Regarding CB2 receptor, no changes have been detected in its mRNA levels in prefrontal cortex of depressed patients [71] (Table 1).

The role of the ECS in the effect of antidepressant drugs has also been evidenced in studies reporting an increased CB1 receptor expression [74], and a lack of changes in CB1-mediated activation of Gi/o proteins in prefrontal cortex (Mato et al., in this issue) in the antidepressant-treated group. Other areas such as the hippocampus only elicited increased CB1 receptor density after chronic monoamine oxidase inhibitors (MAOI) treatment [74]. On contrast, studies in human brain samples have shown a reduction in CB1 receptor immunoreactivity in the anterior cingulate cortex of patients treated with serotonin selective reuptake inhibitors (SSRIs) [73].

The presence of different single nucleotide polymorphisms in the cannabinoid receptor 1 (*CNR1*) gene appears to modulate either the depressive phenotype, and/or the response to antidepressant treatment. The carriers of *CNR1* gene variants influences the vulnerability to suffer mental disorders, including major depression [75]. The frequency of the G allele of the *CNR1* gene polymorphism rs806371 is higher in patients with major depression showing comorbid psychotic symptoms, while the haplotype C-G-T (rs806368, rs1049353, rs806371) is associated with an increased risk for melancholic and psychotic symptoms of major depression [76]. This is consistent with the melancholic depressive-like symptoms observed in animal models with pharmacological or genetic blockade of the ECS [4]. Other studies report a lower incidence of depression in Parkinsons' disease patients carrying two long alleles of the *CNR1* gene polymorphism (AAT)n [77]. The C allele carriers of the *CNR1* gene polymorphism rs2023239 present a lower incidence of major depression within a group of methadone-responder patients [78].

In patients with the *CNR1* gene polymorphism rs1049353, carriers of one or more copies of the minor allele (AA/AG) exhibit a buffering effect to anhedonia and depression after early childhood trauma [79]. Moreover, the G allele of the rs1049353 polymorphism is associated to the resistance to the antidepressant treatment in females diagnosed with major depression that present comorbid anxiety [80]. Patients with the G allele of the *CNR1* rs1049353 also present a subcortical hypo-responsiveness in the bilateral amygdala, putamen, and pallidum activity and left lateralized caudate and thalamus activity, to specific cues, which might be linked to a deficient effect on the processing of emotional and social behavior [80]. On contrast, other authors report a better response to treatment of male presenting the GG genotype [76]. In patients treated with citalopram, TT homozygous carriers for the rs806368 and rs806371 polymorphisms, show a higher incidence of no remission, compared to the G carriers [76]. Moreover, the response in the rs806368 G carriers was different depending on the gender, presenting a better antidepressant outcome in men than in women [76].

Although the CB2 receptor subtype is less abundant than CB1 receptor subtype in the brain, some studies also describe an association with mental disorders. In this sense, the RR genotype of the Q63R polymorphism in the *CNR2* gene presents a higher association with depression in the Japanese population [81]. This Q63R polymorphism presents also a high incidence in patients with eating disorders (anorexia nervosa and bulimia nervosa) [82] and schizophrenia [83]. Studies in cells expressing this mutated form of the *CNR2* gene showed that the functional relevance of this polymorphism is due to changes in CB2 ligand affinity, constitutive activity and a reduced 2-AG-induced adenylyl cyclase inhibition [84]. Regarding the *CNR2* gene polymorphism rs2501431, the AA carriers present a higher severity of the disease, compared to the G carriers [85].

The activity of cannabinoid receptors is associated to the cross-regulation that the CB1 receptors exert over the NMDA receptors mediated by their interaction via the histidine triad nucleotide binding protein 1 (HINT-1), in which a reduction in the number of CB1 receptors may be associated to the NMDA hyperfunction observed in depression [86]. In this sense, molecular studies have shown an increase in the HINT-1 protein and the NR1 subunit of the NMDA receptors in the prefrontal cortex of depressed patients, in parallel to results obtained in CB1 receptor knockout mice [86].

3.2. Endocannabinoid metabolizing enzymes in depression

One of the most frequent polymorphism of FAAH in humans is a functional non-synonymous single-nucleotide polymorphism (C385A; rs324420) associated with a reduced cellular expression of this enzyme. The C385A polymorphism of the *FAAH* gene, presents a greater association in A allele carriers with pathologies such as depression and bipolar disorder [87]. Moreover, the presence of this polymorphism constitutes a susceptibility factor to develop depressive and anxious phenotypes in adult individuals that have been exposed to childhood trauma [88]. The high AEA levels because the reduced FAAH activity in

the A allele carriers, induce the desensitization of CB1 receptors. This reduction in CB1 receptors promotes a glutamatergic hyperactivity that, together with high cortisol levels due to childhood trauma in critic neurodevelopmental periods, results in anxious and/or depressive disorders [88]. The CC carriers of the rs324420 polymorphism in the *FAAH* gene present a reduction in the WM integrity of fibers that connect with the anterior cingulate cortex and the orbital cortex, and greater incidence of self-reported depressive symptoms [67].

