UNIVERSITY OF SÃO PAULO

SCHOOL OF PHARMACEUTICAL SCIENCES OF RIBEIRÃO PRETO

AARHUS UNIVERSITY

Behavioural and molecular effects induced by Cannabidiol in animal models of depression

Efeitos comportamentais e moleculares induzidos pelo Canabidiol em modelos animais de depressão

GABRIELA PANDINI SILOTE

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Doctoral thesis presented to the Graduate Program of Pharmaceutical Sciences, School of Pharmaceutical Sciences of Ribeirão Preto (University of São Paulo, Brazil) and Faculty of Health (Aarhus University, Denmark) to obtain the double PhD degree.

Concentration Area: Natural and Synthetic Products/Health

Supervisor: Prof. Sâmia R. L. Joca Wegener

Co-supervisor: Prof. Gregers Wegener

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To my beloved parents and brother
for the love and constant support.

Aos meus amados pais e irmão pelo
amor e constante apoio.

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SUMMARY

SILOTE, G. P. Behavioural and molecular effects induced by Cannabidiol in animal models of depression. 2021. 296f. Thesis (Doctoral). School of Pharmaceutical Sciences of Ribeirão Preto – University of São Paulo, Ribeirão Preto, 2021.

Introduction: Major depressive disorder (MDD) is a chronic and severe psychiatric disorder, which is more prevalent in women. Cannabidiol (CBD) is a compound isolated from the plant *Cannabis sativa* L., which produces an antidepressant-like effect in animal models. However, only a few studies investigated the effect of such compounds in females, and it is unclear the influence of gender on CBD effects. The antidepressant effect induced by CBD involves the activation of BDNF-TrkB-mTOR signaling in the hippocampus and prefrontal cortex, an effect also demonstrated for ketamine. **Aims:** The present study aimed to: investigate the influence of strain and gender of mice in CBD antidepressant-like effects (Study 1A); investigate CBD effects in male and female FSL rats, tested at different time points (Study 1B); investigate the molecular mechanisms involved in CBD and ketamine antidepressant effect in the prefrontal cortex (PFC) and hippocampus of FSL rats (Study 2). **Methods:** Study 1: Adult male and female Swiss and C57BL/6 mice and adult male and female FSL and Flinders Resistant Line (FRL) rats were used. Mice received the systemic injection with CBD (3, 10, and 30 mg/kg, i.p.), imipramine (IMIP; 20 mg/kg, i.p.) or vehicle 30 minutes before the elevated plus maze (EPM) and tail suspension test (TST). FSL rats were treated with CBD (10, 30, and 60 mg/kg, i.p.), S-ketamine (15 mg/kg, i.p.) or vehicle, 1 or 2 hours before the open field test (OFT) and forced swim test (FST). An independent experiment was conducted with female FSL rats that received S-ketamine (10, 15, and 20 mg/kg, i.p.) or vehicle 1h before OFT and FST to select ketamine effective dose. Study 2: Adult male FSL and FRL rats received intraperitoneal treatment with CBD (30 mg /kg), S-Ketamine (15 mg/kg) or vehicle (Saline and Tween 80 3%), 1h before behavioral tests in the OFT (5 min) and FST (5 min). Immediately after the behavioral tests, the PFC, dorsal hippocampus (DH), and ventral (VH) were dissected. To investigate the molecular mechanisms involved in the antidepressant-type effect induced by CBD and S-Ketamine, the analysis of gene expression (Fluidigm) and synaptosome protein levels by WB were performed on PFC, DH, and VH for the glutamatergic, neurotrophic signaling and synaptic plasticity. **Results:** Study 1A: CBD produced an antidepressant-like effect in male, but not in female Swiss mice in the TST. Furthermore, CBD did not induce any significant effect in C57BL/6 mice, both males and females. Study 1B: Surprisingly, in FSL rats, CBD (30 mg/kg) induced a depressive-like effect in females 1 hour after the treatment, but an antidepressant-like effect after 2 hours. In males, CBD (30 mg/kg) produced an antidepressant-like effect 1 hour after the injection; no effect could be observed following 2 hours. Ketamine induced a significant antidepressant-like effect in female FSL rats submitted to FST 1 hour after the injection (15 and 20 mg/kg). Study 2: We replicated the behavioural results from Study 1B, the injection of CBD and ketamine reduced the immobility time in FSL rats exposed to FST, which reinforces our findings. There was no correlation between the CBD blood levels and the immobility exhibited in the FST. In the molecular analysis, the effect of CBD was associated with increased expression of the EAAT3, Nr2a, Nr2b, BDNF transcript in the PFC. In contrast, ketamine effect was associated with downregulation in VEGF and sortilin levels and

increased protein levels of Nr2b, Nr2a and pGluR1 (S831) in the same region. However, in DH, CBD increased the levels of VEGF and Nr2b and decreased the expression of Sort1 and pGluR1 (S831), and ketamine reduced the expression of pGluR1 (S831) and increased the levels of Nr2b protein. In VH, CBD reduced the expression of mGluR5 and pGluR1 (S831 and S845) and increased the expression of GluR2, and ketamine reduced the levels of pGluR1 (S831) in the same limbic region. **Conclusion:** Based on the present findings, we conclude that CBD effects can be influenced by species, strain, gender, and time of administration. The molecular mechanisms involved on CBD antidepressant-like effect involves the regulation of the neurotrophic and glutamatergic signaling pathway in the PFC, DH and VH. In contrast, the effect of ketamine seems to involve mainly the restoration of normal glutamatergic function in the limbic brain areas.

Keywords: Cannabidiol; S-ketamine; gender; FSL/FRL rats; mice; forced swim test; gene expression; synaptosome.

RESUMO

SILOTE, G. P. **Efeitos comportamentais e moleculares induzidos pelo Canabidiol em modelos animais de depressão.** 2021. 296f. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2021.

Introdução: O transtorno depressivo maior (TDM) é um transtorno psiquiátrico crônico e grave, mais prevalente em mulheres. O canabidiol (CBD) é um composto isolado da planta *Cannabis sativa L.*, que produz um efeito antidepressivo em modelos animais. No entanto, apenas alguns estudos investigaram o efeito de tais compostos em fêmeas, e ainda não está claro sobre a influência do sexo nos efeitos do CBD. O efeito antidepressivo induzido pelo CBD envolve à ativação da via de sinalização do BDNF-TrkB-mTOR no hipocampo e no córtex pré-frontal (PFC), efeito também demonstrado para a ketamina. **Objetivos:** O presente estudo teve como objetivo: investigar a influência da linhagem e do sexo de camundongos no efeito tipo-antidepressivo do CBD (Estudo 1A); investigar os efeitos do CBD em ratos FSL machos e fêmeas, testados em diferentes momentos (Estudo 1B); investigar os mecanismos moleculares envolvidos no efeito antidepressivo do CBD e da ketamina no PFC e no hipocampo de ratos FSL (Estudo 2). **Métodos:** Estudo 1: Foram utilizados camundongos adultos machos e fêmeas das linhagens Swiss e C57BL/6 e ratos e ratas adultos FSL e Flinders Resistant Line (FRL). Os camundongos receberam a injeção sistêmica com CBD (3, 10 e 30 mg/kg, ip), imipramina (IMIP; 20 mg/kg, ip) ou veículo 30 minutos antes do labirinto em cruz elevado (EPM) e teste de suspensão da cauda (TST) Os ratos FSL foram tratados com CBD (10, 30 e 60 mg/kg, ip), S-ketamina (15 mg/kg, ip) ou veículo, 1 ou 2 horas antes do teste de campo aberto (OFT) e teste de natação forçada (FST). Um experimento independente foi conduzido com ratas FSL que receberam S-ketamina (10, 15 e 20 mg/kg, i.p.) ou veículo 1h antes de OFT e FST para selecionar a dose eficaz de ketamina. Estudo 2: Ratos adultos FSL e FRL receberam tratamento intraperitoneal com CBD (30 mg/kg), S-ketamina (15 mg/kg) ou veículo (solução salina e Tween 80 3%), 1h antes dos testes comportamentais no OFT (5 min) e FST (5 min). Imediatamente após os testes comportamentais, o PFC, o hipocampo dorsal (HD) e o ventral (VH) foram dissecados. Para investigar os mecanismos moleculares envolvidos no efeito do tipo antidepressivo induzido por CBD e S-ketamina, a análise da expressão gênica (Fluidigm) e dos níveis de proteína do sinaptossoma por WB foram realizadas no PFC, DH e VH para a sinalização glutamatérgica, neurotrófica e plasticidade sináptica. **Resultados:** Estudo 1A: o CBD produziu um efeito tipo-antidepressivo em camundongos Swiss machos, mas não em fêmeas no TST. Além disso, o CBD não induziu nenhum efeito significativo em camundongos C57BL / 6, tanto machos quanto fêmeas. Estudo 1B: Surpreendentemente, em ratas FSL, o CBD (30 mg/kg) induziu um efeito do tipo-depressivo 1 hora após o tratamento, mas efeito do tipo-antidepressivo após 2 horas. Nos ratos, o CBD (30 mg/kg) produziu um efeito tipo- antidepressivo 1 hora após a injeção; nenhum efeito pode foi observado após 2 horas. A ketamina induziu um efeito antidepressivo significativo em ratas FSL submetidas ao FST 1 hora após a injeção (15 e 20 mg/kg). Estudo 2: Nós replicamos os resultados comportamentais do Estudo 1B, a injeção de CBD e ketamina reduziram o tempo de imobilidade em ratos FSL expostos ao FST, o que reforça os nossos achados. Não houve correlação entre os níveis sanguíneos de CBD e a

imobilidade exibida no FST. Na análise molecular, o efeito do CBD foi associado ao aumento da expressão do transcrito de EAAT3, Nr2a, Nr2b, BDNF no PFC. Em contraste, o efeito da ketamina foi associado a uma downregulation em VEGF e sortilina e aumento nos níveis protéicos de Nr2b, Nr2a e pGluR1 (S831) na mesma região. No entanto, no DH, o CBD elevou os níveis de VEGF e Nr2b e diminuiu a expressão de Sort1 e pGluR1 (S831), e a ketamina reduziu a expressão de pGluR1 (S831) e aumentou os níveis de proteína Nr2b. No VH, o CBD reduziu a expressão de mGluR5 e pGluR1 (S831 e S845) e aumentou a expressão de GluR2, e a ketamina reduziu os níveis de pGluR1 (S831) na mesma região límbica. **Conclusão:** Com base nos presentes achados, concluímos que os efeitos do CBD podem ser influenciados pela espécie, linhagem, sexo e tempo de administração. No PFC, a análise molecular revelou que o CBD modula principalmente o BDNF e a via de sinalização glutamatérgica, enquanto a ketamina regula as moléculas associadas à neurotransmissão glutamatérgica, VEGF e vias de sinalização da sortilina. No entanto, para a DH, o CBD regula a Sortilina, VEGF, sistemas glutamatérgicos e ketamina regulados exclusivamente a neurotransmissão glutamatérgica.

Palavras-chave: Canabidiol; S-ketamina; gênero; Ratos FSL / FRL; teste de natação forçada; expressão gênica; sinaptossoma.

RESUMÉ

SILOTE, G. P. **Adfærdsmæssige og molekylære effekter induceret af Cannabidiol i dyremodeller for depression.** 2021. 296f. PhD afhandling. School of Pharmaceutical Sciences of Ribeirão Preto - University of São Paulo, Ribeirão Preto, 2021.

Indledning: Depression er en kronisk og alvorlig psykiatrisk lidelse, som er mere udbredt hos kvinder. Cannabidiol (CBD) er et kemisk stof isoleret fra planten *Cannabis sativa L.*, som producerer en antidepressiv-lignende virkning i dyremodeller. Imidlertid har kun få studier undersøgt effekten af sådanne forbindelser hos kvinder, og det er uklart hvilken indflydelse køn har på den mulige effekt af CBD. Den antidepressive effekt induceret af CBD involverer aktivering af BDNF-TrkB-mTOR-signaleringskaskaden i hippocampus og præfrontale cortex, en effekt, der også er vist for ketamin. **Formål**: Dette studium havde til formål at: undersøge indflydelsen af stamme og køn hos mus på CBD antidepressiva-lignende effekter (Undersøgelse 1A); undersøge CBD-effekter hos FSL-hanrotter, testet på forskellige tidspunkter (Studie 1B); undersøge de molekylære mekanismer, der er involveret i effekten af CBD og ketamin antidepressiv-lignende effekter i præfrontale cortex (PFC) og hippocampus hos FSL rotter (Studie 2). **Metoder**: Undersøgelse 1: Voksne Swiss og C57BL/6-mus samt voksne FSL- og Flinders Resistant Line-rotter (begge køn) blev brugt. Mus modtog en systemiske injektion med CBD (3, 10 og 30 mg/kg, ip), imipramin (IMIP; 20 mg/kg, ip) eller vehikel 30 minutter før Elevated Plus Maze (EPM) og Tail Suspension Test (TST). FSL-rotter blev behandlet med CBD (10, 30 og 60 mg/kg, ip), S-ketamin (15 mg/kg, ip) eller vehikel 1 eller 2 timer før open field test (OFT) og Forced Swim Test (FST). Et uafhængigt eksperiment blev udført med FSL-hunrotter, der modtog S-ketamin (10, 15 og 20 mg / kg, i.p.) eller vehikel 1 time før OFT og FST, for derigennem at vælge effektiv ketamin dosis. Undersøgelse 2: Voksne FSL- og FRL-hanrotter fik intraperitoneal behandling med CBD (30 mg/kg), S-ketamin (15 mg/kg) eller vehikel (saltvand og Tween 80 3%), 1 time før adfærdstest i OFT (5 min) og FST (5 min). Umiddelbart efter adfærdstestene blev PFC, dorsal hippocampus (DH) og ventral (VH) dissekeret. For at undersøge de molekylære mekanismer, der er involveret i den antidepressiv-lignende effekt induceret af CBD og S-ketamin, blev der foretaget en analyse af genekspression (Fluidigm) og synaptosomprotein-niveauer ved hjælp af Western Blot på væv fra PFC, DH og VH. Proteiner og gener indenfor glutamaterg, neurotrofiske signalering og synaptisk plasticitet blev udvalgt. **Resultater**: Undersøgelse 1A: CBD producerede en antidepressiv-lignende virkning hos hanner, men ikke hos Swiss hunmus i TST. Endvidere inducerede CBD ikke nogen signifikant effekt i C57BL/6-mus, både hanner og hunner. Undersøgelse 1B: CBD (30 mg/kg) inducerede hos FSL-rotter en depressiv-lignende virkning i hunner 1 time efter behandlingen, men en antidepressiv-lignende virkning efter 2 timer. I hanner producerede CBD (30 mg/kg) en antidepressiv-lignende virkning 1 time efter injektionen; ingen virkning kunne observeres efter 2 timer. Ketamin inducerede en signifikant antidepressiv-lignende virkning hos FSL-hunrotter, i FST 1 time efter injektionen (15 og 20 mg/kg). Undersøgelse 2: Vi replikerede adfærdsmæssige resultater fra undersøgelse 1B, injektion af CBD og ketamin reducerede immobilitetstiden i FSL-rotter i FST. Der var ingen sammenhæng mellem CBD-blodniveauerne og immobiliteten

i FST. I molekylæranalysen var effekten af CBD forbundet med øget ekspression af EAAT3, Nr2a, Nr2b, BDNF-transkript i PFC. I modsætning hertil var ketamineffekten forbundet med nedregulering i VEGF- og sortilinniveauer og øgede proteinniveauer af Nr2b, Nr2a og pGluR1 (S831) i samme region. Imidlertid øgede CBD i DH niveauerne af VEGF og Nr2b og nedsatte ekspressionen af Sort1 og pGluR1 (S831), og ketamin reducerede ekspressionen af pGluR1 (S831) og øgede niveauerne af Nr2b-protein. I VH reducerede CBD ekspressionen af mGluR5 og pGluR1 (S831 og S845) og øgede ekspressionen af GluR2, og ketamin reducerede niveauerne af pGluR1 (S831) i samme limbiske region. **Konklusion**: Baseret på de nuværende fund konkluderer vi, at effekterne af CBD kan påvirkes af art, stamme, køn og administrationstidspunkt. De molekylære mekanismer, der er involveret i den antidepressiv-lignende virkning af CBD involverer regulering af den neurotrofiske og glutamatergiske signalvej i PFC, DH og VH. I modsætning hertil synes effekten af ketamin hovedsageligt at involvere glutamaterg funktion i de limbiske hjerneområder.

Nøgleord: Cannabidiol; S-ketamin; køn; FSL / FRL rotter; mus; tvungen svømmetest genekspression; synaptosom.

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ABBREVIATIONS LIST

Δ9-THC	delta-9-tetrahydrocannabinol
2-AG	2-arachidonoylglycerol
5-HT	5-hydroxy tryptamine or serotonin
5-HT1A	serotonin receptor type 1A
5-HT2A	serotonin receptor type 2A
5-HT3	serotonin receptor type 3
A2A	Adenosine A2A receptor
ACTH	adrenocorticotrophic hormone
AEA	anandamide
Akt	protein kinase B
ANOVA	analysis of variance
BCA	bicinchoninic acid
BDNF	brain-derived neurotrophic factor
BSA	bovine sérum albumin
Ca2+	calcium
CaMKII	calmodulin kinase II
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CBD	cannabidiol
cDNA	complementar deoxyribonucleic acid
Cmax	Maximum drug concentration
CNS	central nervous system
COMT	Cathecol-O-methyltransferase
COX	cyclooxygenase
CREB	cAMP response elemento-binding protein
CRH	corticotrophin releasing fator

Ct	cycle thereshold
CUMS	chronic umpredictable mild stress
DA	dopamine
DAG	diacylglicerol
DAT	dopamine transporter
DFP	diisopropyl fluorophosphate
DH	dorsal hippocampus
DNA	deoxyribonucleic acid
DSM-5	Diagnostic and Statistical Manual of Mental Disorders – fifth edition
EA	enclosed arm
EAAT	glutamate transporter
ECT	electroconvulsive therapy
EPM	elevated plus maze
ERK	extracelular signal regulated kinase
FAAH	fatty acid amide hydrolase
FRL	Flinders resistant line rats
FSL	Flinders sensitive line rats
FST	forced swim test
GABA	gamma-aminobutyric acid
GABAA	gamma-aminobutyric acid receptor type A
GPR55	G protein-coupled receptor 55
GluR	Glutamate receptor
HPA	hypothalamic-pituitary-adrenal
HPC	hippocampus
HTR1A	serotonin receptor 1A gene
HTR2A	serotonin receptor 2A gene
i.c.v.	intracerebroventricular
i.p.	intraperitoneal

IBA1	ionized calcium binding adaptor molecule 1
IL-1β	interleukin 1 beta
IL-6	interleukin 6
IMIP	imipramine
INF	interferon
iNOS	inducible nitric-oxide synthase
IP3	inositol triphosphate
LH	learned helplessness
LPS	lipopolysccharide
LTD	long-term depression
LTP	long-term potentiation
MAGL	monoacylglycerol lipase
MAO	monoamine-oxidase
Mapk	microtube-associated protein kinase
MDD	major depressive disorder
MEK	MAP/ERK kinase
mGluR	Metabotropic glutamate receptor
mPFC	Medial pré-frontal cortex
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NA	noradrenaline
NET	Noradrenaline transporter
NF-κB	Fator nucelar kappa B
NMDA	N-methyl-D-aspartate
OA	open arm
OBB	Odyssey Blocking Buffer
OFT	open field test
PCR	polymerase chain reaction

PFC	prefrontal cortex
PI3K	phosphatidyl inositol-3 phosphate
PKA	protein kinase A
PKC	protein kinase C
Pick1	protein interating with C kinase
PPARγ	peroxisome proliferator-activated receptor gamma
PSD-95	Postsynaptic density protein 95
PT	pre-test session
qPCR	quantitative polymerase chain reaction
REM	rapid eye moviment
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SNRI	selective noradrenaline reuptake inhibitor
SPF	specific pathogen free
SPT	sucrose preference test
SSRI	selective serotonin reuptake inhibitor
Syn 3	synapsine 3
Syp	synaptophysin
TBS	tris-buffer saline
TBST	tris-buffer saline and tween 20
TCA	tricyclic antidepressant
TNF-α	tumor necrosis fator alpha
TPH1	tryptophan hydroxylase 1
TPH2	tryptophan hydroxylase 2
TrkB	tropomyosin-related kinase B
TRPA1	transient receptor potential cation channel subfamily A member 1

TRPM8	transient receptor potential cation channel subfamily M member 8
TRPV1	transient potential vanniloid type 1
TRPV2	transient potential vanniloid type 2
TST	tail suspension test
VEH	vehicle
VH	Ventral hippocampus
WB	Western blotting
WKY	Wistar-kyoto rats

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INTRODUCTION

1 INTRODUCTION

1.1 Major Depressive Disorder (MDD)

1.1.1 Diagnosis and epidemiology

Major depressive disorder (MDD) is a mood disorder that affects 322 million people worldwide, according to the World Health Organization (WHO, 2017a, 2017b). The prevalence varies across the different countries, raging in between 3 to 6%. In Brazil, about 11 million people are affected by depression, corresponding to 5.8% of the population (WHO, 2017a). Unfortunately, depression is a severe, chronic, debilitating, and disabling psychiatric disorder, which significantly impact the social, physical, and occupational aspects of the life of affected individuals (KUEHNER, 2017; KYU et al., 2018; OTTE et al., 2016; WHO, 2017a). This results in several years lived with disability, and considerable global burden of diseases, making MDD one of the leading cause of disability worldwide (information published in January 2020 and accessed in September 2020: https://www.who.int/news-room/fact-sheets/detail/depression). MDD increases the risk of suicide and produces an enormous social and economic impact on society (WHO, 2017a). MDD is twice more prevalent in women than in men, but the mechanisms involved in gender differences are still unknown (OTTE et al., 2016; WHO, 2017a).

Given the subjectivity and complexity of the symptoms observed in depression, firm diagnostic criteria are necessary to guarantee the correct diagnosis. The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders from the American Psychiatric Association (DSM-5, APA) established that MDD is defined by the presence of five or more symptoms, including one of the core symptoms, depressed mood or loss of interest (anhedonia), accompanied by other symptoms not related to other medical conditions for at least two weeks (APA, 2013). The current diagnostic criteria for MDD are described in Table 1.

Table 1. Diagnostic criteria for MDD disorder, according to DSM-5 (APA, 2013).

	Symptoms	Frequency
Core symptoms	1. Depressed mood (feels sad, empty, hopeless) and, in children and adolescents, irritable mood evidenced by subjective report or observation made by the others.	Most of the day, nearly every day
	2. Anhedonia (a reduction in interest or pleasure in the activities).	Most of the day, nearly every day
Other symptoms	3. Weight disturbances, considerable weight loss when not dieting, weight gain (more than 5% in one month), or increase or decrease appetite.	Nearly every day
	4. Insomnia or hypersomnia.	Nearly every day
	5. Psychomotor agitation or retardation .	Nearly every day
	6. Fatigue or loss of energy.	Nearly every day
	7. Feelings of worthlessness or excessive or inappropriate guilt.	Nearly every day
	8. Decreased ability to think or concentrate, or indecisiveness.	Nearly every day
	9. Thoughts of death (not just fear of dying), suicide ideation with or not a specific plan, or a suicide attempt or a detailed plan.	Recurrent

1.1.2 Etiology

The etiology of MDD is complex and multifactorial, and it results from the interactions between environmental factors (e.g. stress exposure), genetic/epigenetic factors, and personality traits (CASPI et al., 2003; KENDLER; GARDNER, 2016; OTTE et al., 2016). The stress from chemical, physical, or psychological origin triggers several physiological and behavioral responses to promote adaptation to the new internal or external demands. However, prolonged and intense exposure to stress can lead to excessive exposure to mediators of the stress response and can potentially impair adaptation to the aversive environment, allowing the development of physical and emotional disorders, such as depression (KENDLER; GARDNER, 2016; KENDLER; KARKOWSKI; PRESCOTT, 1999; KENDLER et al., 1995; MAHAR et al., 2014; POST, 1992; RAVINDRAN et al., 1995). In line with that, it has been suggested that exposure to a stressful event may precipitate the first

depressive episode in 60% of cases, with less importance of the environmental factor for the following episodes, thus suggesting that the depressive episode itself can sensitize the organism for the development of new episodes (POST, 1992). Different stressful life events in adulthood, such as unemployment, exposure to violence, financial insecurity, chronic health problems, divorce, and grief can increase the risk of MDD development, (KENDLER; GARDNER, 2016; KENDLER et al., 1995; KESSLER, 1997). Moreover, exposure to traumatic events in childhood (psychological neglect, physical and sexual violence, exposure to domestic violence, or separation from the parents) can also increase the likelihood of developing MDD later in life (DUBE et al., 2001; KESSLER et al., 2010; KIM et al., 2020; STARR et al., 2020; WANG, 2020)(ENTRINGER; BUSS; WADHWA, 2017; STEIN et al., 2014).

The core response to stress involves the activation of the hypothalamic-pituitary-adrenal (HPA) axis (HERMAN et al., 2016; NICOLAIDES et al., 2014; RUSSELL; LIGHTMAN, 2019). During the exposure to stress, corticotrophin-releasing factor (CRF) is released in the paraventricular nucleus of the hypothalamus (PVN), which leads to the release of adrenocorticotropic hormone (ACTH) from the pituitary, triggering the secretion of cortisol (corticosterone in rodents) by the adrenal cortex. Glucocorticoids act through activation of MR and GR receptors, which then mobilizes energy resources and prepares the body to face a stressful situation (HERMAN et al., 2016; NICOLAIDES et al., 2014; RUSSELL; LIGHTMAN, 2019). In healthy individuals, the HPA axis activation is limited through negative feedback, which involves the activation of GR localized in the PVN, prefrontal cortex (PFC) and hippocampus (HPC) (HERMAN et al., 2016; NICOLAIDES et al., 2014; OTTE et al., 2016; RUSSELL; LIGHTMAN, 2019). Under chronic stress exposure, this feedback mechanism can become impaired, due to down-regulation of GR receptors, for example, resulting in dysregulation of the HPA axis activation, which has been associated with the development of MDD (HERMAN et al., 2016; OTTE et al., 2016; RUSSELL; LIGHTMAN, 2019; STARR et al., 2020).

The interaction between environmental factors and the genetic background seems to play an essential role in the etiology of depression (KENDLER; GARDNER, 2016; KENDLER et al., 1995; WICHERS et al., 2009, 2007). Indeed, genetic vulnerability accounts for ~35%-40% of depression (SULLIVAN; NEALE; KENDLER, 2000). Accordingly, previous studies have shown that first-degree relatives of patients with major depression were

at three times higher risk of developing MDD (GESCHWIND; FLINT, 2015). Furthermore, a large-scale study with monozygotic and dizygotic twin estimated 38% heredity for MDD (KENDLER et al., 2006). Several genetic mutations have been implicated in the pathophysiology of depression and in response to antidepressant treatment, including: BDNF, HTR1A, HTR2A, COMT, CLOCK, SLC6A4, SLC6A3, SLC6A2, and TPH, although they have only moreate role in vulnerability to depression (FLINT; KENDLER, 2014). It is thought that MDD development involves the participation of several genes producing small effects that contribute to the phenotype exhibited. These genes have important roles in brain neurochemistry and neurplasticity and can, therefore, impact stress adaptation and disease vulnerability.

Not only genetic, but also epigenetic factors have proven to be important in vulnerability to stress and depression neurobiology. As the current work did not focus on epigenetics, a comprehensive discussion lies beyond the scope of this text, and has been reviewed elsewhere (NESTLER, 2014; PARK et al., 2019; PENNER-GOEKE; BINDER, 2019). Briefly, epigenetics is a dynamic process derived from the interaction between gene x environmental factors that affects the remodeling and function of chromatin, resulting in altered gene expression and protein translation without changing the base sequence of deoxyribonucleic acid (DNA) s (HOLLIDAY, 2006; NESTLER et al., 2016). Many epigenetic mechanisms, including phosphorylation, acetylation, deacetylation, methylation and demethylation in the histone tails and DNA methylation, are modified by stress exposure and antidepressant treatment (HOLLIDAY, 2006). Growing evidence has identified an association between epigenetics alterations with aetiology of major depressive and treatment response (ROBISON et al., 2013; TALAROWSKA, 2020; VIALOU et al., 2013).

1.2 Animal models of depression

Animal models of depression play an essential role in exploring the mechanisms involved in the pathophysiology of MDD and in investigating novel potential compounds for its treatment (NESTLER; HYMAN, 2010; WANG et al., 2018). However, there are some symptoms (such as recurrent thoughts of death or suicide, or excessive guilt) and psychological concepts (like low self-esteem and the ability to perceive the future) which are impossible to model in rodents (CRYAN; SLATTERY, 2007). Despite these limitations, the

use of animal models of depression remains as important experimental tools to understand the molecular mechanisms and new treatment options of depression.

Based on the etiology involved in depression, animal models may involve different approaches, such as genetic alterations, pharmacological manipulation, environmental challenges (stress), and brain injuries. These manipulations induce physiological and behavioral changes in animals, which are attenuated by effective antidepressant treatment (HAO et al., 2019; NESTLER; HYMAN, 2010; PLANCHEZ; SURGET; BELZUNG, 2019). Animal models based on environmental manipulation usually consist of exposing animals to uncontrollable stressors that trigger physiological and behavioral changes similar to the ones observed in depressed patients, such as hypercortisolemia, anhedonia, and cognitive deficits (HAO et al., 2019; PLANCHEZ; SURGET; BELZUNG, 2019; STREKALOVA et al., 2011). Exposure to stress in adulthood, such as chronic unpredictable mild stress (CUMS), learned helplessness (LH), social defeat, or early in life (maternal separation) can induce behavioral changes that can reflect different aspects of MDD symptomatology: sucrose preference (anhedonia), social interaction (sociability), novelty-induced suppressed feeding (anxiety), forced swimming test (despair, helpless), amongst others (DUMAN, 2010; NESTLER; HYMAN, 2010; PLANCHEZ; SURGET; BELZUNG, 2019; SÖDERLUND; LINDSKOG, 2018; WANG et al., 2018). Of note, the Forced Swim Test (FST) and the Tail Suspension Test (TST) were originally developed to assess the behavioral changes produced by exposure to an inescapable stressful situation that could be attenuated by antidepressant treatment and, despite increasing criticism, they remain widely used tests to investigate both stress and antidepressant effects (COMMONS et al., 2017; CRYAN; MOMBEREAU; VASSOUT, 2005; CRYAN; VALENTINO; LUCKI, 2005; DUMAN, 2010; NESTLER; HYMAN, 2010).

It has been a consensus that animal models of depression do not represent all the complexity underlying the human condition (GURURAJAN et al., 2019; HAO et al., 2019; MONTEGGIA; HEIMER; NESTLER, 2018; PLANCHEZ; SURGET; BELZUNG, 2019), and itmay be difficult to model a human situation where the diagnosis is solely based on phenomological observations and not biological correlates. Nevertheless, the animal models are useful tools that allows for the evaluation of behavioral endpoints that resemble depression, allowing us to study its neurobiology and treatment. It is therefore, crucial that any given animal model of depression would fulfill or partly fulfill specific validity criteria, classified as following: 1) Homological validity; 2) Pathogenic validity; 3) Mechanistic

validity; 4) Face validity; 5) Predictive validity (BELZUNG; LEMOINE, 2011), See deatials in Table 2. The "ideal model" would fulfill all of these criteria, but since only some aspects of illness can be modeled in animals, it is important to take into consideration the limitations of each model when interpreting their results (BELZUNG; LEMOINE, 2011; GURURAJAN et al., 2019).

Table 2. Validity criteria for animal models of psychiatry disorders (BELZUNG; LEMOINE, 2011).

Validity Criteria	*Description*
1. Homological validity	Proper choice of strain and species of animal to understand the disease (Species and strain validity).
2. Pathogenic validity	The similarity of processes leading to disease identical to humans (Ontopathogenic and triggering validity).
3. Mechanistic validity	Cognitive or biological mechanisms underlying the disorder are identical in humans and animals.
4. Predictive validity	The aetiological factors and therapeutic agents are identical to the human condition (Induction and remission validity).
5. Face validity	The similarity of observable behaviours or biological outcomes (Ethological and biomarker validity) in humans and animals.

The FST, developed by Porsolt and colleagues in 1977, (PORSOLT; LE PICHON; JALFRE, 1977; PORSOLT; BERTIN; JALFRE, 1978), has been widely used to detect novel antidepressant compounds and understand the biological brain substrates involved in depression (BORSINI; MELI, 1988; CRYAN; MOMBEREAU, 2004; CRYAN; MOMBEREAU; VASSOUT, 2005; CRYAN; VALENTINO; LUCKI, 2005). It consists of submitting the rodent to inescapable stress (swimming in a cylinder filled with water), which triggers active behaviors oriented to escape followed by the prevalence of an immobile posture (BOGDANOVA et al., 2013; PORSOLT; LE PICHON; JALFRE, 1977; PORSOLT; BERTIN; JALFRE, 1978). There are differences in the protocol depending on the species of rodent used (CRYAN; VALENTINO; LUCKI, 2005; PORSOLT; BERTIN; JALFRE, 1978; SLATTERY; CRYAN, 2012). The majority of clinically effective antidepressant drugs reduce immobility and increase or prolong the active escape behaviours (climbing and swimming) during the test (DETKE; JOHNSON; LUCKI, 1997; DETKE; RICKELS; LUCKI, 1995; PORSOLT; LE PICHON; JALFRE, 1977). The advantages of the FST are the

following: a) the low cost; b) ease to execute the method; c) high sensitivity to screening the efficiency of antidepressants with strong predictive validity (BOGDANOVA et al., 2013; CRYAN; SLATTERY, 2007; HAO et al., 2019; PLANCHEZ; SURGET; BELZUNG, 2019).

The tail suspension test (TST) is another important test for detecting potential antidepressant drugs with the same construct as the FST (STERU et al., 1985). In this test, the mice are subjected to an inescapable stressful situation (hung by their tail), and after a struggling period, they assume an immobile response. However, if treated with antidepressant drugs, the immobility is decreased, resulting in more escape-oriented behaviours (CRYAN; MOMBEREAU; VASSOUT, 2005; STERU et al., 1985). Despite the similarities with the FST, it is noteworthy that the TST has the following advantages: a) avoid any possible complications induced by hypothermic exposure in FST; b) it is a useful tool to study genetically modified mice with compromised motor activity; c) increased sensibility to detect SSRIs treatments; d) the animal is immobile faster, but cannot remain at this posture for an extended period (CRYAN; MOMBEREAU; VASSOUT, 2005). However, both TST and FST have been criticized for detecting the antidepressant effect after acute drug administration, unlike depressed patients who need chronic treatment to show the therapeutic effect (CRYAN; MOMBEREAU; VASSOUT, 2005). Despite both FST and TST exhibit the same construct, evidence indicates different neural substrates' activation in the tests (RENARD et al., 2003). Even though they have important limitations in terms of vailidity, the FST and TST remain the most widely tests to study the stress effects associated to depression and the screening of promising antidepressant substances.

In addition to the models based on exposure to stress, models resulting from genetic manipulation have also become important experimental tools to investigate depression neurobiology and treatment. Behavioral changes associated with depression have been observed in transgenic animals which present mutations in genes associated to neuroplasticity, such as BDNF and TrkB, and serotonin signalling (5-HT1A receptors SERT), amongst others (COWEN; EDITORS, 2013; PLANCHEZ; SURGET; BELZUNG, 2019). Genetic models can also result from selective breeding, such as the Flinders Sensitive Line (FSL) rats, which were developed to investigate the mechanisms involved in resistance to anticholinesterase agents, organophosphates, specifically to diisopropyl fluorophosphate (DFP; OVERSTREET et al., 1988). However, these rats present altered sensitivity to cholinergic agonists, what is also observed in depressive patients (OVERSTREET; RUSSELL, 1982, 1984;

OVERSTREET et al., 2005; OVERSTREET; WEGENER, 2013; RISCH et al., 1980). Besides that, it was evidenced that this rat strain has several characteristics resembling depression, such as disrupted sleep pattern (BENCA et al., 1996), low body weight (OVERSTREET, 1993, 2002), reduced appetite (BUSHNELL; LEVIN; OVERSTREET, 1995), psychomotor retardation (OVERSTREET, 1986; RUSSELL et al., 1982) and significant sensitivity/vulnerability to stress (OVERSTREET, 1986; OVERSTREET et al., 1986).

It is important to note that subchronic treatment (14 days) with classical antidepressants (including SSRI, SNRI, MAOI, and TCA) is normally required to induce a pronounced antidepressant-like effect in FSL rats, which is an advantage when compared to other rats lines exposed to FST (OVERSTREET et al., 1995, 2005; OVERSTREET; KEENEY; HOGG, 2004; OVERSTREET; WEGENER, 2013; PUCILOWSKI et al., 1993; SCHILLER et al., 1992). However, treatment with Ketamine (KET) and other potential fast-acting antidepressant substances can modify the behavioural response of FSL rats in the FST after acute administration (DU JARDIN et al., 2018, 2016a; LIEBENBERG; JOCA; WEGENER, 2014). (DU JARDIN et al., 2016a)(SANCHEZ; ASIN; ARTIGAS, 2015). The use of FSL rats has, thus, significantly contributed to the understanding of the participation of gene x environment interaction in the aetiology of depression and investigation of new potential antidepressants.

1.3 Neurobiological hypotheses for MDD

Multiple hypotheses regarding the underlying pathophysiology of MDD exist, which does not necessarily exclude – but rather supplement each other. Some of the more established hypotheses will be presented briefly below.

1.3.1 Monoaminergic hypothesis

The discoveries about the mechanism of action of antidepressant drugs in the sixties set the first biological basis for the neurobiology of MDD, the ‘Monoaminergic Hypothesis of depression’. This hypothesis was mainly based on the following observations: 1) Drugs that

inhibit the metabolism of monoamines, such as monoamine oxidase (MAO), promotes mood improving effects (ZELLER et al., 1952); 2) Tricyclic drugs, such as imipramine, which were able to block the reuptake of monoamines also induced antidepressant effects in humans (AXELROD et al., 1961; AXELROD; INSCOE, 1963; AXELROD; WHITBY; HERTTING, 1960; HERTTING; AXELROD; GORDON, 1961; KUHN, 1958); 3) Reserpine, a drug used as antihypertensive medication inhibits monoamine storage in vesicles and depletes them from the synapse, induces depressive episodes in some patients (LEMIEUX; DAVIGNON; GENEST, 1956); 4) Imipramine reverses the effects of reserpine and the administration on psychostimulant amphetamines induced transient mood elevating effects e (BUNNEY; DAVIS, 1965; COPPEN et al., 1967; LEMIEUX; DAVIGNON; GENEST, 1956; SCHILDKRAUT, 1965). Based on that, the monoaminergic hypothesis postulated that MDD results from a reduction in the monoamines levels in the synaptic cleft in important limbic brain regions and the antidepressant effect would be associated to the restoration of the levels of these neurotransmitters (COPPEN, 1967; COPPEN et al., 1967; SCHILDKRAUT, 1965).

However, the basis of the monoaminergic hypothesis has been challenged, due to several limitations. Of note, the mood elevating effect induced by antidepressant drugs are only observed after several weeks of treatment, usually 4 to 6 weeks, even though the blockage of the monoamine transporter or MAO inhibition reaches the steady-stage within a few hours or days after injection (BLIER; DE MONTIGNY, 1983; BLIER; LISTA; DE MONTIGNY, 1993; BLIER; CHAPUT; DE MONTIGNY, 1988; BLIER; WARD, 2003). To explain this latency for the antidepressant effect, it was proposed that the acute treatment with antidepressants promotes a rapid increase of 5-HT levels in the synaptic cleft and subsequent activation of 5HT1A receptors located in the cell bodies of serotoninergic neurons in raphe nuclei, inhibiting the neuronal firing and diminishing 5-HT release in target limbic regions. The chronic treatment promotes desensitization and/or downregulation of somatodendritic 5-HT1A auto-receptors, allowing the recovery of neuronal firing and consequent increased release of monoamines in limbic structures, which coincides with the therapeutic effect of the drugs (BLIER; EL MANSARI, 2013; HAMON; BLIER, 2013). Therefore, signaling through post-synaptic 5-HT1A heteroreceptors in the PFC and hippocampus (HPC) after chronic antidepressant treatment would be associated with the antidepressant action (ALTIERI et al., 2013; GARCIA-GARCIA; NEWMAN-TANCREDI; LEONARDO, 2014). Other serotonin

and noradrenalin receptors are known to be up or down-regulated after chronic antidepressant treatment, as reviewed by (KÖHLER et al., 2016).

In support of the monoaminergic theory, several genetic mutations in genes related to monoamines have been implicated in the pathophysiology of depression and in response to treatment, such as HTR1A, HTR2A, COMT, MAOA, 5HTTLPR/SLC6A4, DAT/SLC6A3, NET/SLC6A2, TPH1, TPH2 (FLINT; KENDLER, 2014). However, monoamine depletion in healthy patients did not produce depressive symptoms, but it impaired the treatment response in depressed patients (RUHÉ; MASON; SCHENE, 2007). Thus, monoamines certainly play an important modulatory role in mood regulation and are still the main target of the available pharmacological treatment in MDD (CIPRIANI et al., 2018; MAFFIOLETTI et al., 2020; SRAMEK; MURPHY; CUTLER, 2016). Nevertheless, it is important to consider that monoaminergic antidepressant also induce several changes in different neurotransmitter systems, especially after chronic administration, which point to additional mechanisms involved in the antidepressant effect (BALLESTEROS-ZÉBADÚA; MANJARREZ-MARMOLEJO; FRANCO-PÉREZ, 2013; LAMMERS et al., 2000; MARTÍNEZ-TURRILLAS; DEL RÍO; FRECHILLA, 2007; MARTINEZ-TURRILLAS; FRECHILLA; DEL RÍO, 2002; PRATT; BOWERY, 1993) . The non-monoaminergic mechanisms have been the source of intense investigation in the past decades as a way to better understand depression neurobiology and identify novel and more effective pharmacological treatments.

1.3.2 Glutamatergic hypothesis

Glutamate is the major excitatory neurotransmitter and widely distributed in the mammalian brain, with an essential physiological role in synaptic plasticity, learning, and memory. Its action occurs through the interaction with two superfamilies of receptors located pre and postsynapticly, including the ionotropic receptors (AMPA, NMDA, Kainate) and the metabotropic receptors (mGluR) (MURROUGH; ABDALLAH; MATHEW, 2017a; NICIU; KELMENDI; SANACORA, 2013). Once released in the synaptic cleft, glutamate is reuptaken by excitatory amino acid transporters 1, 2, and 3 (EAAT1, 2, and 3) which limit its action (MURROUGH; ABDALLAH; MATHEW, 2017b; WATKINS; PEI; NEWBERRY, 1998).

The excess of glutamate has been implicated in excitotoxic damage, neurodegeneration and impairments in the synaptic integrity (MURROUGH; ABDALLAH; MATHEW, 2017b; NICIU; KELMENDI; SANACORA, 2013; PEREIRA; HIROAKI-SATO, 2018; SANACORA; TRECCANI; POPOLI, 2012). Patients diagnosed with MDD present high glutamate levels in the serum (ALTAMURA et al., 1995; KIM et al., 1982), cerebrospinal fluid (LEVINE et al., 2000), and brain (HASHIMOTO; SAWA; IYO, 2007; MCEWEN et al., 2012a; SANACORA et al., 2004), which have implicated glutamate in the neurobiology of MDD. Accordingly, chronic treatment with antidepressant drugs normalizes excessive brain glutamate levels induced by stress exposure and modulates the expression of glutamate receptors (POPOLI et al., 2013; TOKARSKI et al., 2008). Thus, the investigation of potential new antidepressant drugs that act in the glutamatergic system seems has been intensively studied in the past three decades.

In fact, drugs that modulate the glutamatergic system have shown promising effects in depression treatment. In 1990, Trullas and Skolnick were the first to report that the administration of NMDA receptor antagonists produced an antidepressant-like effect in the FST (TRULLAS; SKOLNICK, 1990). In this initial study, and later several other studies it was shown that systemic administration of NMDA antagonists induce a behavioral response similar to those induced by antidepressant drugs in animals submitted to different animal models (BURGDORF et al., 2013; LI et al., 2010b; MAENG et al., 2008; MOSKAL et al., 2014; SHIRAYAMA; HASHIMOTO, 2017). Furthermore, injection with NMDA antagonist into a specific brain area related to depression, such as PFC and HPC, also produced an antidepressant-like effect, thus implicating dysfunctional glutamatergic signaling in this brain regions in depression neurobiology (FUKUMOTO et al., 2017a; FUKUMOTO; IIJIMA; CHAKI, 2016; PADOVAN; GUIMARÃES, 2004; PEREIRA et al., 2015; PHAM et al., 2017a).

Clinical studies also show that the infusion of a subanesthetic dose of a non-competitive NMDA antagonist, namely KET exerts robust rapid (within 2 h following administration) and sustained antidepressant effects, both in humans and in animal models (BERMAN et al., 2000) (AUTRY et al., 2011; FRANCESCHELLI et al., 2015; FUKUMOTO et al., 2017a; KOIKE; IIJIMA; CHAKI, 2011; LI et al., 2010a; LIEBENBERG; JOCA; WEGENER, 2014; MAENG et al., 2008). Recently, an intranasal formulation of s-ketamine was approved by the FDA in treating treatment resistant depression

(FDA, 2019). The antidepressant effect induce by ketamine and its molecular mechanism will be discussed in subsequent section 1.4.2 and also in the study 2. Furthermore, recently, other drugs that modulate the NMDA receptor activity are under investigation as fast-acting antidepressant drugs, such as Rapastinel, AV-101, NRX-1074 (for review see (MURROUGH; ABDALLAH; MATHEW, 2017a).

1.3.3 Inflammation hypothesis

Accumulating evidence has associated increased inflammatory response to MDD neurobiology (BRUNO et al., 2020; MILLER; RAISON, 2016). The immune hypothesis proposes that chronic exposure to stress is associated with the activation of the inflammatory response and leads to increased levels of proinflammatory cytokines, especially interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor alfa (TNF-α), and interferon (INF), which can modulate brain neurochemistry and neuroendocrine responses, ultimately leading to behavioral changes (BRUNO et al., 2020; MILLER; RAISON, 2016).

In line with this hypothesis, administration of inflammatory cytokines or their inducers (for example, endotoxin or typhoid vaccination) causes depressive symptoms in healthy patients (BONACCORSO et al., 2002; CAPURON et al., 2002; HARRISON et al., 2009; REICHENBERG et al., 2001). In addition, individuals affected by chronic inflammatory diseases (rheumatoid arthritis, lupus erythematosus, influenza virus infection) often present depression as a comorbidity (MEIJER; ZAKAY -RONES; 2020), and the blockade of cytokines or inflammatory signaling reverses the depressive symptoms in patients (ABBOTT et al., 2015; KÖHLER et al., 2014, 2015; SUN et al., 2017; TYRING et al., 2006). However, the modulatory effect of antidepressant drugs in immune response is still controversial (KUBERA; BASTA-KAIM; PAPP, 1995; MUSSELMAN et al., 2001; WEIZMAN et al., 1994).

Furthermore, a growing body of evidence has shown that the activation of the immune system can play a modulatory role in the HPA axis (FELGER et al., 2016; MILLER; RAISON, 2016), promotes changes in the neurochemical profile (FELGER; LI; MARVAR, 2013; KORTE-BOUWS et al., 2019; MAES, 1995; MAES et al., 1994; SPERNER-UNTERWEGER; KOHL; FUCHS, 2014; ZOLLER et al., 2012), alterations in neurotrophin signaling (CARLOS et al., 2017; KENIS et al., 2011; LOTRICH; ALBUSAYSI; FERRELL,

2013; MILLER; RAISON, 2016; RAGE; SILHOL; TAPIA-ARANCIBIA, 2006; TONG et al., 2008), and impairment of neuroplastic mechanisms (HAYLEY, 2014; WU et al., 2012; YIRMIYA; GOSHEN, 2011), leading to the development of major depression.

1.3.4 Neuroplastic hypothesis

Neuroplasticity is a crucial adaptive function of the brain to perceive, assess, adapt, and select the appropriate response to internal and external stimuli. This response occurs through different mechanisms, including alterations in dendritic function, synaptic remodeling, long-term potentiation, dendritic arborization, synaptogenesis, and neurogenesis (DUMAN et al., 2000, 2016a; MANJI; DREVETS; CHARNNEY, 2001). This brain function plays an essential role in memory, cognition, learning, and stress adaptation (DUMAN et al., 2016a; PITTENGER; DUMAN, 2008; PRICE; DUMAN, 2020). Brain-derived neurotrophic factor (BDNF) is considered a key neurotrophin responsible for neuronal survival, growth, and differentiation of neurons, and regulation of the synaptic plasticity (DINIZ et al., 2018; PARK; POO, 2013). The neuroplasticity hypothesis postulates that the development of MDD results from the impairments in neurotrophin signaling and subsequently in neuroplasticity processes, which would be restored by treatment with antidepressants (CASTRÉN, 2005).

Imaging studies revealed that depressed patients present reduced hippocampus (HPC) and frontal cortex regions, important limbic areas related to depression (DREVETS, 2001, 2000; DREVETS; PRICE; FUREY, 2008; HORNE; NORBURY, 2018; TAE et al., 2011; WU et al., 2018). Moreover, postmortem studies have revealed smaller size and density of neurons and lower synapses in the PFC of depressed subjects (DREVETS, 2000; KANG et al., 2012). Besides, the morphological alterations are accompanied by reduced levels of BDNF (DUNHAM et al., 2009; GUILLOUX et al., 2012; KAREGE et al., 2005a, 2005b; KOBAYASHI et al., 2005; RAY et al., 2011; SEN; DUMAN; SANACORA, 2008; SHIMIZU et al., 2003). However, the treatment with several antidepressant classes prevents or normalizes these morphological deficits (CASTRÉN; ANTILA, 2017; CASTRÉN, 2005), accompanied by the increased BDNF levels (SEN; DUMAN; SANACORA, 2008; SHIMIZU et al., 2003). The volume reduction extension is correlated with the duration of disorder, time of treatment, and the severity of depression (DUMAN et al., 2016b).

Corroborating with the clinical findings, in animals, exposure to inescapable stress reduced BDNF levels in brain regions related to depression, the PFC and HPC (DUMAN; MONTEGGIA, 2006; LARSEN et al., 2010; SMITH et al., 1995), causing atrophy, and the loss of neurons and glial cells in these brain region (DUMAN; AGHAJANIAN, 2012; MCEWEN et al., 2012b). Furthermore, rodents exposed chronically to glucocorticoids presented decreased synaptic number and function and atrophy in neurons located in limbic structures (LIU; AGHAJANIAN, 2008; MAGARIÑOS; MCEWEN, 1995) and diminished BDNF levels in the same structure (LI et al., 2019). Chronic, but not acute, antidepressant treatment restores the the impairment of synaptic plasticity (ARDALAN et al., 2020; CASTRÉN; ANTILA, 2017; LI et al., 2010b; TRECCANI et al., 2019) and also increased BDNF and TrkB receptor levels in the HPC (AUTRY; MONTEGGIA, 2012; CASTRÉN; ANTILA, 2017; DUMAN; MONTEGGIA, 2006; NIBUYA; MORINOBU; DUMAN, 1995a). However, fast-acting antidepressants, such as ketamine, produces rapid increases in BDNF levels and the dendritic arborization in the HPC (LI et al., 2010b). Since the selective loss of BDNF in HPC attenuates the antidepressant effect produced by monoaminergic antidepressant drugs (desipramine and escitalopram) and the fast-acting antidepressant KET (ADACHI et al., 2008; AUTRY et al., 2012), BDNF-TrkB signaling is considered necessary for the antidepressant effect.

The interaction between BDNF and its receptor TrkB activates its intracellular cascades that regulate neuronal survival, development, and differentiation, playing a fundamental role in the neuroplasticity process (CASTRÉN; ANTILA, 2017; CUNHA; BRAMBILLA; THOMAS, 2010a). The detailed discussion about the interaction between BDNF and TrkB is described elsewhere (CASTRÉN; KOJIMA, 2017; CUNHA; BRAMBILLA; THOMAS, 2010b).

Furthermore, it is noteworthy that the impairment of neuroplastic and synaptogenesis mechanisms resulting from a complex interaction between the other factors involved in the neurobiology of depression, such as monoaminergic neurotransmission imbalance (MAFFIOLETTI et al., 2020; PEREIRA; HIROAKI-SATO, 2018), increased levels of glutamate (MURROUGH; ABDALLAH; MATHEW, 2017a; NICIU; KELMENDI; SANACORA, 2013), the activation of HPA axis (MCEWEN et al., 2012a), immune response activation (BRUNO et al., 2020; MILLER; RAISON, 2016) and neurotrophin signaling (CASTRÉN; ANTILA, 2017; CASTREN; VOIKAR; RANTAMAKI, 2007; CASTRÉN;

KOJIMA, 2017; CASTRÉN; RANTAMÄKI, 2010a; DUMAN; DEYAMA; FOGAÇA, 2019).

1.3.5 Conclusion

Although many hypothesis have been postulated about depression neurobiology, there is no unifying theory that can explain such a complex and heterogenous disorder. Dysfunctions in different pathways and neurotransmitter systems could explain the diversity set of symptoms presented by depressed patients. Therefore, each of the hypothesis can represent an oversimplification of one of the dysfunctions associated with MDD.

1.4 Pharmacological treatment of MDD

As mentioned a variety of current treatment options exist for treatment of MDD. However, none of them are able to relieve the symptoms in more than 60-70% of the patients, and only 25-30% of the patients achieve remission. Below, a brief summary of the established treatment options for MDD is listed.

1.4.1 Monoaminergic antidepressants

The discovery of the first antidepressant occurred by serendipity. The tuberculosis patients with simultaneous diagnose of depression treated with iproniazid reported a general improvement in the mood (ZELLER et al., 1952). In the same period, imipramine was synthesized from the chemical modifications in the structure of the antipsychotic agent, chlorpromazine, that was ineffective for psychosis but displayed a remarkable improvement of depression symptoms after chronic treatment for 1 to 6 weeks (AXELROD et al., 1961; AXELROD; INSCOE, 1963; AXELROD; WHITBY; HERTTING, 1960; KUHN, 1958). The elucidation of the mechanism of action for both drugs revealed that iproniazid acts inhibiting the enzyme monoamine oxidase (MAO) (ZELLER et al., 1952), and imipramine blocks the reuptake of noradrenaline and 5-HT (tricyclic antidepressant (TCA), increasing the monoamines levels in the brain (AXELROD et al., 1961; AXELROD; INSCOE, 1963;

AXELROD; WHITBY; HERTTING, 1960; HERTTING; AXELROD; GORDON, 1961). Due to due to the lack of selectivity in their action, both drugs cause several significant side effects (MAFFIOLETTI et al., 2020; PEREZ-CABALLERO et al., 2019). Subsequently, the search for new antidepressants with similar mechanisms of action was initiated, and more selective compounds were developed, including the selective serotonin reuptake inhibitor (SSRI), selective noradrenaline reuptake inhibitors (SNRI), and serotonin and norepinephrine reuptake inhibitor (SNRI), resulting in safer compounds with fewer side effects (MAFFIOLETTI et al., 2020; PEREZ-CABALLERO et al., 2019). The SSRI and SNRI are considered the first choice pharmacological treatment of depression nowadays (CIPRIANI et al., 2018; MAFFIOLETTI et al., 2020).

Although monoaminergic antidepressants are effective to treat depression, these compounds have several limitations, including a latency to initiate the therapeutic effect (4 to 6 weeks) (CIPRIANI et al., 2018), and low-efficacy rates, in which 40-50% of the patients respond partially or do not respond to treatment (CIPRIANI et al., 2018; KEKS et al., 2007; OTTE et al., 2016). Furthermore, at the begging of the treatment, the conventional antidepressants may worsen depression or induce suicidal ideation (CIPRIANI et al., 2018; OTTE et al., 2016). In fact, it is fundamental to understand the pathophysiology enrolled in the disorder to investigate new substances with potential antidepressant effects. In particular, compounds with rapid onset of action and effective in individuals who do not respond to currently available treatments.

1.4.2 Ketamine

Ketamine is a non-competitive antagonist of the N-methyl-D-aspartate glutamate receptor (NMDA; KOHRS; DURIEUX, 1998; WHITE et al., 1980), commonly used as a dissociative anesthetic in humans. In the begging of 2000, Berman et al. (BERMAN et al., 2000) were the first to reveal that the infusion of a subanesthetic dose of ketamine exerts robust rapid (within 2 h following administration) and sustained antidepressant effect lasting for 3 days in the depressed patient (BERMAN et al., 2000). Subsequent clinical studies evidenced similar rapid and sustained (7 days on average) antidepressant effect in patients (CUSIN et al., 2016; DIAZGRANADOS et al., 2010; FREEMAN et al., 2020; GHASEMI et al., 2013; KRYSTAL et al., 1994; O’BRIEN et al., 2019; RODRIGUES et al., 2020;

ZARATE et al., 2006; ZARATE JR et al., 2012). Moreover, ketamine exerts an antidepressant effect in treatment-resistant depression patients and reduces suicide ideation (DIAZGRANADOS et al., 2010; MURROUGH et al., 2015; O'BRIEN et al., 2019; RODRIGUES et al., 2020). In this sense, ketamine opens a new era for the treatment of depression with a fast-acting antidepressant class, with non-monoaminergic mechanism.

Besides, preclinical studies have described similar findings. A single injection of ketamine produces a rapid and sustained antidepressant-like effect in several animal models of depression, including chronic unpredictable mild stress (CUMS), learned helplessness (LH), social defeat, forced swim test (FST), and tail suspension test (AUTRY et al., 2011; FUKUMOTO et al., 2017b; KOIKE; IIJIMA; CHAKI, 2011; LI et al., 2010a; MAENG et al., 2008; PHAM et al., 2017a; SUN et al., 2016). Ketamine can also produce an antidepressant-like effect in genetic animal models depression, FSL, and Wistar-Kyoto rats, as well as treatment-resistant models (DU JARDIN et al., 2016a; LIEBENBERG; JOCA; WEGENER, 2014; PEREIRA et al., 2019; SOWA et al., 2019). Corroborating with prior clinical findings, ketamine induces an antidepressant-like effect which lasts for 7 days in different paradigms (AUTRY et al., 2011; FUKUMOTO et al., 2014, 2017b; LIEBENBERG; JOCA; WEGENER, 2015; MAENG et al., 2008; TRECCANI et al., 2012; ZANOS et al., 2019a). In this sense, preclinical studies are essential tools that allow us to investigate the ketamine antidepressant effect mechanisms and possibly translate those findings to humans.

The molecular mechanism responsible for ketamine antidepressant effect is complex. It involves multiple the antagonism of the NMDA receptor localized on GABAergic interneurons promoting glutamate release in synaptic cleft, which activates α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor. Once activated the AMPA receptor leads the release BDNF, which activates its receptor tropomyosin related kinase B (TkB) signaling pathway, activating mammalian target of rapamycin (mTOR) other downstream molecules, resulting synaptogenesis and synaptic plasticity induction. The detailed molecular mechanism involved in ketamine antidepressant effect is reviewed elsewhere (DUMAN; SANACORA; KRYSTAL, 2019; PHAM; GARDIER, 2019; ZANOS; GOULD, 2018), and also elaborated on in the discussion of study 2 later in this thesis.

Notwithstanding, ketamine can induce undesirable side-effects, such as abuse and addiction (WHITE; WAY; TREVOR, 1982). Among which, the main side effects psychotomimetic effects, such as delusions, hallucinations, dissociative or extracorporeal

effects (feeling of being outside the body), and vivid dreams (DIAZGRANADOS et al., 2010; JANSEN, 2000; KRYSTAL et al., 1994; SHORT et al., 2018; SINGH et al., 2016; WHITE; WAY; TREVOR, 1982; ZARATE et al., 2006), that can produce abuse, which, therefore, would limit its use in depressed patients. Therefore, it is still fundamental to develop new therapeutic approaches with antidepressant effects similar to those produced by ketamine (fast and sustained).

1.4.3 Cannabidiol

The *Cannabis sativa L.* (cannabis) is a plant that has been used for medical and religious purposes by several civilizations in the world for thousands of years (ZUARDI, 2006). The use of cannabis in medicine by ancient Chinese was reported in the pharmacopeia around 2.700 B.C. (TOUW, 1981). Notably, in India, folk medicine reported cannabis preparation with flowers and resin to treat anxiety, mania, hysteria, and depression thousands of years before the Christian era (RUSSO, 2005). Beyond the medicinal use, the plant is widely used for recreational purposes because of its psychoactive properties, including the alteration of conscious perception, euphoria, and relaxation (RUSSO, 2005). Thereby, the medicinal use spread gradually worldwide, first to the Middle East and Europe in the 18th century, reaching Africa and America later. Indeed, the actual introduction of cannabis for medical propose occurred in the 19th century from William B. O'Shaughnessy, who described the cannabis preparations, methodically investigated its toxic effect on animals and the therapeutic effect on humans different diseases (O'SHAUGHNESSY, 1843). In the first decade of the 20th century, there was a decline in the plant's use for therapeutic purposes due to the difficulty to replicate the effects, variable efficacy of different sample of the plant (ZUARDI, 2006). The active compounds were still unknown, and the extract has varying concentration and potency, which produce considerable side effects (ZUARDI, 2006). Advances in isolation techniques allowed to isolate the cannabinoids present in plant extracts in the 1940s (ADAMS; HUNT; CLARK, 1940; GAONI; MECHOULAM, 1964; MECHOULAM; SHVO, 1963) and to identify endogenous receptors and ligands, leading to the identification of the endocannabinoid system in the central nervous system (CNS). Understanding the molecular basis and the endocannabinoid system's role has increased scientific interest in exploring the potential effect of cannabinoids.

The endocannabinoid system is constituted by two G protein-coupled receptors (GPCR), cannabinoid type 1 and 2 receptors (CB1 and CB2), two main endogenous ligands arachidonoyl ethanolamide (AEA, anandamide) and 2-arachidonoylglycerol (2-AG) that acts in the receptors; the enzymes involved in the endocannabinoid biosynthesis and degradation, AEA (other N-acylethanolamines) are respectively synthesized and hydrolyzed by N-acylphosphatidylethanolamine (NAPE)-specific phospholipase D-like hydrolase (NAPE-PLD) and fatty acid amide hydrolase (FAAH)(CRAVATT et al., 1996; OKAMOTO et al., 2004). Whereas 2-AG, diacylglycerol lipase α (DAGLα), and DAGLβ catalyze the biosynthesis, and monoacylglycerol lipase (MAGL) is responsible for its hydrolysis (BISOGNO et al., 2003; DINH et al., 2002). Besides, the endocannabinoids may act through orphan GPCR 55, known as GPR55 receptor (LAUCKNER et al., 2008; PERTWEE, 2007; RYBERG et al., 2007) and vanilloid receptor, transient receptor potential cation channel subfamily V member 1 (TRPV1) (BISOGNO et al., 2001; CRISTINO et al., 2006; ROSS, 2003).

The endocannabinoid belongs to the class of atypical neurotransmitters. Thus, the neurotransmitter is not stored in vesicles; however, it is synthesized in the postsynaptic neuron on demand, in response to physiological neuronal depolarization. Once the neurotransmitter is released in the synaptic cleft, it may act as a CB1 agonist located in a presynaptic neuron, in a retrograde manner. The receptor is coupled with G inhibitory protein resulting in the inhibition of neuronal depolarization modulating neurotransmitter releases, mainly glutamate and GABA(AZAD et al., 2008; HÄRING et al., 2007; HERMANN; LUTZ, 2005; KANO et al., 2009; MOROZOV; TORII; RAKIC, 2009). Endocannabinoids can also act on CB2 receptors located in the glial cells in the central nervous system, which modulates the release of cytokines participating in the synaptic activity and pruning (CRISTINO; BISOGNO; DI MARZO, 2020; DI MARZO, 2018).

Abnormalities in the endocannabinoid neurotransmission have been implicated in the pathophysiology of stress-related disorders, such as anxiety and MDD (MICALE et al., 2013; NAVARRETE et al., 2020). Post mortem study revealed that CB1 receptor expression was reduced in the anterior cingulate cortex (ACC) of the depressed patient (KOETHE et al., 2007). In the same way, the 2-AG blood levels were reduced in women diagnosed with depression (HILL et al., 2008). Similarly, non-treated depressed patients present low basal serum levels of AEA and 2-AG (HILL et al., 2009). In contrast, patients treated with SSRI

have increase plasmatic levels of AEA and 2-AG (ROMERO-SANCHIZ et al., 2019). Similar to the clinical findings, knockout mice for CB1 receptors exhibit a depressive- and anxiety-like phenotype in different behavioural tests (MARTIN et al., 2002). Also, the systemic administration of the CB1 agonist receptor produces an antidepressant-like effect in rats submitted to FST (BAMBICO et al., 2007). In addition, the MAGL inhibitor, JZL194, has similar antidepressant effects in CUMS (ZHANG et al., 2015).

The plant *Cannabis sativa* L. has over 100 different phytocannabinoids, and the Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) are the two most studied compounds. Δ^9-THC is the primary psychoactive substance responsible for the effect produce by the cannabis, which was isolated and the chemical structure elucidated in the 1964s (GAONI; MECHOULAM, 1964), while CBD a non-active compound isolated in the early 1940s, and the chemical structure determined in 1963 (ADAMS; HUNT; CLARK, 1940; MECHOULAM; SHVO, 1963). In the beginning, curiously, CBD was considered a non-active cannabinoid, but later studies revealed that CBD produces the opposite behavioral effect induced by Δ9-THC on anxiety and psychotic symptoms in healthy patients (MARTIN-SANTOS et al., 2012; ZUARDI et al., 1982). THC acts through agonism of CB1 and CB2 receptors, facilitating the endocannabinoid action (GROTENHERMEN, 2003).

Differently from THC, the molecular mechanism involved in the CBD effect has not been fully elucidated. It is known that CBD acts through several molecular targets and is not restricted only to the endocannabinoid system. It seems that CBD modulates the endocannabinoid system acting as CB1 and CB2 receptors allosteric modulators, AEA uptake inhibitor, FAAH inhibitor, TRPV1 agonist, and GPR55 antagonist, as well as the in serotoninergic, opioidergic, adenosinergic neurotransmission systems, and modulating the inflammatory signaling, as presented in Table 3.

Table 3. Main pharmacological targets for Cannabidiol.

Biological System	***Target***	***References***
eCBD	CB1 receptor antagonist	(CAMPOS et al., 2013; FOGAÇA et al., 2018)
	CB2 receptor inverse agonist	(CAMPOS et al., 2013; FOGAÇA et al., 2018)
	FAAH inhibitor	(BISOGNO et al., 2001; CAMPOS et al., 2013; FOGAÇA et al., 2018; LEWEKE et al., 2012; PETROSINO et al., 2018)
	AEA uptake inhibitor	(BISOGNO et al., 2001; CAMPOS et al., 2013; FOGAÇA et al., 2018; LEWEKE et al., 2012; PETROSINO et al., 2018)
	TRPV1 agonist	(BISOGNO et al., 2001; DE GREGORIO et al., 2018; FONSECA; CORREIA-DA-SILVA; TEIXEIRA, 2018; PETROSINO et al., 2018)
	TRPA1 agonist	(DE PETROCELLIS et al., 2008)
	TRPM8 antagonist	(DE PETROCELLIS et al., 2008)
	TRPV2 agonist	(EUBLER et al., 2018; NABISSI et al., 2015; QIN et al., 2008)QIN et al., 2008; NABISSI et al., 2015;EUBLER et al., 2018.
	GPR55 antagonist	(CHERIF et al., 2015; WALSH et al., 2015)WALSH et al., 2015; CHERIF et al., 2015.
Serotonin	*5-HT1A agosnist*	(CAMPOS; FERREIRA; GUIMARÃES, 2012; DE GREGORIO et al., 2018; FOGAÇA et al., 2014; GOMES; RESSTEL; GUIMARÃES, 2011; MYERS et al., 2018; RESSTEL et al., 2009; RUSSO et al., 2005; SARTIM; GUIMARÃES; JOCA, 2016; ZANELATI et al., 2010)
	5-HT2A agonist	(LONG et al., 2012; PELZ et al., 2017; RUSSO et al., 2005)RUSSo et al., 2005; LONG et al., 2012; PELZ et al., 2017

	5-HT3 agonist	(XIONG et al., 2011)
	Tryptophan degradation inhibitor	(JENNY et al., 2009)
Opioid	Mu- opioid ligand	(KATHMANN et al., 2006; RODRÍGUEZ-MUÑOZ et al., 2012; VIUDEZ-MARTÍNEZ et al., 2018)
	Delta- opioid allosteric modulator	(KATHMANN et al., 2006)
	Sigma-opioid ligand	(RODRÍGUEZ-MUÑOZ et al., 2012)
Adenosine	Adenosine uptake inhibitor and indirect A2A agonist	(CARRIER; AUCHAMPACH; HILLARD, 2006; LIOU et al., 2008; MIJANGOS-MORENO et al., 2014; OLÁH et al., 2014; PANDOLFO et al., 2011)
Dopamine	Dopamine uptake inhibitor	(MURILLO-RODRÍGUEZ et al., 2011; PANDOLFO et al., 2011; ROSSIGNOLI et al., 2017)
Other	PPARγ agonist	(GIACOPPO et al., 2017; HIND; ENGLAND; O'SULLIVAN, 2016; VALLÉE et al., 2017)
	GABAA positive allosteric modulator	(BAKAS et al., 2017; LONG et al., 2012)
	α7 nicotinic acetylcholine antagonist	(MAHGOUB et al., 2013)
	Regulator of intracellular calcium	(DRYSDALE et al., 2006; RYAN et al., 2009b)
	iNOS inhibitor	(ESPOSITO et al., 2006)
	NF-κB inhibitor	(ESPOSITO et al., 2006)
	COX-1 and 2 inductor	(WHEAL et al., 2014)

A growing body of evidence has indicated that CBD shows promising effects in several psychiatric disorders, including anxiety (CRIPPA et al., 2011; GUIMARÃES et al., 1990; MOREIRA; AGUIAR; GUIMARÃES, 2006; SOARES et al., 2010; ZUARDI et al., 1982, 2017), psychosis (MOREIRA; GUIMARÃES, 2005; ROTTANBURG et al., 1981; ZUARDI; ANTUNES RODRIGUES; CUNHA, 1991), epilepsy (CRIPPA et al., 2016; DO VAL-DA SILVA et al., 2017; GOBIRA et al., 2015), and major depression (LINGE et al., 2016; SALES et al., 2018b, 2018a; ZANELATI et al., 2010). In the last years, CBD was approved by Food and Drug Administration (FDA) for the treatment of severe forms of

epilepsy, Lennoux-Gastaut syndrome, and Dravet syndrome (FDA, 2018), and also approved National Health Surveillance Agency (ANVISA) a corresponding FDA agency in Brazil for the same purpose (CONSELHO FEDERAL DE MEDICINA, 2016).

Despite its proven efficacy in other conditions, CBD effects in depression remains controversial a largely unexplored. For the first time, our group investigated whether the acute systemic treatment with CBD produced an antidepressant effect in male Swiss mice exposed to a test predictive for antidepressant compounds, the FST. The results revealed that the acute administration with CBD reduces the immobility time in the test, similar to the established antidepressant imipramine (ZANELATI et al., 2010). Interestingly, the behavioural response produced by CBD was counteracted by the pre-treatment with 5-HT1A antagonist receptor (WAY100635) (ZANELATI et al., 2010). For the first time, this study suggested an antidepressant-like effect induced by CBD and evidenced the involvement of 5-HT1A receptor in the CBD effect. A subsequent study corroborated these findings, showing an antidepressant-like response in Swiss mice exposed to FST and TST after acute administration even at a higher dose (200 $mg.kg^{-1}$) (EL-ALFY et al., 2010).

In addition, it was evidenced that CBD is also effective after the repeated treatment in Swiss mice and Wistar rats exposed to TST and FST (RÉUS et al., 2011; SCHIAVON et al., 2016a). Altogether, these findings reinforce that CBD produces antidepressant effects after acute or repeated administration, in both mice and rats. However, varying effects in the effective doses are observed and there is not systematic investigation of CBD effects in different strain to assess possible differences, which can be a source of variability.

Other studies have been evidenced promised antidepressant-like effect of CBD in rodents exposed to different paradigms, including the learned helplessness (LH) (SALES et al., 2018b), the olfactory bulbectomy (OBX) (LINGE et al., 2016), and the chronic unpredictable mild stress (CUMS; (GÁLL et al., 2020; XU et al., 2019). Interestingly, for Wistar rats, only the chronic treatment with CBD (for 28 days) reversed the behavioural response induced by CUMS, not the acute administration (GÁLL et al., 2020). Notably, CBD produced antidepressant and pro-hedonic responses in the genetic rat model based on selective breeding, the Flinders Sensitive Line (FSL; Sales et al., 2018; Shbiro et al., 2019), and the Wistar-Kyoto rats (SHBIRO et al., 2019; SHOVAL et al., 2016). Strikingly, CBD produced the antidepressant effect in both males and females Wistar-Kyoto rats, but only in male FSL rats. Interestingly, it appears that the effectiveness of CBD depends on the gender

and strain of rodent selected. However, the present study evaluated only one dose of CBD (30 mg.kg-1), which it is difficult to conclude about the effect of CBD in female rats. Additional studies are necessary to address this question.

Importantly, CBD promotes rapid antidepressant effect in OBX and LH models (LINGE et al., 2016; SALES et al., 2018b), in contrast to conventional monoaminergic antidepressants, which require chronic treatment, indicating that it may be a fast-acting antidepressant. Furthermore, our group showed for the first time that CBD promotes an antidepressant effect that lasts for one week after a single injection (SALES et al., 2018b), thus suggesting a sustained antidepressant-like effect similar which have been demonstrated for KET (LI et al., 2010b; MAENG et al., 2008). Besides, the serotoninergic system appears to be crucial for the CBD effect, as previously shown for ketamine also (DU JARDIN et al., 2018, 2017; FUKUMOTO et al., 2017a). Accordingly, the co-administration of sub effective doses of CBD with fluoxetine (SSRI) produced a synergic antidepressant-like effect in mice exposed to FST. However, the previous depletion of 5-HT following with PCPA (serotonin synthesis inhibitor) abolished the CBD antidepressant effect (SALES et al., 2018a). Corroborating with the findings, the antidepressant effect observed with CBD treatment in the OBX model was accompanied by increased levels of 5-HT in the ventromedial PFC. However, the previous administration with 5-HT1A antagonist receptor (WAY100635) attenuated the behavioral and neurochemical response induced by CBD (LINGE et al., 2016).

Interestingly, the sub chronic administration of CBD (during 14 days) in diabetic rats reversed the behavioral deficits and increased 5-HT levels in HPC and PFC (CHAVES et al., 2020). Furthermore, the site-specific injection of CBD into limbic brain regions related to depression, including dorsal HPC and ventromedial PFC resulting in an antidepressant effect in FST (SARTIM; GUIMARÃES; JOCA, 2016; SARTIM et al., 2018). The behavioral effect produced by intra-mPFC injection was countered by previous treatment with 5-HT1A (SARTIM; GUIMARÃES; JOCA, 2016). Indeed, the results show the relevance of serotoninergic neurotransmission on mPFC for the CBD behavioral effect. Altogether, these results indicated that CBD effects depend on the intact function of serotonin signaling in limbic brain regions. Further investigations are necessary to understand the mechanism involved in the effect.

Based on the above findings, presented in full in Table 4, it is possible to conclude that CBD produces an antidepressant-like effect in different paradigms, using distinct rodent

strains and species. Furthermore, the regimen of treatment did not influence the final observed outcome of CBD (acute, repeated treatment (14 days) or chronic (28 days)). However, the antidepressant effect in female animals is still unclear and warrants further investigations based on epidemiologic data about MDD prevalence. Moreover, it is essential to determine the molecular mechanism involved in the effect of CBD to develop the most effective antidepressant compounds.

Table 4. Preclinical evidence regarding the antidepressant effect produced by CBD (Modified from SILOTE et al., 2019).

Reference	Animal	Age	Origin	Dose	Route	Test	Effect
ZANELATI et al., 2010	Male Swiss mice	n.s.	Natural	30 mg/kg	i.p.	FST	Antidepressant effect
				3, 10 and 100 mg/kg	i.p.	FST	No effect
EL-ALFY et al., 2010	Male Swiss Webster mice	8 weeks	Natural	200 mg/kg i.p.	i.p.	FST	Antidepressant effect
	Male Swiss Webster mice	8 weeks		20 and 100 mg/kg i.p.	i.p.	FST	No effect
	Male DBA/2	8 weeks		20, 100 and 200 mg/kg i.p.	i.p.	TST	No effect
RÉUS et al., 2011	Male Wistar rats	8 weeks	n.s.	30 mg/kg (Acute)	i.p.	FST	Antidepressant effect
				15 and 60 mg/kg (Acute)	i.p.	FST	No effect
				30 mg/kg (Repeated - 14 days)	i.p.	FST	Antidepressant effect
				15 and 60 mg/kg (Repeated - 14	i.p.	FST	No effect

				days)			
CAMPOS et al., 2013	Male C57BL/6J mice (CUS)	12 weeks	Natural	30 mg/kg (Repeated - 14 days)	i.p.	EPM and NSF	Anti-stress effect
	HiB5 cells	-	Natural	100 nM	culture medium	Immunofluorescence microscopy	Neural progenitor proliferation
					culture medium	Flow citometry	Increase neural progenitor cell in S phase cells
SCHIAVON et al., 2016	Male Swiss albino mice	5-6 weeks	Natural	3 and 10 mg/kg (Acute)	i.p.	TST	Antidepressant effect
				30 mg/kg (Acute)	i.p.	TST	No effect
				3 and 30 mg/kg (Repeated - 15 days)	i.p.	TST	Antidepressant effect
LINGE et al., 2016	Male C57BL6 mice (Olfatory bulbectomy)	12 weeks	Natural	50 mg/kg (Acute)	i.p.	OFT	Antidepressant effect
				50 mg/kg (Repeated- 7 days)	i.p.	OFT	Antidepressant effect
					i.p.	SPT	Prohedonic effect

SHOVAL et al., 2016	Wistar-Kyoto rats	13 weeks	Natural	30 mg/kg	oral (food pellet)	SPT	Prohedonic effect
				15 and 45 mg/kg	oral (food pellet)	SPT	No effect
SARTIM; GUIMARÃES; JOCA, 2016	Male Wistar rats	n.s.	Natural	10, 30 and 60 nmol/0.2 ul/side	intra-PL mPFC	FST	Antidepressant effect
				45 and 60 nmol/0.2 ul/side	intra-IL mPFC	FST	Antidepressant effect
				30 nmol/0.2 ul/side	intra-IL mPFC	FST	No effect
BREUER et al., 2016*.	Male Swiss mice	n.s.	Natural	HUF101: 3 mg/kg	i.p.	FST	Antidepressant effect
				HUF101: 1 and 10 mg/kg	i.p.	FST	No effect
				HUF103: 3 and 10 mg/kg	i.p.	FST	Antidepressant effect
				HUF103: 1 mg/kg	i.p.	FST	No effect
FOGAÇA et al., 2018	Male C57BL6 mice (CUS)	8-9 weeks	Natural	30 mg/kg (Repeated - 14	i.p.	EPM and NSF	Anti-stress effect

				days)			
SARTIM et al., 2018	Male Swiss mice	7-8 weeks	Natural	10 nmol/0.2 ul/side	intra-dHPC	FST	Antidepressant effect
				30 and 60 nmol/0.2 ul/side	intra-dHPC	FST	No effect
				10 mg/kg	i.p.	FST	Antidepressant effect
SALES et al., 2018b	Male Swiss mice	8 weeks	Natural	10 mg/kg	i.p.	FST	Rapid antidepressant effect
				10 mg/kg	i.p.	FST	Sustained antidepressant effect
				7 and 30 mg/kg	i.p.	FST	No effect
				300 nmol/ul	i.c.v.	FST	Antidepressant effect
				50 and 150 nmol/ul	i.c.v.	FST	No effect
	Male Wistar rats	n.s.	Natural	30 mg/kg	i.p.	LH	Rapid antidepressant effect
				10 mg/kg	i.p.	LH	No effect

	Male FSL and FRL rats	n.s.		10 and 30 mg/Kg	i.p.	FST	Rapid antidepressant effect
SALES et al., 2018a	Male Swiss mice	8 weeks	Natural	10 mg/kg	i.p.	FST	Antidepressant effect
				3 and 7 mg/kg	i.p.	FST	No effect
DE MORAIS et al., 2018	Male Wistar rats	n.s.	n.s.	30 mg/kg (Acute)	i.p.	FST	Antidepressant effect
	Male Wistar rats (diabetic)	n.s.		0.3, 3, 10, 30 and 60 mg/Kg (Acute)	i.p.	FST	No effect
				30 mg/Kg (Subchronic; 3 injections 24,5 and 1h before FST)	i.p.	FST	Antidepressant effect
				0.3, 3, 10 and 60 mg/Kg (Subchronic; 3 injections 24,5 and 1h before FST)	i.p.	FST	No effect
SHBIRO et al.,	Male and	70 days	n.s.	30 mg/kg	Oral (food	FST	Antidepressant effect

2019	Female Wistar Kyoto rats				pellet)	SPT	Prohedonic effect
	Male FSL rats			30 mg/kg	Oral (food pellet)	FST	Antidepressant effect
	Female FSL rats			30 mg/kg	Oral (food pellet)	FST	No effect
XU et al., 2019	Male ICR mice (SPF; Animals submitted to CMS 4 weeks)	6 weeks	Natural	10 mg/kg (1 inj./week - 4 weeks)	i.v.	FST	Antidepressant effect
				100 mg/kg (1 inj./week - 4 weeks)	oral	FST	Antidepressant effect
				10 mg/kg (1 inj./week - 4 weeks)	oral	FST	No effect
GÁLL et al., 2020	Male Wistar rats (Animals submitted to CMS 4 weeks)	n.s.	Natural	10 mg/kg	i.p.	SPT	Prohedonic effect

SALES; GUIMARÃES; JOCA, 2020	Male Swiss mice	8 weeks	Natural	3, 7 and 10 mg/kg	i.p.	FST	No effect
				10 mg/kg	i.p.	FST	Antidepressant effect
CHAVES et al., 2020	Male Wistar rats (Diabetic)	n.s.	Synthetic	3 and 10 mg/kg (Repeated - 14 days)	i.p.	FST	No effect
				30 mg/kg (Repeated - 14 days)	i.p.	FST	Antidepressant effect

Abbreviations: EPM- Elevated plus maze; FRL rats - Flinders Resistant Line; FSL rats - Flinders Sensitive Line; FST- Forced swim test; HPC - Hippocampus; IBA1 – Ionized calcium binding adaptor molecule 1; i.c.v - intracerebroventrcular; i.p. - intraperiatoneal; intra-dHPC - Intra-dorsal hippocampus; intra-IL mPFC- infralimbic medial prefrontal cortex: intra-PL mPFC- prelimbic medial prefrontal cortex; LH - Learned helplessness; n.s. - not specified; NSF - Novelty supressed feeding; OFT - Open field test; PFC - Prefronal cortex; SPF – Specific-pathogen-free; SPT - Sucrose preference test; TST - Tail suspension test; * Fluorinate cannabidiol.

AIMS

2 AIMS

As mentioned, several lines of evidence indicate that glutamatergic neurotransmission, neurotrophins are implicated in the etiology of MDD and antidepressant responsiveness. Ketamine and CBD seem to display pharmacological and therapeutic effects that differentiate them from SSRI and SNRI antidepressants.

Despite many efforts, no consensus on the mechanism responsible for ketamine's and CBD's antidepressant activity has been reached. As mentioned, the majority of the studies have concentrated on modifications of glutamatergic neurotransmission leading to neuroplastic changes.

To gain novel insights into the therapeutic potential in the molecular events, this project aimed to i) further characterize CBD effects in rats and mice, both male and female; and ii) in depth investigate the molecular mechanism involved in the CBD fast antidepressant action.

Emphasis was put on effects on behavior associated with MDD as well as relevant gene expression. Studies were conducted in Flinders Sensitive Line (FSL) rats and their control strain Finders Resistant Line (FRL) rats, which constitute a genetic model of depression (discussed above).

Specifically, the following questions were asked:

- **Study 1A:** To investigate CBD antidepressant effects in males and females Swiss and C57BL/6 mice.
- **Study 1B:** To investigate CBD antidepressant-like effect in males and females animals from a selective breeding model of depression, the Flinders Sensitive Line rats, at different time points (1 and 2 hours) exposed to FST.
- **Study 2:** To investigate whether the antidepressant-like effect induced by CBD and KET both would be associated with changes in gene expression patterns related to glutamatergic neurotransmission, neurotrophic signaling and synaptic proteins, in brain regions involved with depression neurobiology (Prefrontal cortex, dorsal, and ventral hippocampus).

STUDY 1

3 STUDY 1

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ABBREVIATIONS LIST

Δ9-THC	delta-9-tetrahydrocannabinol
ANOVA	analysis of variance
CBD	cannabidiol
Cmax	Maximum drug concentration
CUMS	chronic umpredictable mild stress
EA	Enclosed arm
ECT	electroconculsive therapy
EPM	elevated plus maze
FRL	Flinders resistant line rats
FSL	Flinders sensitive line rats
FST	forced swim test
i.p.	intraperitoneal
IMIP	imipramine
LH	learned helplessness
MDD	major depressive disorder
OA	open arm
OFT	open field test
SEM	standard error of the mean
SPT	sucrose preference test
TST	tail suspension test
VEH	vehicle
WKY	Wistar-kyoto rats

ABSTRACT

Introduction: Major depressive disorder (MDD) is a chronic and severe psychiatric disorder, which is more prevalent in women. Despite that, drug effects in females have been poorly explored in the preclinical studies. The currently available antidepressants have a latency to produce a therapeutic response and is not effective in many patients. The development of novel drugs that address these limitations is critical to improving public health. Cannabidiol (CBD) is a compound isolated from the plant *Cannabis sativa* L., which produces an antidepressant-like ad anxiolytic-like effect in male rodents. However, only a few studies have investigated CBD effect in females, and it is uncertain the efficacy in this gender. **Aims:** The present study aimed to i) investigate the influence of strain and gender of mice in CBD antidepressant-like effects; ii) investigate CBD effects in male and female FSL rats, tested at different time points. **Methods:** Adult male and female Swiss and C57BL/6 mice and adult male and female Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats were used. Mice received the systemic injection with CBD (3, 10, and 30 mg/kg, i.p.), imipramine (IMIP; 20 mg/kg, i.p.) or vehicle 30 minutes before the elevated plus maze (EPM) and tail suspension test (TST). FSL rats were treated with CBD (10, 30, and 60 mg/kg, i.p.), S-ketamine (15 mg/kg, i.p.) or vehicle, 1 or 2 hours before the open field test (OFT) and forced swim test (FST). An independent experiment was conducted with female FSL rats that received S-ketamine (10, 15, and 20 mg/kg, i.p.) or vehicle 1h before OFT and FST to select ketamine effective dose. **Results:** CBD produced an antidepressant-like effect in male, but not in female Swiss mice in the TST. Furthermore, CBD did not induce any significant effect in C57BL/6 mice, both males and females. Surprisingly, in FSL rats, CBD (30 mg/kg) induced a depressive-like effect in females 1 hour after the treatment, but an antidepressant-like effect after 2 hours. In males, CBD (30 mg/kg) produced an antidepressant-like effect 1 hour after the injection; no effect could be observed following 2 hours. Ketamine induced a significant antidepressant-like effect in female FSL rats submitted to FST 1 hour after the injection (15 and 20 mg/kg). **Conclusion:** Based on the present findings, we conclude that CBD effects can be influenced by species, strain, gender, and time of administration. In addition, we confirm that ketamine has an antidepressant-like effect in female FSL rats.

Keywords: Cannabidiol; S-ketamine; gender; strain; depression; mice; Flinders Sensitive Line rats; tail suspension test; forced swim test.

RESUMO

Introdução: Transtorno depressivo maior (TDM) é um transtorno psiquiátrico crônico e severo, o qual é mais prevalente m mulheres. Apesar dos efeitos de drogas serem poucos explorados em fêmeas em estudos pré-clínicos. Os antidepressivos disponíveis atualmente têm latência para produzir uma resposta terapêutica e não são eficazes em muitos pacientes. O desenvolvimento de novos medicamentos que atendam a essas limitações é fundamental para melhorar a saúde pública. O canabidiol (CBD) é um composto isolado da planta *Cannabis sativa L.*, que produz um efeito do tipo antidepressivo e ansiolítico em roedores machos. No entanto, somente poucos estudos tem investigado o efeito do CBD em fêmeas, e é incerto a eficácia nesse sexo. **Objetivos:** O presente estudo teve como objetivo i) investigar a influência da linhagem e do sexo de camundongos nos efeitos semelhantes aos antidepressivos do CBD; ii) investigar os efeitos do CBD em ratos e ratas FSL, em diferentes momentos. **Métodos:** Camundongos Suíço e C57BL/6 adultos machos e fêmeas, e ratos e ratas Flinders Sensitive Line (FSL) e Flinders Resistant Line (FRL) adultos. Os camundongos receberam a injeção sistêmica com CBD (3, 10 e 30 mg/kg, i.p.), imipramina (IMIP; 20 mg/kg, i.p.) ou veículo 30 minutos antes do labirinto em cruz elevado (EPM) e teste de suspensão da cauda (TST). Os ratos e rats FSL foram tratados com CBD (10, 30 e 60 mg/kg, i.p.), S-ketamina (15 mg/kg, i.p.) ou veículo, 1 ou 2 horas antes do teste de campo aberto (OFT) e teste de natação forçada (FST). Um experimento independente foi conduzido com ratas FSL que receberam S-ketamina (10, 15 e 20 mg/kg, i.p.) ou veículo 1h antes de OFT e FST para selecionar a dose efetiva da ketamina. **Resultados:** O CBD produziu um efeito tipo-antidepressivo em camundongos suíços machos no TST, mas não em fêmeas. Além disso, o CBD não induziu nenhum efeito significativo em camundongos C57BL/6 machos e fêmeas. Surpreendentemente, em ratos FSL, o CBD (30 mg/kg) induziu um efeito do tipo-depressivo em ratas FSL 1 hora após o tratamento, mas um efeito do tipo antidepressivo após 2 horas. Em machos, o CBD (30 mg/kg) produziu um efeito semelhante ao antidepressivo 1 hora após a injeção; nenhum efeito pode ser observado após 2 horas. A ketamina induziu um efeito antidepressivo significativo em ratas FSL submetidas ao FST 1 hora após a injeção (15 e 20 mg/kg). **Conclusão:** Com base nos presentes achados, concluímos que os efeitos do CBD podem ser influenciados pela espécie, linhagem, sexo e tempo de administração. Além disso, confirmamos que a ketamina tem um efeito tipo-antidepressivo em ratas FSL.

Palavras-chaves: Canabidiol; S-ketamina; gênero; linhagem; depressão; camundongo; ratos Flinders Sensitive Line; teste de suspensão pela cauda; teste da natação forçada.

RESUMÉ

Indledning: Depression er en kronisk og alvorlig psykiatrisk lidelse, som er mest udbredt hos kvinder, men desværre er effekter af lægemidler dårligt undersøgt i hunner i prækliniske studier. De klinisk tilgængelige antidepressiva har et forsinket indtrædende terapeutisk respons, og er ikke effektive hos mange patienter. Udviklingen af nye lægemidler, der imødekommer disse begrænsninger, er kritisk for at forbedre folkesundheden. Cannabidiol (CBD) er en forbindelse isoleret fra planten *Cannabis sativa* L., der producerer en antidepressiv-lignende og angstdæmpende virkning i gnavere. Desværre har kun få studier undersøgt effekten af CBD hos hunner, og det er usikkert om effektiviteten modsvare effekten i hanner. **Formål:** Den nuværende undersøgelse sigter mod at i) undersøge indflydelsen af stamme og køn i mus involveret i de antidepressivligenende effekter af CBD; ii) undersøge effekter af CBD hos FSL han og hun rotter, testet på forskellige tidspunkter. **Metoder:** Voksne hanner og hunner, Swiss og C57BL / 6 mus, og voksne FSL/FRL (begge køn) blev anvendt. Musene modtog en systemisk injektion med CBD (3, 10 og 30 mg / kg, ip), imipramin (IMIP; 20 mg / kg, ip) eller vehikel 30 minutter før Elevated Plus MAze (EPM) og Tail Suspension Test (TST). FSL-rotter blev behandlet med CBD (10, 30 og 60 mg / kg, ip), S-ketamin (15 mg / kg, ip) eller vehikel 1 eller 2 timer før Open Field Test (OFT) og Forced Swim Test (FST). Et uafhængigt eksperiment blev udført med FSL hunrotter, der modtog S-ketamin (10, 15 og 20 mg / kg, i.p.) eller vehikel 1 time før OFT og FST for derigennem at vælge den effektive ketamindosis. **Resultater:** CBD producerede en antidepressiv-lignende virkning hos hanner, men ikke hos hun Swiss mus i TST. Endvidere inducerede CBD ikke nogen signifikant effekt i C57BL / 6-mus, både hos hanner og hunner. Overraskende nok inducerede CBD (30 mg / kg) i FSL-rotter en depressiv-lignende virkning hos hunner 1 time efter behandlingen, men en antidepressiv-lignende virkning efter 2 timer. Hos hanner producerede CBD (30 mg / kg) en antidepressiv-lignende virkning 1 time efter injektionen; ingen virkning kunne observeres efter 2 timer. Ketamin inducerede en signifikant antidepressiv-lignende virkning hos FSL hunrotter, der blev underkastet FST 1 time efter injektionen (15 og 20 mg / kg). **Konklusion:** Baseret på de nuværende resultater konkluderer vi, at CBD-effekter kan påvirkes af art, stamme, køn og administrationstidspunkt. Derudover bekræfter vi, at ketamin har en antidepressiv-lignende virkning hos FSL-hunrotter.

Keywords: Cannabidiol; S-ketamin; køn; stammer; depression; mus; Flinders Sensitive Line rotter; tail suspension test; forced swim test.

3.1 INTRODUCTION

Major depressive disorder (MDD) is a chronic and severe disabling psychiatric disorder (APA, 2013), which affects twice more women than men (WHO, 2017b). Despite that, the influence of gender differences has been neglected in several studies that have investigated new drugs and the neuropathology of MDD (BEERY; ZUCKER, 2011; WILL et al., 2017), producing results that are biased towards males. Therefore, it is urgent to include the females in preclinical studies investigating new drugs to prevent future drug-related problems as well as better understanding of MDD neurobiology.

Cannabidiol (CBD) is a compound isolated from the plant *Cannabis sativa L.* (GAONI; MECHOULAM, 1964; MECHOULAM; SHVO, 1963) that has been shown promising effects an antidepressant drug (SALES et al., 2018a, 2018b; SARTIM et al., 2018; SILOTE et al., 2019; ZANELATI et al., 2010). Studies evidenced, recently, that CBD produces an antidepressant-like effect observed in several paradigms, including the FST (EL-ALFY et al., 2010; RÉUS et al., 2011; SALES et al., 2018a, 2018b; ZANELATI et al., 2010), the TST (SCHIAVON et al., 2016a), the learned helplessness (LH) (SALES et al., 2018b), the olfactory bulbectomy (OBX) (LINGE et al., 2016), and the chronic unpredictable mild stress (CUMS) (XU et al., 2019). Moreover, CBD has proven to be effective in two genetic animal models of depression, the Wistar-Kyoto (WKY) (SHBIRO et al., 2019; SHOVAL et al., 2016) and the FSL rats (SALES et al., 2018b; SHBIRO et al., 2019), supporting that it has antidepressant properties. Besides, interestingly, CBD produced a rapid and sustained antidepressant-like effect in rodents, similar to ketamine (SALES et al., 2018b). However, it is worth to note that the studies reported above investigated CBD effects exclusively in male rodents, and further investigations in female rodents are needed.

Shbiro and colleagues (SHBIRO et al., 2019) investigated, for the first time, CBD effects in both females and males of WKY and FSL rats submitted to FST and sucrose preference test (SPT). The findings revealed that CBD administration increased saccharin preference and decreased the immobility time on FST in both gender of WKY rats but produced an antidepressant-like effect only in male FSL in the FST, not in female FSL rats (SHBIRO et al., 2019). However, an important limitation of that study is that only a single dose of CBD (30mg/kg) was investigated. Besides, it is known that CBD presents a U-shape dose-response curve similar to other cannabinoids (BAMBICO et al., 2007; KIRKEDAL et al., 2017; SALES et al., 2018b; ZANELATI et al., 2010), wherein the effective dose could switch according to the gender. Therefore, it is of significant interest to

investigate whether CBD antidepressant effect in male rats could also be observed in female FSL rats when tested in different doses or at different time points.

Several studies have shown that gender can influence the baseline behavioural response and treatment effect in the FST. For example, female Wistar rats showed more active behaviour in the pretest session of the FST than the corresponding males. However, during the test session, female rats showed greater immobility, while males exhibit more active responses (DROSSOPOULOU et al., 2004). Besides, several studies revealed similar findings in a different strain of rodents, for example, WKY rats (DALLA et al., 2008; PARÉ; REDEI, 1993) and in C57BL/6J, A/J, and NMRI mice (LIU; GERSHENFELD, 2001). Moreover, gender can affect the response to a drug or change the effective dose range in FST and TST (CONSOLI et al., 2005; DAVID et al., 2003; KOKRAS; DALLA, 2017; LIU; GERSHENFELD, 2001; RIPOLL et al., 2003; SIMPSON et al., 2012). Notably, evidence indicates that gender might influence the neurochemical profile in brain areas related to depression (DROSSOPOULOU et al., 2004; KOKRAS et al., 2018). Thus, the present findings highlight the importance of investigating drugs effects in both, male and females, in preclinical studies.

Therefore, in the present study, we aimed to investigate CBD effects in mice and rats, both males and females. For this purpose, we selected the two most commonly employed mice strains in behavioural neuroscience research, an outbred and inbred strains, Swiss and C57BL/6 mice, respectively, from both genders. It is recognized that both strains have been widely used to study depression (DAVID et al., 2003; LIU; GERSHENFELD, 2001, 2003; RIPOLL et al., 2003; VÕIKAR et al., 2001) and, C57BL/6 mice are strain commonly used for behavioral experiments in transgenic and knockout research (BROOKS; PASK; JONES, 2005; LIU; GERSHENFELD, 2001, 2003; VÕIKAR et al., 2001). Interestingly, previous studies show significant differences between these mice's strain in the baseline immobility response in TST (DAVID et al., 2003; LIU; GERSHENFELD, 2001, 2003; MARCHETTE et al., 2018; RENARD et al., 2003; RIPOLL et al., 2003), drug response, biochemical parameters (MARCHETTE et al., 2018) and neurochemical profile (DAVID et al., 2003). Thus, testing the novel compound in different animal strains and paradigms may reinforce the behavioral finding and favor a better selection of potential compounds for clinical investigation.

3.2 AIMS

3.2.1 General aim

The current study aimed to investigate whether species and the strain, as well as the gender of the rodents could influence the behavioural effects of CBD in a test predictive of antidepressant effect.

3.2.2 Specific aims

To investigate CBD antidepressant effects in males and females Swiss and C57BL/6 mice **(Study 1A)**.

To investigate CBD antidepressant-like effect in males and females animals from a selective breeding model of depression, the Flinders Sensitive Line rats, at different time points (1 and 2 hours) exposed to FST (**Study 1B**).

3.3 STUDY 1A

3.3.1 Methods

3.3.1.1 Animals

Adult male and female Swiss and C57BL/6 mice (8 weeks old) were purchased from the breeding facility of the University of São Paulo (USP; Ribeirão Preto, São Paulo; Brazil). The mice were housed in 10 animals per polypropylene cages (200 x 120 x 300 mm). All animals were housed in a temperature-controlled room (23 ± 2 °C) with a 12/12-h light-dark cycle (lights on 6:00 am/lights off 6:00 pm.) with free access to tap water and standard food (commercial rodent chow, Nuvilab – Quimtia – Paraná, Brazil). The bedding material for mice was composed of wood shavings without enrichment material inside the cages. Female and male animals were housed in different rooms to minimize the influence of being exposed to the opposite sex.

The experimental protocols were approved by the local Ethics Committee for the Use of Animals (CEUA, Protocol number: 16.1.1142.60.46, copy attachment). The experimental procedures were conducted following the National Council for Control of Animal Experimentation (CONCEA, Brazil). All behavioural experiments were conducted between 9:00 am and 1:00 pm.

3.3.1.2 Drugs

The following drugs were administered intraperitoneally (i.p.) and were freshly prepared before the experiment:

- Synthetic Cannabidiol (CBD, Prati-Donaduzzi (Brazil)) is stored at 4°C and protected from light. CBD was diluted with sterile saline and 2% polysorbate 80 (Tween® 80; Sigma-Aldrich, USA) and administered at the doses 3, 10 and 30 mg/10mL/kg (SALES et al., 2018a, 2018b; ZANELATI et al., 2010).
- Imipramine hydrochloride (IMIP; Abcam, USA), stored at 4°C, diluted in sterile saline, administered at dose 20 mg/10 mL/kg (MONLEON et al., 1995).

The vehicle (VEH) group received CBD vehicle injections. The animals received the treatment randomly by writing treatment of pieces of paper, folding them, mixing, and then drawing one by one for each animal (BESPALOV; MICHEL; STECKLER, 2020).

3.3.1.3 Behavioural test

3.3.1.3.1 Tail suspension test (TST)

The tail suspension test (TST) is a useful tool used to screen drugs with potential antidepressant effects in mice (CRYAN; MOMBEREAU; VASSOUT, 2005). The animal is suspended 60 cm above the floor with an adhesive tape placed 1 cm at the tip of the tail on the experimentation table for 6 minutes (Figure 1). The test was recorded with a video camera positioned in front of the experimentation table. An experimenter blind to drug treatment scored the immobility time (s) during the test (CRYAN; MOMBEREAU; VASSOUT, 2005; SALES et al., 2011). The immobility was defined when mice hung passively and completely immobile. A plastic cylinder tubing (40 mm length; 16mm diameter) was placed around the animal tail to prevent tail climbing behaviour (CAN et al., 2011).

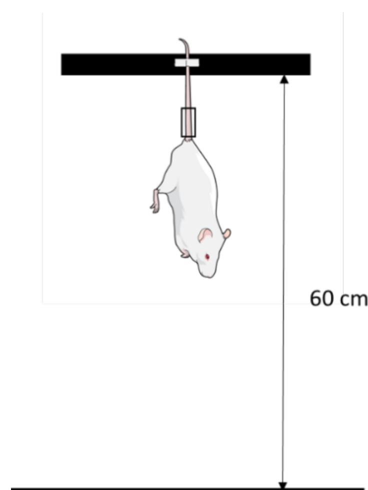


Figure 1. Tail suspension test. Schematic figure with tail suspension test.