3.3. Endocannabinoids levels in depression

The serum content of endocannabinoids is also altered in major depression. Some authors report lower levels of the circulating endocannabinoids AEA and 2-AG in patients with depression [89,90]. Moreover, the 2-AG levels are lower in patients with a longer duration of the depressive episode, while patients with minor depression present higher levels of AEA [89]. In contrast, other authors report no changes in AEA and 2-AG levels in depressed women [91].

The levels of the endocannabinoids AEA and 2-AG were not modified in response to SSRIs such as fluoxetine, while the chronic administration of MAOIs induced a reduction in areas such as prefrontal cortex, hippocampus and hypothalamus [74]. These data are consistent with a study that associates the beneficial effect of exercise in depression with an increase in the plasma levels of AEA, BDNF and cortisol [92].

4. The endocannabinoid system and anxiety-related disorders

Few neurochemical, molecular genetics and neuroimaging studies suggest a potential link between dysregulation of the endocannabinoid signaling and anxiety-related behavior in both healthy and patients with mental disorders in which anxiety is a core symptom (PTSD, social phobia, agoraphobia, etc…).

4.1. Cannabinoid receptors in anxiety

Two single nucleotide polymorphism (SNP) variants (C-A and C-G) of the haplotype formed by the polymorphisms rs806368 and rs1049353 at the *CNR1* gene showed a significant association with PTSD [93]. Moreover, a higher risk to suffer anxiety is observed when homozygous 'SS' of the polymorphism of serotonin transporter (5-HTTLPR) in the SLC6A4 promoter is combined with the homozygous 'GG' rs2180619 of *CNR1* gene [94]. This highlights the strong interaction between the serotonergic system and the ECS on anxiety disorders as extensively described in many preclinical and clinical studies, using pharmacological and genetic approaches [37,95,96].

Moreover, Heitland et al. [97] published the first evidence, in healthy medication-free human subjects, of the implication of ECS in the fear extinction phenomenon, a relevant mechanism underlying the pathophysiology of human anxiety disorders. These authors describe the effect of the *CNR1* gene polymorphism rs2180619 in the response to fear conditioning and extinction. They found that both homozygote (G/G) and heterozygote (A/G) G-allele carriers of this polymorphism showed a clear extinction of fear, whereas this response was absent in homozygotes (A/A).

Recent findings suggest that during childhood and adolescence, the ECS is critical to mediate a correct balance between excitatory and inhibitory neurotransmission, especially within the prefrontal cortex [98]. This makes the endocannabinoid signaling quite sensitive for developmental fluctuations due to environmental causes, which may increase the risk of anxiety and other stress-related disorders. Interestingly, a recent study shows the impact of the variation in the *CNR1*, *CNR2*, and *FAAH* genes in a sample of children with a primary anxiety disorder diagnosis [99]. These authors nicely reported an association between two SNPs (rs12133557 and rs6454676) in the *CNR1* gene and the change in symptom severity in both the entire sample and a subset of patients with fear based diagnoses [99]. Moreover, a favorable and a poorer response during the active treatment period were associated with minor allele of rs12133557 and rs6454676, respectively. Regarding the *CNR2* gene, unlike to previous findings in depression [81], the rs2501431 genotype was not associated with anxiety symptoms or treatment response [99].

In vivo neuroimaging studies also support the existence of an abnormal CB1 receptor-mediated signaling especially in PTSD. Using positron emission tomography (PET) with [^{11}C]OMAR, a CB1-selective tracer, Neumeister and colleagues [100] reported a higher CB1 receptor availability in untreated individuals with PTSD, relative to control subjects (with or without lifetime histories of trauma), which was most pronounced in women. This up-regulation of CB1 receptors was present in anxiety-related brain areas, especially the amygdala-hippocampal-cortico-striatal neural circuit. In a later report, the same group [101] assessed the attentional bias to threat, which is considered one of the main endophenotypic characteristics of trauma-related mental disorders. In line with their previous findings, they reported a positive correlation between an increased CB1 receptor availability ([^{11}C]OMAR binding) in the amygdala and increased in both attentional bias to threat and the severity of threat. Interestingly, this greater CB1 receptor availability in the amygdala was associated with lower plasma levels of AEA.

4.2. Endocannabinoid metabolizing enzymes in anxiety

Pharmacological strategies that reduce the activity of either FAAH or MAGL have been reported to reduce anxiety-like behaviors in rodents and humans [37]; however, dual FAAH/MAGL inhibitors did not reduce stress-related affective dysfunction regardless of treatment timing [102]. There are several studies linking the activity of FAAH, especially in the amygdala, with stress-reactivity and risk to suffer anxiety-disorders.