3.3.1.3.2 Elevated plus maze (EPM)

Anxiety is a common comorbidity presented in a depressed patient (GASPERSZ et al., 2018). To investigate if the gender would also influence CBD effects in anxiety, animals were exposed to the EPM, before the TST. The test is an essential tool to screen potential compounds with anxiety-related effects (CAROBREZ; BERTOGLIO, 2005; PELLOW et al., 1985). The apparatus is built based on rodents' natural behaviour, which avoids the open and elevated place. The equipment is a plus-shaped maze made of wood and consisted of 2 equals enclosed arms (EA; 30 cm x 6 cm; surrounded by walls 15 cm high) disposed perpendicularly to a 2 equals open arms (OA; 30 cm x 6 cm; Figure 2). In the test, the animals were placed in the center of the equipment facing one of the EA and allowed to explore the maze freely for 5 minutes. A video camera recorded the test session from the top of the equipment. Then, an experimenter blind to drug treatment analysed the anxiety-like behaviours: OA entries, time, and percentage of time spent in the OA. EA entries were assessed as an index of exploratory behaviour. The treatment with anxiolytic drugs increases the OA exploration in the EPM (CAROBREZ; BERTOGLIO, 2005).

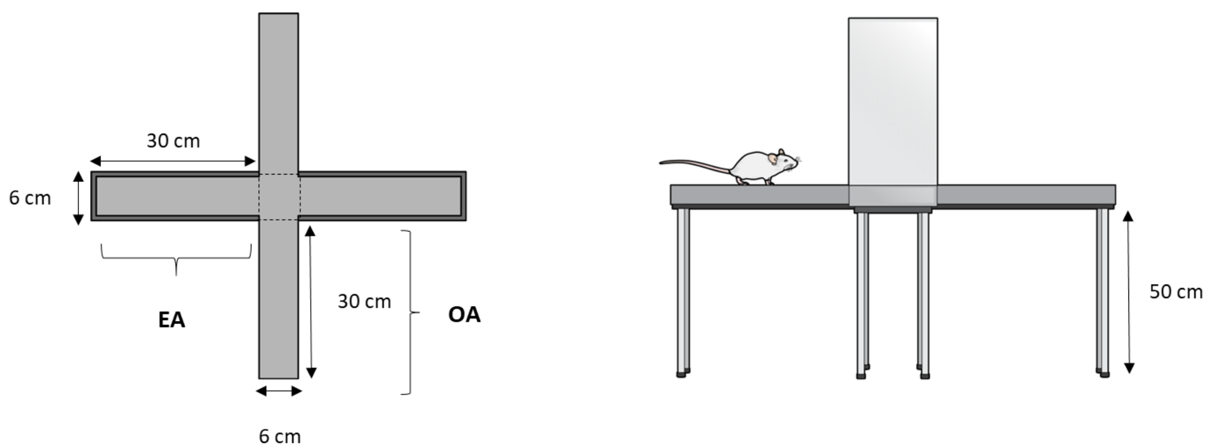


Figure 2. Elevated plus maze. Schematic figure with elevated plus maze test dimensions (EA and OA: 30 cm x 6 cm) elevated 50 cm from the floor.

3.3.1.4 Experimental design

3.3.1.4.1 Effect produced by CBD in male and female Swiss mice exposed to TST

Thirty minutes after the habituation in the experimental room, the mice received the intraperitoneal injection with VEH, IMIP, or CBD (3, 10, and 30 mg/kg). Thirty minutes later, the animals were exposed to EPM (5 min) and TST (6 min).

To avoid interference in the behavioural response in the tests, the experiment carried out with the females was performed independently and on different days of males. The female reproductive cycle status was not considered (PRENDERGAST; ONISHI; ZUCKER, 2014). The experimental design can be seen in Figure 3.

3.3.1.4.2 The effect produced by CBD in male and female C57BL/6 mice exposed to TST

To assess the influence of the gender and strain influence on CBD effect in C57BL/6 mice, the same experimental procedure, and drug treatment as described in section 3.4.1. was used in males and females C57BL/6 mice. The experiments with male and female mice were performed independently and on different days. The experimental design is provided in Figure 3.

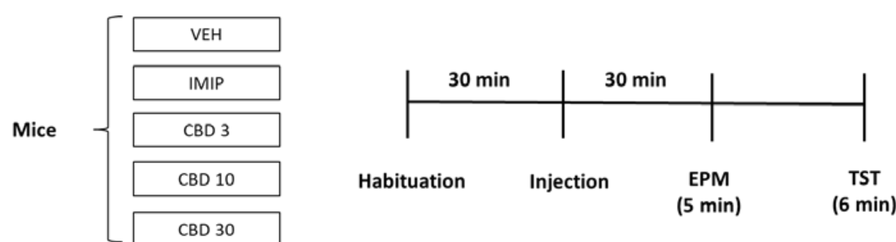


Figure 3. Effect of CBD in male and female Swiss and C57BL/6 mice. After 30 min habituation period, mice were treated with VEH, IMIP, and CBD. Thirty minutes later the animals were exposed to EPM and TST. CBD, Cannabidiol; EPM, elevated plus maze; IMIP, imipramine; VEH, vehicle; TST, tail suspension test.

3.3.1.5 Data analysis and statistical methods

The analysis of immobility time (s), OA percentage of spent time and OA spent time, OA and EA entries (EPM) data were performed using one-way analysis of variance (ANOVA) followed by Fisher's LSD post-hoc test to compare differences between mice treated with VEH, IMIP and

CBD. When the variances between the groups were not homogenous the following statistical analysis was applied, Kruskal-Wallis followed by Dunn's postdoc test (to compare mice treated with VEH and IMIP and CBD). We calculated and reported the effect size of TST using G*Power (FAUL; ERDFELDER; BUCHNER, 2007). Results in the graphs are expressed as the mean ± standard error of the mean (SEM). The p-value to indicate a significant difference between groups was 5% ($p < 0.05$). Statistical analyses and the graphs were created using GraphPad Prism 8.4.2. version for Windows (GraphPad Software Inc., San Diego, CA, USA).

3.3.2 Results

3.3.2.1 Swiss Mice

3.3.2.1.1 CBD effects in male Swiss mice exposed to TST

Male Swiss mice treated with IMIP and all doses of the CBD showed decreased immobility time in TST (One-way ANOVA: $F(4, 25)= 8,657$; $p=0.0002$; Fisher's LSD test: IMIP, $p<0.0001$; CBD 3 mg/kg, $p= 0.005$; CBD 10 mg/kg, $p=0.0011$; CBD 30 mg/kg, $p=0.0057$; Cohen test: VEH x IMIP: $d= 3.983$; VEH x CBD groups: $f=0.70$; Figure 4A), suggesting an antidepressant-like effect. None of the drug treatment modified the parameters analysed on EPM (OA entries: Kruskal-Wallis test: $H(5)= 5.042$; $p=0.2830$; OA time: Kruskal-Wallis test: $H(5)= 4.792$; $p=0.3094$; % OA time: One-way ANOVA: $F(4, 35)=0.852$; $p=0.5019$; Entrance EA: One-way ANOVA: $F(4, 35)=1.379$; $p=0.2611$; Figure 4B-E), which suggest that the CBD did not affect the anxiety-like behaviour nor changed the locomotion.

The animals treated with IMIP presented high variability in the EA entries (Figure 4E). A correlation analysis was performed to check whether the locomotor changes in the EPM test affects the immobility time in TST. As a result, there is a significant positive correlation between immobility time and EA entries in the group treated with IMIP (Correlation: $r=0.8143$; $p=0.0258$; Fig. 4F), but altered locomotion did not affect the response displayed in the TST.

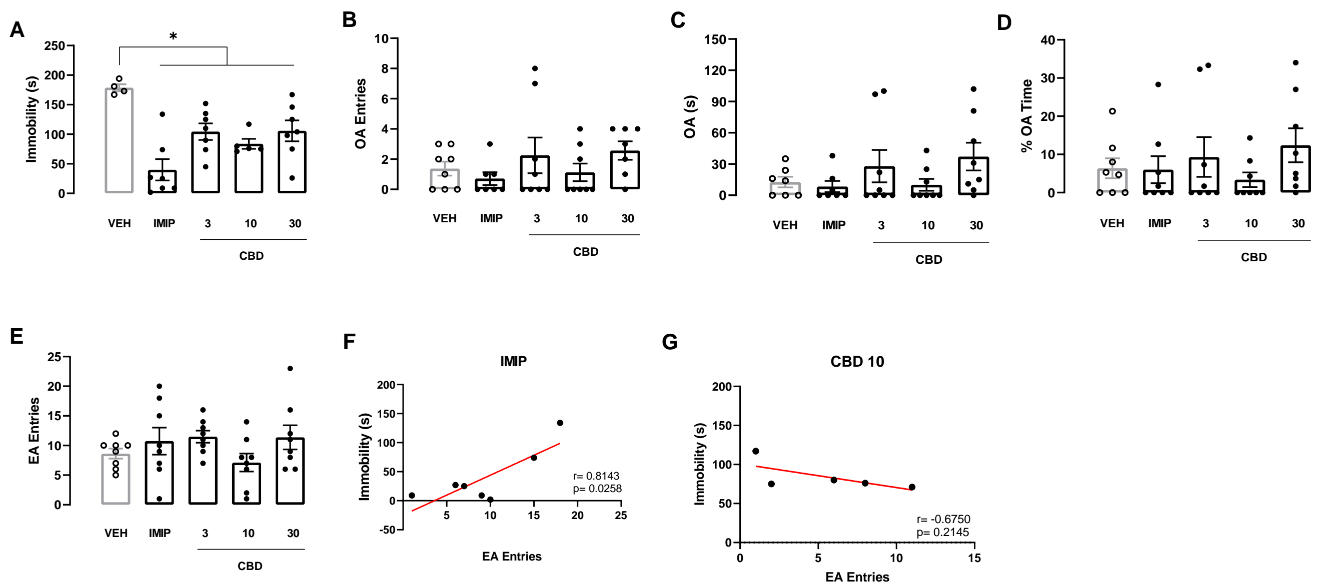


Figure 4. Effect of CBD in male Swiss mice submitted to TST and EPM. Effect of CBD in male Swiss mice administered 30 min before the exposure to TST (A), EPM (B, C, D, E), and correlation analysis between immobility and EA entries (F, G). Bars represent the immobility time (s) in TST, or the OA spent time and percentage spent time, OA, and EA entries in the EPM. Values are mean ± SEM; Asterisk represents significant treatment difference from control (**p*< 0.05; One-way ANOVA followed by Fisher's LSD posthoc test), *n*= 4-8 animals/group. EA: enclosed arm; OA. Open arm.

3.3.2.1.2 CBD effects in female Swiss mice exposed to TST

For female Swiss mice, none of the drug treatments modified the parameters analysed on TST (Kruskal-Wallis test: H(5)= 6.153; *p*=0.188; Figure 5A) and EPM (OA entries: One-way ANOVA: F(4, 33)= 1.064; *p*=0.3897; OA time: One-way ANOVA: F(4, 33)= 1.646; *p*=0.1862; % OA time: One-way ANOVA: F(4, 33)=1.342; *p*=0.2752; Figure 5B-D). However, IMIP and CBD 10 mg/kg displayed a reduction on the EA entries (One-way ANOVA: F(4, 33)=8.305; *p*<0.0001; Fisher's LSD test: IMIP, p=0.0015; CBD 10 mg/kg, p=0.0090; Figure 5E), impairing locomotor activity.

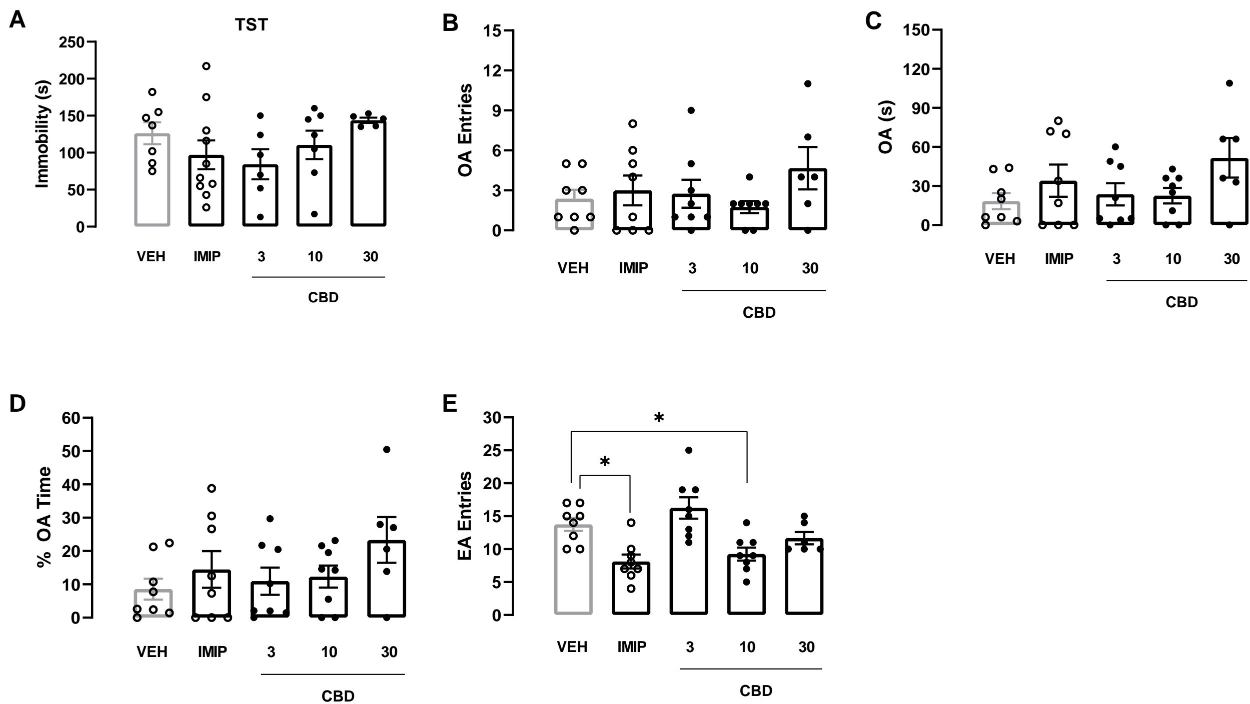


Figure 5. Effect of CBD in female Swiss mice submitted to TST and EPM. Effect of cannabidiol (CBD) in female Swiss mice administered 30 min before the exposure to TST (A) and EPM (B, C, D, E). Bars represent the immobility time (s) in TST, or the OA spent time and percentage spent time, OA, and EA entries in the EPM. Values are mean ± SEM; Asterisk represents significant treatment difference from control ($^{*}p< 0.05$; One-way ANOVA followed by Fisher's LSD posthoc test), n= 5-10 animals/group. EA: enclosed arm; OA. Open arm.

3.3.2.2 C57BL/6 Mice

3.3.2.2.1 CBD effects in male C57BL/6 mice exposed to TST

In male C57BL/6 none of the drug treatments affected the parameters analysed on TST (Kruskal-Wallis test: H(5)= 10.53; p=0.0323; Dunn's test: p> 0.05; Figure 6A) and on EPM (EA entries: Kruskal-Wallis test: H(5)= 2.478; p=0.6486; OA entries: One-way ANOVA: F(4, 35)= 0.9833; p=0.4293; OA time: One-way ANOVA: F(4, 35)= 0.4509; p=0.771; % OA time: One-way ANOVA: F(4, 35)=0.4508; p=0.7711; Figure 6B-E).

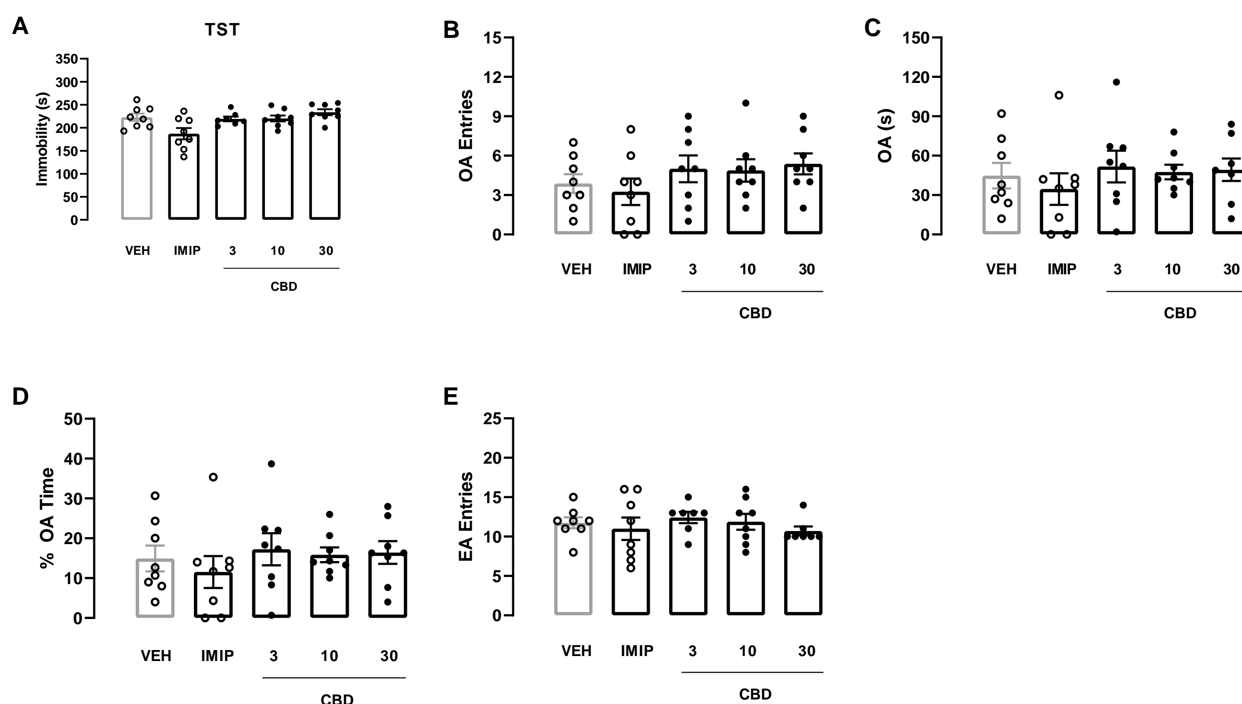


Figure 6. Effect of CBD in male C57BL/6 mice submitted to TST and EPM. Effect of cannabidiol (CBD) in male C57BL/6 mice administered 30 min before the exposure to TST (A) and EPM (B, C, D, E). Bars represent the immobility time (s) in TST, or the OA spent time and percentage spent time, OA, and EA entries in the EPM. Values are mean ± SEM; *n*= 7-8 animals/group. EA: enclosed arm; OA. Open arm.

3.3.2.2.2 CBD effects in female C57BL/6 mice exposed to TST

For female C57BL/6, none of the drug treatments affected the parameters analysed on TST (One-way ANOVA: $F(4, 51)= 0.4893$; $p=0.7435$; Figure 7A) and EPM (EA entries: Kruskal-Wallis test: $H(5)= 1.986$; $p=0.7384$; OA entries: Kruskal-Wallis test: $H(5)= 3.488$; $p=0.4797$; OA time: One-way ANOVA: $F(4, 47)= 0.3677$; $p=0.8304$; % OA time: One-way ANOVA: $F(4, 47)= 0.3677$; $p=0.8304$; Figure 7B-E). As a result, CBD did not affect behaviours in the TST and EPM in the female C57BL/6 mice.

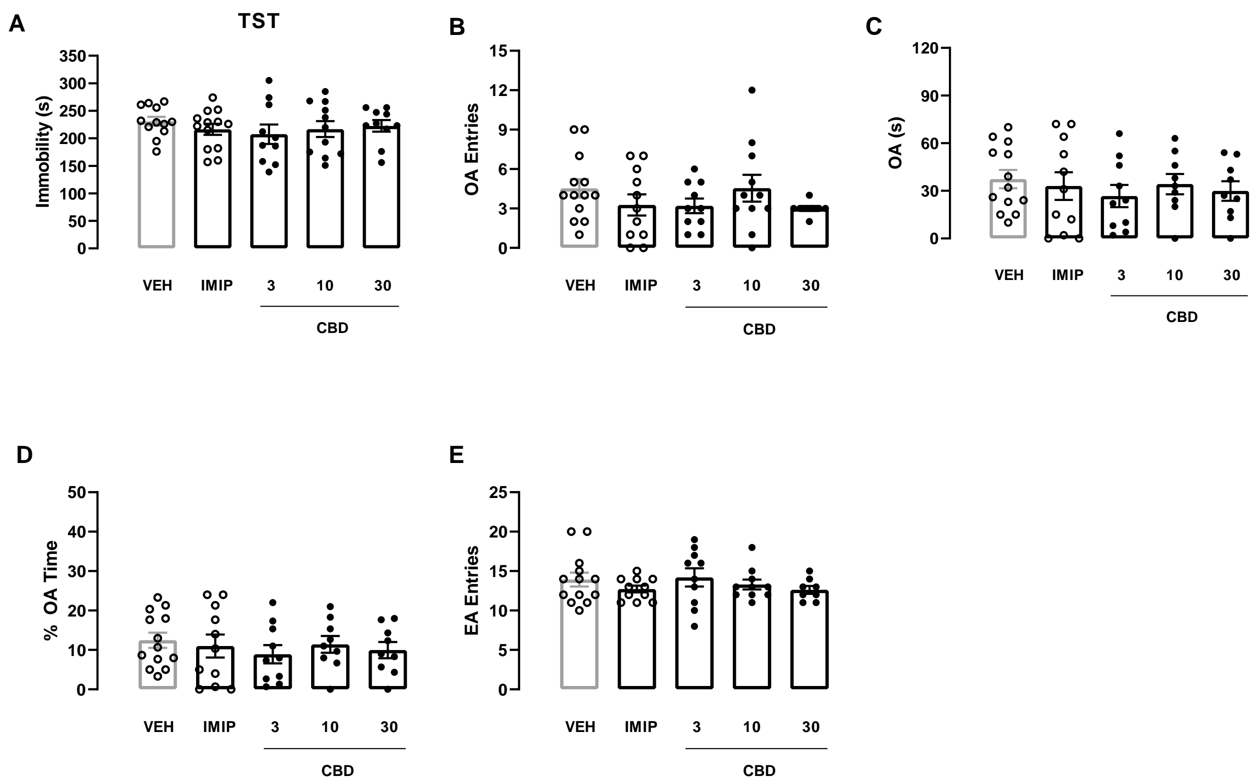


Figure 7. Effect of CBD in female C57BL/6 mice submitted to TST and EPM. Effect of cannabidiol (CBD) in female C57BL/6 mice administered 30 min before the exposition to TST (A) and EPM (B, C, D, E). Bars represent the immobility time (s) in TST, or the OA spent time and percentage spent time, OA, and EA entries in the EPM. Values are mean ± SEM; *n*= 9-13 animals/group. EA: enclosed arm; OA. Open arm.

3.3.3 Discussion

The present study investigated the influence of gender and strain of mice (Swiss or C57BL/6) on the antidepressant-like effect produced by CBD in the TST. The main findings evidenced in the present study were as follow: in male Swiss mice, CBD and IMIP decreased the immobility time in the TST, which is suggestive of an antidepressant-like effect. In contrast, CBD did not affect the parameters analysed on the EPM. On the other hand, in female Swiss mice and in C57BL/6 mice (male and female), CBD did not change the behavioural responses elicited in the TST.

Our results showed that all tested doses of CBD administered systemically produced an antidepressant-like effect in male Swiss mice submitted to TST. Previous studies found similar results in different predictive tests, such as FST (SALES et al., 2018a, 2018b; ZANELATI et al., 2010), TST (EL-ALFY et al., 2010; SCHIAVON et al., 2016a), and also in paradigms that present

better face and construct validity, like the olfactory bulbectomy (LINGE et al., 2016) and the chronic mild stress (CMS) (XU et al., 2019). Thus, our results reinforce the antidepressant effect produce by CBD in Swiss mice.

In C57BL/6 mice, both CBD and IMIP did not change the behavioural response in TST. Although the CBD antidepressant effect has been described in other mice strain, this is the first time that the effect of a single injection of CBD was investigated in C57BL/6 mice submitted to TST. Interestingly, several studies demonstrated that the strain selected affects the baseline behaviour in the TST (LIU; GERSHENFELD, 2001; RIPOLL et al., 2003; TRULLAS; JACKSON; SKOLNICK, 1989; VAN DER HEYDEN; MOLEWIJK; OLIVIER, 1987; VÕIKAR et al., 2001), the response to antidepressant drugs (LIU; GERSHENFELD, 2001; RIPOLL et al., 2003; VAN DER HEYDEN; MOLEWIJK; OLIVIER, 1987), and also interferes with the their neurochemical profile (DAVID et al., 2003). This can help explaining the contradictory results with experiments run in Swiss mice submitted to FST (SALES et al., 2018b, 2018a; SCHIAVON et al., 2016b; ZANELATI et al., 2010). However, other studies revealed that acute (50 mg/kg) and repeated treatment (30 mg/kg for 14 days) with CBD was able to produce its behavioural effect in male C57BL/6 mice in animals submitted to olfactory bulbectomy (LINGE; PAZOS; DÍAZ, 2013) and subjected to a chronic stress model (CAMPOS et al., 2013; FOGAÇA et al., 2018). Therefore, CBD effects in C57BL/6J mice might be dependent on the experimental paradigm and treatment duration (acute vs chronic).

Although the depressive disorder is more prevalent in women than in men, most preclinical studies have used only male animals to investigate potential new compounds to treat depression (BEERY; ZUCKER, 2011; WILL et al., 2017). Based on this, the present study was the first to investigate the antidepressant effect produced by CBD in female mice from different strains. In the present study, we found that CBD and IMIP did not change the immobility time in female Swiss and C57BL/6 mice submitted to TST. Earlier evidence demonstrated that CBD treatment (food pellet; dose: 30 mg/kg) produced antidepressant-like effect in the saccharine preference test and the FST in both female and male WKY rats (SHBIRO et al., 2019), whereas, it was ineffective in female FSL rats (SHBIRO et al., 2019). This is in line with previous studies demonstrating that gender is a critical factor affecting the antidepressant drug response in several paradigms in different rodent species (DAVID et al., 2001; FERNÁNDEZ-GUASTI et al., 2017; FRANCESCHELLI et al., 2015; GÓMEZ et al., 2014; SIMPSON; KELLY, 2012; WRIGHT; KABBAJ, 2018).

An earlier study demonstrated that CBD reaches the peak of maximum drug plasma concentration (Cmax) only 2 hours after the i.p. injection in mice accompanied by an anti-compulsive-like effect in the marble-burying test (DEIANA et al., 2012). The time for CBD to reach Cmax was influenced by several variables, including the route of administration chosen, the vehicle, the animal species, and gender selected (DEIANA et al., 2012). Therefore, the assessment of CBD effect at later time points could have allowed for the detection of its antidepressant properties in C57 and in females. However, this remains to be tested.

Another point to consider as an important source of discrepant results in our study is that we investigated CBD effects only in the TST whereas most of have been performed in mice to submitted to FST (SILOTE et al., 2019). Although the TST present some advantages in comparison to FST, like greater sensitivity to detect the antidepressant effect produced by the drug treatment (CRYAN; VALENTINO; LUCKI, 2005) and avoid the possible complications induced by hypothermia exposure, commonly seen in the FST (CRYAN; VALENTINO; LUCKI, 2005), it activates different neural substrates in comparison to FST (RENARD et al., 2003). This could help explaining differences in results obtained from TST and FST.

It is important to note that, in the present study, the positive control (IMIP group) did not modify the immobility in male C57BL/6 mice and in females from both strains submitted to TST. The treatment with imipramine is commonly effective in C57BL/6 and Swiss mice and is used widely as a positive control in different animal tests, including TST (BAI et al., 2001; KAWAI et al., 2019; RIPOLL et al., 2003). However, the effect in C57BL/6 mice is controversial, with evidence of no effect of the acute injection of IMIP (30 mg/kg i.p.) in the TST (LIU; GERSHENFELD, 2001, 2003).It is known that the gender might influence the effective dose for IMIP in Swiss mice and the same could be expected for C57BL/6 mice (DAVID et al., 2001). Therefore, the lack of effect of imipramine in C57BL/6 (males and females) and in female Swiss mice could be a result of the dose chosen and the time of testing (DAVID et al., 2001; RIPOLL et al., 2003). In fact, to exclude the absence of the effect in both strains of mice and genders, further investigations are necessary to conduct a dose-response curve with IMIP in both genders of Swiss and C57BL/6 mice in the TST.

Anxiety disorders are frequently observed as a comorbidity in depressed patients (KAUFMAN; CHARNEY, 2000; OTTE et al., 2016). In the present study, the EPM has been employed to evaluate the anxiety-related behaviours and locomotion, which can affect the behaviour in the TST. Our results showed that acute injection of CBD did not change the assessed

parameters in the EPM in male Swiss and C57BL/6 mice. In the same line of evidence, a single injection with CBD in male C57BL/6J mice did not change the behavioural exhibited in different paradigms, including EPM, open field test, and light-dark test (KASTEN; ZHANG; BOEHM, 2019; LONG et al., 2010). Similarly, repeated CBD treatment did not change the Swiss's anxiety-related behaviour (SCHIAVON et al., 2016b) and C57BL/6J mice subjected to EPM (KASTEN; ZHANG; BOEHM, 2019). However, a previous study demonstrated that chronic treatment with CBD (21 days) produced an anxiolytic effect in the open field test in male C57BL/6JArc mice (LONG et al., 2010). Also, CBD's acute injection had an anxiolytic-like effect in male Swiss exposed to EPM (SCHIAVON et al., 2016a). Notably, CBD effect seems to depend on the level of stress in rodents. For example, the repeated treatment with CBD reverted the effect induced by chronic unpredictable stress in male C57BL/6J mice submitted to NSF and EPM, resulting in an anxiolytic-like effect (CAMPOS et al., 2013; FOGAÇA et al., 2018). In fact, the CBD effect on male mice's anxiety-related behaviours seems to depend on the selected strain of mice; the treatment regimen adopted, previous stressful experience, and behavioural test chosen.

Furthermore, we found that a single injection of CBD did not change females' behavioural responses from both mice’s strains submitted to EPM. Corroborating with our results, a previous work evidenced that acute and repeated administration with CBD did not produce an anxiolytic effect in adolescent and adult female C57BL/6J mice (KASTEN; ZHANG; BOEHM, 2019). Besides, it is essential to highlight that no study investigated CBD anxiety-related behaviour previously in female Swiss mice. A growing body of evidence has shown that the strain of mice (Swiss and C57BL/6) influences the sensitivity to stress and anxiety-related behaviour and biochemistry parameters, such as corticosterone levels, glucocorticoid receptor expression, and glial fibrillary acid protein levels in the hippocampus and frontal cortex (MARCHETTE et al., 2018).

In conclusion, a single injection with CBD reduced the immobility time in male Swiss mice in the TST, similarly to the positive control, imipramine, without affecting the locomotor activity, suggesting an antidepressant-like effect. In contrast, CBD did not affect the parameters analysed on the EPM. Furthermore, for female Swiss and both gender of C57BL/6 mice, CBD did not modify the animals' behavioural response to TST and EPM.

3.4 STUDY 1B

3.4.1 Methods

3.4.1.1 Animals

Adult male and female Flinders Sensitive Line (FSL) and Flinders Resistant Line rats (FRL; control of genetic background) (Weighing: male: 200.1 to 405.27 g; female: 138.15 to 216 g; 8 to 10 weeks old) from breeding colonies at Translational Neuropsychiatry Unit (Aarhus University; Denmark). The rats were housed in pairs in standard cages (Cage 1291H Eurostandard Type III H, 425 × 266 × 185 mm, Techniplast, Italy). All animals were housed in a temperature-controlled room (23 ± 2 °C) with a 12/12-h light-dark cycle (lights on 6:00 am./lights off 6:00 pm.) with free access to tap water and standard chow diet (Altormin, Brogaarden, Lynge, Denmark). The bedding material for rats (Tapvei Estonia OÜ) was made of wood chips with access to tunnel shelter, nesting material, and a wooden stick. Female and male animals were allocated in different rooms to avoid interference with the behavioural results.

The experimental protocols were approved by the Danish Animal Experiments Inspectorate (Protocol number: 2016-150201-001105, copy in attachment). The experimental procedures were conducted in accordance with the European Community Council Directive 2010/63/EU. All behavioural experiments were conducted between 9:00 am and 1:00 pm.

3.4.1.2 Drugs

The following drugs were administered intraperitoneally (i.p.) and freshly prepared before the experiment:

- Synthetic Cannabidiol (CBD; THC-Pharma (Germany)) is stored at 4°C and protected from light. For rats, diluted with sterile saline and 3% polysorbate 80 (Tween® 80; Sigma-Aldrich, USA), administered at the doses 10, 30, and 60 mg/2mL/kg (SALES et al., 2018b).
- S-Ketamine hydrochloride (KET; Pfizer Ltd., Denmark) stored at 4°C, diluted in sterile saline, administered at doses 10, 15, and 20 mg/2mL/kg (LIEBENBERG; JOCA; WEGENER, 2015; SALES et al., 2018b).

The vehicle (VEH) group received CBD vehicle injections. The animals received the treatment randomly by writing treatment of pieces of paper, folding them, mixing, and then drawing one by one for each animal (BESPALOV; MICHEL; STECKLER, 2020).

3.4.1.3 Behavioral test

3.4.1.3.1 Open field test (OFT)

The open field test (OFT) was conducted as described previously to investigate unspecific alteration in the locomotor activity produced by the drug treatment (PEREIRA et al., 2019; RIBEIRO et al., 2019). Before the FST, the rats were submitted individually to an open field square (100 cm x 100 cm x 50cm) for 5 minutes. The apparatus consists of four squared arenas that allow test four rats simultaneously (Figure 8). The light was adjusted manually to reach the intensity of 40 lux inside each arena. The experiment was recorded, and the total distance travelled (meter; m) was automatically measured by the software EthoVision® XT14 (Noldus Information Technology, The Netherlands).

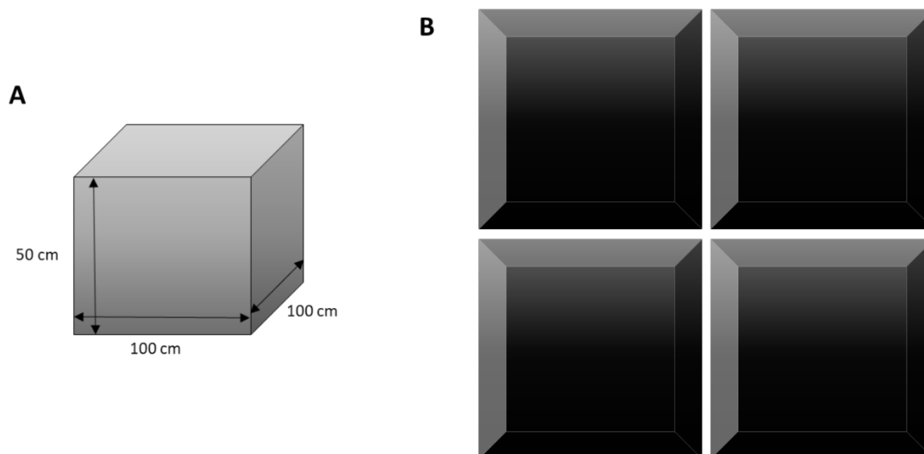


Figure 8. Open field test. A) Schematic figure with open field arena dimensions (100 cm x 100 cm x 50 cm). B) The apparatus arrangement with four arenas in a square format.

3.4.1.3.2 Forced Swim Test (FST)

The FST was performed to evaluate depressive-like behaviour as previously described (RIBEIRO et al., 2019; SALES et al., 2018b). The FSL and FRL rats were exposed to a 10 minutes test in the Perspex cylinder (height 60 cm, diameter 24 cm) filled with tap water at 24±1 °C, up to

40 cm height. The cylinders were positioned side by side with physical barriers between them that allow testing four rats in each section (Figure 9). The water was changed between each rat to avoid the interference of olfactory cues during the test. The immobility time (s) was measured during the first 5 minutes by an experimenter blind to rat strain and treatment groups. The immobility was defined as a floating posture with only minimal movements to keep the head above the water.

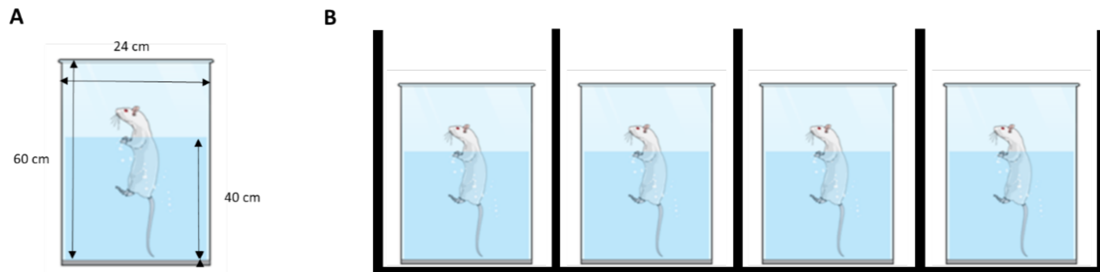


Figure 9. Forced Swim Test. A) Schematic figure of the cylinder with dimensions (60 cm x 24 cm x 40 cm water depth). B) The apparatus arrangement with four cylinders.

3.4.1.4 Experimental design

3.4.1.4.1 CBD effects in male FSL rats exposed to the OFT and FST

To evaluate whether CBD produced an antidepressant-like effect at 1 or 2 hours after the injection, the following experiment was conducted. A) One hour after the habituation in the experimental room, the male rats received the intraperitoneal (i.p.) injection with VEH or ketamine (15 mg/kg) or CBD (10, 30, and 60 mg/kg) and after 50 minutes or 1h50min, the animals were exposed to OFT (5 minutes) and, immediately after, they were submitted to FST (10 minutes). The experimental design is shown in Figure 10.

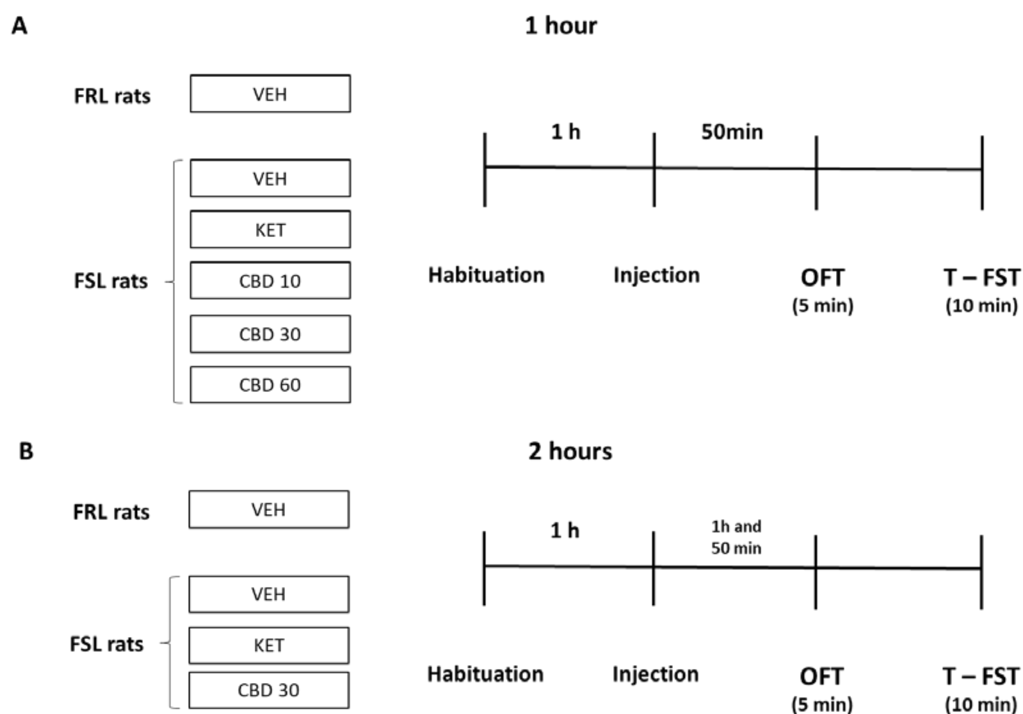


Figure 10. Effect of CBD in males and females FSL rats. After a one-hour habituation period, FRL rats were treated with vehicle, Ketamine or CBD. One hour (A) or 2 h later (A), the animals were exposed to OFT and FST. CBD, Cannabidiol; FST, Forced swimming test; OFT, open field test; Ket, S-Ketamine; VEH, vehicle.

3.4.1.4.2 Ketamine effects in female FSL rats exposed to OFT/FST

Since the effective dose for ketamine was not established in female FSL rats, we performed an initial experiment with the aim to determine the effective dose of ketamine that produced an antidepressant-like effect in female FSL rats. One hour after the habituation in the experimental room, the FRL rats were treated with an intraperitoneal injection of VEH (sterile saline), and FSL rats received the injection with VEH or ketamine (10, 15, and 20 mg/kg i.p.). Fifty minutes later, the animals were exposed to OFT (5 min) and FST (10 min). The experimental design is provided in Figure 11. The reproductive cycle status was not considered, as it in an earlier study was shown not to influence basal FST behaviour (BECKER; PRENDERGAST; LIANG, 2016; ESKELUND et al., 2016).

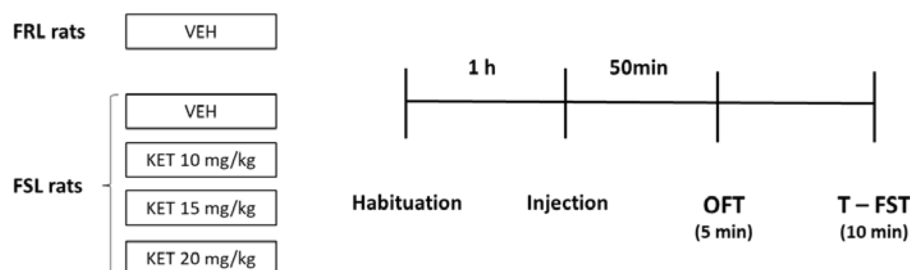


Figure 11. Dose-response curve ketamine with female FSL rats. After one-hour habituation, FRL rats were treated with a vehicle, and FSL was treated with VEH or Ketamine (10, 15, and 20 mg/kg). One hour later, the rats were exposed to OFT and FST. FST, Forced swimming test; OFT, open field test; Ket, S-Ketamine; VEH, vehicle.

3.4.1.4.3 CBD effects in female FSL rats exposed to the OFT/FST

A similar design was used for males, as described in section 5.4.1, except for ketamine dose (20 mg/kg). The experimental setup is provided previously in Figure 10.

According to a previous experiment with female mice, the test carried out with female rats was conducted independently and on different days of the experiment with the males. The reproductive cycle status was not considered (BECKER; PRENDERGAST; LIANG, 2016; ESKELUND et al., 2016).

3.4.1.4.4 Data analysis and statistical methods

The analysis of immobility time (s; FST) and total distance travelled (m; OFT) were performed using: i) Student's *t*-test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; ii) One-way analysis of variance (ANOVA) test followed by Fisher's LSD posthoc test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. When the groups' variances were not homogenous, the following statistical analysis was applied, Mann-Whitney (for comparisons between FSL and FRL vehicle-treated groups) or Kruskal-Wallis followed by Dunn's postdoc test (to compare between FSL treated with VEH, KET). We calculated and reported the effect size of data from FST using G*Power (FAUL; ERDFELDER; BUCHNER, 2007). Results in the graphs are expressed as the mean ± standard error of the mean (SEM). The *p*-value to indicate a significant difference between groups was 5% ($p < 0.05$). Statistical analyses and the graphs were created using GraphPad Prism 8.0 version for Windows (GraphPad Software Inc., San Diego, CA, USA).

3.4.2 Results

3.4.2.1 CBD effects in male FSL rats exposed to the OFT/FST

One hour after treatment, male FSL rats treated with CBD (30 mg/kg) or ketamine (15 mg/kg) displayed reduced immobility time (One-way ANOVA: $F(4,29)=3.178$; $p= 0.0279$; Fisher's LSD test: CBD 30 mg/kg, $p= 0.0256$; KET, $p= 0.0157$; Cohen d: FSL-VEH x FSL-KET, $d=1.218$; FSL-VEH x FSL-CBD 30 mg/kg, $d=1.153$; Figure 12A). FSL treated with VEH showed significantly increased immobility time in comparison to FRL rats treated with vehicle (Student's T test: $t(17)= 5.126$; $p< 0.0001$; Figure 12A). Neither rat strain ($t(17)= 0.5769$; $p= 0.5716$) nor drug treatment in FSL rats (One-way ANOVA: $F(4,29)=0.3576$; $p= 0.8366$; Figure 12B) changed the locomotor activity on the OFT.

Two hours after vehicle injection, male FSL presented increased immobility (Student's T test: $t(16)= 5.241$; $p< 0.0001$; Figure 12C) and increased locomotor activity (Student's T test: $t(16)= 2.722$; $p= 0.0151$; Figure 12D) in comparison with FRL vehicle animals. As demonstrated before, ketamine (15 mg/kg) injected 1 hour before FST reduced immobility in FSL rats (Kruskal-Wallis test: $H(3)= 14.52$; $p= 0.0007$; Dunn's: $p=0.0007$; $d= 1.749$). CBD did not change immobility in the test 2 hours later (Kruskal-Wallis test: $H(3)= 14.52$; $p= 0.0007$; Dunn's: $p>0.9999$; Figure 12C). None of treatment changes the distance travelled in the OFT in FSL rats (One-way ANOVA: $F(2, 26)= 1.382$; $p= 0.2690$; Figure 12D).

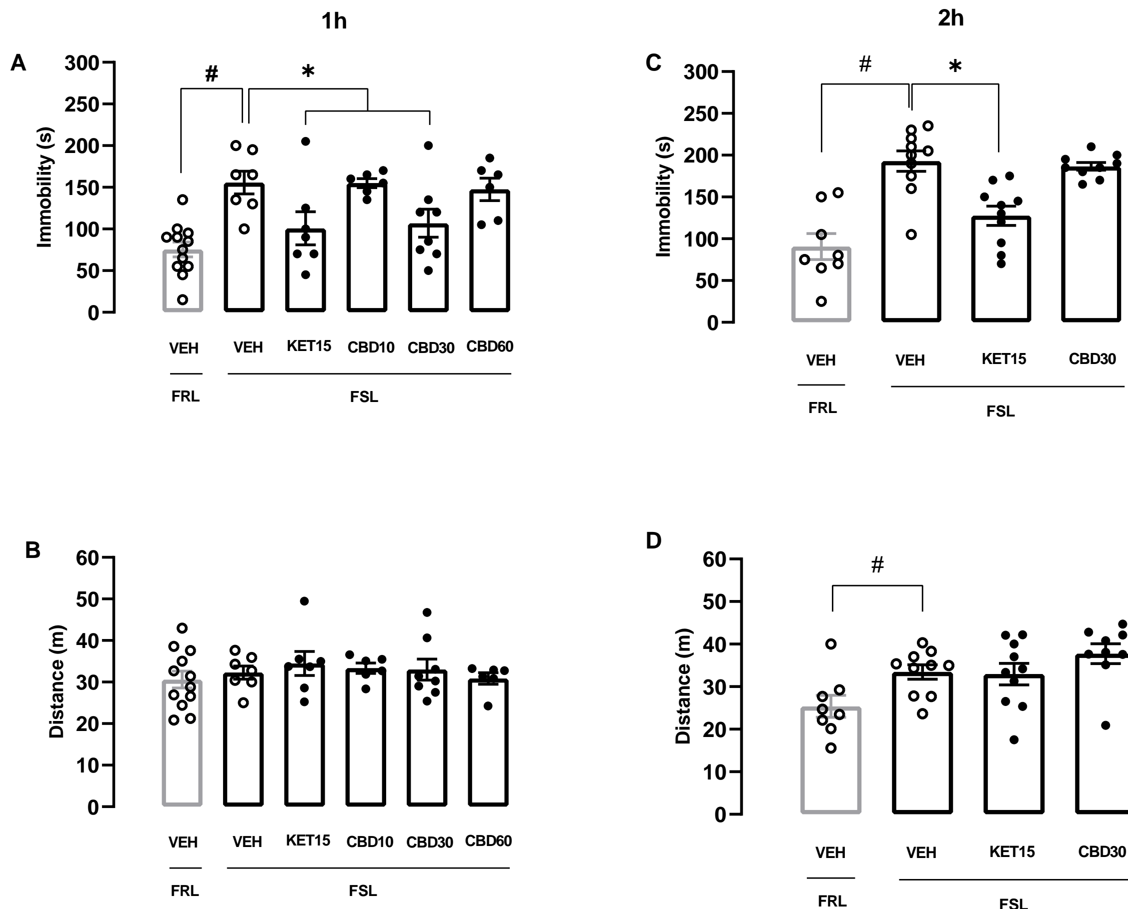


Figure 12. Effect of CBD in male FSL rats submitted to OFT and FST. Effect of cannabidiol (CBD) in male FSL rats administered 1 (A, B) and 2 hours (C, D) before the exposure to FST and OFT. Bars represent the immobility time (s) in FST or the travelled distance (m) in the OFT. Values are mean ± SEM; hash indicates significant differences between FSL and FRL vehicle-treated groups ($^{\#}p < 0.05$, Student's *t*-test); Asterisk represents significant treatment difference from FSL control ($^{*}p < 0.05$; One-way ANOVA followed by Fisher's LSD posthoc test or Kruskal-Wallis followed by Dunn's posthoc), *n*= 6-12 animals/group.

3.4.2.2 Ketamine effects in female FSL rats exposed to OFT/FST

As expected, FSL rats treated with vehicle displayed significantly increased immobility time in FST in comparison to FRL rats treated with vehicle (Mann-Whitney test: U= 6.5 ; *p*= 0.0037; Figure 13A), which characterized depressive-like phenotype. Ketamine (15 and 20 mg/kg) decreased the immobility in FSL (Kruskal-Wallis test: H(4)= 10.60; *p*= 0.0141; Dunn's: Ketamine 15 mg/kg, *p*= 0.0198, d= 1.838; Ketamine 20 mg/kg, *p*= 0.0108, d=3.350; Figure 13A), suggesting an antidepressant-like effect. Neither rat strain (Student's *t*-test: t(17)= 0.5769; *p*= 0.5716) nor drug treatment affected the locomotor activity on the OFT in FSL rats (One-way ANOVA:

F(2,24)=0.6495; *p*= 0.5910) (Figure 13B). Based on the results, the dose of 20 mg/kg of ketamine was chosen for the next experiments.

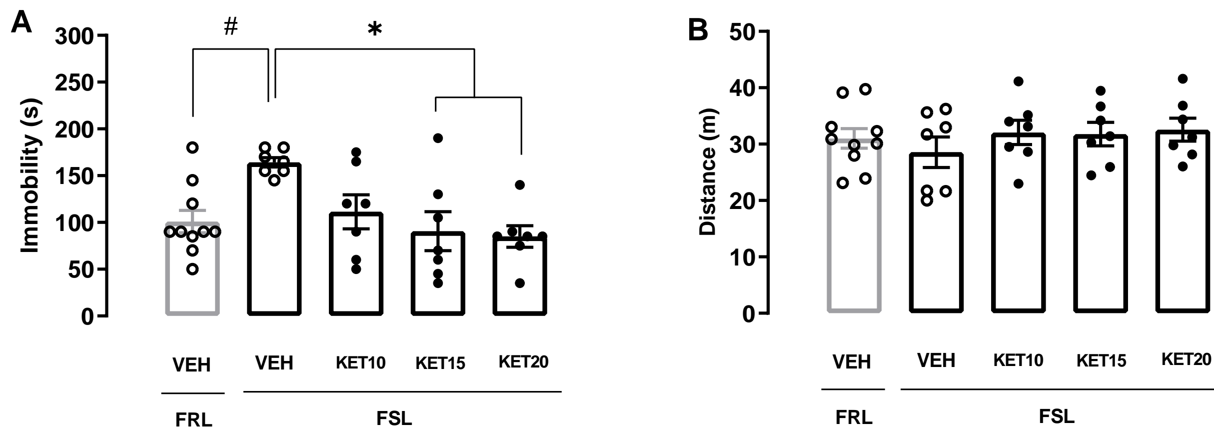


Figure 13. Dose-response curve ketamine with female FSL rats. The rats received the treatment with VEH or ketamine (10, 15, and 20 mg/kg) 1 hour before the exposition to FST (A) and OFT (B). Bars represent the immobility time (s) in FST or the travelled distance (m) in the OFT. Values are mean ± SEM; Hash indicate significant differences between FSL and FRL vehicle-treated groups ($^{\#}p< 0.05$, Student's *t*-test or Mann-Whitney test); Asterisk represents significant treatment difference from FSL control ($^{*}p< 0.05$; One-way ANOVA followed by Fisher LSD posthoc or Kruskal-Wallis followed by Dunn's posthoc), *n*= 7-10 animals/group.

3.4.2.3 CBD effects in female FSL rats exposed to the OFT/FST

One hour after injection, female FSL treated with vehicle displayed higher immobility time when compared with FRL rats treated with vehicle (Student's T test: t(12)= 2.954; p= 0.0120; Figure 14A), and significant increase in the locomotion (Mann-Whitney: U=8; p=0.0426; Figure 14B). As shown previously, ketamine (20 mg/kg) significantly reduced immobility time in FSL rats (One-way ANOVA: F(4,24)=9.464; p< 0.0001; Fisher's LSD test: p= 0.0027; Figure 14A). However, CBD (30mg/kg) increased the immobility in female FSL rats (One-way ANOVA: F(4,24)=9.464; p< 0.0001; Fisher's LSD test: p= 0.0281), suggesting a depressive-like effect. None of the drug treatments modified the locomotor activity in FSL rats (Kruskal-Wallis test: H(5)= 5.917; p= 0.2054; Figure 14B).

At two hours after vehicle injection, FRL displayed significantly decreased immobility time in comparison to FSL rats treated with VEH (t(8)=3.076; p= 0.0152; Figure 14C), and decreased distance travelled in OFT (t(8)= 1.861; p= 0.0998; Figure 14D). Interestingly, a significant reduction in the immobility time was showed 2 hours after CBD (30 mg/kg) treatment in female

FSL rats (One-way ANOVA: F(2, 15)= 4.439; p= 0.0306; Fisher's LSD test: p = 0.0.0097; d= 1.621; Figure 14C). Ketamine (20 mg/kg) administered 2 hours before FST did not change the behaviour in the test (One-way ANOVA: F(2, 15)= 4.439; p= 0.0306; Fisher's LSD test: p = 0.2549; Figure 14C). None of the treatments changed the distance travelled in the OFT in FSL rats (One-way ANOVA: F(2, 15)= 0.9503; p= 0.4087; Figure 14D).

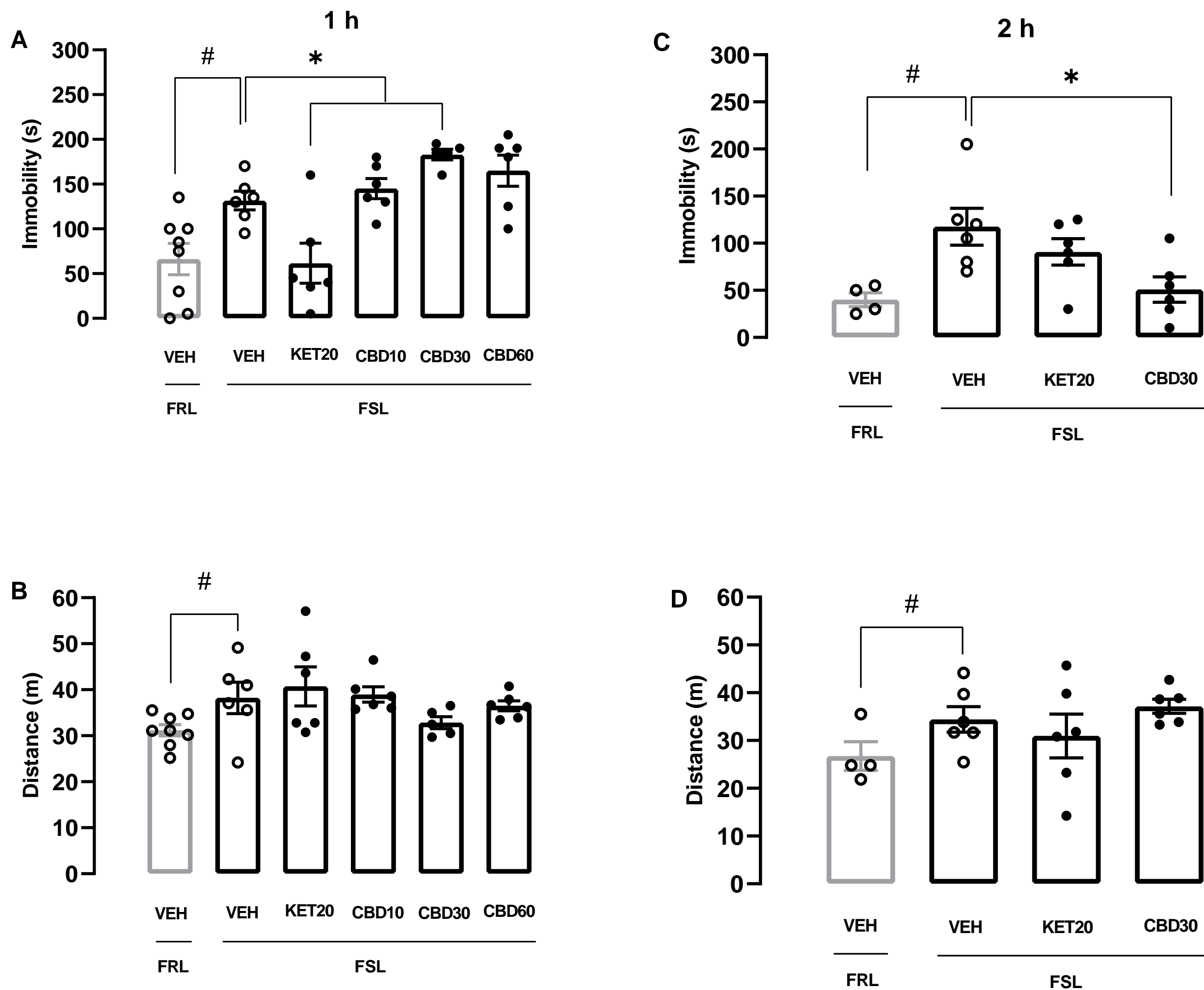


Figure 14. Effect of CBD in female FSL rats submitted to OFT and FST. Effect of cannabidiol (CBD) in female FSL rats administered 1 (A, B) and 2 hours (C, D) before the exposure to FST and OFT. Bars represent the immobility time (s) in FST or the travelled distance (m) in the OFT. Values are mean ± SEM; hash indicates significant differences between FSL and FRL vehicle-treated groups ($^{\#}p < 0.05$, Student's *t*-test); asterisk represents significant treatment difference from FSL control rats ($^{*}p < 0.05$; one-way ANOVA followed by Fisher's LSD posthoc), $n = 4$-10 animals/group.

3.4.3 Discussion

This study aimed at investigating CBD effects in both male and female FSL rats. The main finding in the present study is that CBD differentially modulates depressive-like behaviour in a time and gender-dependent manner. The study is the first to investigate the behavioural and temporal effects produced by CBD and ketamine in both genders of FSL rats. Interestingly, while CBD treatment produced an antidepressant effect at 1 hour CBD in male FSL rats, it induced a bimodal effect in females, with a prodepressive effect at 1h post-injection and an antidepressant effect 2h after its administration. The antidepressant effect of ketamine was also observed at 1h post-injection, but not 2h later.

The present study is also the first study to perform a dose-effect relationship in female FSL rats, where we show that a single dose of ketamine (15 and 20 mg/kg) produces an antidepressant-like effect in female FSL rats exposed to FST. Our finding strengthens previous results showing the efficacy of the treatment with ketamine in females rats, even though the effective doses vary in comparison to males (CARRIER et al., 2015; CHOU et al., 2018; RINCÓN-CORTÉS; GRACE, 2017; SARKAR; KABBAJ, 2017; TIZABI et al., 2012). Even though studies demonstrated that the gender and the oestrous cycle affects the mechanism involved in the ketamine antidepressant effect (DOSSAT et al., 2018; RINCÓN-CORTÉS; GRACE, 2017; SARKAR; KABBAJ, 2017; THELEN et al., 2016, 2019), we did not investigate the influence of the oestrous cycle in our analysis because we have previously shown that it does not modulate the FST behaviour in female FSL rats (ESKELUND et al., 2016).

Surprisingly, we found that ketamine fails to induce an antidepressant-like effect 2 hours after the administration in female FSL, which is suggest a lack of sustained effects. Contrasting with our findings, previous studies demonstrated that ketamine induces a sustained antidepressant effect in female mice exposed to CUMS (FRANCESCHELLI et al., 2015) and Wistar-Kyoto rats exposed to FST (TIZABI et al., 2012), 7 days after a single administration, even though the effective dose varied between males and females animals. These discrepant results can be due to differences in the species and paradims used. In addition, the lack of effect on ketamine at 2 hours in female FSL rats can be due to the small number of animals in this group (n=5).

Male rats treated with CBD presented an antidepressant effect at 1h, but not at 2h after administration. In FSL rats, a previous study from our group has shown that CBD was effective (10 and 30 mg/kg), 1h after ip administration, but presented no sustained effect 7 days later (SALES et

al., 2018b). The observed antidepressant-like effect induced by CBD in male, at 1h post-administration, is consistent with previous studies which reported that acute CBD injection decreased the immobility time in rats exposed to FST (DE MORAIS et al., 2018; RÉUS et al., 2011) and reduced the number of failures in the learned helplessness (LH) (SALES et al., 2018b), one hour after ip administration, at similar doses. On the other hand, CBD produced a hedonic and antidepressant-like effect in WKY and FSL rats 2h after oral administration, 30 mg/kg (SALES et al., 2018b; SHBIRO et al., 2019; SHOVAL et al., 2016). The disparity in behavioural results can be explained by the differences in the absorption profile in administration routes. The oral route presents irregular absorption and may suffer interference from first-pass metabolism. In addition, the feeding condition may affect gastric emptying and, consequently, it will reflect non-validated plasmatic concentration (HUESTIS, 2007; MILLAR et al., 2018, 2020; TURNER et al., 2011). However, the i.p. route produces more reliable plasmatic concentration, the absorption occurs faster and, the metabolism occurs less significantly (AL SHOYAIB; ARCHIE; KARAMYAN, 2020; TURNER et al., 2011). Second, it is well known that CBD is a lipophilic compound. Thus the solubility is not easy, and it can be challenging to make the right dose (ADAMS; HUNT; CLARK, 1940; MECHOULAM; SHVO, 1963; MILLAR et al., 2020). However, as the CBD pharmacology is complex, this warrants further studies and testing different doses of CBD, different routes of administration, different time-points, and concomitant measurements of actual CBD exposure could show us a distinct response profile.

Interestingly, CBD produced opossite effects in female FSL rats, showing increased immobility at 1h post-injection and decreased immobility after 2h. In a previous study with female FSL rats, CBD did not change the saccharin preference and immobility in the FST after oral treatment (food pellet; 30 mg/kg, 2 hours before the test), but induced antidepressant effects in male rats of the same strain (SHBIRO et al., 2019). It is crucial to notice that CBD's time to reach the Cmax depends on the vehicle, administration route, and the animal species used (DEIANA et al., 2012b). The gender-differences observed in our work may be explained due to the physio-chemical properties of CBD. As mentioned before, CBD has a chemical structure that confers high lipophilicity similar to delta-9-tetrahydrocannabinol (Δ9-THC) (ADAMS; HUNT; CLARK, 1940; MECHOULAM; SHVO, 1963), and, as a consequence, the compound rapidly penetrates highly vascularized tissues in a short time, redistribute and accumulates in fat tissue, modifying the plasmatic concentration of the drug (HARVEY, 1999). This process can be affected by body weight and composition, which varies between gender (GROTENHERMEN, 2003; HARVEY, 1999;

LUCAS; GALETTIS; SCHNEIDER, 2018; MCGILVERAY, 2005). In fact, CBD presents a similar molecular structure to Δ9-THC (HARVEY; BROWN, 1991; HARVEY; SAMARA; MECHOULAM, 1991; SAMARA; BIALER; HARVEY, 1991), and studies are showing the profound influence of gender on Δ9-THC metabolism in the rat liver (NARIMATSU et al., 1991; WILEY; BURSTON, 2014). Adult female rats present high hydroxylated metabolite (11-hydroxy-Δ9-THC) blood levels compared to the corresponding male (WILEY; BURSTON, 2014). Similar results were evidenced in the adolescent female rats (WILEY; BURSTON, 2014). Therefore, it is likely that these factors can influence by plasmatic and brain concentration of CBD and, consequently, differentially influence the behavioural effects observed in the test among male and female rats.

Besides that, a recent study evidenced that CBD could be converted in Δ9-THC depending on the storage conditions. The copresence of water and carbon dioxide from the air could create an acidic environment that favours the conversion to Δ9-THC (CITTI et al., 2020). However, the Δ9-THC behavioural effect in animal paradigms related to depression is controversial (BAMBICO et al., 2012; EL-ALFY et al., 2010; ELBATSH et al., 2012; SANO et al., 2009; SCHREIBER et al., 2019). Therefore, the high variability observed inside the CBD treated groups in our results could reflect the increased concentration of Δ9-THC in the animals. Thus, the measurements of plasma CBD and Δ9-THC levels could clarify these results.

There are a few limitations of the present work. Most importantly, it would have been relevant to know the exact exposure to CBD in males and females. CBD is, as mentioned before, difficult to dissolve and can be converted in Δ9-THC. The measurement of plasma CBD and Δ9-THC would be a better way to stratify the test's behaviour. Additionally, it will be crucial to consider the presence of the oestrous cycle in females and how it may affect the test's baseline response and the drug effect.

3.5 OVERALL CONCLUSION

In summary, our findings indicate that gender, strain, species, and chosen time of the administration may interfere with the behavioural response produced by CBD in rodents exposed to animal models of depression. For mice, CBD had an antidepressant-like effect only in male Swiss mice in the TST and no effect in female Swiss mice and both gender of C57BL/6 mice in the test, when injected 30 minutes before the behavioral testing. In FSL rats, a bimodal effect of CBD was observed in females, since CBD produced an antidepressant-like effect 2 hours after the treatment, but a depressive-like effect, at 1 hour. In male FSL, CBD produced an antidepressant-like effect 1 hour after the injection and no effect at 2 hours, suggestive of lack of sustained effects. These findings point to the fact that it is necessary to consider gender, strain, species of rodents chosen, compound chemistry, exposure to a previous stressful condition, and behavioural test to plan the most appropriate experimental design when evaluating CBD antidepressant properties.

3.6 ATTACHMENTS

Attachment 1

Certificate of approval of the Brazilian Council for Animal Experimentation.

UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas de Ribeirão Preto
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

AUTORIZAÇÃO

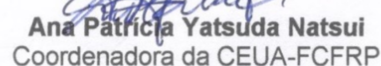
Certificamos que o adendo da proposta intitulada "Investigação do canabidiol em machos e fêmeas submetidos a diferentes modelos animais de transtornos psiquiátricos", registrada sob nº 17.1.537.60.6, sob a responsabilidade de Gabriela Pandini Silote e Sâmia Regiane Lourenço Joca, que envolve a manutenção e utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto º 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi aprovada *ad referendum* em 05/07/2019 pela Comissão de Ética no Uso de Animais da Faculdade de Ciências Farmacêuticas de Ribeirão Preto (CEUA FCFRP).

Lembramos da obrigatoriedade de apresentação do relatório de atividades, em modelo da CEUA, para emissão do certificado, como disposto nas Resoluções Normativas do CONCEA.

Colaboradores: Michelle Carvalho Gatto, Ariandra G. Sartim, Kennia Moura da Silveira.

Finalidade	( ) Ensino (x) Pesquisa Científica			
Vigência da Autorização	03/04/2018 a 31/08/2020			
Espécie/Linhagem/Raça	Camundongo isogênico		Camundongo heterogênico	
	C57BL/6		Swiss	
Nº de animais	69	121	Fêmea	Macho
Peso/Idade	18-20 g/ 6-7 semanas		30-50 g/ 8 semanas	
Sexo	Fêmea	Macho	872	215
Origem	Biotério Central da PUSP-RP		ANILAB	

Ribeirão Preto, 10 de julho de 2019.



Ana Patrícia Yatsuda Natsui
Coordenadora da CEUA-FCFRP

Avenida do Café S/Nº - Monte Alegre – CEP 14040-903 – Ribeirão Preto – SP
Comissão de Ética no Uso de Animais – ceua@fcfrp.usp.br
Fone: (16) 3315-8559 – Fax: (16) 33152-4892

Attachment 2

Certificate of approval of the Danish Animal Experiments Inspectorate is attached in the Study 2 section 4.10.

STUDY 2

4 STUDY 2

ABSTRACT

Introduction: Cannabidiol (CBD) is a compound extracted from the Cannabis sativa L. plant, which produces an antidepressant-like effect similar to ketamine in preclinical studies, without inducing undesirable side effects. CBD's antidepressant effect involves the activation of BDNF-TrkB-mTOR signaling and the induction of synaptogenesis, which has also been demonstrated for the fast acting antidepressant ketamine. However, further shared molecular mechanisms remains unknow.
Aims: The present study aimed to investigate the molecular mechanisms involved in the CBD antidepressant-like effect in the prefrontal cortex (PFC) and hippocampus of FSL rats, in order to investigate whether CBD's effects share molecular mechanisms with s-ketamine (S-KET), using a genetic animal model of depression, the Flinders Sensitive Line (FSL) rats.
Methods: Adult male FSL and Flinders Resistant Line (FRL) rats, were bred and obtained from Translational Neuropsychiatric Unit (TNU), Aarhus University (Aarhus, Denmark). FSL and FRL animals received intraperitoneal treatment with CBD (30 mg /kg), S-Ketamine (15 mg/kg, positive control) or vehicle (Saline and Tween 80 3%), 1h before the behavioural tests in the open field test (OFT, 5 min) and forced swimming test (FST, 5 min). Immediately after the behavioural tests, the brain was extracted, and the PFC, dorsal hippocampus (DH), and ventral hippocampus (VH) were dissected, and further processed as outlined below. Whole blood was collected to measure CBD concentration using HPLC coupled to mass spectrometry. To investigate the molecular mechanisms involved in the antidepressant-type effect induced by CBD and S-Ketamine, the analysis of gene expression (Fluidigm) and synaptosome protein levels by Western Blot (WB) were performed on PFC, DH, and VH for the for targets genes and proteins related to glutamatergic neurotransmission, the neurotrophic signaling pathway and synaptic proteins. **Results:** As expected, FSL-VEH animals displayed elevated immobility time in the test compared to FRL-VEH, suggesting a depressive-type phenotype. CBD and S-ketamine treatment reduced the immobility time in FSL rats exposed to FST, which characterizes an antidepressant-like effect. There was no correlation between CBD blood levels and immobility displayed in the FST. In the molecular analysis, the effects of CBD in the PFC was associated with increased the levels of EAAT3 (mRNA), Nr2a (mRNA and protein), Nr2b (protein), BDNF (protein), while ketamine elicited a downregulation in VEGF(mRNA) and sortilin (mRNA) and, and elevated protein expression of Nr2b, Nr2a, and pGluR1 (S831). In DH, CBD elevated the levels of VEGF (mRNA) and decreased expression of Sort1(mRNA) and pGluR1 (S831 and S845; protein), whereas ketamine reduced the protein expression of Nr2b, pGluR1 (S845) (DH), and pGluR1 (S845). In VH, CBD decreased the expression of mGluR5 (mRNA) and GluR2 (protein); and reduced pGluR1 (S831 and S845, protein) expression, whereas ketamine lowered the levels of pGluR1 (S845, protein). Furthermore, changes in gene expression and protein levels related to neurotrophic neurotransmission, neuroplasticity signaling, and glutamatergic neurotransmission in all brain structures (PFC, DH, and VH) between FSL and FRL rats were observed. **Conclusion:** The results show that CBD produces an antidepressant-like effect in FSL rats in the FST. However, CBD and S-ketamine do not share the common molecular expression patterns in the examined genes and proteins. In the PFC, CBD mainly modulates the BDNF and glutamatergic signaling pathway, whereas in the DH, CBD regulates the Sortilin, VEGF and glutamatergic systems. On the other hand, the effect of ketamine seems to involve mainly the

restoration of normal glutamatergic function in the limbic brain areas. Changes in the BDNF-TrkB, glutamatergic systems, and synaptic proteins were observed among FSL and FRL rats.

Keywords: Cannabidiol; S-Ketamine; depression; Flinders Sensitive Line; Gene expression analysis; Proteins; Glutamatergic neurotransmission; BDNF; VEGF.

RESUMO

Introdução: O canabidiol (CBD) é um composto extraído da planta *Cannabis sativa* L., que produz um efeito antidepressivo semelhante à ketamina em estudos pré-clínicos, sem induzir efeitos colaterais indesejáveis. O efeito antidepressivo do CBD envolve a ativação da sinalização do BDNF-TrkB-mTOR e a indução da sinaptogênese, o que também foi demonstrado para o antidepressivo de ação rápida ketamina. No entanto, outros mecanismos moleculares compartilhados permanecem desconhecidos. **Objetivos:** O presente estudo teve como objetivo investigar os mecanismos moleculares envolvidos no efeito do antidepressivo do CBD no córtex pré-frontal (PFC) e no hipocampo de ratos FSL, a fim de investigar se os efeitos do CBD compartilham mecanismos moleculares com a S-ketamina (S-KET), usando um modelo animal genético de depressão, ratos Flinders Sensitive Line (FSL). **Métodos**: Ratos adultos FSL e Flinders Resistant Line (FRL), foram criados e obtidos na Translational Neuropsychiatric Unit (TNU), Universidade de Aarhus (Aarhus, Dinamarca). Animais FSL e FRL receberam tratamento intraperitoneal com CBD (30 mg / kg), S-ketamina (15 mg / kg, controle positivo) ou veículo (Saline e Tween 80 3%), 1h antes dos testes comportamentais no teste de campo aberto (OFT, 5 min) e teste de natação forçada (FST, 5 min). Imediatamente após os testes comportamentais, o cérebro foi removido, e o PFC, o hipocampo dorsal (DH) e o hipocampo ventral (VH) foram dissecados e posteriormente processados conforme descrito abaixo. O sangue total foi coletado para medir a concentração de CBD usando HPLC acoplado a espectrometria de massa. Para investigar os mecanismos moleculares envolvidos no efeito do tipo antidepressivo induzido por CBD e S-ketamina, a análise da expressão gênica (Fluidigm) e dos níveis de proteína de sinaptossoma por Western Blot (WB) foi realizada em PFC, DH e VH para genes e proteínas relacionados à neurotransimissão glutamatérgica, a via de sinalização neurotrófica e proteínas sinápticas. **Resultados:** Conforme esperado, os animais FSL-VEH apresentaram elevado tempo de imobilidade no teste FST em comparação com FRL-VEH, sugerindo um fenótipo do tipo-depressivo. O tratamento com CBD e S-ketamina reduziram o tempo de imobilidade em ratos FSL expostos ao FST, o que caracteriza um efeito do tipo-antidepressivo. Não houve correlação entre os níveis sanguíneos de CBD e a imobilidade exibida no teste. Na análise molecular, os efeitos do CBD no PFC foram associados ao aumento dos níveis de EAAT3 (mRNA), Nr2a (mRNA e proteína), Nr2b (proteína), BDNF (proteína), enquanto a ketamina induziu uma regulação negativa em VEGF (mRNA) e sortilina (mRNA) e, e expressão de proteína elevada de Nr2b, Nr2a e pGluR1 (S831). Na DH, o CBD elevou os níveis de VEGF (mRNA) e diminuiu a expressão de Sort1 (mRNA) e pGluR1 (S831 e S845; proteína), enquanto a ketamina reduziu a expressão da proteína de Nr2b, pGluR1 (S845) (DH) e pGluR1 (S845). Em VH, o CBD diminuiu a expressão de mGluR5 (mRNA) e GluR2 (proteína); e reduziu a expressão de pGluR1 (S831 e S845, proteína), enquanto a ketamina baixou os níveis de pGluR1 (S845, proteína). Além disso, foram observadas alterações na expressão gênica e nos níveis de proteína relacionados à neurotransmissão neurotrófica, sinalização de neuroplasticidade e neurotransmissão glutamatérgica em todas as estruturas cerebrais (PFC, DH e VH) entre ratos FSL e FRL. **Conclusão:** Os resultados mostram que o CBD produz um efeito do tipo antidepressivo em ratos FSL no FST. No entanto, o CBD e a S-ketamina não compartilham os padrões de expressão molecular comuns nos genes e proteínas examinados. No PFC, o CBD modula principalmente o BDNF e a via de sinalização glutamatérgica, enquanto no DH, o CBD regula os sistemas Sortilin, VEGF e glutamatérgico. Por outro lado, o efeito da ketamina parece

envolver principalmente a restauração da função glutamatérgica normal nas estruturas límbicas do cérebro. Alterações na via de BDNF-TrkB, sistemas glutamatérgicos e proteínas sinápticas foram observadas entre ratos FSL e FRL.

Palavras-chave: Canabidiol; S-ketamina; depressão; ratos Flinders Sensitive Line; Análise de expressão gênica; Proteínas; Neurotransmissão glutamatérgica; BDNF; VEGF.

RESUMÉ

Introduktion: Cannabidiol (CBD) er en forbindelse ekstraheret fra *Cannabis sativa L.*-planten, der i prækliniske studier besidder en antidepressiv-lignende virkning, svarende til effekten af det hurtigtvirkende antidepressiva ketamin, men- uden at fremkalde uønskede bivirkninger. CBDs antidepressive virkning involverer aktivering af BDNF-TrkB-mTOR-signalering og induktion af synaptogenese, hvilket tilsvarende er blevet demonstreret for ketamin. Imidlertid er viden om eventuelt fælles molekylære mekanismer ukendte. **Formål**: Dette studium havde til formål at undersøge de molekylære mekanismer, der er involveret i den antidepressiv-lignende virkning af CBD og ketamin i præfrontale cortex (PFC) og hippocampus fra rotter, for derigennem at undersøge om effekterne af CBD deler molekylære mekanismer med s-ketamin. Der blev anvendt en genetisk dyremodel for depression, Flinders Sensitive Line (FSL) rotter. **Metoder**: Voksne FSL/FRL hanrotter blev opdrættet på Translational Neuropsychiatry Unit (TNU), Aarhus University (Aarhus, Danmark). FSL- og FRL-dyr modtog intraperitoneal injektion med CBD (30 mg / kg), S-ketamin (15 mg / kg, positiv kontrol) eller vehikel (Saltvand og Tween 80 3%), 1 time før adfærdstest i den Open Field Test (OFT, 5 min) og Forced Swim Test (FST, 5 min). Umiddelbart efter adfærdstestene blev hjernen ekstraheret, og PFC, dorsal hippocampus (DH) og ventral hippocampus (VH) blev dissekeret og yderligere behandlet som beskrevet nedenfor. Blod blev opsamlet for at måle CBD-koncentration ved hjælp af HPLC koblet til massespektrometri. Til undersøgelse af de molekylære mekanismer, der er involveret i den antidepressive effekt, induceret af CBD og S-ketamin, blev genekspression i vævet analyseret (Fluidigm) og synaptosomprotein-niveauer blev målt med Western Blot (WB) på PFC, DH og VH for det følgende molekylære targets: 1) Gener: Sort1, VEGF, BDNF, TrkB-receptor, GSK-3, Nr2a, GluR2, EAAT3, mGluR5, Pick1; 2) Proteiner: pGSK3, GSK3, pGluR1 (S831 og S845); GluR1, Sortilin, BDNF, Nr2b, GluR2. **Resultater**: Som forventet havde FSL-VEH-dyr forhøjet immobilitetstid i FST sammenlignet med FRL-VEH, hvilket viser en depressiv fænotype. Behandling med CBD og S-ketamin reducerede immobilitetstiden hos FSL-rotter i FST, hvilket karakteriserer en antidepressiv-lignende virkning. Der var ingen sammenhæng mellem CBD-blodniveauer og immobilitet vist i FST. I molekylæranalysen var virkningerne af CBD i PFC forbundet med øgede niveauer af EAAT3 (mRNA), Nr2a (mRNA og protein), Nr2b (protein), BDNF (protein), mens ketamin fremkaldte en nedregulering i VEGF (mRNA) og sortilin (mRNA) og forhøjet proteinekspression af Nr2b, Nr2a og pGluR1 (S831). I DH hævede CBD niveauerne af VEGF (mRNA) og nedsate ekspression af Sort1 (mRNA) og pGluR1 (S831 og S845; protein), mens ketamin reducerede proteinekspressionen af Nr2b, pGluR1 (S845) (DH) og pGluR1 (S845). I VH reducerede CBD ekspressionen af mGluR5 (mRNA) og GluR2 (protein); og reducerede pGluR1 (S831 og S845, protein) ekspression, hvorimod ketamin sænkede niveauerne af pGluR1 (S845, protein). Ændringer i genekspression og proteinniveauer relateret til neurotrof neurotransmission, neuroplasticitetssignalering og glutamaterg neurotransmission blev observeret i alle hjernestrukturer (PFC, DH og VH) mellem FSL og FRL-rotter. **Konklusion**: Resultaterne viser, at CBD producerer en antidepressiv-lignende virkning hos FSL-rotter i FST. Dog deler CBD og S-ketamin ikke fælles molekylære ekspressionsmønstre i de undersøgte gener og proteiner. I PFC modulerer CBD hovedsagelig BDNF og glutamaterg signalvej, mens CBD i DH regulerer Sortilin, VEGF og glutamaterge systemer. Effekten af ketamin synes hovedsageligt at involvere normalisering af glutamaterg funktion i de limbiske hjerneområder. Ændringer i BDNF-TrkB, glutamaterge systemer og synaptiske proteiner blev observeret blandt FSL- og FRL-rotter.

Nøgleord: Cannabidiol; S-ketamin; depression; Flinders Sensitive Line; Genekspressionsanalyse; Proteiner; Glutamaterg neurotransmission; BDNF; VEGF.

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ABBREVIATIONS LIST

Δ9-THC	delta-9-tetrahydrocannabinol
2-AG	2-arachidonoylglycerol
5-HT	5-hydroxy tryptamine or serotonin
5-HT1A	serotonin receptor type 1A
5-HT2A	serotonin receptor type 2A
5-HT3	serotonin receptor type 3
A2A	Adenosine A2A receptor
ACTH	adrenocorticotrophic hormone
AEA	anandamide
Akt	protein kinase B
ANOVA	analysis of variance
BCA	bicinchoninic acid
BDNF	brain-derived neurotrophic factor
BSA	bovine sérum albumin
Ca2+	calcium
CaMKII	calmodulin kinase II
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CBD	cannabidiol
cDNA	complementar deoxyribonucleic acid
Cmax	Maximum drug concentration
CNS	central nervous system
COMT	Cathecol-O-methyltransferase
COX	cyclooxygenase
CREB	cAMP response elemento-binding protein
CRH	corticotrophin releasing fator

Ct	cycle thereshold
CUMS	chronic umpredictable mild stress
DA	dopamine
DAG	diacylglicerol
DAT	dopamine transporter
DFP	diisopropyl fluorophosphate
DH	dorsal hippocampus
DNA	deoxyribonucleic acid
DSM-5	Diagnostic and Statistical Manual of Mental Disorders – fifth edition
EA	enclosed arm
EAAT	glutamate transporter
ECT	electroconvulsive therapy
EPM	elevated plus maze
ERK	extracelular signal regulated kinase
FAAH	fatty acid amide hydrolase
FRL	Flinders resistant line rats
FSL	Flinders sensitive line rats
FST	forced swim test
GABA	gamma-aminobutyric acid
GABAA	gamma-aminobutyric acid receptor type A
GPR55	G protein-coupled receptor 55
GluR	Glutamate receptor
HPA	hypothalamic-pituitary-adrenal
HPC	hippocampus
HTR1A	serotonin receptor 1A gene
HTR2A	serotonin receptor 2A gene
i.c.v.	intracerebroventricular
i.p.	intraperitoneal

IBA1	ionized calcium binding adaptor molecule 1
IL-1β	interleukin 1 beta
IL-6	interleukin 6
IMIP	imipramine
INF	interferon
iNOS	inducible nitric-oxide synthase
IP3	inositol triphosphate
LH	learned helplessness
LPS	lipopolysccharide
LTD	long-term depression
LTP	long-term potentiation
MAGL	monoacylglycerol lipase
MAO	monoamine-oxidase
Mapk	microtube-associated protein kinase
MDD	major depressive disorder
MEK	MAP/ERK kinase
mGluR	Metabotropic glutamate receptor
mPFC	Medial pré-frontal cortex
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NA	noradrenaline
NET	Noradrenaline transporter
NF-κB	Fator nucelar kappa B
NMDA	N-methyl-D-aspartate
OA	open arm
OBB	Odyssey Blocking Buffer
OFT	open field test
PCR	polymerase chain reaction

PFC	prefrontal cortex
PI3K	phosphatidyl inositol-3 phosphate
PKA	protein kinase A
PKC	protein kinase C
Pick1	protein interating with C kinase
PPARγ	peroxisome proliferator-activated receptor gamma
PSD-95	Postsynaptic density protein 95
PT	pre-test session
qPCR	quantitative polymerase chain reaction
REM	rapid eye moviment
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SNRI	selective noradrenaline reuptake inhibitor
SPF	specific pathogen free
SPT	sucrose preference test
SSRI	selective serotonin reuptake inhibitor
Syn 3	synapsine 3
Syp	synaptophysin
TBS	tris-buffer saline
TBST	tris-buffer saline and tween 20
TCA	tricyclic antidepressant
TNF-α	tumor necrosis fator alpha
TPH1	tryptophan hydroxylase 1
TPH2	tryptophan hydroxylase 2
TrkB	tropomyosin-related kinase B
TRPA1	transient receptor potential cation channel subfamily A member 1

TRPM8	transient receptor potential cation channel subfamily M member 8
TRPV1	transient potential vanniloid type 1
TRPV2	transient potential vanniloid type 2
TST	tail suspension test
VEH	vehicle
VH	Ventral hippocampus
WB	Western blotting
WKY	Wistar-kyoto rats

4.1 INTRODUCTION

Depression is a chronic and debilitating psychiatric disorder that affects more than 300 million people in the whole world (WHO, 2017b). Limbic brain structures, such as prefrontal cortex (PFC), amygdala, hypothalamus, hippocampus (HPC) and nucleus accumbens have been consistently implicated in depression and stress neurobiology (BERTON; NESTLER, 2006; NESTLER et al., 2002; PRICE; DREVETS, 2010), and abnormalities in the morphology and function in these brain structures have been described in depressed patients and animals exposed to stress models of depression (KEMPTON, 2011; NESTLER et al., 2002; OTTE et al., 2016; PRICE; DREVETS, 2010; SCHMAAL et al., 2016). However, despite progress in neuroscience research, the pathophysiology of major depression is not fully elucidated.

The PFC is a brain structure that plays a role in high-order functions, such as decision making, emotional control, learning, and regulation of autonomic, endocrine and behavioral responses to stress (CZÉH et al., 2008; MCKLVEEN; MYERS; HERMAN, 2015; MCKLVEEN et al., 2017; PRICE; DREVETS, 2010; RESSTEL et al., 2006). Patients diagnosed with depression have changes in functionality (DREVETS; PRICE; FUREY, 2008; SCHMAAL et al., 2016), and in morphology of the PFC (DREVETS, MD, 1998; KEMPTON, 2011; SCHMAAL et al., 2016). Similar findings are found in preclinical studies, where animals exposed to inescapable stress show morphological, functional, and neurochemical changes in the PFC, correlating with anhedonia, considered a core symptom in depression (MCKLVEEN; MYERS; HERMAN, 2015).

Another key brain region involved in stress response and major depression is HPC. In several studies, depressed patients display smaller hippocampal volumes (KEMPTON, 2011; SCHMAAL et al., 2016), which seems to be attenuated in patients that have undergone antidepressant treatment (CASTRÉN; RANTAMÄKI, 2010a; SCHMAAL et al., 2016). In a similar way, antidepressants attenuate the effects of exposure to chronic inescapable stress in animals (MONLEON et al., 1995; WILLNER et al., 1987). Of note, the HPC can be anatomically and functionally divided into two main regions, the dorsal and ventral HPC (DH and VH, respectively), which exhibit distinct functions, anatomical projections patterns, gene expression and epigenetic profile (FANSELOW; DONG, 2010; FLORIOU-SERVOU et al., 2018; LEE et al., 2017; ZHANG et al., 2018). In this sense, the DH has been implicated in cognitive functions, including learning/memory and spatial navigation (BERTOGLIO; JOCA; GUIMARÃES, 2006; FANSELOW; DONG, 2010; MOSER; MOSER, 1998), whereas the VH is linked to emotional behaviour and regulating neuroendocrine response (BANNERMAN et al., 2003; FANSELOW; DONG, 2010;

KJELSTRUP et al., 2002). Importantly, a growing body of evidence indicate that sub-regions of the HPC differentially modulate the response to stress and antidepressant drug treatment (FLORIOU-SERVOU et al., 2018; TANTI; BELZUNG, 2013a, 2013b). For example, it was shown that bilateral lesions in VH, but not of the DH, aggravate gastric erosions in rats following stress exposure (HENKE, 1990) and attenuate various anxiety-related behaviours (BANNERMAN et al., 2003; KJELSTRUP et al., 2002; PENTKOWSKI et al., 2006). Moreover, the nature of stress can influence distinct proteomic, transcriptomic, and epigenetic signatures and promote changes in morphology and electrophysiology in sub-areas of the HPC (FLORIOU-SERVOU et al., 2018). In addition, it was shown that the degree of hippocampal neurogenesis induced by antidepressant drugs varies along the rostroventral axis. Indeed, chronic treatment with fluoxetine (TANTI et al., 2012), agomelatine (BANASR et al., 2006) and lithium (O'LEARY; O'CONNOR; CRYAN, 2012) elevated the neurogenesis and proliferation in VH, but not DH, in mice submitted to CUMS. Therefore, it is important to consider the sub-regions the HPC to investigate new compounds for stress-related disorders, including MDD.

Diverse evidence point that MDD results from a dysfunction in multiple systems, including monoamines (noradrenaline, serotonin, and dopamine), glutamatergic, endocannabinoid, GABAergic neurotransmission, neurotrophic, neuroplasticity, and neuroinflammation signaling pathways (CASTRÉN, 2005; DEAN; KESHAVAN, 2017; MICALE et al., 2013; MÜLLER, 2013; OTTE et al., 2016; PEREIRA; HIROAKI-SATO, 2018). Strikingly, these alterations are not present in every patient, reflecting the heterogeneity of this illness (DEAN; KESHAVAN, 2017; OTTE et al., 2016). Another crucial issue is that antidepressant drugs focus on only one system that could limit treatment efficacy (DEAN; KESHAVAN, 2017; OTTE et al., 2016). Thus, it is important to explore the complexity of the mechanisms to understand the neurobiology and delineate the appropriate multi-target treatment for this condition.

The clinically available monoaminergic antidepressant drugs used for the treatment of depression acts pharmacologically, basically, through increasing the monoamines levels, primarily, noradrenaline and serotonin (5-HT) in the brain (BOKU et al., 2017; COPPEN; ECCLESTON; PEET, 1973; MAFFIOLETTI et al., 2020; PARE; SANDLER, 1959; SCHILDKRAUT, 1965). Although effective in restoring mood, these drugs all have deleayed onset of action of the therapeutic effect (about 4 to 6 weeks) (ABDALLAH et al., 2016; OTTE et al., 2016), may induce important side effects, reducing adherence to treatment (BLIER, 1999; OTTE et al., 2016). Also, these drugs have high rates of non-responder patients (corresponding 45%) and often leaving residual symptoms with the

patient. This constitutes an enormous global health problem (see also introduction section 1.4.1. (KEKS et al., 2007). Given these facts, it becomes clear that it is important to study novel substances with a potential antidepressant effect, a rapid onset of action, and effective in individuals that are non-responders to the current available treatments.

Importantly, during the past 20 years, the anaestethic ketamine has received considerable attention as a potential fast-acting antidepressant drug (AUTRY et al., 2012; BERMAN et al., 2000; DIAZGRANADOS et al., 2010; FREEMAN et al., 2020; GERHARD; DUMAN, 2018; MAENG et al., 2008; ZARATE et al., 2006). Ketamine primarily acts as a non-competitive antagonist of the N-methyl-D-aspartate glutamate receptor (NMDA; KOHRS; DURIEUX, 1998; WHITE et al., 1980), and have the ability to induce a rapid and sustained antidepressant effect in animal models (AUTRY et al., 2012; GARCIA et al., 2008; KOIKE; IIJIMA; CHAKI, 2011; LI et al., 2010b; MAENG et al., 2008) and patients (BERMAN et al., 2000; DIAZGRANADOS et al., 2010; FREEMAN et al., 2020; O'BRIEN et al., 2019; RODRIGUES et al., 2020; ZARATE et al., 2006; ZARATE JR et al., 2012). In addition, KET produces antidepressant action in treatment-resistant patients (FREEMAN et al., 2020; MESSER et al., 2010; PAUL et al., 2009; ZARATE et al., 2006).

The molecular mechanisms behind ketamine's effects are complex and involves a large array of multistep molecular cascades. The proposed mechanisms of ketamine's antidepressant action can be divided into three main chains of events, as follows: 1) ketamine blocks the NMDA receptors primarily located at GABAergic interneurons, thus disinhibiting glutamate release from the primary neurons (HOMAYOUN; MOGHADDAM, 2007; MOGHADDAM et al., 1997; ZANOS; GOULD, 2018). Subsequently, the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor (AMPA) is activated (FUKUMOTO; IIJIMA; CHAKI, 2016; KOIKE; IIJIMA; CHAKI, 2011; MAENG et al., 2008; TIZABI et al., 2012; ZHOU et al., 2014), promoting an increase in BDNF release (AUTRY et al., 2012; LI et al., 2010b; PARK et al., 2014; ZHOU et al., 2014). Once released, BDNF acts through its tyrosine kinase receptor, tropomyosin related to kinase B (TrkB), activating the downstream molecular MEK/ERK and phosphatidylinositide 3-kinase (PI3K)/Akt signaling pathways, culminating with activation of the mechanistic target of rapamycin (mTOR), a key protein that participates in the regulation of cell growth and proliferation (AUTRY et al., 2012; LEPACK et al., 2016; LI et al., 2010a; PARK et al., 2014; PHAM et al., 2017a); 2) Ketamine and its metabolites (norketamine, dehydronorketamine, hydroxyketamine, and hydroxynorketamine) may directly activate AMPA receptors, and consequently increase BNDF release and its action in TrkB receptor, culminating on the activation of mTOR, as described above (CAN et al., 2016;

PHAM et al., 2017b; ZANOS et al., 2019a, 2019b); 3) Ketamine facilitates serotoninergic neurotransmission, through the indirect regulation of the activity/firing of serotoninergic neurons of the dorsal raphe nucleus (DRN), where it activates 5-HT1A and 5-HT1B (DU JARDIN et al., 2018, 2016a; PHAM et al., 2017a; PHAM; GARDIER, 2019). In conclusion, the effect of ketamine rapidly increases BDNF-TrkB-mTOR signaling (AUTRY et al., 2011; LI et al., 2010a; PARK et al., 2014; YANG et al., 2013), and subsequently induces synaptic plasticity, including increased synaptogenesis in the PFC and HPC (LI et al., 2010b; MONTEGGIA et al., 2007; TRECCANI et al., 2012; YANG et al., 2013; ZHOU et al., 2014).

Despite these promising findings, KET administration produce several unpleasant side effects, such as psychotomimetic symptoms, which may induce abuse and dependence in certain individuals, thereby limiting its use in depressed patients (DIAZGRANADOS et al., 2010; JANSEN, 2000; KRYSTAL et al., 1994; SHORT et al., 2018; SINGH et al., 2016; WHITE; WAY; TREVOR, 1982; ZARATE et al., 2006). It is important to develop new treatment approaches that can offer similar therapeutic profile as ketamine, but with less pronounced side effects.

Interestingly, recent studies have described that the phytocannabinoid cannabidiol (CBD) produce rapid and sustained antidepressant-like effect, similar to what have been demonstrated with KET (LINGE et al., 2016; SALES et al., 2018b). CBD has a complex pharmacology which involves multiple targets from distinct neurotransmitter systems, including endocannabinoid receptors and enzymes, serotonin receptors, monoamine transporters, opioid receptors as described previously in the introduction in section 1.5. However, the mechanisms responsible for the antidepressant effect have not been fully elucidated.

Intriguingly, there is evidence that the antidepressant effect of CBD involves the release of glutamate and serotonin in ventromedial PFC (LINGE et al., 2016), activation of 5-HT1A receptors (SARTIM; GUIMARÃES; JOCA, 2016; ZANELATI et al., 2010), CB1 and CB2 receptors (CAMPOS et al., 2013; FOGAÇA et al., 2018); as well as rapid increase of brain-derived neurotrophic factor (BDNF) and synaptic proteins with subsequent increase in dendritic arborization in the PFC (SALES et al., 2018). The activation of 5-HT1A, as well as, increased levels of BDNF and synaptogenesis are commonly related to the behavioral effect of antidepressant drugs (BJÖRKHOLM; MONTEGGIA, 2016; BLIER; DE MONTIGNY, 1994; CASTRO et al., 2003; MONTEGGIA et al., 2004; NIBUYA; MORINOBU; DUMAN, 1995a; SAARELAINEN et al., 2003). As already mentioned, KET triggers some of these mechanisms acutely while monoaminergic antidepressants need chronic treatment to attenuate stress effects in brain

neuroplasticity and behaviour (ARDALAN et al., 2020; AUTRY et al., 2012; CASTRÉN; RANTAMÄKI, 2010b; CHEN et al., 2001; LARSEN et al., 2010; LI et al., 2010a, 2010c; MAENG et al., 2008; MATVEYCHUK et al., 2020; NIBUYA; MORINOBU; DUMAN, 1995a; RANTAMÄKI et al., 2007; SANTARELLI et al., 2003).

As the mechanisms involved in the putative rapid antidepressant effect induced by CBD are not known, a broad exploratory investigation on the molecular profile associated with CBD effects could help understanding its molecular mechanism of action. Moreover, a comparison of the effects of CBD with the effects of KET could unravel potential common molecular targets associated with the rapid antidepressant effect. Therefore, this study focused at investigating changes in the level of protein and gene expression that could be associated with the rapid antidepressant effect induced by both CBD and ketamine in different brain regions (DH, VH and PFC) of a rat model of depression, the Flinders Sensitive Line (FSL) rats.

Based on the aforementioned information, we hypothesized that the rapid antidepressant effect of CBD and KET would be associated to changes in the expression of genes and proteins associated with neurotrophic (Sortilin, BDNF, VEGF, TrkB, Mapk1, eEF2K, mTOR, and GSK3), glutamatergic signaling (Cnih2, Cnih3, GluR1, GluR2, Nr1, Nr2a, Nr2b, mGluR5, EAAT3, EAAT2, CaMKII, PKA, PKC, and Pick1) and synaptic proteins (Homer3, PSD-95, Neuroligin 1, Neurexin 2, Spinophilin, Synapsine 3, and Synaptophysin).

4.2 AIMS

The aim of the current study was to investigate whether the antidepressant-like effect induce by CBD and KET both would be associated with changes in gene expression patterns related to glutamatergic neurotransmission, neurotrophic signaling and synaptic proteins, in brain regions involved with depression neurobiology (PFC, DH and VH).

4.3 METHODS

4.3.1 Animals

Adult male Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats (8 to 10 weeks old; weighing 154.8 to 361.26 g) were obtained from the breeding colonies at the Translational Neuropsychiatry Unit (Aarhus University; Denmark). The rats were pair-housed in standard cages (Cage 1291H Eurostandard Type III H, 425 × 266 × 185 mm, Tecniplast, Italy) at 20 ± 2 °C and 60 ± 5% relative humidity on a standard light/dark cycle (lights on at 6 a.m.) with free access standard food (Altormin, Brogaarden, Lynge, Denmark) and tap water. The housing consisted of wooden bedding material for rats (Tapvei Estonia OÜ; Estonia), access to tunnel shelter, nesting material, and a wooden stick. The rats were randomly selected to experimental groups. The behavioral tests and the killing were performed in specially equipped rooms in the animal facility between 9:00 a.m. and 1:00 p.m.

The experimental protocols were approved by Danish Animal Experiments Inspectorate (Protocol number: 2016-150201-001105, copy in attachment). The experimental procedures were conducted in accordance with European Community Council Directive 2010/63/EU. All behavioural experiments were conducted between 9:00 am and 1:00 pm.

4.3.2 Drugs

The following drugs were administered intraperitoneally (i.p.) and freshly prepared before the experiment:

- Synthetic Cannabidiol (CBD; THC-Pharma (Germany)) stored at 4°C and protected from light, diluted in sterile saline and 3% polysorbate 80 (Tween® 80; Sigma-Aldrich, USA), administered at the dose 30 mg/2mL/Kg (SALES et al., 2018b).
- S-Ketamine hydrochloride (KET; Pfizer A/S, Denmark) stored at 4°C, diluted in sterile saline, administered at dose 15 mg/2mL/Kg (LIEBENBERG; JOCA; WEGENER, 2015; SALES et al., 2018b).

The vehicle (VEH) group received the injection with CBD vehicle. The animals received the treatment randomly by writing treatment options on pieces of paper, folding them, mixing, and then drawing one by one for each animal (BESPALOV; MICHEL; STECKLER, 2020).

4.3.3 Behavioral tests

4.3.3.1 Open field test (OFT)

The OFT was performed as previously described in the Study 1B, section 3.4.1.3.1.

4.3.3.2 Forced swim test (FST)

The FST was performed as previously described in the Study 1B, section 3.4.1.3.2.

4.3.4 Molecular assay

4.3.4.1 Sample collection

Immediately after the FST, the FRL and FSL rats were brought to another room and killed by decapitation without anaesthesia. The prefrontal cortex (PFC), dorsal and ventral hippocampus (DH and VH, respectively) were rapidly dissected, frozen on powered dry ice, and stored at -80ºC until further analysis.

4.3.4.2 Gene expression analysis

4.3.4.2.1 Sample preparation

The mRNA was isolated from dissected brain tissue using High Pure RNA Tissue Kit (Roche Diagnostics; Thermofisher) following the manufacturer instructions with few modifications. The amount of mRNA extracted was measured in a nanophotometer (NanoDrop 1000 Spectrophotometer; Thermo Scientific, USA) and the purity was determined by the ratio between absorbance at 260 and 280 nm (A_{260}/A_{280}). To consider the isolated mRNA appropriate for use, the purity should have an A_{260}/A_{280} value between 2 and 2.2.