In healthy volunteers, there are some studies examining the impact of the *FAAH* gene polymorphism C385A (rs324420) on threat- and reward-related human brain function. Using imaging genetics, a decreased threat-related amygdala reactivity but increased reward-related ventral striatal reactivity was detected in carriers of the A allele of the *FAAH* enzyme gene [103]. In a later study, a quicker habituation of amygdala reactivity to threat and lower scores on the personality trait of stress-reactivity was found to be associated with carriers of a low-expressing FAAH variant (385A allele; rs324420) [104]. More recently, an enhanced fear extinction was demonstrated in both mouse and human A-allele carriers of this *FAAH* gene C385A polymorphism, highlighting again the association of the increased fronto-amygdala connectivity with enhanced stress-reactivity [105]. This genetic alteration appears to have functional consequences since a markedly reduced FAAH protein expression was detected in many cortico-limbic areas using the first available PET radiotracer ([^{11}C]CURB) in human brain [106]. Regarding the role of FAAH on PTSD, Pardini et al. [107] reported an association of the rs2295633 SNP of *FAAH* gene with PTSD diagnosis in male Vietnam War veterans without lesions in the ventromedial prefrontal cortex. Even more, the C allele was present in subjects that had a more negative reported experience of trauma.

4.3. Endocannabinoid levels in anxiety

Preclinical findings suggest that the pharmacological manipulation of endogenous either AEA or 2-AG levels under stressful conditions could represent a good strategy for treatment of anxiety-related disorders [108]. Thus, it is plausible to hypothesize the existence of altered endocannabinoid levels in the brain of patients diagnosed of psychiatric diseases in which anxiety is either core or a comorbid symptom.

Decreased plasma AEA levels are found in PTSD patients relative to healthy control subjects without trauma history [109], though elevations are also reported in chronic PTSD [110]. The AEA deficiency

seems to be specific of PTSD patients since healthy subjects with lifetime histories of trauma, but without PTSD, exhibit normal AEA plasma levels [100]. As mentioned above, a positive correlation between lower plasma levels of AEA and increased CB1 receptor availability in the amygdala has been reported; in addition, AEA levels were negatively associated with attentional bias to threat [101]. Intriguingly, this inverse relationship between AEA levels and anxiety appears to be disease-specific since acute stress increases the circulating levels of AEA and other endocannabinoids, in parallel with cortisol [111]. Regarding the 2-AG, reduced [112] and increased [110] levels among individuals meeting diagnostic criteria for PTSD were described. Healthy humans that were subjected to prolonged stress (520-day isolation period and simulating a flight to Mars) showed reduced blood levels of 2-AG, but not AEA [113].

All these findings suggest the interaction between the ECS and HPA axis activity in response to stress, especially at the level of the amygdala [19]. In line with this relationship, a recent imaging genetics study [114] revealed a molecular interaction between genetic polymorphisms associated with differential AEA levels (FAAH rs324420) and corticotrophin releasing hormone (*corticotropin-releasing hormone receptor 1*, *CRHR1* rs110402) signaling. and amygdala function. However, in a very recent study, the levels of circulating endocannabinoids were measured in subjects with and without history of psychiatric disorder and the relationships with categorically DSM-5 defined disorders or state dimensional measures of depression or anxiety were studied. Surprisingly, neither AEA nor 2-AG levels differed as a function of any syndromal/personality disorder and neither correlated significantly with state depression or state anxiety scores [115]. Therefore, we must be cautious since peripheral endocannabinoid levels may be not well correlated with brain concentrations.

5. The endocannabinoid system and schizophrenia

Schizophrenia is one of the main psychiatric syndromes together with Major Depression. It is a chronic and devastating disorder affecting 1% of the population worldwide. Individuals diagnosed with schizophrenia have impaired social and occupational functioning. Thus, the combined economic and social costs of schizophrenia place it as the world's 15th cause of disease-related disability [116]. The clinical features of schizophrenia are clustered in positive symptoms (i.e. hallucinations and delusions); negative symptoms (i.e. social withdrawal and blunted affect) and cognitive deficits (i.e. impaired working memory and cognitive flexibility). Current antipsychotic drugs, which are the main treatment for schizophrenia, are not effective in all patients and their benefits are restricted to the amelioration of the positive symptoms, having none or limited impact in negative symptoms and cognitive impairment.

Despite the efforts of the scientific community in the last decades to elucidate the etiological basis of schizophrenia, the etiopathogenesis of the disease remains unknown. Since many years, the predominant focus of studies concerning the biological substrates of schizophrenia has been primarily centered on unique neurotransmitters including dopamine, serotonin, glutamate and γ-aminobutyric acid (GABA). Nonetheless, the limited efficacy of current antipsychotic drugs to treat some of the symptoms of schizophrenia has lead up researchers to investigate other potential neurotransmitter systems that may be altered in this disease.