4.3.4.2.2 Reverse transcription-specific target amplification (RT-STA)

The reverse transcription-specific target amplification was carried out to synthesize the complementary DNA (cDNA) and, simultaneously, to increase the number of copies from lower expression gene levels (REINERT et al., 2016). First, all the samples were diluted at the same concentration (12.5 ng/µL) and the reaction mix, consisting of a pool of 48 primers, was prepared (0.2x TaqMan assay probe; SuperScript III RT/Platinum Taq Mix; RNA-free water and Cells direct 2X Reaction Buffer). In sequence, the samples and reaction mix were added to standard 96-well plate to perform the PCR reaction. Pre-amplification parameters were 50°C for 15 min, 95°C for 2 min, followed by 12 cycles at 95°C for 15 sec and 60 °C for 4 min (REINERT et al., 2016). Next, the cDNA pre amplified was diluted 1:30 (PFC) and 1:45 (DH and VH) in elution buffer.

4.3.4.2.3 Real-time quantitative PCR using 48.48 dynamic microfluidic array (Fluidigm®)

The gene expression analysis was performed through BioMark™ DH System using TaqMan® Gene Expression Assay. Each PCR reaction contained diluted sample (diluted cDNA (section 1.4.2.2); TaqMan Universal PCR Master Mix (2X); 20X GE Sample Loading Reagent) and assays (TaqMan Gene Expression Assay; 2X Assay Loading Reagent) in duplicate for each gene. Samples without cDNA (no template control, NTC) or without RT enzyme (Minus RT) were included as a negative control. After loading the chip with the samples and assays on specific inlets, the qPCR was conducted following these cycling parameters: 60 sec at 95 °C, followed by 30 cycles at 96 °C for 5 sec and 20°C for 20 sec, and, finally, the melting curve 60 °C for 3 sec (REINERT et al., 2016). The expression of 46 target genes (Bdnf, CamK2a, Cnih2, Cnih3, Dgl4, Eef2k, Gria1, Gria2, Grin1, Grin2a, Grin2b, Grm5, Gsk3b, Homer3, Htr1a, Htr1b, Htr2a, Htr2b, Htr2c, Mapk1, Mtor, Nlgn1, Ntrk2, Pick1, Ppp1r9b, Prkaca, Syn3, Prkcd, S100a10, Slc1a1, Slc1a2, Slc6a4, TRPV1, MgII, Faah, Cnr1, Cnr2, Gpr55, Oprm1, Vegfa, Gad2, Gabrd, Sort1, Ngfr, Syp and Nrnx2) were investigated and essential gene description and Assay ID are given in Table 1.

The data was analyzed using Fluidigm Real-Time PCR Analysis Software (Biomark instrument, Fluidigm Corporation, USA) and an Excel spreadsheet. The expression stability of two reference genes Beta-actin and HPRT1 was compared under the experimental conditions. The target gene levels were analyzed using the $2^{-\Delta\Delta Ct}$ method (LIVAK; SCHMITTGEN, 2001), and HPRT1

(#Rn01527840_ml) was selected as a housekeeping gene (HKG) for all structures. The results were expressed as fold change relative to FRL-VEH group (Control group).

Table 5. Characteristics of gene-specific real-time qPCR primers.

Symbol	Alias	Description	Assay ID[1]
Reference genes			
ActB		Beta-actin	Rn00667869_m1
Hprt1		Hypoxanthine phosphoribosyltransferase 1	Rn01527840_m1
Target genes			
Bdnf	Bdnf exon IV	Brain-derived neurotrophic factor exon (IV)	Rn01484927_m1
Camk2a	CamkIIα	Calcium/calmodulin-dependent protein kinase II alpha	Rn01258147_m1
Cnih2		Cornichon family AMPA receptor auxiliary protein 2	Rn00515551_g1
Cnih3		Cornichon family AMPA receptor auxiliary protein 3	Rn01412227_mH
Dlg4	Psd95	Discs, large homolog 4 (Drosophila)	Rn00571479_m1
Eef2k		Eukaryotic elongation factor 2 kinase	Rn00564087_m1
Gria1	Glur1	Glutamate receptor, ionotropic, AMPA 1	Rn00709588_m1
Gria2	Glur2	Glutamate receptor, ionotropic, AMPA 2	Rn00568514_m1
Grin1	Nr1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	Rn01436038_m1
Grin2A	Nr2a	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A	Rn00561341_m1
Grin2B	Nr2b	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	Rn00680474_m1
Grm5	mGluR5	Glutamate receptor, metabotropic 5	Rn00566628_m1
Gsk3b		Glycogen synthase kinase 3 beta	Rn00583429_m1
Homer3		Homer scaffolding protein 3	Rn00584023_m1
Htr1a	5-ht1a	5-hydroxytryptamine (serotonin) receptor 1A	Rn00561409_s1
Htr1b	5-ht1b	5-hydroxytryptamine (serotonin) receptor 1B	Rn01637747_s1
Htr2a	5-ht2a	5-hydroxytryptamine (serotonin) receptor 2A	Rn00568473_m1
Htr2b	5-ht2b	5-hydroxytryptamine (serotonin) receptor 2B	Rn00568450_m1
Htr2c	5-ht2c	5-hydroxytryptamine (serotonin) receptor 2C	Rn00562748_m1
Mapk1	Erk2	Mitogen activated protein kinase 1	Rn00587719_m1
Mtor		Mechanistic target of rapamycin (serine/threonine kinase)	Rn00693900_m1
Nlgn1		Neuroligin 1	Rn01642900_m1
Ntrk2	Trkb	Neurotrophic tyrosine kinase, receptor, type 2	Rn01441749_m1

Pick1		Protein interacting with PRKCA 1	Rn00584954_m1
Ppp1r9b	Spinophilin	Protein phosphatase 1, regulatory subunit 9B	Rn01464011_m1
Prkaca	Pkα	Protein kinase, cAMP-dependent, catalytic, alpha	Rn01432300_g1
Syn3	Synapsin III	Synapsin 3	Rn01509259_m1
Prkcd	Pkcδ	Protein kinase C, delta	Rn00440891_m1
S100a10	P11	S100 calcium binding protein A10	Rn00821296_g1
Slc1a1	Eaat3	Solute carrier family 1 (glial high affinity glutamate transporter), member 1	Rn00564705_m1
Slc1a2	Eaat2	Solute carrier family 1 (glial high affinity glutamate transporter), member 2	Rn00691548_m1
Slc6a4	Sert	Solute carrier family 6 (neurotransmitter transporter), member 4	Rn00564737_m1
TRPV1	TRPV1	transient receptor potential cation channel, subfamily V, member 1	Rn00583117_m1
MgII	MAGL	monoglyceride lipase	Rn00593297_m1
Faah	FAAH	fatty acid amide hydrolase	Rn00577086_m1
Cnr1	CB1	cannabinoid receptor 1 (brain)	Rn00562880_m1
Cnr2	CB2	cannabinoid receptor 2 (macrophage)	Rn04342831_s1
Gpr55	GPR55	G-protein coupled receptor 55 isoform X2	Rn03037213_s1
Oprm1		opioid receptor, mu 1	Rn00561699_m1
Vegfa		vascular endothelial growth factor A (isoform 2)	Rn01511602_m1
Gad2	GAD65	glutamate decarboxylase 2	Rn00561244_m1
Gabrd	$GABA_A$	gamma-aminobutyric acid (GABA) A receptor, delta	Rn01517017_g1
Sort1	Sortilin 1	Sortilin 1	Rn01521847_m1
Ngfr	P75NTR	tumor necrosis factor receptor superfamily member 16 precursor	Rn00561634_m1
Syp	Synaptophysin	Synaptophysin	Rn00561986_m1
Nrxn2		Neurexin 2	Rn01454541_m1

1 Assay ID according to custom TaqMan™ Gene Expression Assay (Applied Biosystems™).

4.3.4.3 Protein analysis

4.3.4.3.1 Crude synaptosome preparation

The crude synaptosomes were purified according to previous works (KOHTALA et al., 2019; MÜLLER et al., 2013). The PFC, DH and VH samples were homogenized in 10% (w/v) ice-cold sucrose buffer (40 mM HEPES pH 7.4, 2 mM EDTA, 2 X protease inhibitor cocktail (Roche, Mannheim, Germany), 10 mM NaF, 0.64 M sucrose, 2mM Na_3VO_4, and 5 mM Na_2HPO_4) and, subsequently, the mixture was centrifuged. The collected homogenate was centrifuged at 2800 rpm for 10 min at 4 °C, the supernatant removed and put into a new tube for centrifugation (12 000 rpm for 10 min). Subsequently, the supernatant (cytosolic fraction) was removed and the resultant pellet, crude synaptosomal fraction, was resuspended in 50 µL lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 1 X protease inhibitor cocktail, 2 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4, and 5 mM Na_2HPO_4).

4.3.4.3.2 Total protein analysis by bicinchoninic acid (BCA) method

The concentration (µg/µL) of total proteins was determined in each sample using the Pierce BCA Protein Kit (Thermo Scientific, # 23227) following to the manufacturer instructions. Briefly, the synaptosome fraction from each sample was diluted 1:20 in water. Next, 25µL of standard or diluted sample with a concentration within the standard curve (0 to 1000 µg/mL) was individually added to a 96-well plate in duplicate. In sequence, 200µl of BCA solution was added to each well, the plate was gently shaken for 30 seconds, incubated at 37°C for 30 minutes, cooled to RT and read by spectrophotometry at 570nm.

4.3.4.3.3 Western blotting (WB)

After determination of total protein concentration, the samples were diluted to 3000 µg/mL in Bio Plex Cell Lysis Buffer supplemented with protease inhibitor (1:25), Na3VO4 (1:100) and NaF (1:20). Then, sample buffer (lysis buffer supplemented with 125mM dithiothreitol (DTT)) was added to sample. The samples were agitated and heated on heating block 50 °C for 15 minutes, to induce protein denaturation. The polyacrylamide gel (Criterion TGX Precast Gel, 26 well comb, 15

μL, 1.0 mm; Bio Rad) was loaded with prepared samples (10 μL) and standard molecular weight in two different wells. The electrophoresis procedure was conducted for 1h and 30 minutes at 100V. In sequence, the proteins were transferred to Nitrocellulose membrane (Midi format, 0.2 μm nitrocellulose, single application; Bio Rad, #1704159) using the Trans-Blot Turbo system (Bio Rad, USA; 7 minutes, 25V).

Following the sequence, the membrane was washed in TBS buffer and incubated with the blocking solution Odyssey Blocking Buffer (OBB; LICOR Bioscience, # 927-5000) for 1 hour at RT. After, the primary antibody for the following target genes were incubated overnight at 4 °C: Beta-actin; GluR1(phosphorylated and total); GluR2; Nr2a; Nr2b; mGluR5; EAAT3; CaMKII (phosphorylated and total); Sortilin; BDNF; TrkB; ERK1/2 (phosphorylated and total); mTOR (phosphorylated and total); eEF2 (phosphorylated and total); GSK3 (phosphorylated and total); PSD-95; spinophilin; p11 and detailed description of the used antibodies is given in the Table 2. The primary antibodies were diluted in OBB solution in 0.1% TBST (1:2).

On the next day, the membrane was washed in 0.1% TBST buffer (4 times for 5 minutes) and incubated with secondary antibody (Goat anti-mouse: 1:10.000 dilution, Licor, IRDye 680RD; Goat anti-mouse: 1:10.000 dilution, Licor, IRDye 800CW; Goat anti-rabbit: 1:10.000 dilution; Licor; IRDye 680RD; Goat anti-rabbit: 1:10.000 dilution; Licor; IRDye 800CW) diluted in OBB solution in 0.1% TBST plus 0.01% SDS at RT for 1 hour protected from light. Under the protection from the light, the membrane was washed with 0.1 % TBST and TBS, then, scanned for further analyses.

Table 6. Antibodies used for western blotting analysis of proteins involved in glutamatergic, serotoninergic neurotransmission, neurotrophin pathway and synaptic proteins.

MW (kDa)	Primary antibodies
Target proteins	
Glutamatergic neurotransmission	
110	Mouse anti-GluR1 (Millipore MAB2263)(1:1000)
110	Rabbit anti-GluR1(Ser831) (Abcam ab109464)(1:500)
110	Rabbit anti-GluR1(Ser845) (Abcam ab76321)(1:500)
90-110	Rabbit anti-GluR2 (Abcam ab52932)(1:1000)
180-190	Rabbit anti-NMDAR2A (Cell Signaling #4205)(1:1000)
180-190	Rabbit anti-NMDAR2B (Cell Signaling #14544)(1:1000)
150	Rabbit anti-mGluR5 (Abcam ab76316)(1:5000)
50	Rabbit anti-CaMKII (Thr286)(Cell Signaling #3361)(1:1000)
50	Mouse anti-CaMKII (Millipore 05-532)(1:1000)
Neurotrophin signaling	
100	Rabbit anti-Sortilin (ANT 009)(1:500)
14/25	Rabbit anti-BDNF (Abcam ab108319)(1:1000)
90-140	Goat anti-TrkB (R&D AF1494)(1:500)
44/42	Rabbit anti-Erk1/2 (Thr202/Tyr204)(Cell Signaling #4370)(1:1000)
44/42	Mouse anti-Erk1/2(Cell Signaling #9107)(1:1000)
289	Rabbit anti-mTOR (Ser2448)(Cell Signaling #2971)(1:500)
289	Mouse anti-mTOR (Cell Signaling #4517)(1:1000)
95	Rabbit anti-eEF2 (Thr56) (Cell Signaling #2331)(1:500)
95	Mouse anti-eEF2 (Abcam ab131202)(1:4000)
46	Rabbit anti-GSK3beta (Ser9) (Cell Signaling #5558)(1:500)
46	Mouse anti-GSK3beta (Cell Signaling #9832)(1:1000)
Synaptic proteins	
95	Rabbit anti-PSD95 (Cell Signaling #2507)(1:500)
130	Goat anti-Spinophilin (Santa Cruz; sc-14774)(1:200)
Serotoninergic system	
43	Rabbit anti-5HT1b (Alomone ASR-022) (1:400)

10-12	Goat anti-P11 (R&D AF2377)(1:250)
Normalizer proteins	
42	Rabbit anti-actin (Licor 926-42210)(1:3000)
42	Mouse anti-actin (Licor 926-42212)(1:3000)

The fluorescence was analyzed with Image Studio™ Lite Quantification Software (LI-COR Biosciences; Version 5.2) and an Excel spreadsheet. The results of target proteins were normalized by Beta-actin and corresponding total protein for phosphorylated protein. The results were expressed as percentage of control group (FRL vehicle-treated rats).

4.3.4.4 Sample preparation and whole blood CBD measurement by liquid chromatography-tandem mass spectrometry (UPLC+MS).

The CBD was quantified using a validated analytical method, as described previously (SØRENSEN; HASSELSTRØM, 2017, 2018). For sample preparation, the protein was first precipitated with acidic MeCN, following by the removal of phospholipid through filtration a sorbent with Lewis acid properties. Subsequently, 100 µL of whole blood was mixed with 50 µL of MeOH, 50 µL of SIL HIS solution, 5
sequence, 350 µL of 1% FA in MeCN was added to the sample, and 350 µL of the sample suspension was filtered through a HybridSPE plate. 150 µL of the filtered mix was mixed with 10 µL of 10% FA. Finally, 10 µL of the prepared sample was injected directly onto the analytical column. The liquid chromatography system was a Waters Acquity UPLC system that consisted of a binary pump, a flow through needle sample manager set at 10 ± 2°C and a column oven set at 45 ± 2°C (Waters, Milford, MA, USA). The mass spectrometer was a Waters Xevo TQS triple quadrupole instrument with an ESI ion source. The separation was performed using an Acquity UPLC HSS C18 column (1.8 µm, 2.1 mm I.D. × 100 mm; Waters, Milford, MA, USA). The lower limit of quantification was 0.2 µg/L for CBD.

4.3.4.5 Experimental design

The FSL and FRL rats were brought to the experimental room 1h before the start of the experiment and allowed to habituate. After that period, FRL rats received a systemic injection with VEH. FSL rats were treated with VEH, ketamine (15 mg/Kg i.p.) or CBD (30 mg/Kg i.p.). Fifty minutes later, the rats were exposed to OFT (5 min) and after that the FST (10 min). Immediately after the tests, the rats were decapitated without prior anesthesia. Whole blood was collected from the trunk and following brain extraction, the PFC, DH, and VH were rapidly dissected. The brain structures were immediately frozen on dry ice and all samples were stored at -80°C until further analysis.

An independent experiment was performed according to the same experimental design to collect the brain samples (PFC, DH, and VH) for protein analysis. Total of 66 animals was used in the present study, consisting of 50 FSL and 16 FRL rats.The complete experimental design is depicted in Figure 15.

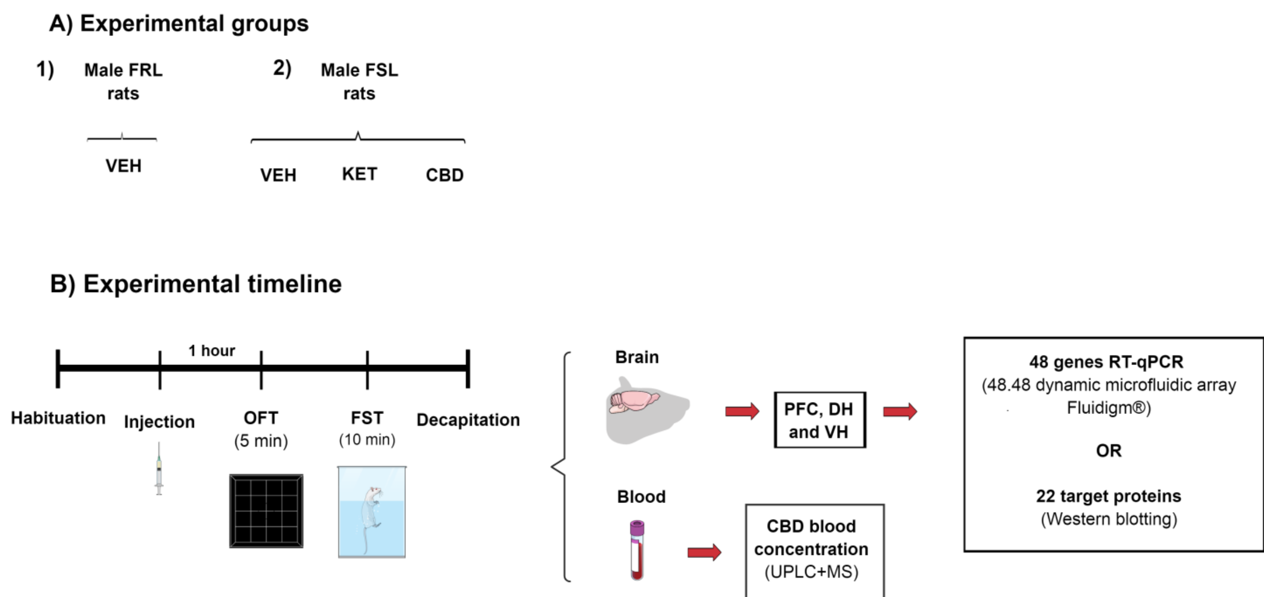

Figure 15. Experimental protocol used to evaluate the effect of the treatment with CBD and ketamine on mRNA and protein levels in the prefrontal cortex, dorsal and ventral hippocampus of FSL and FRL rats. (A) Experimental groups. (B) Experimental timeline. One hour after habituation period, FRL rats were treated with VEH, and FSL treated with VEH, S-ketamine (15 mg/kg), and CBD (30 mg/kg) 1 hour before the exposition to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis of the transcripts levels by real-time qPCR (48.48 dynamic microfluidic array) and proteins by western blotting. CBD, Cannabidiol; FST, Forced swimming test; KET, S-Ketamine; OFT, open field test; VEH, vehicle.

4.3.4.6 Data analysis and statistical analysis

The analysis of immobility time (s), total distance traveled (m), relative mRNA levels (Fold change relative to FRL-VEH group) and protein levels (percentage of the control (FRL-VEH group) data were performed using the following tests: i) Student's *t*-test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; ii) One-way analysis of variance (ANOVA) followed by Fisher's LSD post hoc test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. When variances between the groups were not homogenous the following statistical analysis was applied, Mann-Whitney (for comparisons between FSL and FRL vehicle-treated groups) or Kruskal-Wallis followed by Dunn's post hoc test (to compare between FSL treated with VEH, KET, and CBD) was carried out. Results in the graphs are expressed as mean ± standard error of the mean (SEM). The correlation analysis was performed between the CBD blood concentration and immobility time (s). The *p*-value indicates a significant difference between the groups was 5% ($p<0.05$). The *p*-value between 0.05 and 0.1 was interpreted as a statistical trend (TILLMANN et al., 2019). Statistical analyses and the graphs were created using GraphPad Prism 9.0.0 version for Windows (GraphPad Software Inc., San Diego, CA, USA).

4.4 RESULTS AND DISCUSSION

4.4.1 Behaviour, CBD blood concentration and general aspects

4.4.1.1 Antidepressant-like effect produced by CBD in FSL rats submitted to FST

As expected, FSL rats treated with VEH displayed significantly increased immobility time in comparison to FRL rats treated with vehicle (Mann-Whitney test: U= 2; p= 0.0008; Figure 16A), and significantly higher locomotion (Student's *t*-test: t(15)= 4.458; *p*= 0.005; Figure 16B). As previously shown, CBD and KET significantly reduced the immobility time of FSL rats (One-way ANOVA: F(2, 22)=15.61; *p*< 0.0001; Fisher's LSD test: CBD 30 mg/kg, *p*= 0.0189; KET, *p*< 0.0001; Figure 16A), producing an antidepressant-like effect. None of drug treatments changed the locomotion of FSL rats in the OFT (One-way ANOVA: F(2, 23)=0.1848; *p*= 0.8325; Figure 16B).

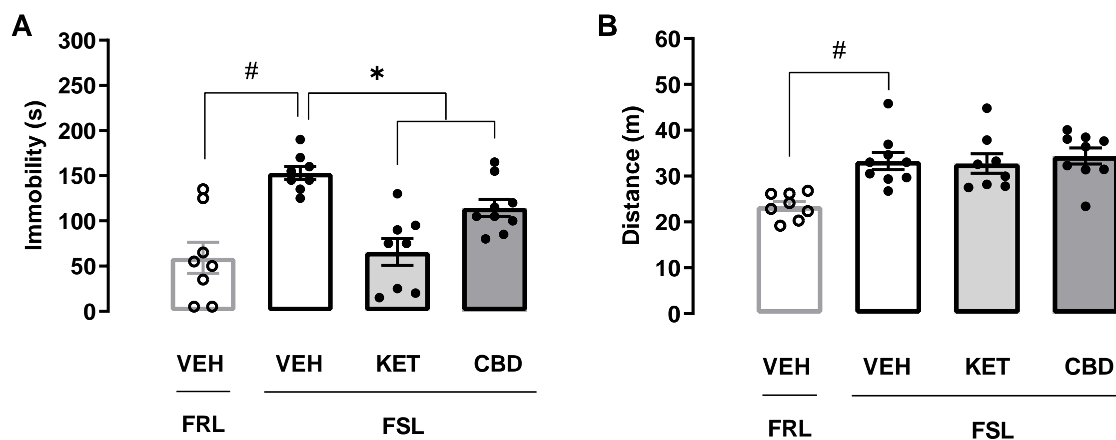

Figure 16. Effect of CBD and ketamine in male FSL rats submitted to FST (A) and OFT (B). FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposition to FST and OFT, the immobility time and traveled distance were registered. Bars represent the immobility time (s) in FST or the traveled distance (m) in the OFT. Values are mean ± SEM; Hash indicates significant differences between FSL and FRL vehicle-treated groups (#p< 0.05, Student's t-test or Mann-Whitney test); Asterisks represents significant treatment difference from FSL control (*p< 0.05; One-way ANOVA followed by Fisher's LSD post hoc test), n= 9-8 animals/group.

4.4.1.2 Correlation between the CBD blood levels and behaviour displayed in FST

A correlation analysis was performed to check whether there is a relationship between the concentration of CBD in the blood and the behaviour displayed by the FSL rats in the FST. As it

can be seen in Figure 17, there is no statistically significant correlation between the CBD blood level and immobility time (Correlation: r=0.1299; *p*=0.7390).

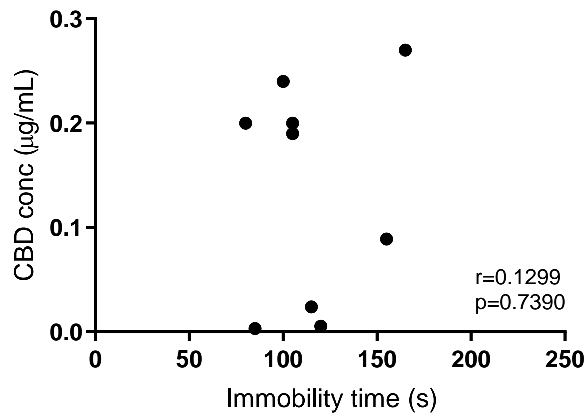

Figure 17. Correlation analysis between the blood concentration of CBD and immobility time in FSL rats. Graph representation of blood concentration of CBD (µg/mL) versus immobility time (s) exhibited by FSL rats treated with CBD (30 mg/kg) submitted to FST.

4.4.1.3 General aspects of gene expression and protein levels changes induced by CBD and KET in the PFC, DH and VH of FSL/FRL rats.

To ensure uniformity of gene expression results and reduce noise in the results, two rats with higher immobility time from FSL CBD-treated group were removed from the transcript analysis according to previously depicted in Figure 17. Furthermore, in order to focus the presentation of this work, gene expression results from targets genes related to serotoninergic (5-HT1A; 5-HT1B; 5-HT2A; 5-HT2B; 5-HT2C; Slc6a4), endocannabinoid neurotransmission (MAGL; FAAH; CB1; CB2; GPR55) and other genes (diverse genes; Opmr1; GAD65; GABAA) are not presented in the current work. The gene p75 neurotrophic factor (p75NTR) was not satisfactorily amplified for all brain regions (PFC, DH, and VH) in the gene expression analysis.

4.4.1.4 Housekeeping gene analysis

The housekeeping genes beta-actin and HPRT1 were selected to analyze the data obtained from the gene expression analysis, as we observed a tendency to decreased levels of Beta-Actin in the PFC of FSL-VEH rats in comparison to FRL-VEH (Student t-test: t(15)= 1.857; *p*= 0.083;

Figure 18D). On the other hand, the drug treatment and strain of rats did not affect HPRT1 relative levels in all the brain regions investigated, PFC, DH and VH (Student's t-test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. PFC: HPRT1: $t(14)= 1.054$; $p= 0.3098$; $F(2, 22)=0.9108$; $p= 0.4168$; Beta-actin: $t(15)= 1.857$; $p= 0.083$; $F(2, 23)=0.4537$; $p= 0.6409$. DH: HPRT1: $t(15)= 1.218$; $p= 0.2422$; $F(2, 23)=2.159$; $p= 0.1383$; Beta-actin: $t(15)= 1.086$; $p= 0.2448$; $F(2, 23)=1.137$; $p= 0.3380$. VH: HPRT1: $t(15)= 0.3543$; $p= 0.7280$; $F(2, 23)=2.237$; $p= 0.1295$; Beta-actin: $t(15)= 0.843$; $p= 0.4124$; $F(2, 23)=1.250$; $p= 0.3054$; Figure 18). Therefore, the HPRT1 was selected as a reference gene to use to normalize the data from gene expression analysis for all brain regions.

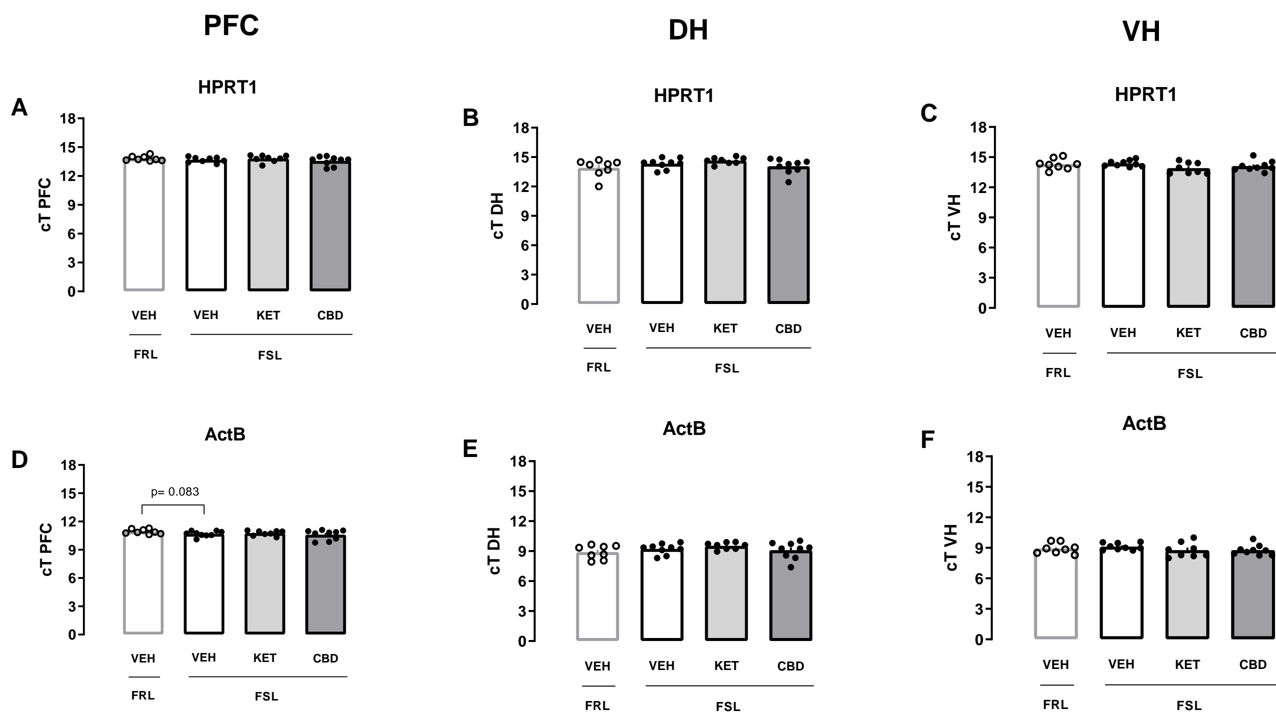

Figure 18. Housekeeping gene analysis in prefrontal cortex, dorsal, and ventral hippocampus. Ct value for HPRT1 and Beta-actin expression in PFC (A, D), DH (B, E) and VH (C, F) from FSL rats treated with VEH, ketamine (15 mg/kg), or CBD (30 mg/kg) via i.p. one hour before exposition to FST/OFT. Bars represent the cT values. Values are mean ± SEM; n= 8-9 animals/group. ActB: Beta-actin; DH: Dorsal hippocampus; HPRT1: Hypoxanthine phosphoribosyltransferase 1; PFC: Prefrontal cortex; VH: Ventral hippocampus.

4.4.1.5 Discussion

In agreement with our previous results shown in Study 1B, FSL vehicle-treated rats displayed increased immobility compared to FRL controls in the FST, suggesting a depressive-like

phenotype of this strain. Importantly, this behaviour cannot be attributed to reduced locomotor activity, since FSL rats displayed a longer traveled distance in the open field test compared to FRL rats. Furthermore, single injection of both CBD (30 mg/Kg) or ketamine (15 mg/Kg) significantly reduced the immobility time in FSL rats, indicating an antidepressant-like effect, as also reported earlier. Interestingly, we failed to observe any correlation between the behaviour displayed in the FST and CBD levels in the blood. Such information could be of relevance, given the physio-chemical properties of CBD, being very difficult to dissolve in an aquous solution. As reviewed elsewhere, the effective doses of CBD used in the literature varies enormously (5-200 mg/kg), probably reflecting differences in bioavailability in the different drug-preparations used in the studies (SILOTE et al., 2019).

Our average blood concentration of CBD was 0.136 µg/mL one hour after administration, contrasting a previous study which found that the maximum plasma concentration of 2.4-2.6 µg/mL 2 hours after the administration (DEIANA et al., 2012a). However, in the mentioned study, th evaluation was done at another time point (2 h vs 1 h), and CBD was diluted using another vehicle (Cremophor and solutol), compared to ours (sterile saline and 3% polysorbate 80 (Tween® 80)). The reason we failed to demonstrate any correlation remains obscure. However, it should be noted that CBD have a very complex metabolism, which involve several active metabolites, as well as tissue compartments (LUCAS; GALETTIS; SCHNEIDER, 2018; MILLAR et al., 2018). While it is generally believed that the behavioural response induced by CBD treatment will depend on the CBD brain concentration achieved to act on its putative molecular targets (CALAPAI et al., 2020; DEIANA et al., 2012a; MILLAR et al., 2020), it cannot be excluded that other important pharmacokinetic and pharmacodynamic factors plays a major role. As we used all brain samples collected to perform molecular and biochemical analysis, it was unfortunately not possible also to assess levels of CBD in the brain. This remains to be investigated in future studies.

4.4.2 Analysis of mRNA and protein levels in prefrontal cortex (PFC)

The following section is stratified based on clusters with overlapping biology.

4.4.2.1 Glutamatergic neurotransmission

4.4.2.1.1 Relative mRNA analysis

On Figure 19, one-way ANOVA revealed a trend for difference between drug treatments in the transcript levels of EAAT3 (Excitatory amino acid transporter type 3) ($F (2, 20) = 2.958$; $p = 0.0749$) and the post-test showed that a significant increase in the transcript levels of CBD treated rats in the PFC (Fisher's LSD test: $p=0.0245$; Figure 19I). Moreover, one-way ANOVA indicated a tendency for difference between treatment in the mRNA levels of Pick1 (One-way ANOVA: $F(2, 20)= 2.615$; $p= 0.098$) and the post-test showed that a significant reduction in CBD treated animals (Fisher's LSD test: $p= 0.0348$; Figure 19N). However, no significant difference between the rat strains was found in the mRNA levels of EAAT3 and Pick1 (FSL vs FRL vehicle treated groups; EAAT3: $U=17$; $p=0.2204$; Figure 5I; Pick1: $t(13)= 0.6762$; $p= 0.5108$; Figure 19D).

Furthermore, one-way ANOVA indicated a tendency for difference between treatments in the mRNA levels of Nr2A (NMDA receptor subunit) ($F(2, 17)=3.020$; $p= 0.0755$) and the post-test showed that a significant increase in this transcript level in ketamine-treated rats and a tendency to increase with CBD treatment in the same limbic region (Fisher's LSD test: KET, $p= 0.0429$; CBD, $p= 0.0580$; Figure 19F). A Mann-Whitney test revealed a significant reduction of Nr2a ($U=9$; $p=0.0289$; Figure 19F) and EAAT2 ($U=10.50$; $p=0.0221$; Figure 19J) mRNA levels in FSL-vehicle rats compared to their counterparts. Student's t-test showed a significant enhancement on GluR2 transcript levels, a subunit of AMPA receptor, in comparison to FRL-vehicle rats ($t(13)= 2.351$; $p= 0.0352$; Figure 19D).

On the other hand, the drug treatment and strain of rats did not affect the Cnih2, Cnih3, GluR1, Nr1, Nr2b, mGluR5, CaMKII, PKA, and PKC relative levels in PFC (Student’s t-test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. Cnih2: $t(13)= 0.8158$; $p= 0.4293$; $F(2, 17)=1.572$; $p= 0.2363$; Cnih3: $t(14)= 0.07561$; $p= 0.9408$; $F(2, 19)=0.5921$; $p= 0.5389$; GluR1: $t(13)= 0.791$; $p= 0.4431$; $F(2, 17)=1.178$; $p= 0.3318$; Nr1: $t(14)= 0.8081$; $p= 0.4326$; $F(2, 20)=0.8599$; $p= 0.785$; Nr2b: $t(13)= 0.7140$; $p= 0.4878$; $F(2, 20)=0.8014$; $p= 0.4626$; mGluR5: $t(14)= 0.3716$; $p= 0.7158$; $F(2, 20)=0.02681$; $p= 0.9736$; CaMKII: $t(13)= 0.3241$; $p= 0.751$; $F(2, 18)=2.128$; $p= 0.1481$; PKA: $t(14)= 0.1501$; $p= 0.8828$; $F(2, 20)=0.01507$; $p= 0.9851$; PKC: $t(14)= 0.5476$; $p= 0.5926$; $F(2, 19)=0.5627$; $p= 0.5789$; Figure 19).

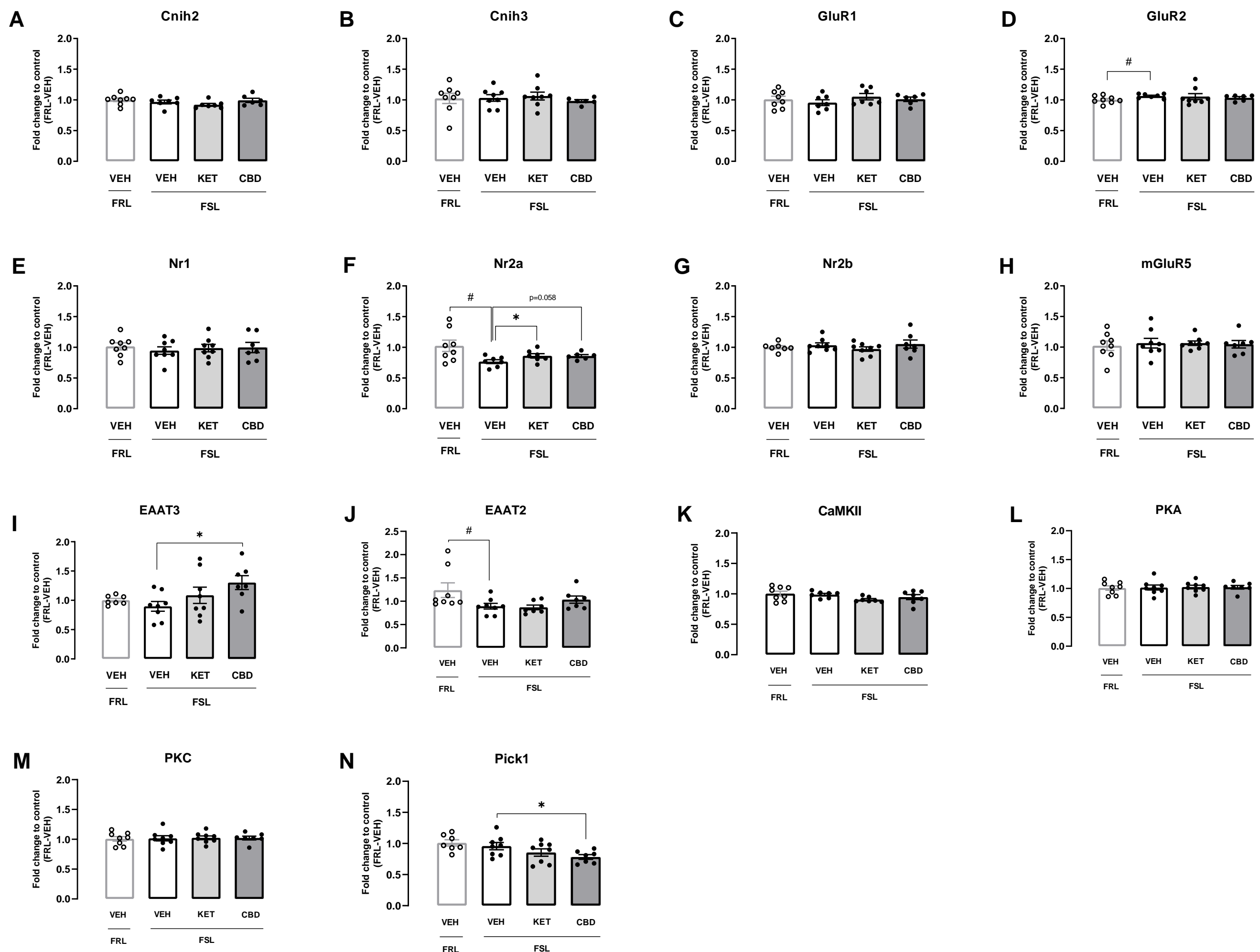

Figure 19. Effect of CBD and ketamine on relative mRNA levels of genes related to glutamatergic neurotransmission in the prefrontal cortex of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposure to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent relative mRNA levels of Cnih2 (A); Cnih3 (B); GluR1 (C); GluR2 (D); Nr1 (E); Nr2a (F); Nr2b (G); mGluR5 (H); EAAT3 (I); EAAT2 (J); CaMKII (K); PKA (L); PKC (M); Pick1 (N) in PFC expressed as fold change to control group (FRL-VEH). Values are mean ± SEM; hash indicate significant differences between FSL and FRL vehicle-treated groups (#p< 0.05, Mann-Whitney test); Asteriks represents significant treatment difference from FSL control (*p< 0.05; One-way ANOVA followed by Fisher's LSD post-hoc test), n= 8-6 animals/group.

4.4.2.1.2 Protein analysis

A one-way ANOVA test showed that both drugs, CBD and ketamine, increased the levels of Nr2b in the PFC compared to FSL-VEH group (Figure 20, $F(2, 15)= 3.713$; $p= 0.0490$; Fisher LSD test: CBD: $p= 0.0243$; KET: $p= 0.0453$; Figure 20E). Student's t-test did not show significant

difference between the condition (FSL vs FRL vehicle treated groups; t(10)= 0.2381; *p*= 0.8166; Figure 20E).

Student's t-test showed that FSL-VEH had low phospho GluR1(pGluR1(S831)) levels (t(9)= 4.940; *p*= 0.0008; Figure 20A) compared to FRL-VEH group, and Kruskal-Wallis test showed that ketamine increased the levels of pGluR1(S831) (Kruskal-Wallis: H(3)= 7.840; *p*=0.0114; Dunn's test: *p*=0129; Figure 20A), reversing the strain effect. Moreover, the Kruskal-Wallis test showed that ketamine had a tendency to increase the Nr2a protein levels (Kruskal-Wallis: H(3)= 6.974; *p*=0.0222; Dunn's test: *p*=0.0509; Figure 20D) compared to FSL vehicle-treated. In contrast, Student's t-test did not show significant difference in the Nr2a levels compared to its counterpart (t(10)= 0.04872; *p*= 0.9621; Figure 20D).

In addition, Student's t-test showed that FSL-VEH had increased levels of GluR2 (t(13)= 2.351; *p*= 0.0352; Figure 20C) and decreased phospho CaMKII (t(13)= 2.351; *p*= 0.0352; Figure 20G) compared to FRL vehicle-treated animals. Otherwise, no differences between the condition (FRL vs FSL vehicle treated groups) and the treatment (FSL-vehicle vs FSL-ketamine and FSL-CBD) on the protein levels of phospho GluR1 (pGluR1(S845) and EAAT3 were found (Student's t-test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. pGluR1(S845): t(10)= 0.9488; *p*= 0.3651; F(2, 14)=1.622; *p*= 0.2325; EAAT3: t(9)= 0.1169; *p*= 0.9095; F(2, 14)=1.393; *p*= 0.288; Figure 20).

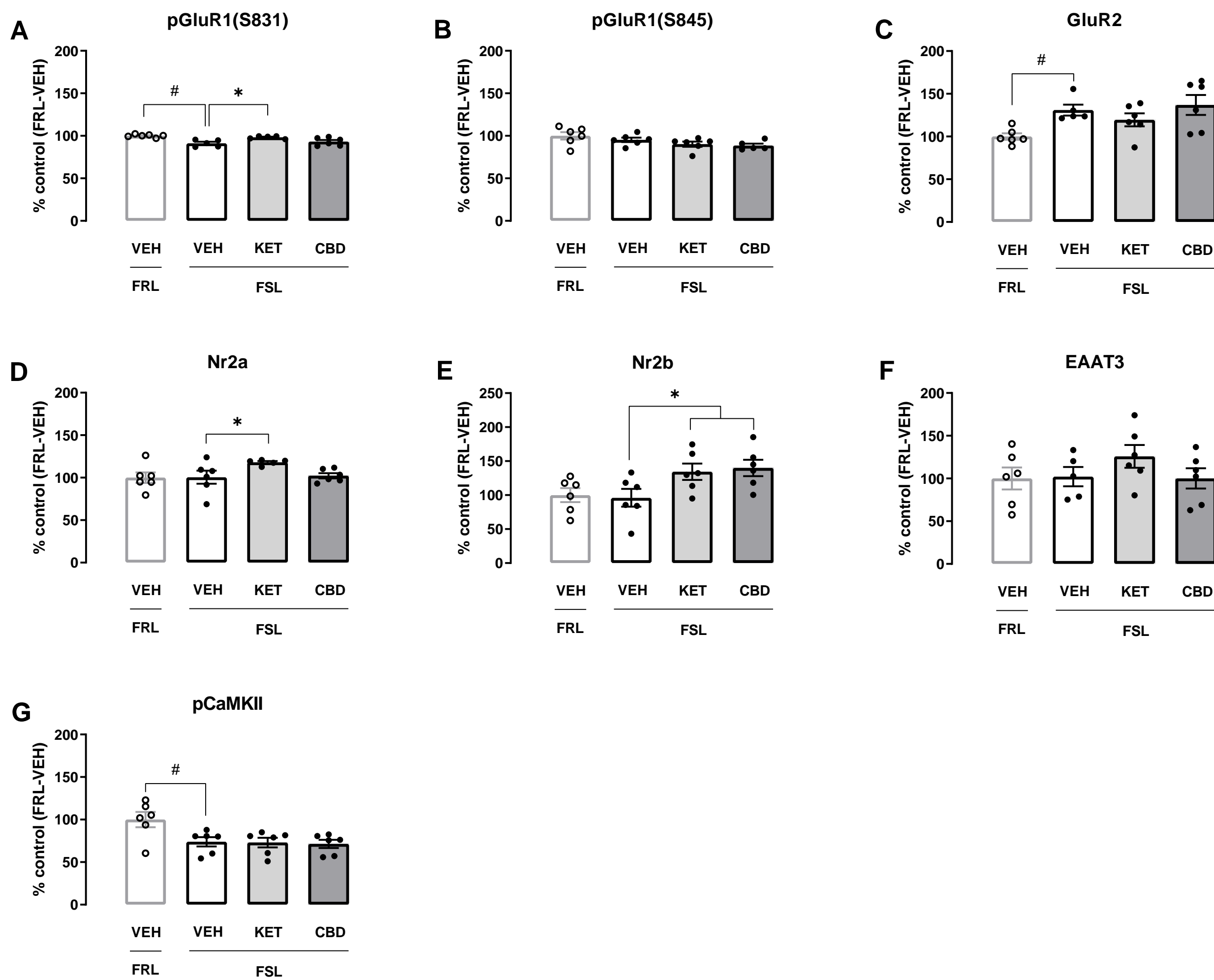

Figure 20. Effect of CBD and ketamine on the **protein levels** related to **glutamatergic neurotransmission in the prefrontal cortex of FSL rats**. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposure to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent fluorescence levels of phospho GluR1(S831) normalized by GluR1 (A); phospho GluR1(S845) normalized by GluR1 (B); GluR2 normalised by actin (C); Nr2a normalised by actin (D); Nr2b normalised by actin (E); EAAT3 normalised by actin (F); phospho CaMKII normalized by CaMKII(G) in PFC expressed as percentage of the control group (FRL-VEH). Values are mean ± SEM; hash indicate significant differences between FSL and FRL vehicle-treated groups (#p< 0.05, Student's t-test); Asteriks represents significant treatment difference from FSL control (*p< 0.05; One-way ANOVA followed by Fisher's LSD post-hoc test and Kruskal-Wallis followed by Dunn's post-hoc test), n= 6-5 animals/group.

The relative transcript and protein levels related to glutamatergic neurotransmission were investigated in the PFC of FSL rats treated with acute systemic injection of CBD (30 mg/kg) and ketamine (15 mg/kg), at dose able to induce the antidepressant-like effect in FST. The main alterations in the glutamatergic system found in PFC are summarized in Table 7.

Table 7. Summary of the effects induced by CBD and ketamine acute treatment on transcript and protein levels related to glutamatergic neurotransmission in the prefrontal cortex of FSL rats.

Molecular Analysis	*Transcripts and proteins of interest*	*FSL-VEH*	*FSL-KET*	*FSL-CBD*
Relative mRNA Levels	Cnih2	NA	NA	NA
	Cnih3	NA	NA	NA
	GluR1	NA	NA	NA
	GluR2	↑ (#p=0.0352)	NA	NA
	Nr1	NA	NA	NA
	Nr2a	↓ (#p=0.0289)	↑ (*p=0.0429)	↑ (*p=0.0580)
	Nr2b	NA	NA	NA
	mGluR5	NA	NA	NA
	EAAT3	NA	NA	↑ (*p=0.0245)
	EAAT2	↓ (#p=0.0221)	NA	NA
	CaMKII	NA	NA	NA
	PKA	NA	NA	NA
	PKC	NA	NA	NA
	Pick1	NA	NA	↓ (*p=0.0348)
Protein levels	pGluR1 (S831)	↓ (#p=0.0008)	↑ (*p=0.0129)	NA
	pGluR1 (S845)	NA	NA	NA
	GluR2	↑ (#p=0.0352)	NA	NA
	Nr2a	NA	↑ (*p=0.0129)	NA
	Nr2b	NA	↑ (*p=0.0129)	↑ (*p=0.0129)
	EAAT3	NA	NA	NA
	pCaMKII	↓ (#p=0.0352)	NA	NA

#: Significant difference presented by FSL-VEH rats compared to FRL-VEH. *: Significant difference induced by treatment with KET or CBD compared with FSL-VEH. ↓: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present lower levels compared to their respective control groups, FRL-VEH and FSL-VEH; ↑: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present higher levels compared to their respective control groups, FRL-VEH and FSL-VEH; NA: No significant alteration was observed.

4.4.2.2 Neurotrophin signaling

4.4.2.2.1 Relative mRNA analysis

Mann-Whitney test revealed that VEGF transcript levels was increased in FSL rats treated with vehicle in comparison to FRL vehicle in the PFC (Mann-Whitney test: U=10; *p*=0.019; Figure 21C). Interestingly, one-way ANOVA followed by Fisher's LSD test revealed that KET treatment reversed the strain effect in FSL rats, reducing VEGF mRNA levels in the same brain region (One-way ANOVA: F(2, 20)=4.393; p= 0.0262; Fisher's LSD test: p= 0.0077; Figure 21C).

Besides, Student's t-test indicated that FSL-vehicle showed a significant reduction of BDNF (Student's *t*-test: t(14)= 2.445; *p*= 0.0283; Figure 21B) and GSK3B (Student's *t*-test: t(14)= 2.461; *p*= 0.0274; Figure 21F), and a tendency to decreased TrkB mRNA levels (Student's *t*-test: t(14)= 2.017; *p*= 0.0633; Figure 21I) in the PFC in comparison to FRL-vehicle rats. None of the drug treatment significantly changes the mRNA levels of these genes (One-way ANOVA test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. BDNF: F(2, 17)=1.572; *p*= 0.2363; GSK3B: F(2, 17)=1.572; *p*= 0.2363; TrkB: F(2, 17)=1.572; *p*= 0.2363).

On the other hand, the drug treatment and strain of rats did not affect the Sort1, Eef2K, Mapk1, and mTOR relative mRNA levels in PFC (Student's t-test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA or Kruskal-Wallis test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. Sort1: t(14)= 0.1598; *p*= 0.8753; F(2, 20)=0.0534; *p*= 0.9481; Eef2K: t(14)= 0.07024; *p*= 0.945; F(2, 20)=1.463; *p*= 0.2552; Mapk1: t(14)= 1.593; *p*= 0.1334; F(2, 20)=0.7587; *p*= 0.4813; mTOR: t(13)= 0.2069; *p*= 0.8393; H(3)=5.247; *p*= 0.0685; Figure 21).