In this sense, the ECS has become a hot-topic in schizophrenia research in the last years. Several studies starting from the 40 s up to nowadays agree that the ECS represents a major neuromodulatory system participating in tones of physiological processes [117,118]. Thereby, deregulation of the ECS has been speculated to be a proximal pathology in some forms of schizophrenia. In this sense, two 'cannabinoid hypotheses' of schizophrenia have been proposed [119]. The endogenous hypothesis refers to the fact that deregulation of the ECS may contribute to the pathophysiology of schizophrenia, whereas the exogenous theory refers to the risk associated with cannabis abuse that could facilitate the onset of the disease in vulnerable individuals or aggravate the symptoms in schizophrenic patients. The psychotomimetic effects of cannabis plant are known since thousands of years, but the first systematic work concerning psychotic-like experiences after acute cannabis use came in the 19th century, by Jacques-Joseph Moreau [120]. In his book he described a plethora of symptoms resembling those of schizophrenia, including delusions, disorganized speech, and other psychotic symptoms. The high expression of CB1 receptors in the central nervous system, as well as the discovery that the psychoactive compound of cannabis THC actually binds to this receptor in the brain, seem sufficient reasons to consider this system as an interesting field of study in the context of psychiatric diseases, such as schizophrenia.

In this sense, different publications have reported alterations of the ECS components in the brain of patients with schizophrenia. The evidence for the implication of different elements of the central ECS in the pathophysiology and treatment of this psychiatric disorder are presented below.

5.1. Cannabinoid receptors in schizophrenia

Several studies have investigated the status of CB1 receptors in the brain of patients with schizophrenia. Both, imaging and postmortem brain studies, have reported alterations on CB1 receptor availability, density and/or mRNA expression but with different outcomes. Thus, the integration of these results seem to be complex and certain potential confounding factors might be underlying these discrepant findings.

Three neuroimaging PET studies have evaluated the CB1 receptor availability in schizophrenic patients compared to controls [121–123]. The first two studies, by Wong et al. [121] and Ceccarini et al. [122] reported a generalized increase in CB1 receptor density in most brain regions of schizophrenic patients compared to controls, being statistically significant only in certain areas (Table 2). Interestingly, these studies also reported that CB1 receptor binding in certain areas correlated with the severity of positive symptoms and inversely correlated with the severity of negative symptoms [121,122]. Opposite to these first reported imaging findings, a recent PET study by Ranganathan et al. [123], showed a significant decrease in CB1 receptor availability in patients with schizophrenia compared to healthy controls (Table 2). Moreover, this study showed a positive global association between both positive and negative symptoms and the availability of CB1 receptors in schizophrenia [104]. Given these discordant findings related to CB1 receptor availability in schizophrenia and its association with different symptoms of the disease, the necessity of further *in vivo* assessments in this regard becomes clear. Explanations for the contradictory results reported in imaging studies published so far, have been discussed in detail [124]. Thus, confounding factors such as sex and age of patients and controls included in the studies, the radiotracers and the procedures used for the analysis, the influence of cannabis and/or tobacco consumption and the impact of antipsychotic medication have been proposed as variables that can account for the discrepancies of these results [124].

Regarding postmortem studies in the brain of patients with schizophrenia, also different outcomes for gene and/or protein expression of CB1 receptors have been shown depending on the technical approach used. Thus, different reports have linked schizophrenia with increased, decreased or unaltered expression/density of CB1 receptors in the postmortem human brain. Postmortem brain radioligand binding studies consistently reported increased density of CB1 receptors in schizophrenia [125–131]. Six out of the eight radioligand binding studies published to date have reported an increase of CB1 receptor density in the brain of schizophrenic subjects compared to controls in areas known to be involved in schizophrenia, including the cingulate cortex and dorsolateral prefrontal cortex (Table 2). The only study that has evaluated CB1 receptor binding density in the superior temporal gyrus,

Table 2
Studies about alterations of the different components of the ECS in the brain of patients with schizophrenia.

ECS element	Finding in Sch (% change)	Brain region	Cohort (n: Sch-Ct)	Method/sample	Reference
CB1 (availability)	↓ (12%)	AM, CD, Insula, PCC, HC, HT	25–18	*In vivo* brain PET scan [^{11}C]OMAR	123
	↑ (10%-5%)	NAcc, Insula, CC, IFC	67–12	*In vivo* brain PET scan [^{18}F]MK-9470	122
	↑ (23%)	BS/pons	9–10	*In vivo* brain PET scan [^{11}C]OMAR	121
CB1	↑ (8%)	DLPC (BA9)	21–21	[^{3}H]-OMAR/PMBT	131
(density)	↑ (20%)	DLPC (BA9,46)	47–43	[^{3}H]MePPEP/PMBT	130
	↑ (22%)	DLPC (BA46)	37–37	[^{3}H]CP-55940/PMBT	129
	≈	STG	8–8	[^{3}H]SR141716A and [^{3}H]CP-55940/PMBT	128
	↑ (25%)	PCC	8–8	[^{3}H]CP-55940/PMBT	127
	↑ (64%)	ACC	10–9	[^{3}H]SR141716A/PMBT	126
	↑ (23%)	DLPC (BA9)	14–14	[^{3}H]CP-55940/PMBT	125
CB1 (protein)	↓ (19–20%)	DLPC (BA46)	26–26	Immunohistochemistry/PMBT	72
	≈ (AP-F)/↓ (29%)(AP-T)	DLPC (BA9)	25–25	Immunoblot/PMBT	137
	↓ (12–14%)	DLPC (BA9)	23–23	Immunohistochemistry/PMBT	136
	≈	ACC	15–15	Immunohistochemistry/PMBT	73
CB1	≈	DLPC (BA46)	37–37	RT-qPCR/PMBT	129
(mRNA)	≈	DLPC (BA9)	20–20	RT-qPCR/PMBT	137
	↓ (15%)	DLPC (BA9)	23–23	In situ hybridization/PMBT	136
CB2 (mRNA)	≈	DLPC (BA9)	23–24	RT-qPCR/PMBT	82
EC enzymes (mRNA)	↑ (18%) ABHD6 in < 40 years (n = 13–13)	DLPC (BA9)	42–42	RT-qPCR/PMBT	156
	≈ FAAH, MAGL, DAGLα, DAGLβ	DLPC (BA9)	42–42	RT-qPCR/PMBT	155

↑ Increase; ↓ decrease; ≈ no significant change. ABHD6: α-β-hydrolase domain 6; AP-F: antipsychotic free; AP-T: antipsychotic treated; BA: Brodmann's area; Ct: controls; DAGL: diacylglycerol lipase; EC: endocannabinoid; FAAH: fatty acid amide hydrolase; MAGL: monoacylglycerol lipase; RT-qPCR: real-time quantitative polymerase chain reaction; Sch: patients with schizophrenia; PET: positron emission tomography; PMBT: post-mortem brain tissue.
Brain regions: ACC: anterior cingulate cortex; AM: amygdala; BS; brain stem; CC: cingulate cortex; CD: caudate; DLPC: dorsolateral prefrontal cortex; HC: hippocampus; HT: hypothalamus; IFC: inferior frontal cortex; NAcc: nucleus acumens; PC: parietal cortex; PCC: posterior cingulate cortex; STG: superior temporal gyrus.

a brain area particularly involved in auditory hallucinations [132], found no differences between schizophrenic subjects and controls [128]. This fact, could suggest that the alterations found in CB1 receptor density in schizophrenia might be more associated to negative and/or cognitive symptoms of the disorder, which are linked with altered cortical functions integrated by the cingulate cortex (emotional processing and selective attention responses) and the dorsolateral prefrontal cortex (DLPC) (motivational responses and executive functions) [133]. Interestingly, the study by Dalton et al. [129] only found increased CB1 receptor binding in the DLPC of schizophrenic patients with a diagnosis of paranoid schizophrenia, characterized by the presence of prominent delusions or hallucinations, while no changes were reported in non-paranoid schizophrenic patients, indicating that also the type of diagnoses can influence the outcomes obtained. This fact highlights the relevance of the source of the diagnoses and the potential influence of changes in the clustering of schizophrenia spectrum disorders in the diagnosis manuals along time. One example of these changes, is the modification in schizophrenia definition in the Diagnostic and Statistical Manual of Mental Disorders (DSM), 5th Edition [134], respect to the previous one, that eliminates the clustering of schizophrenia classic subtypes—disorganized (hebephrenic), catatonic, paranoid, and undifferentiated—adding psychopathological dimensions instead [135]. This decision was made because the classic DSM-IV subtypes of schizophrenia provide poor description of the heterogeneity of schizophrenia, low diagnostic stability, do not exhibit distinctive patterns of treatment response or longitudinal course, and are not heritable [135]. Thus, the comparisons of the outcomes between different schizophrenia subtypes should be interpreted with caution.

The potential influence of antipsychotic medication in CB1 receptor radioligand binding studies has been also taken into account. These reports state that the increases in CB1 receptor density were not related to the antipsychotic treatment given to the patients [125–127,129–131]. However, it must be noticed that in all the studies more than 80% of schizophrenic subjects included were under antipsychotic medication at the time of death. The evaluation of the impact of antipsychotic drugs on CB1 receptors in schizophrenia postmortem studies is difficult to overcome due to the lack of brain tissue from drug naïve patients. Nevertheless, the inclusion of a greater number of schizophrenic patients with a negative toxicology for antipsychotics at the time of death could provide new information in this regard when compared with antipsychotic-treated patients.