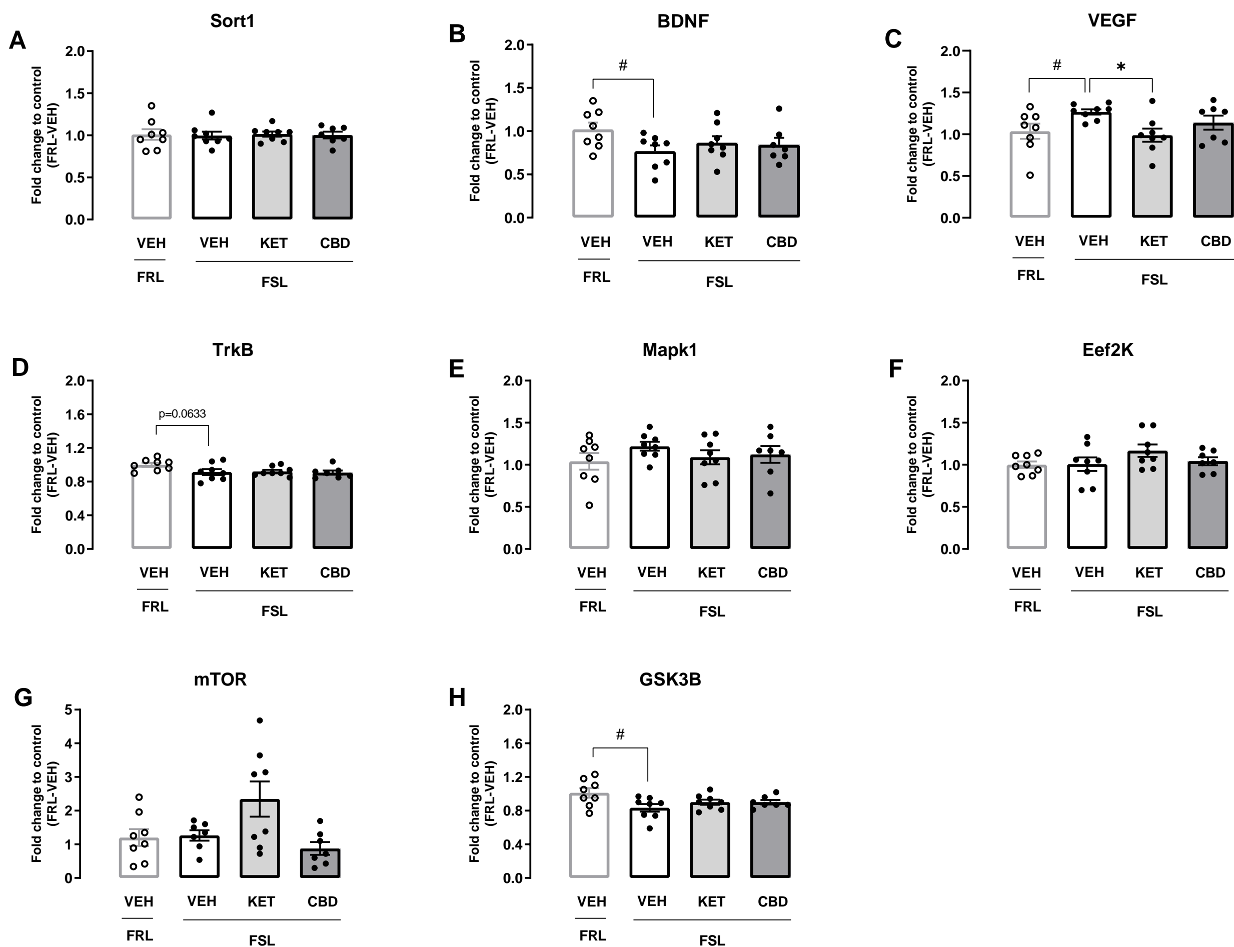

Figure 21. Effect of CBD and ketamine on relative mRNA levels of genes related to neurotrophic signaling in prefrontal cortex of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposure to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent relative mRNA levels of Sort1 (A); BDNF (B); VEGF (C); TrkB (D); Mapk1 (E); Eef2k (F); mTOR (G) and GSK3 (H) in PFC expressed as fold change to control group (FRL-VEH). Values are mean ± SEM; hash indicate significant differences between FSL and FRL vehicle-treated groups (#p< 0.05, Student's t-test or Mann-Whitney test); Asteriks represents significant treatment difference from FSL control (*p< 0.05; One-way ANOVA followed by Fisher LSD post-hoc test), n= 8-7 animals/group.

4.4.2.2.2 Protein analysis

Kruskal-Wallis test showed that CBD increased the levels of BDNF (Kruskal-Wallis: H(3)= 6.889; p=0.0249; Dunn's test: p=0.0401; Figure 22B), and ketamine decreased the levels of sortilin in the PFC (Kruskal-Wallis test: H(3)= 7.729; p=0.0128; Dunn's test: p=0.0110; Figure 22A). However, Student's t-test did not show significant difference between the condition for both

proteins (FSL vs FRL vehicle treated groups; BDNF: (t(10)= 0.2063; *p*= 0.8407; Fig. 11B; Sortilin: t(10)= 0.5357; *p*= 0.6039; Figure 22A)

Furthermore, FSL-VEH group showed a reduction in the levels of phospho mTOR (Student's t-test: t(10)= 2.432; *p*= 0.0354; Figure 22F) and increased levels of phospho ERK1 (Mann-Whitney test: U=3; *p*= 0.0303; Figure 22D) compared to FRL vehicle-treated. However, none of the drug treatments significantly change these proteins level in this brain structure (One-way ANOVA test: pmTOR: F(2, 15)=0.6743; *p*= 0.5243; pERK1: F(2, 14)=0.71; *p*= 0.5085).

Conversely, no differences between the strain (FRL vs FSL vehicle treated groups) and the treatment (FSL-vehicle vs FSL-ketamine and FSL-CBD) on the protein levels of TrkB, phospho ERK2, phospho eEF2, and phospho GSK3 were found in the PFC (Student's t-test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA or Kruskal-Wallis tests to compare differences between FSL-VEH and FSL-KET or FSL-CBD. TrkB: t(10)= 0.3448; *p*= 0.7374; H(3)=0.8706; *p*= 0.6689; pERK2: t(10)= 1.138; *p*= 0.2816; F(2, 15)=1.812; *p*= 0.1974; peEF2: t(10)= 0.7138; *p*= 0.2816; F(2, 15)=1.647; *p*= 0.2256; pGSK3: t(10)= 1.155; *p*= 0.2749; F(2, 15)=2.815; *p*= 0.0916; Figure 22).

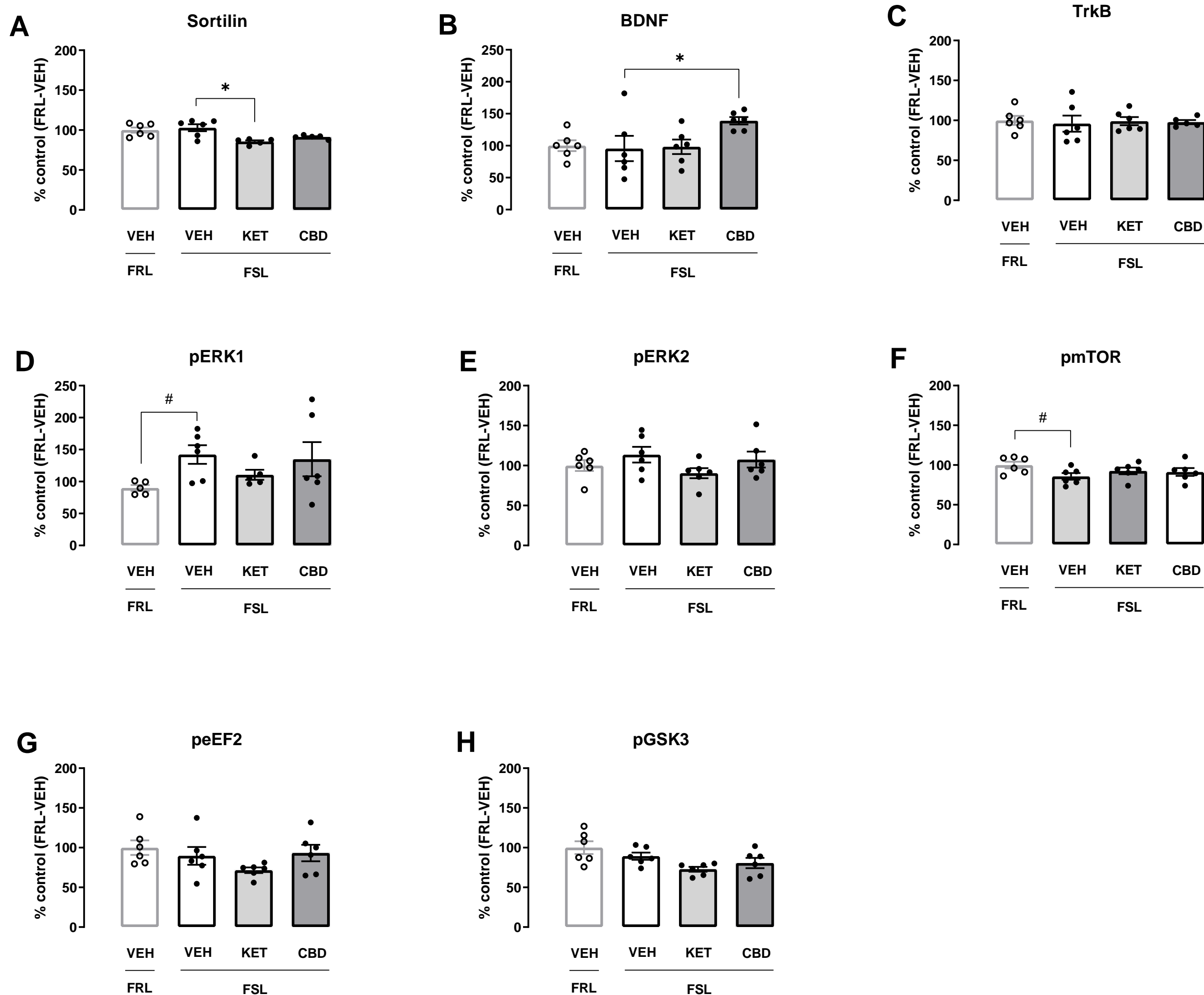

Figure 22. Effect of CBD and ketamine on the protein levels related to neurotrophin signaling in prefrontal cortex of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposition to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent fluorescence levels of sortilin normalized by actin (A); BDNF normalized by actin (B); TrkB normalized by actin (C); phospho ERK1 normalized by ERK1 (D); phospho ERK2 normalized by ERK2 (E); phospho mTOR normalized by mTOR (F); phospho eEF2 normalized by eEF2 (G); phospho GSK3 normalized by GSK3(H) in PFC expressed as percentage of the control group (FRL-VEH). Values are mean ± SEM; hash indicate significant differences between FSL and FRL vehicle-treated groups (#p< 0.05, Mann-Whitney test); Asteriks represents significant treatment difference from FSL control (*p< 0.05; One-way ANOVA followed by Fisher's LSD post-hoc test), n= 6-5 animals/group.

The main changes in neurotrophic signaling pathway found in PFC are summarized in Table 8.

Table 8. Summary of the effects induced by CBD and ketamine acute treatment on transcript and protein levels related neurotrophic signaling pathway in prefrontal cortex of FSL rats.

Molecular Analysis	*Transcripts and proteins of interest*	*FSL-VEH*	*FSL-KET*	*FSL-CBD*
Relative mRNA Levels	Sort1	NA	NA	NA
	BDNF	↓ (#p=0.0283)	NA	NA
	Vegf	↑ (#p=0.019)	↓ (*p=0.0077)	NA
	TrkB	↓ (#p=0.0633)	NA	NA
	Mapk1	NA	NA	NA
	Eef2k	NA	NA	NA
	mTOR	NA	NA	NA
	GSK3	↓ (#p=0.0274)	NA	NA
Protein Levels	Sortilin	NA	↓ (*p=0.0128)	NA
	BDNF	NA	NA	↑ (*p=0.0401)
	TrkB	NA	NA	NA
	pERK1	↑ (#p=0.0303)	NA	NA
	pERK2	NA	NA	NA
	pmTOR	↓ (#p=0.0354)	NA	NA
	peEF2	NA	NA	NA
	pGSK3	NA	NA	NA

#: Significant difference presented by FSL-VEH rats compared to FRL-VEH. *: Significant difference induced by treatment with KET or CBD compared with FSL-VEH. ↓: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present lower levels compared to their respective control groups, FRL-VEH and FSL-VEH; ↑: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present higher levels compared to their respective control groups, FRL-VEH and FSL-VEH; NA: No significant alteration was observed.

4.4.2.3 Synaptic proteins

4.4.2.3.1 Relative mRNA analysis

On Figure 23, for the synaptic proteins target genes, neither the drug treatment nor the strain of rats affect the Homer3, PSD-95, Neuroligin, Neurexin 2, Spinophilin, Syn3 and Syp relative levels in PFC (Student's *t*-test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. Homer3: $t(13)= 0.9714$; $p= 0.3491$; $F(2, 17)=1.443$; $p= 0.2637$; PSD-95: $t(14)= 1.074$; $p= 0.3009$; $F(2, 19)=1.8$; $p= 0.1924$; Neuroligin: $t(14)= 0.5651$; $p= 0.5809$; $F(2, 20)=0.1235$; $p= 0.8845$; Neurexin 2: $t(14)= 1.175$; $p= 0.2596$; $F(2, 20)=0.01217$; $p= 0.9879$; Spinophilin: $t(14)= 0.7137$; $p= 0.4871$; $F(2, 20)=0.7066$; $p= 0.5052$; Syn3: $t(14)= 0.991$; $p= 0.3385$; $F(2, 20)=0.06721$; $p= 0.9352$; Syp: $t(14)= 0.2359$; $p= 0.8169$; $F(2, 20)=0.5913$; $p= 0.563$; Figure 23).

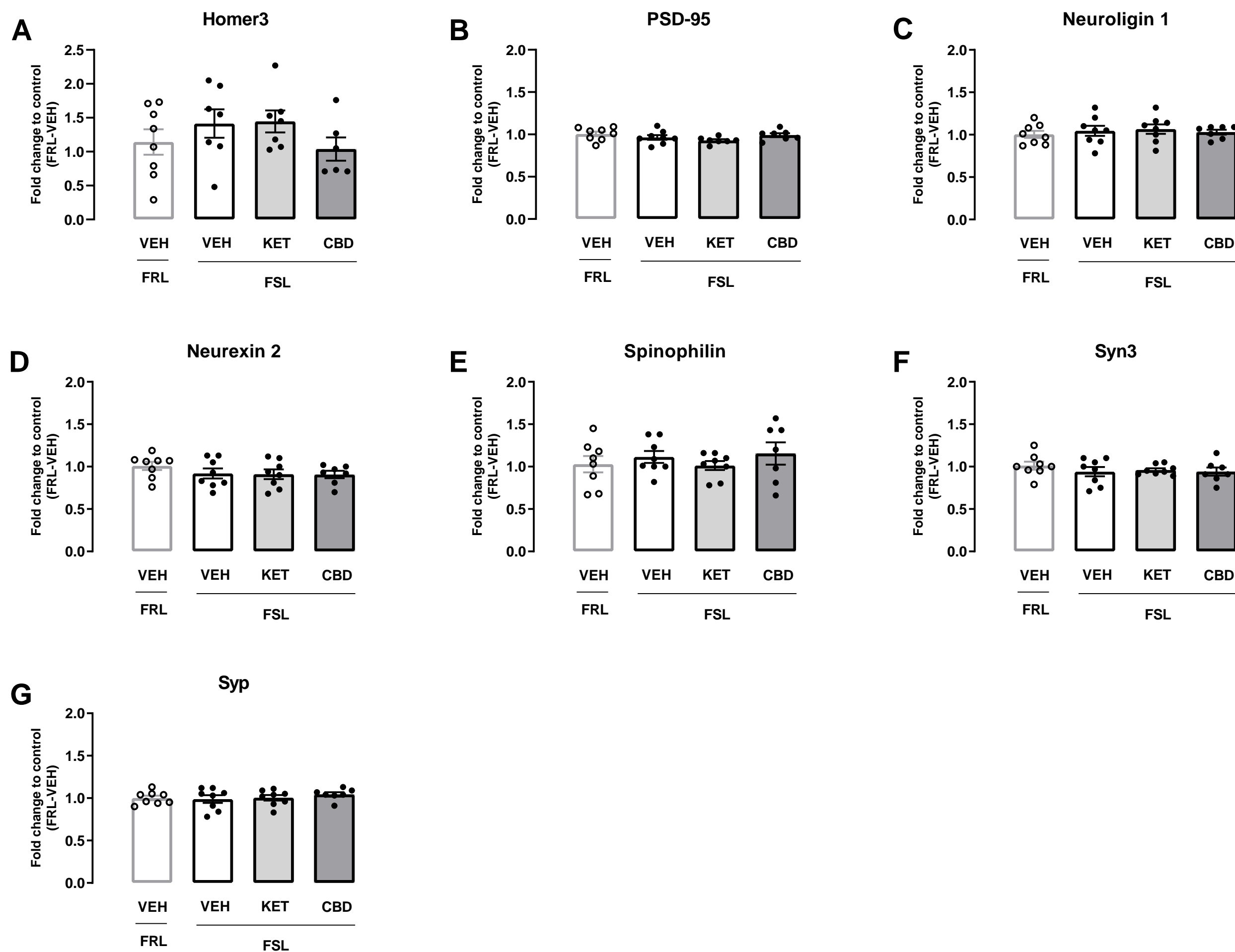

Figure 23. Effect of CBD and ketamine on relative mRNA levels of genes related to synaptic protein in prefrontal cortex of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposure to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent relative mRNA levels of Homer3 (A); PSD-95 (B); Neuroligin (C); Neurexin 2 (D); Spinophilin (D); Syn3 (F) and Syp (G) in PFC expressed as fold change to control group (FRL-VEH). Values are mean ± SEM; Asteriks represents significant treatment difference from FSL control (*p< 0.05; One-way ANOVA followed by Fisher LSD post-hoc test), n= 8-6 animals/group.

4.4.2.3.2 Protein analysis

On Figure 24, Student's t-test revealed a tendency to decreased the levels of spinophilin in FSL vehicle-treated (t(10)= 2.030; *p*= 0.0698; Figure 24B) in comparison to its counterpart. Although, none of the drug treatment significantly change the mRNA levels of this target gene (One-way ANOVA: F(2, 15)= 1.251; *p*= 0.3145). However, neither the drug treatment nor the strain of rats affect the levels of PSD-95 in the PFC (Student's *t*-test: t(10)= 0.2181; *p*= 0.8317; One-way ANOVA: F(2, 15)= 0.7005; *p*= 0.5118; Figure 24A).

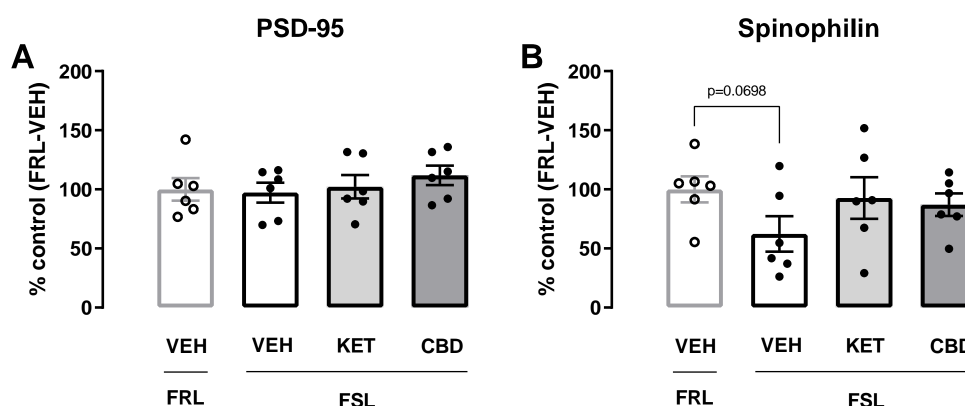

Figure 24. Effect of CBD and ketamine on the protein levels related to synaptic protein in prefrontal cortex of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposition to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent fluorescence levels of PSD-95 normalized by actin (A) and spinophilin normalized by actin (B) in PFC expressed as percentage of the control group (FRL-VEH). Values are mean ± SEM, n= 6 animals/group.

The main alterations in synaptic protein found in PFC are summarized in Table 9.

Table 9. Summary of the effects induced by CBD and ketamine acute treatment on transcript and protein levels related synaptic protein in prefrontal cortex of FSL rats.

Molecular Analysis	*Transcripts and proteins of interest*	*FSL-VEH*	*FSL-KET*	*FSL-CBD*
Relative mRNA Levels	Homer3	NA	NA	NA
	PSD-95	NA	NA	NA
	Neuroligin 1	NA	NA	NA
	Neurexin 2	NA	NA	NA
	Spinophilin	NA	NA	NA
	Synapsine 3	NA	NA	NA
	Synaptophysin	NA	NA	NA
Protein Levels	PSD-95	NA	NA	NA
	Spinophilin	↓(#p=0.0698)	NA	NA

#: Significant difference presented by FSL-VEH rats compared to FRL-VEH. *: Significant difference induced by treatment with KET or CBD compared with FSL-VEH. ↓: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present

lower levels compared to their respective control groups, FRL-VEH and FSL-VEH; ↑: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present higher levels compared to their respective control groups, FRL-VEH and FSL-VEH; NA: No significant alteration was observed.

4.4.2.4 Discussion

In the present study, we found a reduction in the excitatory amino acid transporter 2 (EAAT2) mRNA levels in the PFC of FSL rats, indicating that glutamate reuptake might be impaired, with excess glutamate levels this brain region. The EAATs, such as EAAT2 and EAAT3, are responsible for keeping the neurotransmitter's optimal extracellular concentration, preventing it from reaching neurotoxic levels (BJØRN-YOSHIMOTO; UNDERHILL, 2016; BLACKER et al., 2019), with EAAT2 being the most abundant transporter located on the glial cell membrane (ZHANG et al., 2013) and responsible for regulating the global concentration of glutamate levels (TAKAHASHI; FOSTER; LIN, 2015). Comparatively, EAAT3 is located in postsynaptic neurons (ZHANG et al., 2013) and plays an essential role in regulating regional glutamate levels (BJØRN-YOSHIMOTO; UNDERHILL, 2016). In line with our findings, previous studies have shown that animals submitted to different stress paradigms, such as CUMS (CHEN et al., 2014; LIU et al., 2016, 2019; ZHU et al., 2017), social defeat (VEERAIAH et al., 2014), learned helplessness (ZINK et al., 2010), repeated restraint stress (MIYAGISHI et al., 2017), and prenatal stress (ZHANG et al., 2013) have low levels of EAAT2 in the PFC. Furthermore, similar results were also observed in the brains of depressed patients (CHANDLEY et al., 2013; CHOUDARY et al., 2005; MEDINA et al., 2013; ZHAO et al., 2016). However, as we failed to detect corresponding changes in EATT2 protein levels, the neurobiological relevance of our findings needs to be re-assessed, optimally at another time following injection, as the lacking protein expression can be attributable to delayed transcript.

Interestingly, some studies investigate the role of EAAT3 in the pathophysiology of MDD and antidepressant drug response. We found that CBD-treated FSL rats exhibited an upregulation in transcript levels of EAAT3 in the PFC compared to FSL vehicle-treated, again without a change in the protein levels. Interestingly, beyond maintaining adequate glutamate levels, the EAAT3 transporter is also a key protein involved in synaptic plasticity (JARZYLO; MAN, 2012). Synaptic plasticity is a change of morphology, composition, or signal transduction efficiency at a neuronal synapse over time to adapt to the environment, including long-term potentiation (LTP) and long-term depression (LTD; For review see (CITRI; MALENKA, 2008; DUMAN et al., 2016b; LIU et

al., 2017a, 2017c; VOSE; STANTON, 2017). The AMPA and NMDA receptors play a critical role in this process (ALT et al., 2006; ANGGONO; HUGANIR, 2012; FREUDENBERG; CELIKEL; REIF, 2015). Interestingly, a prior study show that EAAT3 transporter reduced the recruitment of extrasynaptic Nr2b-containing NMDA receptor (SCIMEMI; TIAN; DIAMOND, 2009). Despite our data showing an upregulation in the levels of Nr2b in the PFC, we do not know the exactly synaptic location, whether this subunit is expressed in synaptic or extrasynaptic receptors. However, it is known that the activation of Nr2b in the extrasynaptic NMDA receptor contributes to excitotoxicity, impairing the synaptic strengthening (LAI; ZHANG; WANG, 2014). In this sense, it may be speculated that CBD facilitate EAAT3 expression in the PFC, which in turn regulates the regional glutamate levels and decreased recruitment of extrasynaptic Nr2b-containing NMDA receptor (see below), favoring LTP, which could reflect the observed behavioral response. However, this hypothesis requires further work, confirming that the EAAT2 and EAAT3 transcription results in active protein. It is worthy to note that the correlation between gene expression and protein levels can be 40% and depends on the transcriptional regulations, RNA stability, translational process, protein stability, and modifications (VOGEL; MARCOTTE, 2012).

As mentioned, synaptic plasticity process are closely related to the NMDA receptor. This receptor is a tetrameric complex composed of four different subunits Nr1, Nr2a-d, and Nr3a-b, which varies according to the location and function in the brain (AMIDFAR et al., 2019; CULL-CANDY; BRICKLEY; FARRANT, 2001; CULL-CANDY; LESZKIEWICZ, 2004; LI et al., 2007). NMDA receptors are functionally different due to differences in subunit composition, e.g. between Nr2a and Nr2b containing receptors - especially regarding the synaptic location, channel properties, receptor binding, and downstream signaling pathways (AMIDFAR et al., 2019). In the present study, we found a reduction in Nr2a mRNA in the PFC of the FSL rats compared to FRL rats. Interestingly, both CBD and KET treatment increased the levels of Nr2a (gene and proteins only for FSL KET-treated group) and Nr2b (protein for both drug treatment) in the PFC, indicating that this can be a mechanism associated with the antidepressant-like effect induced by these drugs. Corroborating with our findings, depressed patients have lower protein levels of Nr2a (BENEYTO; MEADOR-WOODRUFF, 2008; FEYISSA et al., 2009) and Nr2b in PFC (FEYISSA et al., 2009). Moreover, similarly, preclinical studies show a reduction in the levels of Nr2a and Nr2b in the HPC and PFC in animals submitted to different models of depression, such as prenatal stress (SUN et al., 2013), FSL rats (MUSAZZI et al., 2010; TRECCANI et al., 2016) and treatment-resistant model of depression, WKY rat (MILLARD et al., 2019). Moreover, it was demonstrated that chronic

treatment with monoaminergic antidepressant drugs promotes adaptive changes in the receptor-binding and composition of NMDA receptors in frontal cortex (BOYER; SKOLNICK; FOSSOM, 1998). Based on that, it suggests that in FSL rats NMDA receptor composition is changed, resulting in impairment in synaptic plasticity, which could be rapidly reversed by KET and CBD treatment.

Calcium/calmodulin-dependent protein kinase II (CaMKII) is an enzyme activated by Ca^{2+} and highly expressed in different brain regions (LISMAN; SCHULMAN; CLINE, 2002; RONGO, 2002). This enzyme plays a vital role in learning, memory, and synaptic plasticity in LTP induction and persistence, as well as apoptosis (AMIDFAR et al., 2019; LISMAN; SCHULMAN; CLINE, 2002; RONGO, 2002). The NMDA receptor's activation promotes the Ca^{2+} influx, consequent activation of CaMKII, and the transcription factor cAMP-response element-binding protein (CREB), enabling synaptic plasticity. Our study evidenced that the FSL vehicle-treated group has low levels of phospho CaMKII in the PFC compared to FRL vehicle-treated groups. Consistent with our results, a reduction in CaMKII levels in the PFC was found in rodents submitted to different paradigms, including, chronic restraint stress (LEEM; YOON; JO, 2020), chronic social defeat stress (JIANG et al., 2015), and chronic mild stress (BARRETO et al., 2012). Thus, a reduction of phospho CaMKII levels in the FSL rats might be associated with their behavioral phenotype. Surprisingly, this change was not rescued in CBD or ketamine-treated animals.

The AMPA receptor composed of four subunits, including GluR1-GluR4 (NICIU; KELMENDI; SANACORA, 2013; SANACORA et al., 2008). In particular, we measured the gene expression and protein analysis of GluR1 (total and phospho S831 and S845) that play a critical role in the channel conductance, opening and amplitude of AMPA receptor currents, regulating the long-term potentiation (LTP) and long-term depression (LTD), and the GluR2 involved in the AMPA receptor internalization (ANGGONO; HUGANIR, 2012; ELHUSSINY et al., 2021). We found that FSL vehicle-treated rats had a reduction in phospho GluR1(S831) levels in the PFC compared to FRL vehicle-treated rats. The treatment with ketamine reversed this effect, increasing the phospho GluR1(S831). Notably, this serine residue 831 is CaMKII phosphorylation site responsible for increasing GluR1-containing AMPA receptor conductance and synaptic transmission (JIANG et al., 2020). Similarly, animals submitted to different paradigms, such as olfactory bulbectomy and CUMS presented decreased levels of phospho GluR1 (S831) (JIANG et al., 2020; POCHWAT et al., 2015). Importantly, both ketamine and CBD increased phospho GluR1 (845), which is associated with the insertion of GluR1 subunit–containing AMPA receptors into synaptic membrane and synaptic plasticity (DERKACH et al., 2007). This is relevant, since insertion of the

GluR1 subunit of AMPA into the synapse is described following administration of ketamine (LI et al., 2010b), fluoxetine (SVENNINGSSON et al., 2002), imipramine (SZABO et al., 2009), and tianeptine (DUMAN; VOLETI, 2012; SVENNINGSSON et al., 2007). Moreover, the rapid antidepressant effect of ketamine is associated with increased phosphor GluR1 (845), but this was shown for the hippocampus (ZHANG et al., 2016) and its behavioral effects can be blocked by AMPA receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulfoamoylbenzo(f)-quinoxaline (NBQX), (MAENG et al., 2008; ZANOS et al., 2016). Despite those changes in phosphorylation, no changes were observed in CaMKII, PKA and PKC levels in the PFC (data not shown). Nevertheless, our results suggest that CBD, as shown for ketamine, might induce a fast antidepressant effect by facilitating AMPA-mediating signaling in the prefrontal cortex. It is, however, not clear how CBD may facilitate AMPA activation.

Protein interacting with C-kinase (PICK1) is widely expressed in the brain (HUGANIR; NICOLL, 2013) and regulates AMPA receptor endocytosis containing subunit GluR2, which reduce plasma membrane levels of GluR2 subunit and, consequently, controlling the synaptic strengthening (HANLEY, 2018; HUGANIR; NICOLL, 2013; PEREZ et al., 2001). We found a downregulation in mRNA Pick1 in FSL CBD-treated rats compared to FSL vehicle-treated animals. As it has been demonstrated that CBD induces LTP in vitro (HUGHES; HERRON, 2019; MAGGIO; SHAVIT STEIN; SEGAL, 2018), this could happen as a result of a decrease in the endocytosis of the AMPA receptor following downrelulation of Pick1. Moreover, even though no significant difference between the condition (FRL vs FSL vehicle-treated groups), a prior work show that FSL rats have reduced plasticity (LTP) in the PFC (SRIVASTAVA et al., 2020), which could be related to the Pick1 expression. This finding is in agreement with a study where rodents were submitted to chronic unpredictable mild stress, and exhibited a depressive-like behaviour and upregulation in mRNA and protein of Pick1 in the brain (JIANG et al., 2020; LIN et al., 2018), reflecting the dysfunction in the synaptic strengthening. However, additional studies are necessary to confirm the participation of Pick1 in CBD effect determining the protein levels of Pick1 and phospho GluR2 subunit of AMPA receptor in this brain region.

In line with the hypothesis of altered glutamatergic synaptic activity and synaptic plasticity in the FSL rats, other previous studies report dysfunctional glutamate reuptake and decreased Long-Term Potentiation (LTP) and Excitatory Post-Synaptic Currents (sEPSC) in FSL rats (GÓMEZ-GALÁN et al., 2013; RYAN et al., 2009a), suggesting a dysfunction on glutamatergic system and synaptic plasticity in the depressive-phenotype exhibited by this rat in the FST.

It was previously demonstrated that the rapid effect of ketamine and of CBD are associated with fast increase in synaptogenesis in the PFC associated with increased BDNF-TrkB-mTOR signaling (SALES et al., 2018b; XU et al., 2019). Results in the present work partially support those findings by demonstrating that BDNF and TrkB mRNA levels were decreased in the PFC of FSL animals, although this was not reflected in corresponding protein changes. CBD rapidly increased BDNF levels in the PFC, whereas ketamine decreased sortilin and VEGF.

Despite the earlier work showing that stress decrease and imipramine, ketamine and CBD increase dendritic branching and synaptogenesis (HVILSOM et al., 2019; MUSAZZI et al., 2019; NAVA et al., 2014, 2017; SALES et al., 2018b), we failed to detect any increase levels of synaptic proteins in the PFC. Several explanations may be given, but it should be noted that the study design of the present work was different from earlier studies.

Sortilin is a protein encoded by the Sort1 gene that plays an important functional and regulatory role in the brain-derived neurotrophic factor (BDNF) signaling (CHEN et al., 2005; EVANS et al., 2011; TENG et al., 2005), which is important for survival, growth, differentiation and maturation of neuronal cells (PARK; POO, 2013). BDNF plays its function through the interaction with tropomyosin receptor kinase B (TrkB) (DUNHAM et al., 2009; GUILLOUX et al., 2012; RAY et al., 2011; TRIPP et al., 2012). Evidence suggested that sortilin forms a complex with proBDNF (immature BDNF) and its receptor, p75NTR, favoring the cellular apoptosis pathway (EVANS et al., 2011; TENG et al., 2005). Additionally, it has been revealed that sortilin regulates BDNF secretion (CHEN et al., 2005). We found that FSL rats treated with ketamine had lower levels of sortilin in the PFC compared with FSL vehicle-treated group. This is in line with finding using other depression models, where repeated treatment with fluoxetine (14 days) in mice submitted to chronic unpredictable mild stress (CUMS), induced a hedonic and antidepressant response, and decreased the sortilin levels in the cortex of mice (YANG et al., 2020). Moreover, one clinical study show that depressed patients responsive to monoaminergic antidepressant drugs have low baseline levels of sortilin compared to non-responders (BUTTENSCHØN et al., 2018), and that depressed patients admitted to acute ECT, and the combination of ECT and antidepressant drug, lowered the plasma sortilin levels (STELZHAMMER et al., 2013).

As expected, we found that FSL rats treated with vehicle showed a downregulation in mRNA BDNF and TrkB expression in PFC compared to FRL vehicle-treated rats, although protein levels were not significantly changed. Interestingly, systemic administration of CBD increased the protein levels of BDNF in this brain region in compared to FSL vehicle-treated rats.

Corroborating our results, previous studies demonstrate that the rapid antidepressant-like effect induced by CBD is accompanied by the increased levels of BDNF in the PFC in mice exposed to FST (SALES et al., 2018b; XU et al., 2019), which was blocked by the previous intracerebroventricular injection of TrkB inhibitor, K252a (SALES et al., 2018b). This indicates that the effects of CBD may depend on intact BDNF-TrkB signaling in the brain. However, we failed to see an effect of ketamine on BDNF, TrkB and mTOR signaling, as described by others (AUTRY et al., 2011; PEREIRA et al., 2017; POPP et al., 2016; ZANOS et al., 2016). The contradictory results could reflect changes in the experimental setup and analysis (brain homogenate vs synaptossomes). It is known that BDNF levels can be rapidly increased in synaptosomes (20 min) due to increased translation resulting from increased neuronal activation (GHARAMI; DAS, 2014). Therefore, it is possible that CBD could increase BDNF translation in synaptossomes, whereas the effects of ketamine could be associated to increasing BDNF levels at different locations (SONG; MARTINOWICH; LEE, 2017). Further work should clarify this issue.

Reduced levels of TrkB receptor in the PFC has been found in depressed patients (TRIPP et al., 2012), and a less (phosphorylated) active form of the receptor in the brain of suicide victims (DWIVEDI et al., 2003, 2009). Chronic treatment with monoaminergic antidepressant or ketamine increase BDNF serum levels in depressed patients (DUMAN et al., 2012; SEN; DUMAN; SANACORA, 2008). A similar alteration in BDNF-TrkB pathway has been reported in rodents submitted to different preclinical models of depression and subjected to chronic treatment with antidepressant drugs and ECT (AUTRY et al., 2011; CASTRÉN; ANTILA, 2017; CASTRÉN; KOJIMA, 2017; LEPACK et al., 2014; NIBUYA; MORINOBU; DUMAN, 1995b). Based on these complex findings and the results in the present work, additional investigations are needed to investigate the participation of the TrkB receptor in the PFC in the antidepressant effect of CBD.

The vascular endothelial growth factor (VEGF) is a neurotrophin responsible for stimulating angiogenesis and vasculogenesis (MILLAUER et al., 1993). Several studies also show that VEGF is present in neuronal cells and play an essential role in neurogenesis and neuroprotection (BECERRIL-VILLANUEVA et al., 2019; JIN et al., 2002). Contrary to our expectations, we found that FSL rats have higher VEGF transcript levels than FRL rats in PFC. However, it was earlier shown that the VEGF protein levels were lower in the frontal cortex of FSL animals compared to FRL (ELFVING; PLOUGMANN; WEGENER, 2010). Interestingly, treatment with ketamine promotes the opposite effect, decreasing the mRNA expression of VEGF. It was also earlier shown that chronic restraint stress increased the VEGF protein levels in the PFC (ELFVING et al., 2015).

Furthermore, FSL ketamine-treated rats showed an increase in total length of microvessels in the brain and, potentially, might influence neuronal activity, synaptic plasticity and glial function (ARDALAN et al., 2016a, 2017b, 2020). These finsings point to a complex relationship between VEGF, depression pathophysiology and recovery. Further studies are warranted.

The activation of TrkB and VEGFR2 receptors by its endogenous ligands (BDNF and VEGF, respectively) triggers the activation of common cascades of signaling pathway: phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinases (ERK), member of the mitogen-activated protein kinase (MAPK) family (CUNHA; BRAMBILLA; THOMAS, 2010a; DURIC; DUMAN, 2013). Interestingly, ERK plays a pivotal role in the neurobiology of depression and drug response (WANG; MAO, 2019). In our work, we found that FSL vehicle-treated rats have an increased level of phospho ERK isoform 1 in the PFC compared to its counterpart. This finding may not be surprising, as a previous study revealed that FSL rats have higher levels of phospho ERK1 and ERK2 in synaptosomal fraction compared to FRL rats (MUSAZZI et al., 2010). Strikingly, it was found that acute stress exposure, such as exposure to FST, TST, and tail shocks, increased phospho ERK1/2 in rat brain acute and sustained (12 hours after the stress exposure) compared to the non-stressed group (GALEOTTI; GHELARDINI, 2012; MELLER et al., 2003; YANG; HUANG; HSU, 2004). Indeed, ERK isoforms' activation depends on the stress nature, duration, and rodents' strain selected.

Furthermore, another important downstream molecule involved in the activation of the AMPA and neurotrophic factor receptor is mTOR (ATHIRA; MOHAN; CHAKRAVARTY, 2020; IGNÁCIO et al., 2015), which regulates the cell growth, proliferation, metabolism, protein synthesis, autophagy and this molecule plays a critical role in the learning, synaptic plasticity and cortical development in the brain (ATHIRA; MOHAN; CHAKRAVARTY, 2020). Disturbances in the mTOR signaling pathway have been implicated in MDD (ATHIRA; MOHAN; CHAKRAVARTY, 2020; IGNÁCIO et al., 2015). In the present study, we found reduced levels of phospho mTOR in the PFC of FSL vehicle-treated rats compared to FRL vehicle-treated group. Consistent with our findings, previous works demonstrated that the mTOR signaling pathway is impaired in the PFC of depressed patients (JERNINGAN et al., 2011). Furthermore, equivalent findings were observed in brains from rodents subjected to stress and several pre-clinical models of depression (ATHIRA; MOHAN; CHAKRAVARTY, 2020; CHANDRAN et al., 2013; IGNÁCIO et al., 2015; LI et al., 2010b; LIU et al., 2015; SU; DAI, 2017; ZHOU et al., 2014; ZHU et al., 2013). In the present work we failed to find effets of ketamine and CBD on mTOR levels in the

PFC. It should be noted that not all previous studies have also not found significant effects of ketamine on mTOR (PEREIRA et al., 2017) and an effect may be attributable to differences in methodology.

Glycogen synthase kinase 3B (GSK3B) is a serine/threonine kinase isoform expressed in the brain (TAKAHASHI et al., 1994) that promotes inhibitory control on protein kinases, such as protein kinase B (Akt) responsible for the regulation of the mTOR activity (BRADLEY et al., 2012; PEINEAU et al., 2009). A growing body of evidence demonstrated that dysfunction in this enzyme activity had been implicated in mood disorders (JOPE, 2011). A postmortem study revealed that depressed suicide victims have GSK3B activity increased in the ventral PFC, without affecting the protein levels (KAREGE et al., 2007). Consistent with human findings, the activation of GSK3B aggravates depressive-like phenotype in mice submitted to chronic stress model (PENG et al., 2018) and maternal separation (BIAN et al., 2015). Besides, animals with a deficiency in the activity of GSK3B showed increased vulnerability to stress-induced depressive-like behaviour in different models (POLTER et al., 2010). On the other hand, inhibiting this enzyme induces an antidepressant-like effect in different paradigms (PENG et al., 2018). Supporting this evidence, the antidepressant effect induced by several classes of antidepressant drugs is accompanied by the GSK3 inhibition and upregulation of Akt (for review see (DUDA et al., 2020). Surprisingly, we failed to replicate the results from other studies, as we saw that GSK3B mRNA was downregulated in FSL vehicle-treated rats compared to FRL rats. There were no influence on the total and phosphorylated protein levels in the PFC. However, as protein – not mRNA - is the final product from the gene responsible for the functional response, we cannot make further conclusions on the findings. In future studies, additional investigations will be interesting to examine the enzyme activity and determine the active form of protein (phosphoprotein) at different time-points to clarify the results.

4.4.3 Analysis of mRNA and proteins levels in dorsal hippocampus (DH)

The following section is stratified based on clusters with overlapping biology.

4.4.3.1 Glutamatergic neurotransmission

4.4.3.1.1 Relative mRNA analysis

On Figure 25, Student's *t*-test and Mann-Whitney test revealed that FSL-vehicle showed a significant upregulation of mRNA levels of GluR2 (Student's *t*-test: t(13)= 2.660; *p*= 0.0196; Figure 25D) and a significant reduction on transcript levels of EAAT2 (Student's *t*-test: t(14)= 3.272; *p*= 0.0056; Figure 25J), PKA (Mann-Whitney test: U= 11; *p*= 0.0488; Figure 25L), and a tendency to decreased expression of Pick1 (Mann-Whitney test: U= 12; *p*= 0.0651; Figure 25N) in the DH compared to FRL-vehicle rats. On the other hand, one-way ANOVA or Kruskal-Wallis test did not find treatment difference in the mRNA levels of GluR2, EAAT2, PKA and Pick1 (One-way ANOVA or Kruskal-Wallis test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. GluR2: F(2, 19)=0.2299; *p*= 0.7968; EAAT2: F(2, 20)=0.5640; *p*= 0.5777; PKA: H(3)=3.296; *p*= 0.1966; Pick1: H(3)= 4.771; *p*= 0.0895).

The drug treatment and strain of rats did not alter the mRNA Cnih2, Cinh3, GluR1, Nr1, Nr2b, mGluR5, EAAT3, CaMKII relative levels in DH (Student's t-test or Mann-Whitney, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA or Kruskal-Wallis test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. Cnih2: t(14)= 0.1097; *p*= 0.9142; F(2, 18)=3.260; *p*= 0.0619; Cnih3: t(14)= 0.180; *p*= 0.8597; F(2, 20)=1.242; *p*= 0.31; GluR1: t(14)= 1.279; *p*= 0.2217; F(2, 19)=0.9334; *p*= 0.4105; Nr1: t(14)= 0.05226; *p*= 0.9591; F(2, 20)=0.141; *p*= 0.8694; Nr2a: t(14)= 0.6993; *p*= 0.4958; F(2, 19)=0.006623; *p*= 0.9934; Nr2b: t(14)= 0.4966; *p*= 0.6272; F(2, 20)=0.4762; *p*= 0.628; mGluR5: U=18.50; *p*= 0.2923; H(3)=0.4065; *p*= 0.8270; EAAT3: t(14)= 1.434; *p*= 0.1736; F(2, 20)=0.5731; *p*= 0.5728; CaMKII: t(14)= 0.2005; *p*= 0.8440; F(2, 20)=1.459; *p*= 0.2562; Figure 25).

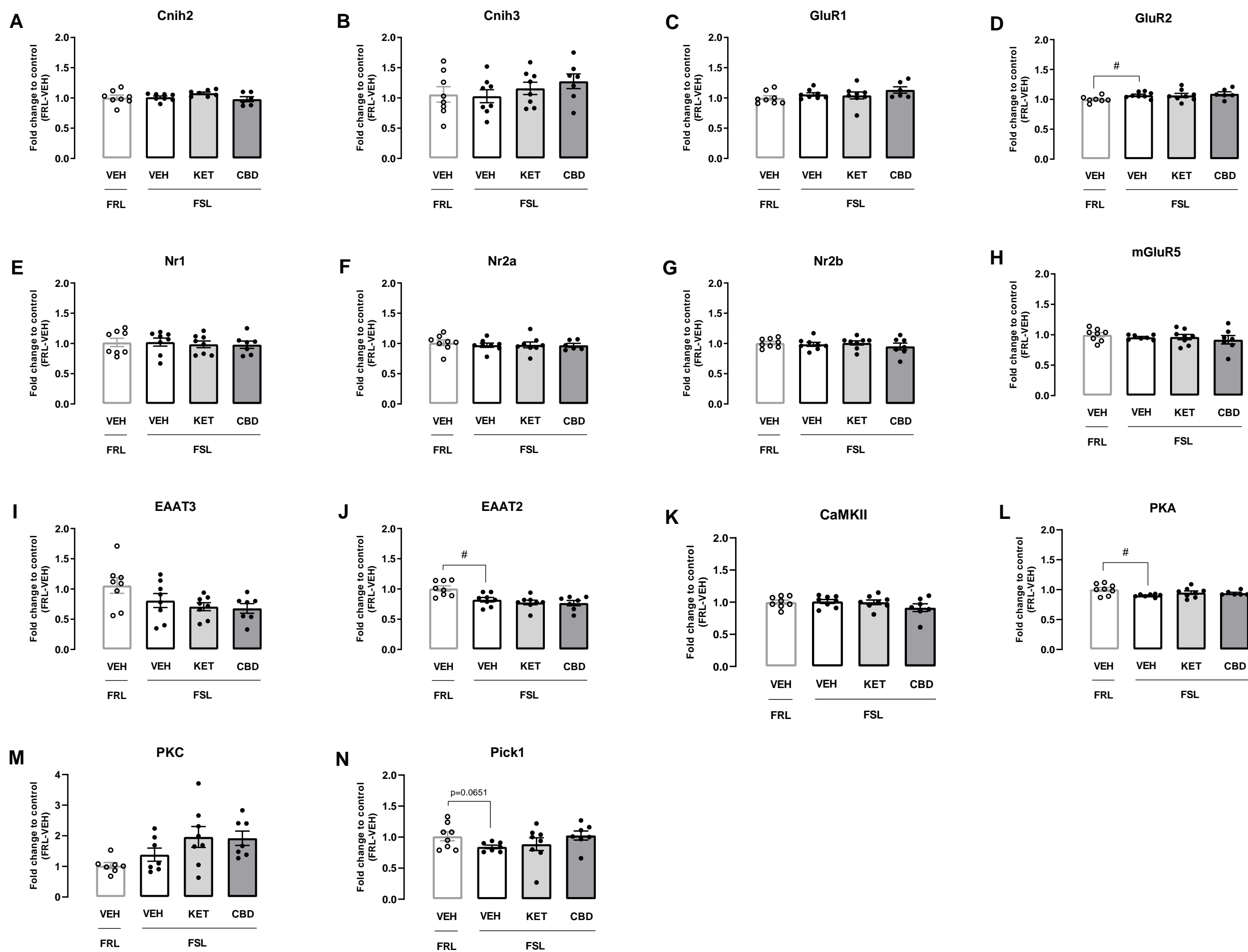

Figure 25. Effect of CBD and ketamine on relative mRNA levels of genes related to glutamatergic neurotransmission in dorsal hippocampus of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposure to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent relative mRNA levels of Cnih2 (A); Cnih3 (B); GluR1 (C); GluR2 (D); Nr1 (E); Nr2a (F); Nr2b (G); mGluR5 (H); EAAT3 (I); EAAT2 (J); CaMKII (K); PKA (L); PKC (M); Pick1 (N) in DH expressed as fold change to control group (FRL-VEH). Values are mean ± SEM; hash indicate significant differences between FSL and FRL vehicle-treated groups (#p< 0.05, Mann-Whitney test), n= 8-6 animals/group.

4.4.3.1.2 Protein analysis

On Figure 26, the one-way ANOVA test showed that both treatments, CBD and ketamine, decreased the levels of Nr2b and phospho GluR1 (S831) in the DH in comparison to FSL-VEH group (One-way ANOVA test: Nr2b: F(2, 14)= 8.223; *p*= 0.0043; Fisher LSD test: CBD: *p*= 0.0012; KET: *p*= 0.0455; Figure 26F; phospho GluR1(S831): F(2, 15)= 4.428; *p*= 0.0308; Fisher LSD test: CBD: p= 0.0137; KET: *p*= 0.0368; Figure 26A). Student's t-test revealed that FSL vehicle-treated rats present a decreased phospho GluR1 (S831) levels (t(10)= 2.474; *p*= 0.0329;

Figure 26A) compared to FRL-VEH group. While, no significant difference between the condition was found in Nr2b protein levels (FSL vs FRL vehicle-treated groups; t(10)= 0.4658; *p*= 0.6513; Figure 26F).

Furthermore, no significant differences between the strain (FRL vs FSL vehicle treated groups) and the treatment (FSL-vehicle vs FSL-ketamine and FSL-CBD) on the protein levels of phospho GluR1 (pGluR1(S845), GluR2, Nr2a, mGluR5 and phospho CaMKII were found in the DH (Student's t-test or Mann-Whitney test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA or Kruskal-Wallis test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. pGluR1(S845): t(10)= 0.7922; *p*= 0.4466; F(2, 14)=1.987; *p*= 0.1740; GluR2: t(10)= 0.1623; *p*= 0.8743; H(3)=0.9825; *p*=0.6343; Nr2a: t(9)= 0.3402; *p*= 0.7415; F(2, 14)= 2.157; *p*= 0.1525; mGluR5: U= 13; *p*= 0.4848; H(3)= 1.205; *p*= 0.5728; pCaMKII: t(10)= 1.580; *p*= 0.1451; F(2, 15)=0.5664; *p*= 0.5792; Figure 26).