Opposite to radioligand binding studies, CB1 receptor immunoreactivity has been found to be decreased in the postmortem DLPC of schizophrenic subjects compared to controls, with or without changes in *CB1* receptor mRNA [72,136,137] (Table 2). Urigüen et al. [137] reported a significant decrease in CB1 receptor immunoreactivity only in the DLPC of schizophrenic subjects that were antipsychotic-treated at the time of death without changes in those with a negative toxicology for antipsychotics. In the two reports by Eggan et al. [72,136] decreased CB1 receptor immunoreactivity was shown in the DLPC of subjects with schizophrenia compared to controls. Authors argued that this decrease was not a consequence of the antipsychotic treatment based on the lack of statistical correlation between CB1 receptor immunoreactivity and the presence of antipsychotic medication, and on the absence of alterations in CB1 expression in the brain of antipsychotic treated monkeys [72,136]. However, more than 75% of the schizophrenic subjects included in these studies were positive for antipsychotic drugs at the time of death, making it difficult to find statistical significance when assessing the potential impact of antipsychotics. CB1 mRNA expression has also been shown decreased in the DLPC of schizophrenic subjects [136], although absence of changes have also been reported [137]. In the cingulate cortex, no alterations in CB1 receptor immunoreactivity nor *CB1* receptor mRNA have been found [73] (Table 2). The reported changes in CB1 receptor immunoreactivity in the DLPC of medicated schizophrenic subjects point to a role of the antipsychotic treatment in the regulation of the ECS in this brain area. However, it is unknown whether this antipsychotic modulation of CB1 receptors could contribute or not to the therapeutic effects of these drugs. Taking into account all the results from postmortem studies regarding CB1 receptors in schizophrenia, showing lower or unchanged levels of mRNA, reduced immunoreactivity and higher receptor binding, two potential hypotheses have been proposed: 1) an altered trafficking of the receptor resulting in higher levels of membrane-bound CB1 receptor, and 2) a higher CB1 receptor affinity [131]. Both situations would entail a greater CB1 receptor availability, something that is not supported in all the neuroimaging studies reported to date [123].

Overall, the imaging and postmortem outcomes regarding CB1

receptor availability, density and expression in the brain of schizophrenic patients, although inconclusive, point towards a role of CB1 receptors in this pathology. Further research including functional assessment of the status of this receptor might help in understanding the potential pathophysiological consequences of the altered CB1 receptor availability, density and/or expression.

Little is yet known about the status of CB2 receptors in schizophrenia. To date, the only study reporting data related to CB2 receptor in the brain of schizophrenic patients found no significant correlation between the diagnosis of schizophrenia and total CB2 mRNA expression in the postmortem DLPC (BA9) [82] (Table 2). The main goal of this study was to test the association between tag SNPs in the *CNR2* gene and schizophrenia. In this regard, authors showed two SNPs associated with schizophrenia that were also related to reduced function of CB2 receptors, thus concluding that people with genetically predetermined lower functioning of CB2 receptors has an increased susceptibility to suffer schizophrenia when combined with other risk factors [82]. Previously, in 2003, De Marchi and co-workers [138] reported that clinical remission in schizophrenic patients was accompanied by a significant decrease in CB2 receptor mRNA in peripheral blood mononuclear cells. This finding does not agree with the observations of potential reduced CB2 receptor function associated with increased risk for schizophrenia [82], but authors also suggested that these peripheral changes might be related to several immunological alterations described in this pathology [138]. Nevertheless, further research is needed to support the potential role of CB2 receptors in schizophrenia.

Several genetic studies investigating different components of the ECS in patients with schizophrenia have been carried out, most of them focusing on different polymorphisms of the *CNR1* gene. Nevertheless, it must be noted that all genetic studies have been carried out in peripheral blood samples, and not in the brain. As these studies go beyond the main scope of this review, only a brief summary of the studies will be provided.

Nineteen studies have addressed different *CNR1* polymorphisms in relation with schizophrenia. Four of them studied the triplet AAT in *CNR1* repeat, finding no linkage when comparing with the general population of schizophrenic patients from different genetic backgrounds [139–142]. Another study from Seifert et al. studied two other SNPs apart from the triplet AAT (rs6454674 and rs1049353), not finding any association with any of them [143]. Nevertheless, a nine-time repetition of the triplet AAT in *CNR1* has been associated with the hebephrenic subtype of schizophrenia in two different studies, carried out in Caucasian and Japanese population [144,145]. Ujike et al. [144] also addressed the association of rs1049353 in their cohort, but, as in the study of Seifert, they did not find any linkage. These data reflect the heterogeneity of the schizophrenia and suggest that variations in the *CNR1* gene may contribute to the pathogenesis of specific subtypes of this disorder.

Some other studies have analyzed the SNP rs1049353 in schizophrenic patients [87,146], none of them showing any significant linkage. Several other studies have failed in trying to find associations between different SNPs of *CNR1* (rs806366, rs806368, rs806376, rs806379, rs806380, rs6454674, sr1535255 among other) and schizophrenia [147–150]. However, some SNPs such as rs6454674 [151], rs2023239 [152] and interactions with rs1049353, rs1535255, and rs2023239 [152] have been associated with positive and negative symptoms. At the same time, a study from Tiwari et al. have found an association between *CNR1* SNP rs806378 and the weight gain as a consequence of antipsychotic treatment [153].

An interesting study evaluated interactions between *CNR1* gene polymorphisms, cannabis use, cerebral volume and cognitive function [154]. They compared patients with schizophrenia or schizoaffective disorder with cannabis abuse/dependency and patients without cannabis use and observed smaller frontotemporal white matter (WM) volumes in those that smoked cannabis. They also observed associations between SNPs rs12720071, rs7766029, rs9450898 and WM volumes, as well as between SNP rs12720071 and processing speed/attention and problem-solving tests. Those results suggest that the use of cannabis in association with specific *CNR1* genotypes can contribute to alterations in WM and cognitive deficits in a subgroup of schizophrenic patients, which supports the hypothesis that both genetic and environmental factors could work together to determine the phenotypic expression in patients with schizophrenia.

Regarding genetic studies involving *CNR2*, data are critically scarce. One study has reported a close relationship between a polymorphism of the *CNR2* and increased susceptibility to schizophrenia in a large Japanese population [83]. This association was also confirmed in postmortem PFC from schizophrenic and control subjects with other ethnicities, being the risk allele also associated with low *CB2* receptor mRNA levels [83]. Furthermore, culture cell experiments showed that this *CNR2* gene polymorphism was linked to a lower functionality of the CB2 receptor, suggesting an increased risk of schizophrenia for people with low CB2 receptor function [83].

5.2. Endocannabinoid synthesizing and metabolizing enzymes in schizophrenia

Only a few studies have evaluated the endocannabinoid enzymes in the brain of patients with schizophrenia (Table 2). In the first study [155], quantitative polymerase chain reaction (PCR) was used to measure mRNA levels of DAGL (DAGLα and DAGLβ), MAGL, and FAAH. The mRNA level quantification of these enzymes was carried out in the prefrontal cortex Brodmann's area 9 of 42 schizophrenia subjects and matched control comparison subjects. No differences between subject groups were found in mRNA levels for endocannabinoid synthesizing and metabolizing enzymes.

In a more recent study, the same authors studied the transcript levels for the recently discovered 2-AG metabolizing enzyme, α-β-hydrolase domain 6 (ABHD6), in the prefrontal cortex of schizophrenia and healthy subjects (n = 84), using quantitative PCR [156]. This study showed that ABHD6 mRNA levels were elevated in schizophrenia subjects who were younger and had a shorter illness duration relative to age-matched comparison subjects. Furthermore, age and illness duration were strongly correlated in schizophrenia subjects, which made it difficult to differentiate between their effects on ABHD6 mRNA levels.

On the other hand, Morita et al. investigated a possible relationship between the non-synonymous polymorphism in Pro129Thr (rs324420) of the *FAAH* gene and schizophrenia. No differences were found in a group of 260 patients with schizophrenia (127 paranoids, 127 hebephrenics and 6 not classified) as compared to 63 controls in a Japanese population, regardless of the disorder subtype [157].

5.3. Endocannabinoid levels in schizophrenia

To date, only one study has evaluated endocannabinoid levels directly in the brain of patients with schizophrenia [158]. In this postmortem study, contents of the two main endocannabinoids, 2-AG and AEA, as well as other endocannabinoid and cannabimimetic compounds were quantified in three brain regions of subjects with schizophrenia and matched controls. The study revealed an opposite pattern for the regulation of endocannabinoids in schizophrenia. Authors found increased levels of 2-AG in cerebellum, hippocampus, and DLPC, whereas decreased levels of AEA and other *N*-acylethanolamines—dihomo-γ-linolenoylethanolamine (LEA), oleoylethanolamide (OEA), palmytoylethanolamide (PEA), and docosahexaenoylethanolamine (DHEA)—were reported [158]. In this way, antipsychotic medications reduced the content of endocannabinoids in the prefrontal cortex and hippocampus, but not in cerebellum, of antipsychotic-treated patients compared to antipsychotic-free subjects [158].

Before this study, several works focused their attention in the link between endocannabinoid levels, in both cerebrospinal fluid (CSF) and blood, and schizophrenia [138,159–162]. Thus, four studies of the same

research group have reported elevated AEA levels in CSF of schizophrenic patients, with no significant differences in serum AEA levels between schizophrenic patients and controls [159–162]. Moreover, in non-medicated acute schizophrenics, a negative correlation was found between CSF AEA levels and psychotic symptoms [160]. The increases of AEA levels reported in CSF from patients with schizophrenia contrast with the reduced AEA found in postmortem human brain of schizophrenics, but the neuronal origin of CSF endocannabinoids remains conjectural and it might reflect peripheral alterations of these signaling messengers [138]. In this sense, an increase in AEA levels in the blood of patients with acute schizophrenia respect to healthy volunteers has been reported [138]. Furthermore, in schizophrenic patients, pharmacologically-induced remission of the symptoms was accompanied by a significant decrease of blood AEA levels and of the mRNA transcripts for the degrading enzyme FAAH [138]. Thus, it has been proposed that the increased blood AEA levels observed in patients with acute schizophrenia might be due to the modified immune response observed during the course of the disease [138]. In fact, patients in initial prodromal states of psychosis with lower levels of AEA in CSF showed a higher risk for transiting to psychosis earlier [162]. Regarding the effect of antipsychotic medication on CSF endocannabinoid levels, AEA concentrations remained increased in patients that were treated with atypical antipsychotics, but not in those treated with typical ones [160].