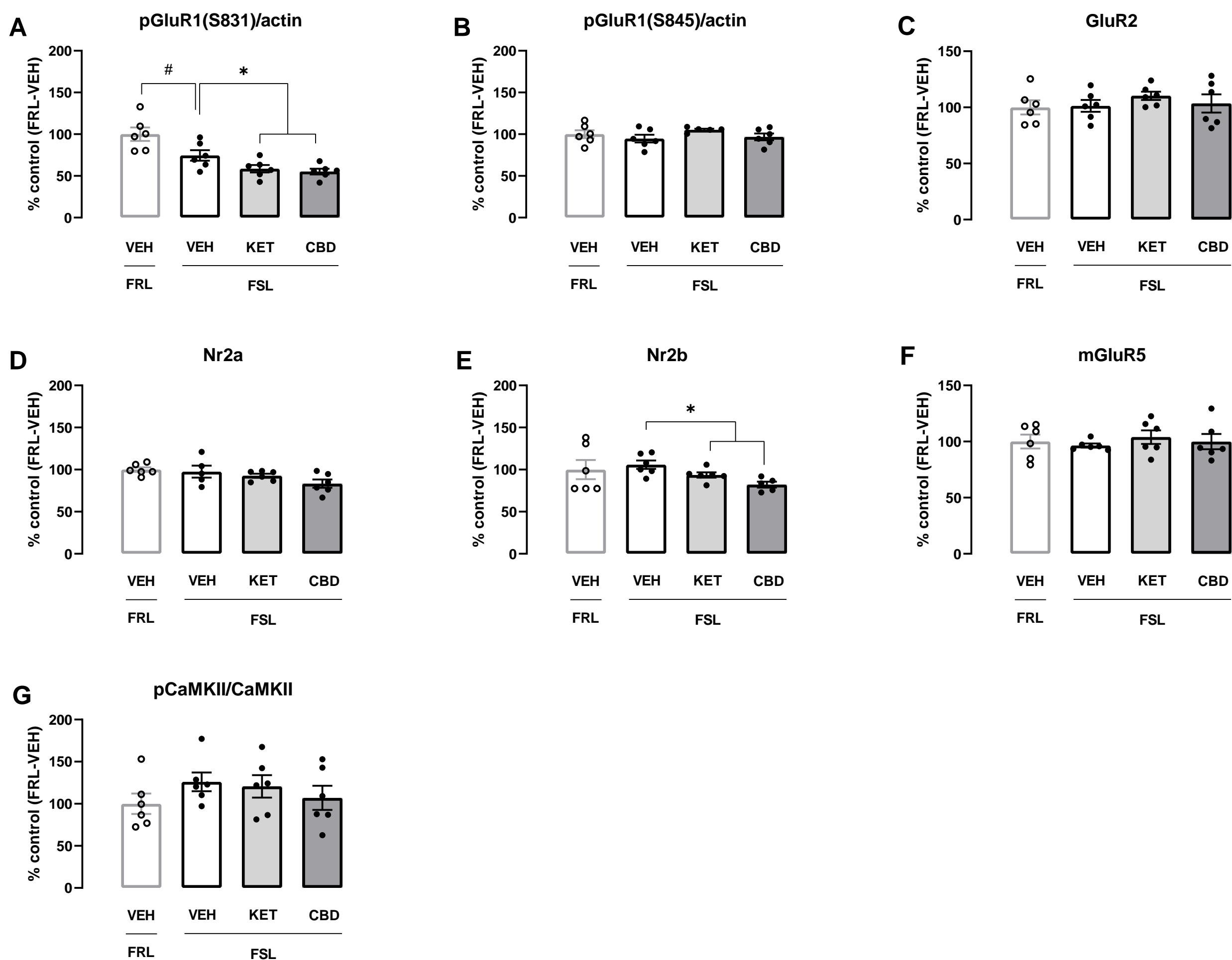

Figure 26. Effect of CBD and ketamine on the protein levels related to glutamatergic neurotransmission in dorsal hippocampus of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or KET (15 mg/kg) 1 hour before the exposure to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent fluorescence levels of phospho GluR1(S831) normalized by actin (A); phospho GluR1(S845) normalized by actin (B); GluR2 normalised by actin (C); Nr2a normalised by actin (D); Nr2b normalised by actin (E); EAAT3 normalised by actin (F); phospho CaMKII normalized by CaMKII(G) in DH expressed as percentage of the control group (FRL-VEH).Values are mean ± SEM; hash indicate significant differences between FSL and FRL vehicle-treated groups (#p< 0.05, Student's t-test); Asteriks represents significant treatment difference from FSL control (*p< 0.05; One-way ANOVA followed by Fisher's LSD post-hoc test and Kruskal-Wallis followed by Dunn's post-hoc test), n= 6-5 animals/group.

The main changes in glutamatergic system found in DH are summarized in Table 10.

Table 10. Summary of the effects induced by CBD and ketamine treatment on transcript and protein levels related to glutamatergic neurotransmission in the dorsal hippocampus of FSL rats.

Molecular Analysis	*Transcripts and proteins of interest*	*FSL-VEH*	*FSL-KET*	*FSL-CBD*
Relative mRNA Levels	Cnih2	NA	NA	NA
	Cnih3	NA	NA	NA
	GluR1	NA	NA	NA
	GluR2	↑ (#p=0.0196)	NA	NA
	Nr1	NA	NA	NA
	Nr2a	NA	NA	NA
	Nr2b	NA	NA	NA
	mGluR5	NA	NA	NA
	EAAT3	NA	NA	NA
	EAAT2	↓ (#p=0.0056)	NA	NA
	CaMKII	NA	NA	NA
	PKA	↓ (#p=0.0488)	NA	NA
	PKC	NA	NA	NA
	Pick1	↓ (#p=0.0651)	NA	NA
Protein Levels	pGluR1 (S831)	↓ (#p=0.0329)	↓ (*p=0.0368)	↓ (*p=0.0137)
	pGluR1 (S845)	NA	NA	NA
	GluR2	NA	NA	NA
	Nr2a	NA	NA	NA
	Nr2b	NA	↓ (*p=0.0455)	↓ (*p=0.0012)
	mGluR5	NA	NA	NA
	pCaMKII	↓ (#p=0.0352)	NA	NA

#: Significant difference presented by FSL-VEH rats compared to FRL-VEH. *: Significant difference induced by treatment with KET or CBD compared with FSL-VEH. ↓: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present lower levels compared to their respective control groups, FRL-VEH and FSL-VEH; ↑: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present higher levels compared to their respective control groups, FRL-VEH and FSL-VEH; NA: No significant alteration was observed.

4.4.3.2 Neurotrophin signaling

4.4.3.2.1 Relative mRNA analysis

On Figure 27, Mann-Whitney test revealed that VEGF transcript levels were decreased in FSL rats treated with vehicle in comparison to FRL vehicle (Student's *t*-test: t(13)= 2.776; *p*= 0.0157; Figure 27C). Interestingly, one-way ANOVA showed that CBD treatment reverted the strain effect in FSL rats, increasing VEGF mRNA levels in DH (One-way ANOVA: F(2, 18)=4.719; p= 0.0225; Fisher's LSD test: p= 0.0101; Figure 27C). Moreover, one-way ANOVA indicated that FSL treated with CBD decreased Sort1 transcript levels in the same brain structure (One-way ANOVA: F(2, 20)=6.689; p= 0.006; Fisher's LSD test: p= 0.0016; Figure 27A), but no changes were observed between the condition (FRL vs. FSL vehicle-treated rats; Student's *t*-test: t(14)= 0.62; *p*= 0.5452; Figure 27A).

Besides, Student's *t*-test indicated that FSL rats present a downregulation on mRNA of BDNF (Student's *t*-test: t(14)= 2.632; *p*= 0.0197; Figure 27B), GSK3B (Student's *t*-test: t(14)= 3.328; *p*= 0.0050; Figure 16F), and TrkB (Student's *t*-test: t(14)= 2.653; *p*= 0.018; Figure 27I) in the DH compared to their counterpart. However, none of the drug treatments significantly changed the mRNA expression (One-way ANOVA test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. <u>BDNF</u>: F(2, 20)=0.8635; *p*= 0.4368; <u>GSK3B</u>: F(2, 20)=0.7829; *p*= 0.4706; <u>TrkB</u>: F(2, 17)=1.572; *p*= 0.2363).

Meanwhile, one-way ANOVA and Student's *t*-test revealed that neither the drug treatment nor strain of rats affect the Eef2K, Mapk1, and mTOR mRNA expression in DH (Student's t-test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. <u>Eef2K:</u> t(14)= 0.8542; *p*= 0.4074; F(2, 20)=0.1885; *p*= 0.8297; <u>Mapk1:</u> t(14)= 0.6936; *p*= 0.4993; F(2, 20)=0.4219; *p*= 0.6615; <u>mTOR:</u> t(13)= 0.8050; *p*= 0.4353; F(2, 19)=0.9534; *p*= 0.4013; Figure 27).

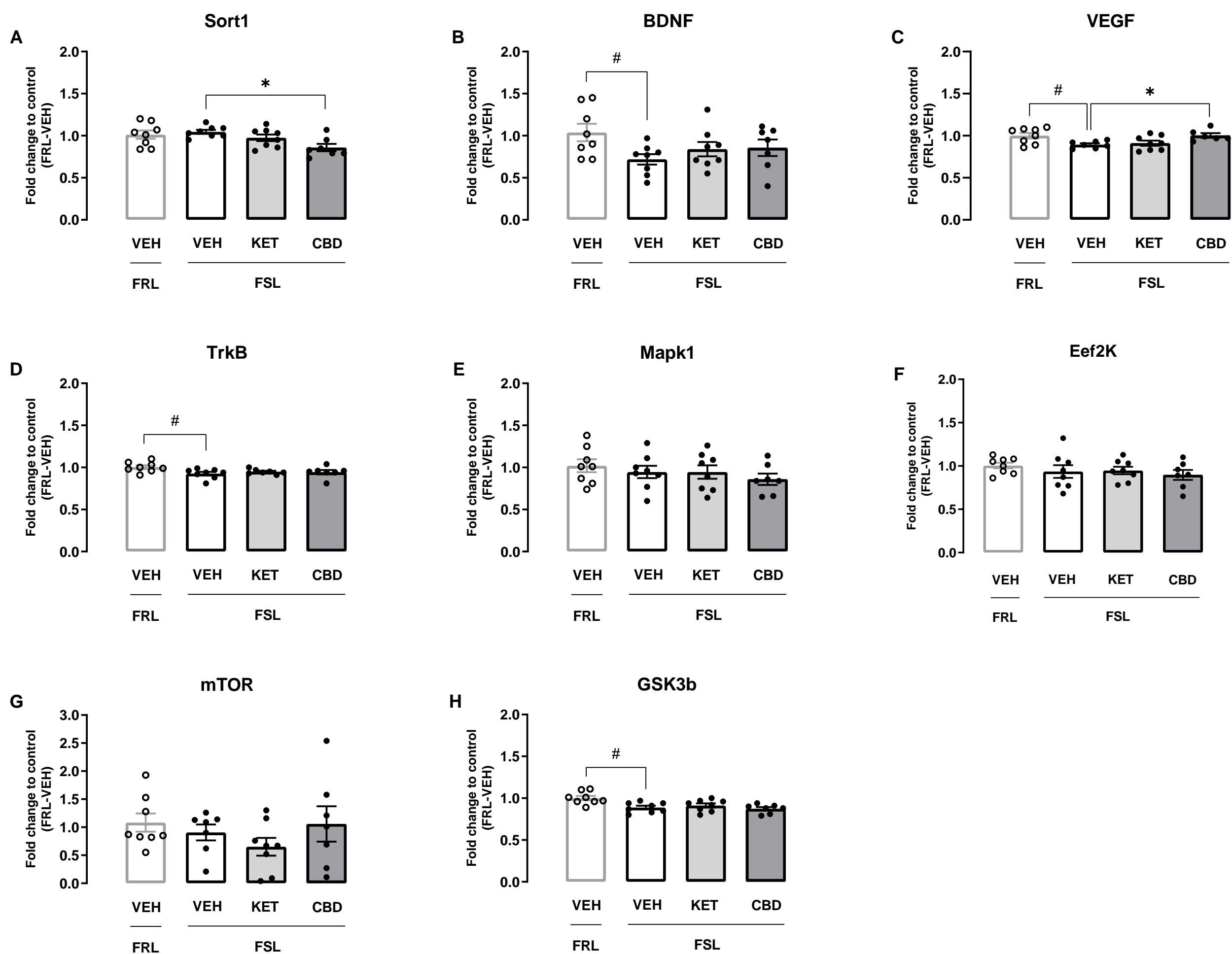

Figure 27. Effect of CBD and ketamine on relative mRNA levels of genes related to neurotrophic signaling in dorsal hippocampus of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposition to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent relative mRNA levels of Sort1 (A); BDNF (B); VEGF (C); CamK2a (D); Eef2K (E); GSK3B (F); Mapk1 (G); mTOR (H); TrkB (I) and p11 (J) in DH expressed as fold change to control group (FRL-VEH). Values are mean ± SEM; hash indicate significant differences between FSL and FRL vehicle-treated groups (#p< 0.05, Student's t-test or Mann-Whitney test); Asteriks represents significant treatment difference from FSL control (*p< 0.05; One-way ANOVA followed by Fisher LSD post-hoc test), n= 8-6 animals/group.

4.4.3.2.2 Protein analysis

On Figure 28, no significant differences between the strain (FRL vs FSL vehicle treated groups) and the treatment (FSL-vehicle vs FSL-ketamine and FSL-CBD) on the protein levels of BDNF and TrkB were found in the DH (Mann-Whitney test, to compare the results between the condition, FRL and FSL vehicle-treated groups; One-way ANOVA test to compare differences

between FSL-VEH and FSL-KET or FSL-CBD. BDNF: U= 15; *p*= 0.6991; F(2, 15)= 0.3766; *p*= 0.6925; TrkB: U= 11; *p*= 0.3095; F(2, 15)= 1.384; *p*=0.2807; Fig. 28).

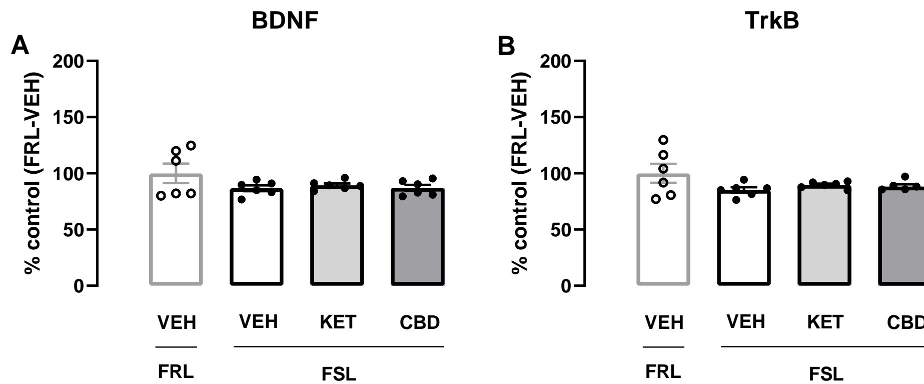

Figure 28. Effect of CBD and ketamine on the protein levels related to neurotrophin signaling in the dorsal hippocampus of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposition to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent fluorescence levels of BDNF normalized by actin (A) and TrkB normalized by actin (B) in VH expressed as percentage of the control group (FRL-VEH). Values are mean ± SEM, n= 6 animals/group.

The main changes in the neurotrophin signaling pathway found in DH are summarized in Table 11.

Table 11. Summary of the effects induced by CBD and ketamine acute treatment on transcript and protein levels related neurotrophic signaling pathway in the dorsal hippocampus of FSL rats.

Molecular Analysis	*Transcripts and proteins of interest*	*FSL-VEH*	*FSL-KET*	*FSL-CBD*
Relative mRNA Levels	Sort1	NA	NA	↓ (*p=0.0580)
	BDNF	↓ (#p=0.0352)	NA	NA
	Vegf	↓ (#p=0.0157)	NA	↑ (*p=0.0225)
	TrkB	↓ (#p=0.018)	NA	NA
	Mapk1	NA	NA	NA
	Eef2k	NA	NA	NA
	mTOR	NA	NA	NA
	GSK3	↓ (#p=0.0050)	NA	NA
Protein Levels	BDNF	NA	NA	NA
	TrkB	NA	NA	NA

#: Significant difference presented by FSL-VEH rats compared to FRL-VEH. *: Significant difference induced by treatment with KET or CBD compared with FSL-VEH. ↓: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present lower levels compared to their respective control groups, FRL-VEH and FSL-VEH; ↑: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present higher levels compared to their respective control groups, FRL-VEH and FSL-VEH; NA: No significant alteration was observed.

4.4.3.3 Synaptic proteins

4.4.3.3.1 Relative mRNA analysis

On Figure 30, Mann-Whitney test and Student's *t*-test indicated that FSL rats present decreased mRNA of Homer3 (Student's *t*-test: t(14)= 2.781; *p*= 0.0147; Figure 29A), and PSD-95 (Student's *t*-test: t(14)= 2.175; *p*= 0.0472; Figure 29B) in the DH compared to FRL-vehicle rats. However, none of the drug treatment significantly change in the mRNA levels of these genes (One-way ANOVA test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. Homer3: F(2, 20)=1.270; *p*= 0.3026; PSD-95: F(2, 20)=0.1743; *p*= 0.8413; as depicted in Figure 29).

However, for the other genes from synaptic proteins, none of the drugs nor the strain of rats affect the Neuroligin 1, Neurexin 2, Spinophilin, Syn3 and Syp relative levels in DH (Student's *t*-

test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. Neuroligin 1: t(14)= 0.1854; *p*= 0.8556; F(2, 20)=0.1534; *p*= 0.8588; Neurexin 2: t(13)=0.6404; *p*= 0.5330; F(2, 20)=0.5924; *p*= 0.3721; Spinophilin: t(14)=1.092; *p*= 0.2933; F(2, 20)=1.164; *p*= 0.3326; Syn3: t(14)= 0.8921; *p*= 0.3874; F(2, 20)=0.8521; *p*= 0.4414; Syp: t(14)= 0.794; *p*= 0.4405; F(2, 19)=0.1578; *p*= 0.8551; Figure 29).

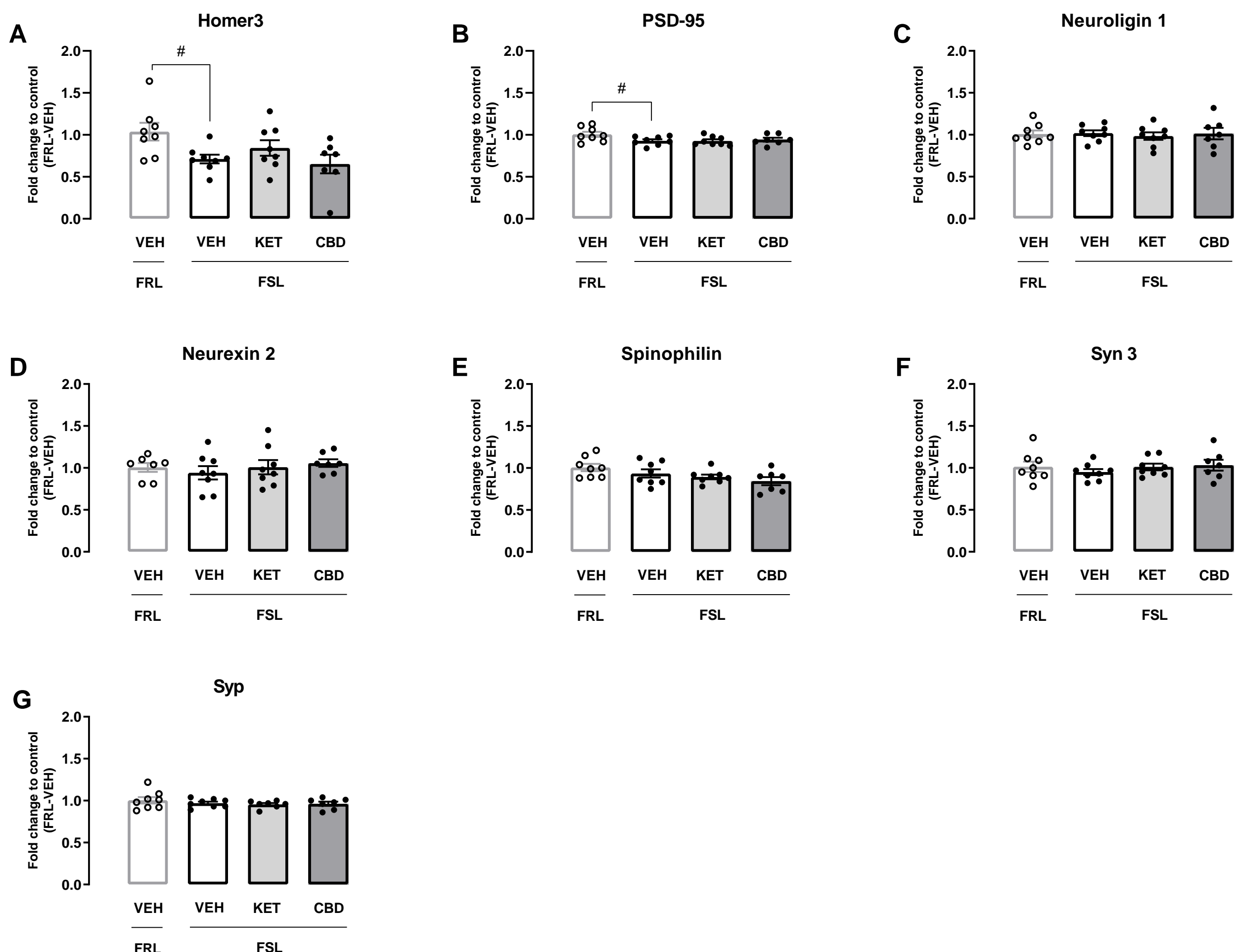

Figure 29. Effect of CBD and ketamine on relative mRNA levels of genes related to neuroplasticity signaling in the dorsal hippocampus of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposure to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent relative mRNA levels of Homer3 (A); PSD-95 (B); Neuroligin (C); Neurexin 2 (D); Spinophilin (E); Syn3 (F); and Syp (G) in DH expressed as fold change to control group (FRL-VEH). Values are mean ± SEM; hash indicate significant differences between FSL and FRL vehicle-treated groups (#p< 0.05, Student's t-test or Mann-Whitney test), n= 8-6 animals/group.

4.4.3.3.2 Protein analysis

On Figure 30, no significant differences between the condition (FRL vs FSL vehicle-treated groups) and the treatment (FSL-vehicle vs FSL-ketamine and FSL-CBD) on the protein levels of PSD-95 and spinophilin were found in the DH (Student's *t*-test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. PSD-95: t(10)= 0.9846; *p*= 0.3481; F(2, 15)= 1.636; *p*= 0.2276; Spinophilin: t(10)= 0.342; *p*= 0.7394; F(2, 15)= 2.708; *p*=0.099; Figure 30).

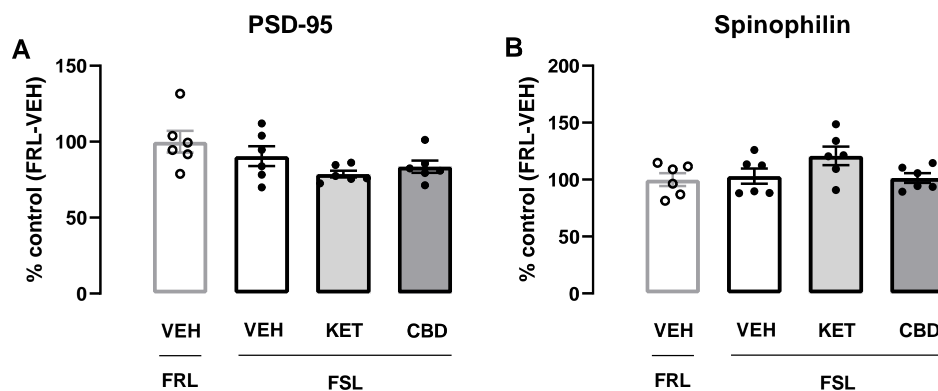

Figure 30. Effect of CBD and ketamine on the protein levels related to synaptic protein in the dorsal hippocampus of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or KET (15 mg/kg) 1 hour before the exposition to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent fluorescence levels of PSD-95 normalized by actin (A) and spinophilin normalized by actin (B) in VH expressed as percentage of the control group (FRL-VEH). Values are mean ± SEM, n= 6 animals/group.

The relative transcript and protein levels related to synaptic protein were investigated in the DH of FSL rats treated with acute systemic injection of CBD (30 mg/kg) and ketamine (15 mg/kg), at dose able to induce the antidepressant-like effect in FST. The main changes found in DH are summarized in Table 12.

Table 12. Summary of the effects induced by CBD and ketamine acute treatment on transcript and protein levels related synaptic protein in the dorsal hippocampus of FSL rats.

Molecular Analysis	*Transcripts and proteins of interest*	*FSL-VEH*	*FSL-KET*	*FSL-CBD*
Relative mRNA Levels	Homer3	↓ (#p=0.0147)	NA	NA
	PSD-95	↓ (#p=0.0472)	NA	NA
	Neuroligin 1	NA	NA	NA
	Neurexin 2	NA	NA	NA
	Spinophilin	NA	NA	NA
	Synapsine 3	NA	NA	NA
	Synaptophysin	NA	NA	NA
Protein Levels	PSD-95	NA	NA	NA
	Spinophilin	NA	NA	NA

#: Significant difference presented by FSL-VEH rats compared to FRL-VEH. *: Significant difference induced by treatment with KET or CBD compared with FSL-VEH. ↓: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present lower levels compared to their respective control groups, FRL-VEH and FSL-VEH; ↑: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present higher levels compared to their respective control groups, FRL-VEH and FSL-VEH; NA: No significant alteration was observed.

4.4.3.4 Discussion

As with the PFC, we also observed that treatment with CBD and ketamine reduced the protein levels of Nr2b in the DH of FSL rats compared to FSL vehicle-treated group. It is well known that HPC is a crucial structure in the neurobiology of depression (see introduction), and although the following may not be specific to HPC, clinical studies show that selective antagonism of Nr2b produces an antidepressant effect in patients diagnosed with depression and treatment-resistant depression (PRESKORN et al., 2008), and preclinical studies have revealed that selective antagonism of Nr2b produces a rapid antidepressant-like effect similar to what has been demonstrated for ketamine (LI et al., 2010b; POLESZAK et al., 2016). Moreover, selective antagonist of Nr2b potentiates the antidepressant effect induced by imipramine and escitalopram in rodents (POLESZAK et al., 2016). It can therefore be speculated that the reduced levels of Nr2b in DH are important factors involved in the antidepressant-like effect produce by CBD and ketamine

in FSL rats. However, further studies with more time-points using more sophisticated techniques, such as genetic manipulation of in vivo levels of Nr2b, are required in order to come to a firm conclusion.

In contrast to PFC results, we found that both CBD and ketamine had significantly lower phospho GluR1 (S831) in the DH. The change in these molecular targets may reflect an impairment of glutamatergic neurotransmission with consequent dysfunction in the neuroplastic processes, as alredy discussed above under PFC. However, despite reduced mRNA levels of BDNF, TrkB, PSD-95 and homer3, no changes were observed in the corresponding protein levels. Moreover, no changes were observed in pCAMKII, which is responsible for phosphorylating the S831 residue of GluR1 receptors. We are not aware of other studies examining similar aspects in FSL animals.

Our findings showed that FSL vehicle-treated rats had reduced levels of PKA in DH compared to FRL rats. Although examined in PFC, this finding parallels postmortem studies showing decreased PKA activation in the PFC of suicide depressed and bipolar patients (DWIVEDI et al., 2002, 2004a), resulting changes in the neuronal and synaptic plasticity. Similarly, consistent with clinical results, animals submitted to stress paradigms, including the learned helplessness model (DWIVEDI et al., 2004b) and CUMS (JIANG et al., 2017), displayed both decreased PKA levels and activity in the HPC, which can be speculated to be related to hippocampal atrophy and cognitive deficits observed in both models (MINEUR; BELZUNG; CRUSIO, 2006; PLANCHEZ; SURGET; BELZUNG, 2019; WILLNER, 2017). Therefore, it can be suggested that the depressive-like phenotype exhibited by FSL rats are associated with a decrement in PKA levels in the brain, resulting in dysfunction in synaptic plasticity observed in FSL rats. Measuremnt of protein levels could help clarifying this issue.

In the current study, we found that the FSL rats presented decreased levels of BDNF, TrkB, VEGF and GSK3 in the dorsal hippocampus, when compared to FSL animals, which is fully consistent with impaired neuroplasticity, as described earlier (ARDALAN et al., 2017a, 2016a, 2017b, 2020; CHEN et al., 2020, 2010, 2013; DU JARDIN et al., 2016a, 2017). However, there was no corresponding changes in protein levels. CBD, however, significantly decreased sortilin levels. In line with our results, repeated treatment with fluoxetine (14 days) induced hedonic and antidepressant effect accompanied by a decrement in the sortilin levels in the cortex of mice submitted to CUMS (YANG et al., 2020). Moreover, prior clinical study reports that depressed patient responder to the antidepressant drug treatment has low baseline levels of sortilin than non-responders individuals (BUTTENSCHØN et al., 2018). In addition, depressed patients submitted to

acute ECT alone or in combination with antidepressant drugs display a decrease in the sortilin levels in the blood (STELZHAMMER et al., 2013). Therefore, in the dorsal hippocampus, CBD antidepressant effect could be associated with downregulation in sortilin levels, facilitating neuronal survival, differentiation, and synaptic plasticity, normally evidenced after chronic administration with classical antidepressant drugs (YANG et al., 2020). However, this hypothesis requires further testing.

Interestingly, VEGF expression in DH of FSL in comparison FRL rats was downregulated. Several studies show that mRNA or protein levels of VEGF are lower in different brain regions in animals submitted to different preclinical models of depression (GREENE et al., 2009; HEINE et al., 2005; NOWACKA-CHMIELEWSKA et al., 2017; WARNER-SCHMIDT; DUMAN, 2007), and also in a genetic model of disease, FSL rats (ARDALAN et al., 2016a; ELFVING; PLOUGMANN; WEGENER, 2010). Consistent with our findings, prior work from our group revealed that FSL rats presented shorter length of the microvessels in the HPC, indicating a possible VEGF deficit in this brain region (ARDALAN et al., 2016b). The treatment with classical monoaminergic antidepressant drugs and KET reverted the effect on VEGF levels in the same brain structure (DEYAMA et al., 2019; GREENE et al., 2009; NOWACKA-CHMIELEWSKA et al., 2017; WARNER-SCHMIDT; DUMAN, 2007). Interestingly, we here show that the FSL CBD-treated rats increased the VEGF transcript levels, which – given the influence of VEGF on vasculature - is in agreement with a recent study showing that CBD treatment increases the blood flow in healthy patients' hippocampus (BLOOMFIELD et al., 2020). Moreover, it was also shown that CBD regulates the VEGF levels in human brain microvascular endothelial cells (HIND; ENGLAND; O'SULLIVAN, 2016), suggesting that VEGF may be involved in the CBD effect in FSL. Indeed, the VEGF signaling pathway is an important molecular target involved in the neurobiology of depression and action of the rapid antidepressant compounds (DEYAMA et al., 2019; KENWOOD et al., 2019). It will be interesting to clarify the involvement of VEGF on the CBD effect, determine the protein levels, and examine the participation of VEGF on the CBD effect.

4.4.4 Analysis of mRNA levels in ventral hippocampus (VH)

The following section is stratified based on clusters with overlapping biology.

4.4.4.1 Glutamatergic neurotransmission

4.4.4.1.1 Relative mRNA analysis

On Figure 31, Kruskal-Wallis showed that CBD treatment decreased the mRNA of mGluR5 expression in the VH (Kruskal-Wallis test: H(3)=7.490; *p*= 0.0236; Dunn's test: p=0.0195; Figure 31H) compared to the FSL control group. No significant difference between the condition was found in the same brain structure (Student's *t*-test: t(14)= 1.054; *p*= 0.3139; Figure 31H). Furthermore, Student's *t*-test or Mann-Whitney test revealed that FSL-vehicle showed a significant reduction on transcript levels of Cnih2 (Student's *t*-test: t(14)= 2.951; *p*= 0.0105; Figure 31A), Nr2a (Student's *t*-test: t(14)= 3.234; *p*= 0.0060; Figure 321), Nr2b (Student's *t*-test: t(15)= 2.797; *p*= 0.0136; Figure 31G), EAAT3 (Student's *t*-test: t(15)= 3.633; *p*= 0.0025; Figure 31I), EAAT2 (Mann-Whitney test: U= 13; *p*= 0.0466; Figure 31J), CamKII (Student's *t*-test: t(15)= 2.024; *p*= 0.0612; Figure 31K), and PKA (Student's *t*-test: t(15)= 3.995; *p*= 0.0012; Figure 31L) in the same limbic region compared to FRL-vehicle rats. None of the drug treatment significantly changes the mRNA levels of these genes (One-way ANOVA test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. Cnih2: F(2, 19)=0.8351; *p*= 0.4492; Nr2a: F(2, 21)=0.4258; *p*= 0.6588; Nr2b: F(2, 21)=0.2162; *p*= 0.8074; EAAT3: F(2, 21)=0.4538; *p*= 0.6413; EAAT2: F(2, 20)=0.4295; *p*= 0.6567; CaMKII: F(2, 21)=0.1922; *p*= 0.8266; PKA: F(2, 20)=0.0017; *p*= 0.9983).

The drug treatment and strain of rats did not affect the Cinh3, GluR1, GluR2, Nr1, PKC and Pick1 relative levels in VH (Student's t-test or Mann-Whitney, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA or Kruskal-Wallis test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. Cnih3: t(15)= 0.7089; *p*= 0.4893; H(3)=0.9541; *p*= 0.6206; GluR1: t(14)= 0.7454; *p*= 0.4683; F(2, 20)=2.125; *p*= 0.1457; GluR2: U= 31; *p*= 0.93923)=1.428; *p*= 0.4897; Nr1: U= 17; *p*= 0.1228; F(2, 20)=0.5792; *p*= 0.5694; PKC: t(15)= 0.4088; *p*= 0.6885; F(2, 20)=1.201; *p*= 0.3218; Pick1: U= 27.5; *p*= 0.4384; F(2, 21)=0.8127; *p*= 0.4571;Figure 31).

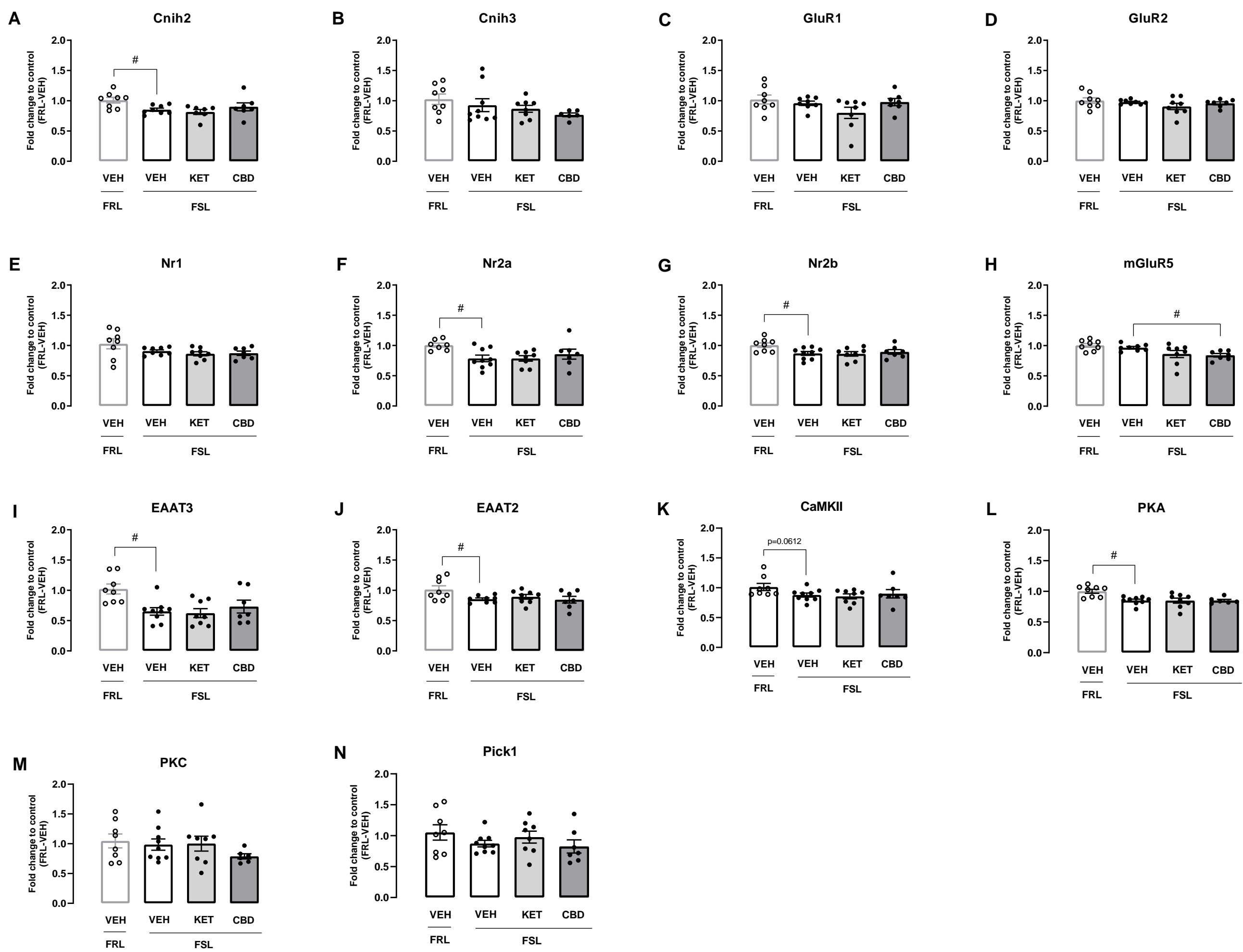

Figure 31. Effect of CBD and ketamine on relative mRNA levels of genes related to glutamatergic neurotransmission in the ventral hippocampus of FSL rats. FRL rats were treated VEH, and FSL rats were treated with VEH or CBD (30 mg/kg) or KET (15 mg/kg) 1 hour before the exposition to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent relative mRNA levels of Cnih2 (A); Cnih3 (B); GluR1 (C); GluR2 (D); Nr1 (E); Nr2a (F); Nr2b (G); mGluR5 (H); EAAT3 (I); EAAT2 (J); CaMKII (K); PKA (L); PKC (M); and Pick1 (N) in VH expressed as fold change to control group (FRL-VEH). Values are mean ± SEM; hash indicate significant differences between FSL and FRL vehicle-treated groups (#$p < 0.05$, Mann-Whitney test); Asteriks represents significant treatment difference from FSL control (*$p < 0.05$; Kruskal-Wallis followed by Dunn's post-hoc test), n= 8-6 animals/group.

4.4.4.1.2 Protein analysis

On Figure 32, the one-way ANOVA test showed that both treatments, CBD and ketamine, decreased the levels of phospho GluR1(S831) in the VH compared to FSL-VEH group (One-way ANOVA test: pGluR1(S831): $F(2, 15)= 12.92$; $p= 0.0005$; Fisher LSD test: CBD: $p= 0.0003$; KET: $p=0.0008$; Figure 32A). Moreover, the One-way ANOVA test showed that CBD injection also decreased the levels of phospho GluR1(S845) (One-way ANOVA test: $F(2, 15)= 4.232$; $p= 0.0349$;

Fisher LSD test: p= 0.0109; Figure 32B) and enhanced GluR2 expression (One-way ANOVA test: F(2, 15)= 4.994; p= 0.0218; Fisher LSD test: p= 0.0065; Figure 32D) in comparison to FSL vehicle-treated group. Although, no significant difference between the condition (FSL vs FRL vehicle treated groups) were found in pGluR1(S831 and S845) and GluR2 protein levels (Student's t-test: pGluR1(S831): t(10)= 1.516; p= 0.1604; Figure 33A; pGluR1(S845): t(10)= 0.1462; p= 0.8867; Figure 33B; GluR2: t(10)= 0.3135; p= 0.7604; Figure 32D).

Moreover, Student's t-test revealed that FSL vehicle-treated rats present a trend to elevate mGluR5 mRNA expression in VH (t(10)= 1.985; p= 0.0752; Figure 32G) compared to its counterpart. However, the one-way ANOVA did not show a significant difference between treatments (FSL-vehicle vs FSL-ketamine and FSL-CBD) for this protein (One-way ANOVA test: F(2, 14)= 1.385; p= 0.2825).

In addition, no significant differences between the strain (FRL vs FSL vehicle treated groups) and the drug treatment (FSL-vehicle vs FSL-ketamine and FSL-CBD) on the protein levels of GluR1, Nr2a, Nr2b and phospho CaMKII were found in the VH (Student's t-test or Mann-Whitney test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA or Kruskal-Wallis test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. GluR1: t(10)= 0.2022; p= 0.8438; F(2, 15)=1.019; p= 0.3847; Nr2a: t(10)= 0.09597; p= 0.9254; F(2, 15)= 2.518; p= 0.1140; Nr2b: t(10)= 0.3449; p= 0.7373; F(2, 15)= 2.276; p= 0.1370; pCaMKII: U= 7; p= 0.1775; F(2, 15)=0.5466; p= 0.59; Figure 32).

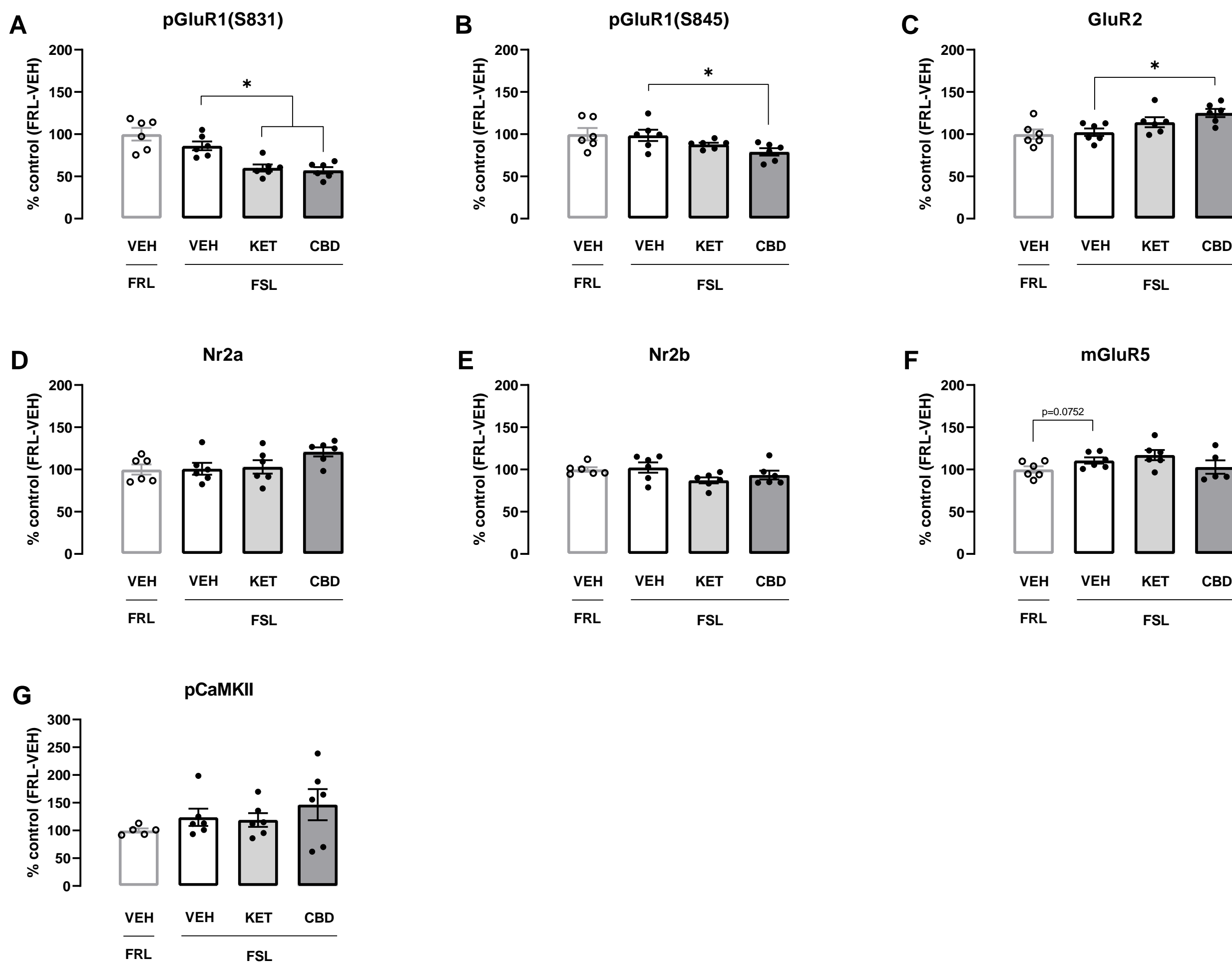

Figure 32. Effect of CBD and ketamine on the protein levels related to glutamatergic neurotransmission in the ventral hippocampus of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposure to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent fluorescence levels of phospho GluR1(S831) normalized by actin (A); phospho GluR1(S845) normalized by actin (B); GluR2 normalized by actin (C); Nr2a normalized by actin (D); Nr2b normalized by actin (E); mGluR5 normalized by actin (F); phospho CaMKII normalized by actin(G) in VH expressed as percentage of the control group (FRL-VEH). Values are mean ± SEM; hash indicate significant differences between FSL and FRL vehicle-treated groups (#p< 0.05, Student's t-test); Asteriks represents significant treatment difference from FSL control (*p< 0.05; One-way ANOVA followed by Fisher's LSD post-hoc test and Kruskal-Wallis followed by Dunn's post-hoc test), n= 6-5 animals/group.

The main changes in glutamatergic system found in VH are summarized in Table 13.

Table 13. Summary of the effects induced by CBD and ketamine acute treatment on transcript and protein levels related glutamatergic neurotransmission in the ventral hippocampus of FSL rats.

Molecular Analysis	*Transcripts and proteins of interest*	*FSL-VEH*	*FSL-KET*	*FSL-CBD*
Relative mRNA Levels	Cnih2	↓ (#p=0.0105)	NA	NA
	Cnih3	NA	NA	NA
	GluR1	NA	NA	NA
	GluR2	NA	NA	NA
	Nr1	NA	NA	NA
	Nr2a	↓ (#p=0.0060)	NA	NA
	Nr2b	↓ (#p=0.0136)	NA	NA
	mGluR5	NA	NA	↓ (*p=0.0195)
	EAAT3	↓ (#p=0.0025)	NA	NA
	EAAT2	↓ (#p=0.0466)	NA	NA
	CaMKII	NA	NA	NA
	PKA	NA	NA	NA
	PKC	NA	NA	NA
	Pick1	NA	NA	NA
Protein Levels	pGluR1 (S831)	NA	↓ (*p=0.0008)	↓ (*p=0.0003)
	pGluR1 (S845)	NA	NA	↓ (*p=0.0109)
	GluR2	NA	NA	↑ (*p=0.0065)
	Nr2a	NA	NA	NA
	Nr2b	NA	NA	NA
	mGluR5	↑ (#p=0.0752)	NA	NA
	pCaMKII	NA	NA	NA

#: Significant difference presented by FSL-VEH rats compared to FRL-VEH. *: Significant difference induced by treatment with KET or CBD compared with FSL-VEH. ↓: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present lower levels compared to their respective control groups, FRL-VEH and FSL-VEH; ↑: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present higher levels compared to their respective control groups, FRL-VEH and FSL-VEH; NA: No significant alteration was observed.

4.4.4.2 Neurotrophin signaling

4.4.4.2.1 Relative mRNA analysis

On Figure 33, Student's *t*-test indicated that FSL rats present decreased mRNA of Sort1 (Student's *t*-test: t(15)= 1.704; *p*= 0.1098; Figure 34A), BDNF (Mann-Whitney test: U= 3; *p*= 0.0011; Figure 33B), VEGF (Student's *t*-test: t(15)= 3.045; *p*= 0.0082; Figure 33C), GSK3B (Mann-Whitney test: U= 9.5; *p*= 0.0086; Figure 33H), and TrkB (Mann-Whitney test: U=9.5; *p*=0.0163; Figure 33D) in the VH in comparison to FRL-vehicle rats. However, none of drug treatment significantly changes in the mRNA levels of these genes (One-way ANOVA or Kruskal-Wallis test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. Sort1: F(2, 21)=0.2697; *p*= 0.7662; BDNF: H(3)=0.5628; *p*= 0.7547; VEGF: F(2, 21)=1.122; *p*= 0.3442; GSK3B: F(2, 21)=0.6455; *p*= 0.5345; TrkB: F(2, 20)=0.2826; *p*= 0.7568).

Meanwhile, one-way ANOVA and Student's *t*-test revealed that neither the drug treatment nor the strain of rats influence the Eef2K, Mapk1, and mTOR relative mRNA expression in the same brain region (Student's t-test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. Eef2K: t(15)=1.609; *p*= 0.1285; F(2, 21)=1.332; *p*= 0.2854; Mapk1: t(15)= 0.8134; *p*= 0.4287; F(2, 21)=1.616; *p*= 0.2225; mTOR: t(13)= 0.9133; *p*= 0.3777; F(2, 19)=1.172; *p*= 0.3312; Figure 33).

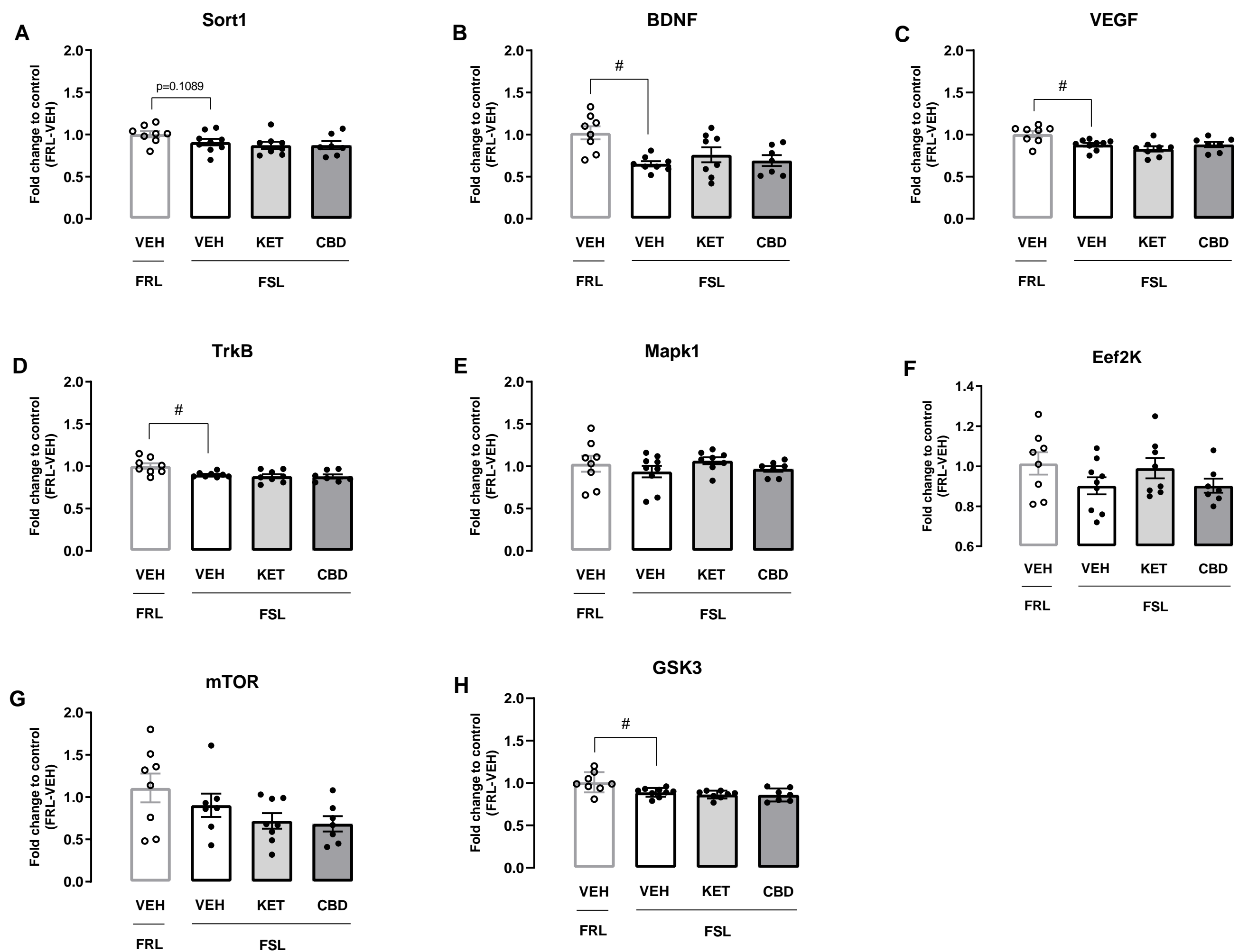

Figure 33. Effect of CBD and ketamine on relative mRNA levels of genes related to neurotrophic signaling in the ventral hippocampus of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposition to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent relative mRNA levels of Sort1 (A); BDNF (B); VEGF (C); TrkB (D); Mapk1 (E); Eef2K (F); mTOR (G); and GSK3 (H) in VH expressed as fold change to control group (FRL-VEH). Values are mean ± SEM; hash indicate significant differences between FSL and FRL vehicle-treated groups (#p< 0.05, Student's t-test or Mann-Whitney test), n= 8-6 animals/group.