In this line, a clinical trial in acute schizophrenia has evaluated the antipsychotic effects of the non-psychoactive phytocannabinoid cannabidiol (CBD) versus the atypical antipsychotic amisulpride, assessing in turn endocannabinoid serum levels along treatments [163]. Either treatment was safe and led to significant clinical improvement, but CBD displayed a markedly superior side effects profile. Results also showed that treatment with CBD, but not with amisulpride, was accompanied by a significant increase in serum AEA levels that were also associated with clinical improvement [163]. These authors suggest that inhibition of AEA deactivation may contribute to the antipsychotic effects of CBD potentially representing a completely new mechanism in the treatment of schizophrenia [163].

Besides AEA, other endocannabinoids have also been evaluated in CSF or serum of patients with schizophrenia. There is only one study which has attempted to determine 2-AG levels in CSF of schizophrenic patients [159]. However, despite being the most abundant endocannabinoid in the brain [164], significant levels of 2-AG could not be detected in any of the samples analyzed, suggesting that these endocannabinoids concentrations are exceedingly low in CSF of both controls and schizophrenic patients. The levels of the endogenous analogues of AEA, OEA and PEA, have also been explored out of the brain in schizophrenia. In observational studies, no differences were found in CSF or serum OEA levels between controls and schizophrenic patients [159–162]. By contrast, a 2-fold increase in PEA CSF levels was found in schizophrenic patients compared to controls [159]. However, this finding was not replicated in subsequent studies [160,161]. In previously mentioned clinical trial with CBD performed in patients with acute schizophrenia, both OEA and PEA serum levels were significantly elevated in schizophrenic patients treated with CBD, compared to those treated with amisulpride [163].

It is also noteworthy that CSF endocannabinoid levels have been shown to be affected depending on the history of cannabis use. Thus, markedly altered AEA concentrations (> 10-fold higher) were reported in CSF of a subgroup of schizophrenic patients who had low frequency cannabis use compared to controls (with high and low frequency use), as well as compared to schizophrenic high-frequency users [161]. However, this impact of cannabis use was not observed in other studies [160,162].

Compiling the information available from these studies is evident that the relationship between levels of endocannabinoids measured in the CSF, peripheral blood and concentrations of endocannabinoids in brain tissue is not clear yet. Thus, the understanding of functional implications of altered levels of endocannabinoids in each type of sample of schizophrenic patients remains to be elucidated.

6. Conclusions

Several evidences suggest the relationship between changes in one or more components of the ECS and some of the symptoms that are present in depression, anxiety-related disorders and schizophrenia. Indeed, recent human postmortem and *in vivo* neuroimaging studies are providing more knowledge about the implication of the ECS in these mental disorders. Most of the findings in depression and anxiety are related to the expression and/or functionality of CB1 receptors and FAAH in brain areas belonging to the amygdala-hippocampal-corticostriatal neural circuit, especially the frontal cortex in depression and the amygdala in anxiety disorders. Regarding schizophrenia, the findings in postmortem and living human brains highlight a deregulation of CB1 receptor in specific brain areas that are highly affected in this disease. The findings on peripheral endocannabinoid levels are in good consonance with these adaptive changes. However, we must be cautious since peripheral endocannabinoid levels may not be well correlated with brain concentrations.

The pharmacological manipulation of the ECS is envisaged as an attractive alternative treatment for these mental disorders. For instance, drugs as the phytocannabinoid compound CBD have been reported to be effective to treat schizophrenia.

The advance in this field, together with the translational preclinical research is opening an attractive research scenario for the development of promising new pharmacological strategies based on drugs targeting the ECS to treat mental disorders.

Acknowledgements

This study was supported by the Spanish Ministry of Economy and Competitiveness (SAF2015-67457-R, MINECO/FEDER), the Plan Estatal de I+D+i 2013-2016, the Instituto de Salud Carlos III-Subdirección General de Evaluación y Fomento de la Investigación, Spanish Ministry of Economy, FEDER (PI13/01529) and the Basque Government (IT616/13). I I-L is a recipient of a Predoctoral Fellowship from the Basque Government. E F-Z is a recipient of a Predoctoral Fellowship from the University of Cantabria. CM is a recipient of a Postdoctoral Marie Skłodowska-Curie Individual Fellowship (H2020-MSCA-IF-2016, ID 747487).

Conflict of interests

There is no conflict of interest to declare.

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