4.4.4.2.2 Protein analysis

On Figure 34, Student's *t*-test indicated that FSL vehicle-treated rats present a tendency to decreased BDNF levels in VH compared to FRL rats (Student's *t*-test: t(10)= 2.025; *p*= 0.0704; Figure 34B). However, Kruskal-Wallis test did not show a significant difference between drugs treatment (Kruskal-Wallis test: H(3)= 0.6082; p=0.7552).

Additionally, no significant differences between the strain (FRL vs FSL vehicle treated groups) and drug treatment (FSL-vehicle vs FSL-ketamine and FSL-CBD) on the protein levels of sortilin and TrkB were found in the same brain region (Student's *t*-test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA or Kruskal-Wallis test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. Sortilin: t(10)= 0.8799; *p*= 0.3996; F(2, 14)= 0.9201; *p*=0.4213; TrkB: t(10)= 1.736; *p*= 0.1133; H(3)= 4.082; *p*=0.1307; Fig. 34).

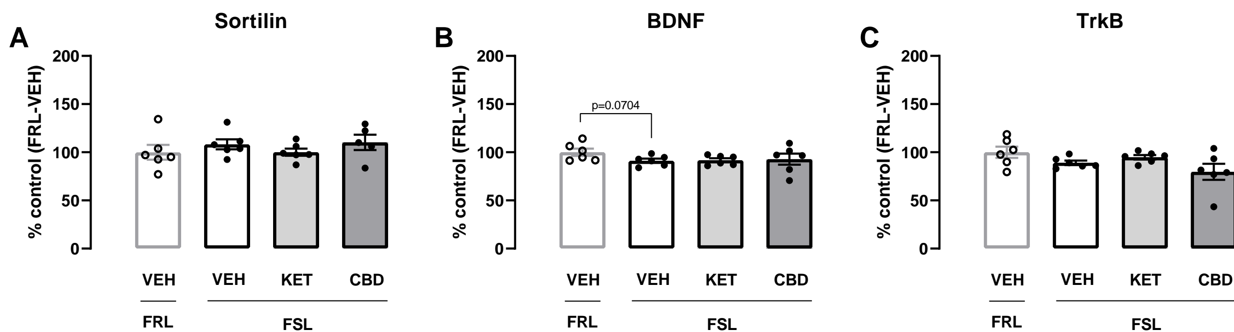

Figure 34. Effect of CBD and ketamine on the protein levels related to neurotrophin signaling in the ventral hippocampus of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposition to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent relative protein levels of sortilin (A), BDNF (B) and TrkB (C) in VH expressed as percentage of the control group (FRL-VEH). Values are mean ± SEM; hash indicate significant differences between FSL and FRL vehicle-treated groups (#p< 0.05, Student's t-test or Mann-Whitney test), n= 6 animals/group.

The relative transcript and protein levels related to neurotrophic signaling were investigated in the VH of FSL rats treated with acute systemic injection of CBD (30 mg/kg) and ketamine (15 mg/kg), at dose able to induce the antidepressant-like effect in FST. The main changes in neurotrophic signaling pathway found in VH are summarized in Table 14.

Table 14. Summary of the effects induced by CBD and ketamine acute treatment on transcript and protein levels related neurotrophic signaling pathway in the ventral hippocampus of FSL rats.

Molecular Analysis	*Transcripts and proteins of interest*	*FSL-VEH*	*FSL-KET*	*FSL-CBD*
Relative mRNA Levels	Sort1	NA (#p=0.1098)	NA	NA
	BDNF	↓ (#p=0.0011)	NA	NA
	Vegf	↓ (#p=0.0082)	NA	NA
	TrkB	↓ (#p=0.0163)	NA	NA
	Mapk1	NA	NA	NA
	Eef2k	NA	NA	NA
	mTOR	NA	NA	NA
	GSK3	↓ (#p=0.0050)	NA	NA
Protein Levels	Sortilin	NA	NA	NA
	BDNF	↓ (#p=0.0704)	NA	NA
	TrkB	NA	NA	NA

#: Significant difference presented by FSL-VEH rats compared to FRL-VEH. *: Significant difference induced by treatment with KET or CBD compared with FSL-VEH. ↓: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present lower levels compared to their respective control groups, FRL-VEH and FSL-VEH; ↑: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present higher levels compared to their respective control groups, FRL-VEH and FSL-VEH; NA: No significant alteration was observed.

4.4.4.3 Synaptic proteins

4.4.4.3.1 Reletive mRNA analysis

On Figure 35, Student's *t*-test indicated that FSL rats present decreased mRNA of Homer3 (Student's *t*-test: t(15)= 4.981; *p*= 0.0002; Figure 35A), PSD-95 (Student's *t*-test: t(15)= 3.337; *p*= 0.0045; Figure 35B), and Spinophilin (Student's *t*-test: t(15)= 2.206; *p*= 0.0434; Figure 35E) in the VH in comparison to FRL-vehicle rats. However, none of drug treatment significantly change in the mRNA levels of these genes (One-way analysis of variance (ANOVA) to compare differences between FSL-VEH and FSL-KET or FSL-CBD. Homer3: F(2, 21)=1.536; *p*= 0.2385; PKA: F (2,

20)=0.001721; p= 0.9983; PSD-95: F(2, 21)=0.6492; p= 0.5326; Spinophilin: F(2, 21)=0.2499; p= 0.7812).

For the other genes related to neuroplasticity signaling, however, none of the drug treatment nor the strain of rats affect the Neuroligin 1, Neurexin 2, Syn3 and Syp relative levels in VH (Student's t-test or Mann-Whitney test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. Neuroligin 1: t(15)= 0.2219; p= 0.8274; F(2, 21)=0.1435; p= 0.8672; Neurexin 2: t(15)=0.9762; p= 0.3444; F(2, 21)=0.5465; p= 0.5870; Syn3: t(14)= 0.8309; p= 0.42; H(3)=1.423; p= 0.4910; Syp: t(15)= 0.6131; p= 0.5490; F(2, 21)=2.184; p= 0.1375; Figure 35).

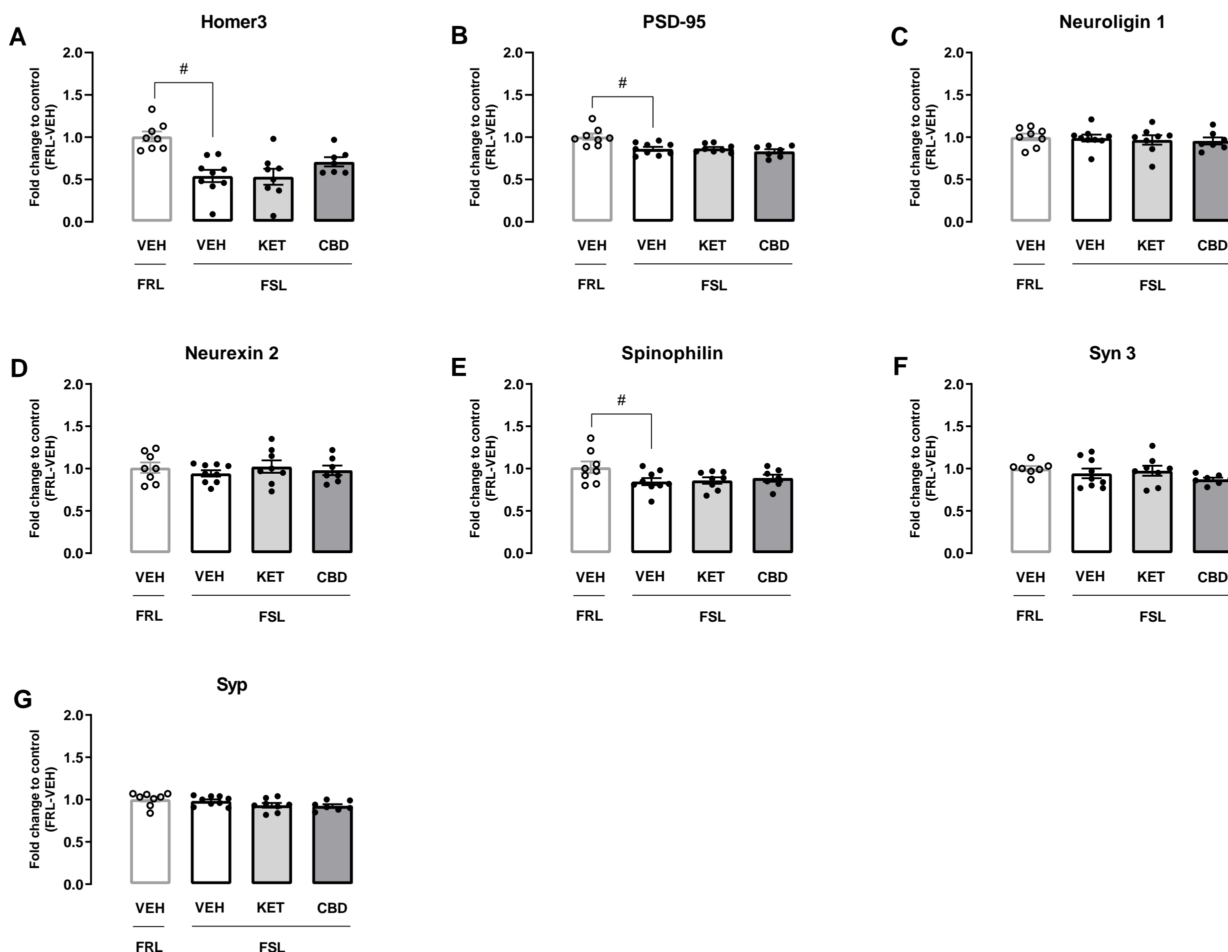

Figure 35. Effect of CBD and ketamine on relative mRNA levels of genes related to synaptic protein the ventral hippocampus of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposition to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent relative mRNA levels of Homer3 (A); PSD-95 (B); Neuroligin 1 (C); Neurexin 2 (D); Spinophilin (E); Syn3 (E) and Syp (G) in DH expressed as fold change to control group (FRL-VEH). Values are mean ± SEM; hash indicate significant differences between FSL and FRL vehicle-treated groups

(#p< 0.05, Student's t-test or Mann-Whitney test). Asterisks represents significant treatment difference from FSL control (*p< 0.05; One-way ANOVA followed by Fisher LSD post-hoc test or Kruskal-Wallis followed by Dunn post-hoc test), n= 8-6 animals/group.

4.4.4.3.2 Protein analysis

On Figure 36, Student's *t*-test indicated that FSL vehicle-treated rats present decreased PSD-95 levels in VH compared to FRL rats (Student's *t*-test: $t(10)= 4.101$; $p= 0.0021$; Figure 36A). However, Kruskal-Wallis test did not show a significant difference between drugs treatment (Kruskal-Wallis test: $H(3)= 0.03509$; $p=0.9896$). Moreover, no significant differences between the strain (FRL vs FSL vehicle treated groups) and the treatment (FSL-vehicle vs FSL-ketamine and FSL-CBD) on the protein levels of spinophilin were found in the same brain region (Student's *t*-test, to compare the results between the rat strain, FRL and FSL vehicle-treated groups; One-way ANOVA test to compare differences between FSL-VEH and FSL-KET or FSL-CBD. Spinophilin: $t(10)= 0.4226$; $p= 0.6815$; $F(2, 14)= 0.6286$; $p=0.5477$; Figure 36).

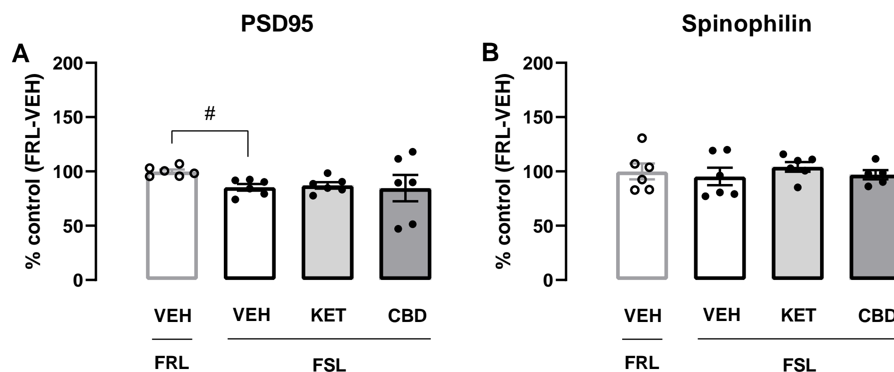

Figure 36. Effect of CBD and ketamine on the protein levels related to synaptic protein in the ventral hippocampus of FSL rats. FRL rats were treated VEH and FSL rats were treated with VEH or CBD (30 mg/kg) or ketamine (15 mg/kg) 1 hour before the exposition to FST, OFT and, prefrontal cortex, dorsal and ventral hippocampus were dissected for posterior analysis. Bars represent relative protein levels of PSD-95 (A) and spinophilin (B) in VH expressed as percentage of the control group (FRL-VEH). Values are mean ± SEM; hash indicate significant differences between FSL and FRL vehicle-treated groups (#p< 0.05, Student's t-test or Mann-Whitney test), n= 6 animals/group.

The main changes in synaptic proteins found in VH are summarized in Table 15.

Table 15. Summary of the effects induced by CBD and ketamine acute treatment on transcript and protein levels related synaptic protein in the ventral hippocampus of FSL rats.

Molecular Analysis	*Transcripts and proteins of interest*	*FSL-VEH*	*FSL-ketamine*	*FSL-CBD*
Relative mRNA Levels	Homer3	↓ (#p=0.0002)	NA	NA
	PSD-95	↓ (#p=0.0045)	NA	NA
	Neuroligin 1	NA	NA	NA
	Neurexin 2	NA	NA	NA
	Spinophilin	↓ (#p=0.0434)	NA	NA
	Synapsine 3	NA	NA	NA
	Synaptophysin	NA	NA	NA
Protein Levels	PSD-95	↓ (#p=0.0021)	NA	NA
	Spinophilin	NA	NA	NA

#: Significant difference presented by FSL-VEH rats compared to FRL-VEH. *: Significant difference induced by treatment with KET or CBD compared with FSL-VEH. ↓: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present lower levels compared to their respective control groups, FRL-VEH and FSL-VEH; ↑: indicate that FSL-VEH, FSL-KET and FSL-CBD rats present higher levels compared to their respective control groups, FRL-VEH and FSL-VEH; NA: No significant alteration was observed.

4.4.4.4 Discussion

The present study shows that FSL treated with CBD had reduced mGluR5 mRNA expression only in VH. This is in agreement with other work showing that pharmacological antagonism of metabotropic glutamatergic receptor mGluR5 with the drug MTEP ([(2- methyl-1,3-thiazol-4-yl)ethynyl]pyridine) (LEGUTKO et al., 2006; MOLINA-HERNÁNDEZ et al., 2006; PALUCHA et al., 2001; PAŁUCHA et al., 2005; PALUCHA; PILC, 2007; WIEROŃSKA et al., 2001) or negative modulator of this receptor (HIROSE et al., 2016; HUGHES et al., 2013) induce an antidepressant-like effect in rodents subjected to several animal models of depression. Likewise, electroconvulsive therapy reduces the levels of mGluR5 mRNA in the dentate gyrus of the HPC in rats (WATKINS; PEI; NEWBERRY, 1998). The mGluR5 is located at the postsynaptic terminal and glial cells (LUJÁN et al., 1996, 1997) and expressed in several brain regions, including HPC, cerebral cortex, caudate/putamen, thalamus and, cerebellum (ABDALLAH et al., 2017; DAGGETT

et al., 1995; OHNUMA et al., 1998). Preclinical studies have implicated mGluR5 in the pathogenesis of MDD (PALUCHA et al., 2001; PALUCHA; PILC, 2007; WIERONSKA et al., 2002).

Although no significant alteration on protein analysis was evidenced in our results, additional investigations of mGluR5 level at different time-poinst are needed to determine the participation of mGluR5 in the CBD effect. Moreover, it will be interesting to increase the experimental group size to confirm the difference between the conditions (FSL vehicle-treated vs FRL vehicle-treated rats). Besides, the evaluation of receptor functionality could clarify the molecular mechanism involved in the effect.

Furthermore, we found that CBD decreased the phosphorylation of GluR1 (S831 and S845) and increased the protein levels of the GluR2 AMPA subunit in the VH compared to FSL vehicle-treated rats. However, ketamine only decreased the phosphorylated GluR1 (S831) in the same limbic region. This is in agreement with an earlier study showing that a single administration of ketamine reduced phospho GluR1 (S845) levels in the HPC. However, the tricyclic antidepressant drug imipramine did not affect this serine residue (MAENG et al., 2008). Beyond this observation, we found that CBD increased GluR2 levels in the VH, which is in agreement with other work administering monoaminergic antidepressants showing that chronic treatment with desipramine and paroxetine increased the membrane expression of GluR2/3 in HPC (MARTINEZ-TURRILLAS; FRECHILLA; DEL RÍO, 2002). Altogether, based on the present findings, it can be hypothesized that CBD treatment leads to GluR1 (S831 and S845) dephosphorylation, and activation of GluR2-containing AMPA receptors, promoting internalization of the AMPA receptor that could modulate the neuronal plasticity in FSL rats. In contrast, as ketamine reduced phosphorylated GluR1 on the serine-831 residue of the AMPA, which could be important for its effect. However, further investigations are necessary to investigate the role of this specific phosphorylation serine residues in the CBD and ketamine antidepressant effect.

The cornichon family AMPA receptor auxiliary protein 2 (Cnih2) is an auxiliary component of the AMPA receptor complex, regulating the AMPA receptor's trafficking, surface delivery, and gating properties, increasing the stabilization of the open state (SCHWENK et al., 2009). In our work, we saw a significant reduction in Cnih2 mRNA expression in VH of FSL vehicle-treated rats compared to FRL vehicle-treated group. Interestingly, previous studies show that the stress exposure decrease AMPA receptors activation (ALEKSANDROVA; PHILLIPS; WANG, 2017; KOIKE; IIJIMA; CHAKI, 2011; MAENG et al., 2008), which could reflect Cnih2 deficits in the

brain. Further investigations on AMPA receptor trafficing will help to determine the role of the participation of Cnih2 auxiliary protein in the depressive-like phenotype of FSL rats.

Similar to our our results shown for PFC and DH, we found a downregulation in the expression of Nr2a, Nr2b, EAAT3, EAAT2, CaMKII, and PKA in VH of FSL vehicle-treated rats in comparison with their FRL-VEH rats. These molecular changes suggest a general impairment of the glutamatergic system, probably resulting in an overall imbalance of glutamatergic neurotransmission in this brain region, reflecting the depressive-like behaviour presented in the FST. The detailed discussion is described in the PFC and DH results in sections 4.4.2.4. and 4.4.3.4.

Notably, it is worth note that more robust dysfunctions in the neurotrophin signaling pathway were observed in the VH of the depressive rat strain, FSL. Briefly, similar to the results showed for DH, we found a downregulation in Sort1, BDNF, TrkB, VEGF, and GSK3B in the VH of the FSL vehicle-treated group compared to the FRL group. These molecular changes most likely reflect the impairment in the neurotrophic signaling pathway, resulting in dysfunction in neuronal proliferation, survival and plasticity, and associated with the depressive-like phenotype displayed by the FSL rats in FST.

Spinophilin is a protein present in dendritic spines involved in regulating spine density and glutamatergic synaptic transmission (ALLEN; OUIMET; GREENGARD, 1997; FENG et al., 2000). Here we found that FSL vehicle-treated rats had reduced mRNA spinophilin expression compared to FRL rats in VH, without any change in the protein levels in the same limbic region. This finding is consistent with studies on spinophilin knockout mice, showing a reduced brain size, especially in the HPC, and reduced AMPA and NMDA receptors activity (FENG et al., 2000). Moreover, these transgenic mice showed a reduction in AMPA receptors currents and more pronounced LTD than wild-type animals (FENG et al., 2000). Similarly, rats subjected to the chronic unpredictable stress protocol, show a decrease in the density of spinophilin and the number of dendritic spines in the CA1, CA3 and dentate gyrus of HPC, and it is associated to anhedonic response in the sucrose preference test (LIANG et al., 2019). In contrast to prior work, no significant difference between FSL and FRL rats in the spinophilin protein levels in the HPC was found (TRECCANI et al., 2019). However, the gene expression was not investigated in the latter study. Besides, an important difference between studies is the exposure to an inescapable stressful situation (for example, FST) that could modulate the brain's spinophilin gene expression. Furthermore, it is important to determine the protein levels at different time points due to the

translational cellular machinery's limitations and perform morphological studies to investigate the neuroplasticity alterations.

4.5 OVERALL DISCUSSION

According to our prior results shown in Study 1B, a single injection of CBD (30 mg/Kg) or S-ketamine (15 mg/Kg) significantly reduced the immobility time in FSL rats without affecting the locomotor activity in the OFT, indicating rapid antidepressant-like effect. Moreover, FSL vehicle-treated rats displayed increased immobility compared to FRL controls in the FST, suggesting a depressive-like phenotype of this strain. Importantly, this behaviour cannot be attributed to reduced locomotor activity since FSL rats displayed a longer traveled distance in the open field test compared to FRL rats. Thus, in the present study, we confirm that a single administration with CBD and ketamine produced a rapid antidepressant-like effect in male FSL rats, an animal model of depression based on selective breeding.

FSL rats present several neurochemical, molecular and morphological differences compared to FRL rats (ARDALAN et al., 2016b, 2017b; BLAVERI et al., 2010; CHEN et al., 2010; ELFVING et al., 2010; ELFVING; PLOUGMANN; WEGENER, 2010; KOVACEVIC et al., 2012; NISHI; KANEMARU; DIKSIC, 2009; OVERSTREET; WEGENER, 2013; PIUBELLI et al., 2011; SKELIN; KOVAÈEVIĆ; DIKSIC, 2011). In addition, these behavioural and molecular changes are reversible by the subchronic treatment with conventional antidepressant drugs and with a fast-acting antidepressant drug, including ketamine (ARDALAN et al., 2017a, 2020; CHEN et al., 2010; DU JARDIN et al., 2018, 2016b; LIEBENBERG; JOCA; WEGENER, 2015; OVERSTREET et al., 1995, 2005; OVERSTREET; WEGENER, 2013; PUCILOWSKI et al., 1993; SCHILLER et al., 1992; TRECCANI et al., 2019). Therefore, the FSL rats represent a valid preclinical model of depression that resembles several behavioural and molecular mechanisms present in this psychiatry disorder, which is reversible by antidepressant treatment.

Our study measured, for the first time, CBD concentration in whole blood in the same animal subjected to behavioural tests (OFT and FST) for subsequent correlation analysis. However, we failed to observe any correlation between the behaviour displayed in the FST and CBD levels in the blood. Several explanations for this exist, as already discussed above, in particular related to the physio-chemical properties of CBD. However, further analysis measuring the CBD levels in the brain to determine the possible correlation with behavioural displayed in the test is required.

The study was also the first to explore in-depth in male FSL rats some important molecular pathways, which could be implicated in the antidepressant-like effect produced by CBD and ketamine. We studied genes and proteins levels related to glutamatergic neurotransmission,

neurotrophic signaling, and synaptic proteins in three brain regions, the PFC, DH, and VH, believed to be essential in the depression neurobiology and treatment response. As already outlined in the introduction, dysfunctions in these brain areas have been associated with cognitive and behavioural abnormalities produced by stress and depressive disorder (LIU et al., 2017c).

In the PFC, we found that the acute administration with CBD elevated the levels of BDNF (protein), Nr2a (gene), EAAT3 (gene), and Nr2b (protein) in FSL rats. As described in figure 37, this may lead to a series of events facilitating synaptic function.

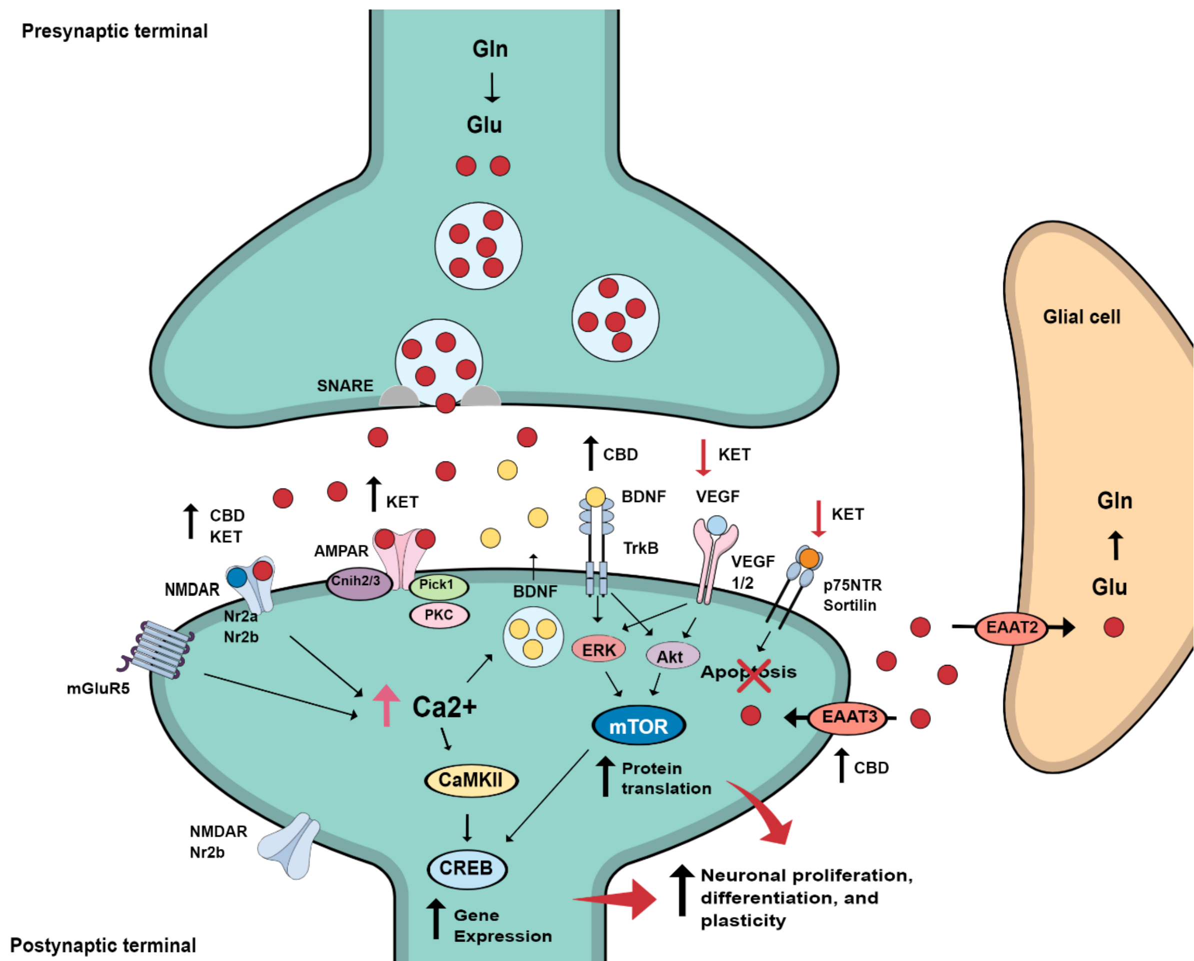

Figure 37. The possible molecular mechanism involved on CBD and ketamine antidepressant effect in the prefrontal cortex of FSL rats. CBD increases BNDF levels in the PFC, which activates its receptor (TrkB), which is responsible for activating a series of second messengers, which lead to the activation of CREB by increasing gene expression and translation of synaptic proteins, resulting in increased neuronal proliferation and synaptic plasticity. CBD also increases the glutamate transporter (EAAT3) in the postsynaptic terminal, reducing the excess of glutamate available and decreasing recruitment of extrasynaptic Nr2b-cotaining NMDA receptor, which prevents the apoptosis. In addition, CBD and ketamine increase levels of Nr2a and Nr2b, subunits of the NMDA receptor, which are closely related to increased synaptic strengthening, including LTP, resulting in the observed behavioral effects. Moreover, ketamine increases the phosphorylation of GluR1 subunit that is also important in the synaptic transmission. In addition, ketamine decreases sortilin levels, which prevents from apoptosis and facilitating the neuronal survival pathway. Abbreviations: Akt: Protein kinase B; AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor;

BDNF: brain-derived neurotrophic factor; Ca2+: Calcium ion; CaMKII: Calcium/calmodulin-dependent protein kinase II; CBD: Cannabidiol; Cnih2/3: Cornichon family AMPA receptor auxiliary protein 2 and 3; CREB: cAMP response element-binding protein; EAAT2: Excitatory amino acid transporter 2; EAAT3: Excitatory amino acid transporter 2; ERK: Extracellular signal-regulated kinase; Gln: Glutamine; Glu: Glutamate; GSK3: Glycogen synthase kinase-3; KET: Ketamine; Mapk1: Mitogen-activated protein kinase; mGluR5: Metabotropic glutamate receptor 5; mTOR: mammalian target of rapamycin; NMDA: N-methyl-D-aspartate; p75NTR: neurotrophin receptor P75; PKA: Protein kinase A; PKC: Protein kinase C; PI3K: Phosphoinositide 3-kinase; Pick1: Protein interacting with C-kinase; SNARE: soluble NSF attachment receptor; TrkB: Tropomyosin receptor kinase B; VEGF: vascular endothelial growth factor; VEGFR-1/2: vascular endothelial growth factor receptor 1 and 2.

Briefly, CBD may be hypothesized to induce an antidepressant-like effect by facilitating glutamatergic neurotransmission and, consequently, the release of BDNF in the postsynaptic terminal, leading to the synaptic protein translation and synaptic plasticity, in the PFC. The hypothesis is based on current and previous findings with CBD treatment, which induce a behavioural response which depends on the involvement of release of glutamate, intact BDNF-TrkB signaling pathway and increased dendritic spine density in the medial PFC (LINGE et al., 2016; SALES et al., 2018b; XU et al., 2019). Intriguingly, this has also been demonstrated as a common molecular pathway implicated in other fast-acting antidepressant compounds, including ketamine, GLYX-13 (NMDA receptor partial agonist) and scopolamine (muscarinic receptor antagonist) (DUMAN, 2015; GERHARD; DUMAN, 2018; LIU et al., 2017b).

As already discussed, accumulating evidence suggests an interplay between neurotrophic (BDNF) and glutamatergic systems are critical for neuroplasticity and its modulation under normal and pathological conditions, including major depression (GULYAEVA, 2017). It is important to note that the two systems are mutually regulated (BLACK, 1999; GULYAEVA, 2017; JARVIS et al., 1997; MATTSON, 2009). Thus, BDNF modulates the glutamatergic signaling by changing the expression of glutamate receptors subunits and Ca2+-regulating proteins or inducing the antioxidant enzymes production, energy-regulating and antiapoptotic proteins (BLACK, 1999; JARVIS et al., 1997; MATTSON, 2009). Conversely, glutamate regulates the production of BDNF, modifying the neuronal glutamate sensitivity, Ca2+ homeostasis, and plasticity (GULYAEVA, 2017; MATTSON, 2009). As mentioned above, BDNF may modulates the NMDA receptor, as acute BNDF selectively increases synaptic evoked NMDA currents dependent on the presence of Nr2b-containing receptors (KOLB; TRETTEL; LEVINE, 2005) and elevates NMDA single-channel open probability (LEVINE; KOLB, 2000). Furthermore, acute exposure to BDNF rapidly and reversibly elevated the amplitude and frequency of excitatory postsynaptic currents (EPSCs) dependent on the activation of NMDA receptor (MADARA; LEVINE, 2008; SONG et al., 1998). This study found that CBD treatment upregulates Nr2a and Nr2b mRNA expression in the PFC of FSL rats, and it may

therefore be speculated that CBD may increase BDNF levels which regulate the NMDA receptor opening and activation. As a result, activation of the NMDA receptor promotes a calcium influx that activates kinases and transcription factors including cyclic AMP response element-binding protein (CREB), which elevates gene expression facilitating neuronal survival and synaptic plasticity. However, this hypothesis, warrants additional studies. EAAT3 plays an essential role in removing excess glutamate from the synaptic cleft, preventing its harmful effects (BJØRN-YOSHIMOTO; UNDERHILL, 2016). However, studies revealed that EAAT3 is important in the synaptic transmission (JARZYLO; MAN, 2012; SCIMEMI; TIAN; DIAMOND, 2009). We found that CBD-treated FSL rats exhibited upregulation in transcript levels of EAAT3 in the PFC compared to FSL vehicle-treated but without a change the protein levels. A prior study revealed that EAAT3 transporter reduced extrasynaptic Nr2b-containing NMDA receptor (SCIMEMI; TIAN; DIAMOND, 2009). Despite our data showing upregulation in the expression levels of Nr2b in the PFC, we do not know the exact synaptic location, and whether this subunit is expressed in synaptic or extrasynaptic receptors. It is known that the activation of Nr2b in the extrasynaptic NMDA receptor contributes to excitotoxicity, impairing the synaptic strengthening (AMIDFAR et al., 2019; HANSEN et al., 2018; LAI; ZHANG; WANG, 2014). However, as it was demonstrated that CBD reverted deficits in LTP (HUGHES; HERRON, 2019; MAGGIO; SHAVIT STEIN; SEGAL, 2018), it can be speculated that CBD increase the EAAT3 expression in the PFC, regulating the regional glutamate levels and decreased recruitment of extrasynaptic Nr2b-containing NMDA receptor, facilitating LTP, which subsequently could be reflect the observed behavioural response. However, further investigations are needed to test this hypothesis.

We also found that a single injection of ketamine modifies the expression of the ion channel glutamate receptors, as reflected in the increasing in the levels of Nr2a (gene and protein), Nr2b (protein), and phospho GluR1(S831 protein) in PFC of FSL rats. This is in agreement with prior work demonstrating that ketamine increases the protein levels of Nr2b and GluR1 in mPFC, and is associated with a robust increase in LTP facilitation (BURGDORF et al., 2013)..

Importantly, in the DH, we evidenced that both drug treatments (CBD and ketamine) decreased phospho GluR1 (S831) levels and elevated the levels of Nr2b of FSL rats. As seen in figure 38, this may lead to a series of events facilitating synaptic function in the dorsal hippocampus.

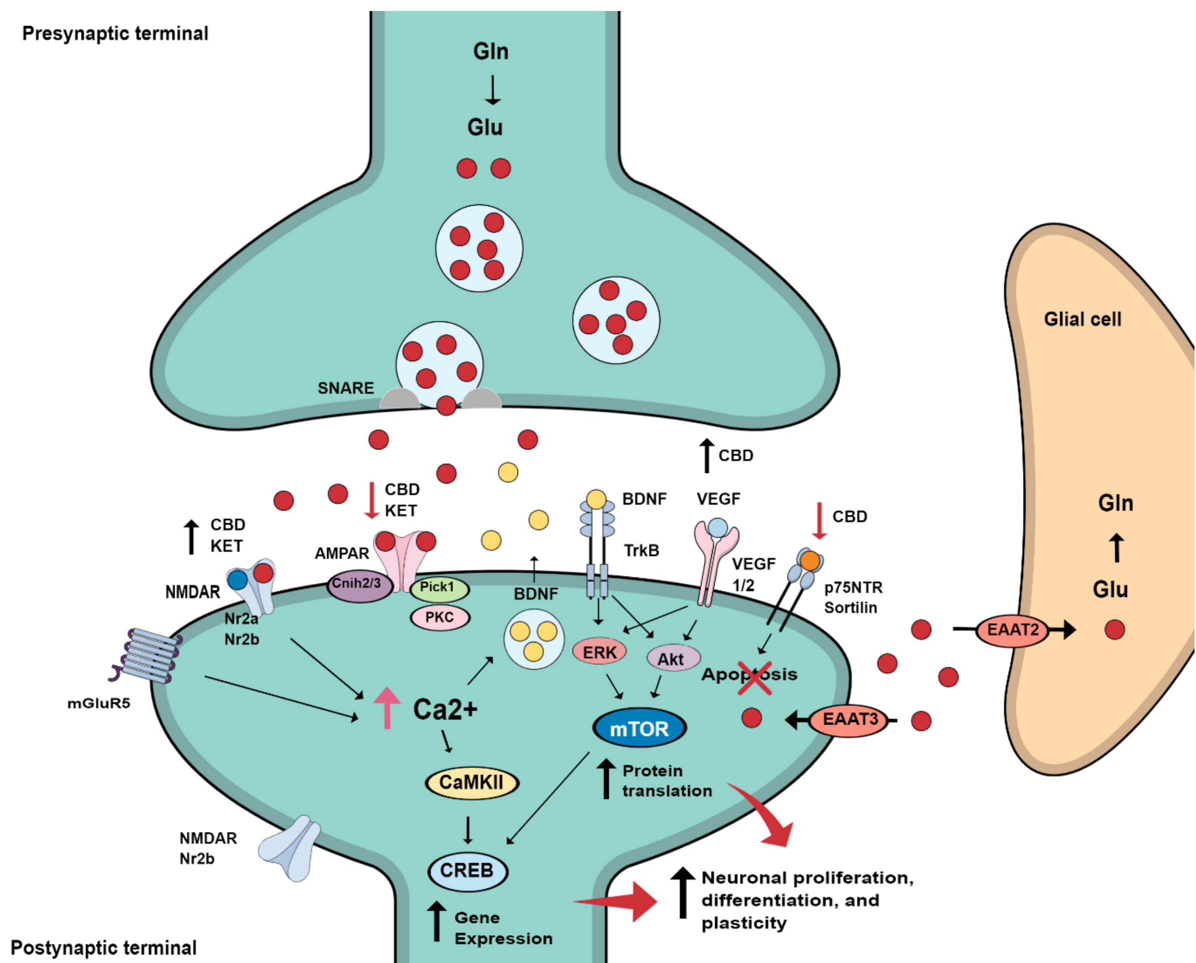

Figure 38. The possible molecular mechanism involved on CBD and ketamine antidepressant effect in the dorsal hippocampus of FSL rats. CBD increases VEGF levels in the DH, which activates its receptor (VEGF-1/2), which is responsible for activating a series of second messengers, which lead to the activation of CREB by increasing gene expression and translation of synaptic proteins, resulting in increased neuronal proliferation and synaptic plasticity. In the same way, CBD decreases the Sortilin levels, preventing apoptosis. In addition, CBD and ketamine decrease the phosphorylation of GluR1 (S831) and Nr2b in the same limbic region, leading to the increased of synaptic strengthening that results in the antidepressant-like effect. Abbreviations: Akt: Protein kinase B; AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BDNF: brain-derived neurotrophic factor; Ca2+: Calcium ion; CaMKII: Calcium/calmodulin-dependent protein kinase II; CBD: Cannabidiol; Cnih2/3: Cornichon family AMPA receptor auxiliary protein 2 and 3; CREB: cAMP response element-binding protein; EAAT2: Excitatory amino acid transporter 2; EAAT3: Excitatory amino acid transporter 2; ERK: Extracellular signal-regulated kinase; Gln: Glutamine; Glu: Glutamate; GSK3: Glycogen synthase kinase-3; KET: Ketamine; Mapk1: Mitogen-activated protein kinase; mGluR5: Metabotropic glutamate receptor 5; mTOR: mammalian target of rapamycin; NMDA: N-methyl-D-aspartate; p75NTR: neurotrophin receptor P75; PKA: Protein kinase A; PKC: Protein kinase C; PI3K: Phosphoinositide 3-kinase; Pick1: Protein interacting with C-kinase; SNARE: soluble NSF attachment receptor; TrkB: Tropomyosin receptor kinase B; VEGF: vascular endothelial growth factor; VEGFR-1/2: vascular endothelial growth factor receptor 1 and 2.

CBD is the only one to change neurotrophin signaling molecule, upregulating the VEGF mRNA expression and downregulation of Sort1 transcript. As discussed previously, sortilin forms a complex with p75NTR that leads to apoptosis. Repeated administration with the monoaminergic antidepressant drug (YANG et al., 2020) and ECT (STELZHAMMER et al., 2013) decreases

sortilin levels in the brain. Depressed patients who respond to the antidepressant treatment have low sortilin baseline levels in the blood than non-responders (BUTTENSCHØN et al., 2018). Our finding suggests that the antidepressant effect of CBD involves a downregulation in sortilin levels in this limbic region in order to prevent apoptosis, which in turn could facilitate other signaling pathways enrolled in neuronal survival, differentiation, and plasticity. In fact, it will be interesting to perform a pharmacological blockade of sortilin receptor to confirm the results and reinforce the role of this molecule in CBD effect.

In addition, CBD injection increased the VEGF transcript levels, reversing the changes in the condition (FSL vs FRL rats). As previously mentioned above, CBD modifies the blood flow in the HPC of healthy patients suggesting a possible VEGF involvement in this effect(BLOOMFIELD et al., 2020). Moreover, it has been demonstrated that VEGF could modulate synaptic plasticity (TILLO et al., 2012). For example, VEGF overexpression in rodents enhanced hippocampal spatial memory formation (CAO et al., 2004; LICHT et al., 2011). Evidence suggests that VEGF interact with its receptor VEGFR2 modulating the Ca2+ influx through AMPA and NMDA receptors (BOGAERT et al., 2010; KIM et al., 2008; ROSSI et al., 2016). VEGF increases Ca2+ influx in the rat hippocampal neuron culture, which activates CaMKII and mTOR, leading to LTP (KIM et al., 2008). However, the CBD injection failed to change the gene and protein levels of CaMKII and mTOR 1 hour after the injection. Thus, it will be interesting to evaluate the time-dependent molecular alterations.

Furthermore, we found that both treatments with CBD and ketamine in FSL rats showed reduced phospho GluR1 (S831) levels in the DH. Corroborating with our findings, a prior study revealed that single administration of ketamine reduced the levels of GluR1 phosphorylated in the HPC, however, the tricyclic antidepressant drug imipramine did not affect the phosphorylation of this serine residue (MAENG et al., 2008). Thereby, the presented findings suggest that reduced phosphorylation GluR1 on the serine-831 residue of the AMPA receptor in the DH is common molecule involved in the mechanism of fast-acting antidepressant drugs, ketamine and CBD.

In VH, CBD-treated rats have decreased the gene-expression levels of mGluR5, which was already discussed above. Not many other changes were detected, as seen in figure 39 for the ventral hippocampus.

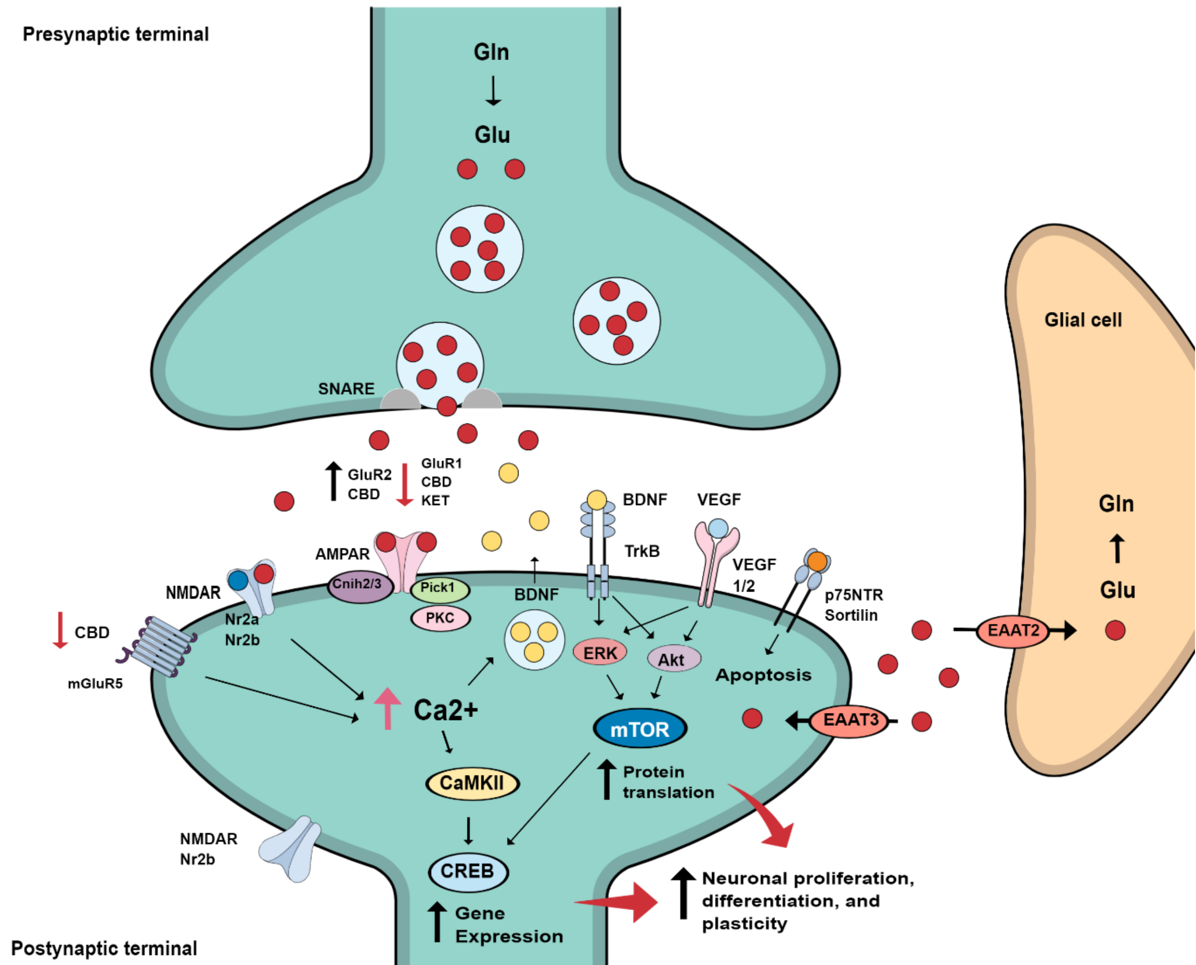

Figure 39. The possible molecular mechanism involved on CBD and ketamine antidepressant effect in the ventral hippocampus of FSL rats. CBD decreases mGluR5 gene expression in the VH that probably could facilitates BDNF signaling pathway, resulting in neuronal survival and synaptic transmission, and its behavioral effect. In addition, CBD and ketamine decrease the phosphorylation of GluR1 (S831 and S845 (CBD)) in the same limbic region, which could modulate the synaptic strengthening. Moreover, CBD increases the GluR2 expression, increasing the internalization of AMPAR in the synapse. Abbreviations: Akt: Protein kinase B; AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BDNF: brain-derived neurotrophic factor; Ca^{2+}: Calcium ion; CaMKII: Calcium/calmodulin-dependent protein kinase II; CBD: Cannabidiol; Cnih2/3: Cornichon family AMPA receptor auxiliary protein 2 and 3; CREB: cAMP response element-binding protein; EAAT2: Excitatory amino acid transporter 2; EAAT3: Excitatory amino acid transporter 2; ERK: Extracellular signal-regulated kinase; Gln: Glutamine; Glu: Glutamate; GSK3: Glycogen synthase kinase-3; KET: Ketamine; Mapk1: Mitogen-activated protein kinase; mGluR5: Metabotropic glutamate receptor 5; mTOR: mammalian target of rapamycin; NMDA: N-methyl-D-aspartate; p75NTR: neurotrophin receptor P75; PKA: Protein kinase A; PKC: Protein kinase C; PI3K: Phosphoinositide 3-kinase; Pick1: Protein interacting with C-kinase; SNARE: soluble NSF attachment receptor; TrkB: Tropomyosin receptor kinase B; VEGF: vascular endothelial growth factor; VEGFR-1/2: vascular endothelial growth factor receptor 1 and 2.

However, we found that CBD decreased phospho GluR1 (S831 and S845) levels and increased the protein levels of the GluR2 AMPA subunit in the VH compared to FSL vehicle-treated rats. However, ketamine treatment decreased only protein levels of phospho GluR1 (S831) in the same brain region. The meaning of these findings remains obscure. However, taking findings from other brain areas into consideration, a prior study revealed that a single administration of

ketamine reduced the levels of GluR1 phosphorylated in the HPC, wheras the tricyclic antidepressant drug imipramine failed to affect the phosphorylation of this receptor subunit (MAENG et al., 2008). Moreover, the chronic treatment with desipramine and paroxetine increased the membrane expression of GluR2/3 in HPC (MARTINEZ-TURRILLAS; FRECHILLA; DEL RÍO, 2002). Given these findings it can be hypothesized that the rapid antidepressant effect produced by ketamine depends on the reduction of the phosphorylation GluR1 on the serine-831 residue of the AMPA receptor in the VH, in order to exert its effect. In contrast, the effect of CBD seems to decrease the phosphorylation of GluR1 (S831 and S845) and increases in the number of GluR2-containing AMPA receptors, suggesting that CBD may enhance the internalization of AMPA receptor. Further studies are needed to measure the phospho GluR2 to clarify the mechanism involved in the effects of CBD.

4.6 LIMITATIONS

A number of limitations are evident from the present work. First, as we were unable to quantify the CBD and metabolite levels in the blood and the brain, it is difficult to fully account for the pharmacokinetic variables in the study, which may affect the findings. Future work should take this into account. Second, performing the gene expression analysis through RT-qPCR using 48.48 dynamic microfluidic arrays (Fluidigm®), the samples were diluted equally using the same dilution factor determined previously in a pilot study. As a result, genes with low expression were not amplified in the samples because they were below the detection threshold. Third, using synaptosomes, an important tool for studying physiological functions in the synapse (GULYÁSSY et al., 2020; JHOU; TAI, 2017), the purification method can contribute to undesired variance. Normally, it is desirable to purify only the nerve terminal, however, it contamination with extrasynaptosomal mitochondria and glial cell type can be observed (GULYÁSSY et al., 2020), and may interfere in the protein results. Indeed, further western blot analyses are needed to evaluate the protein levels from mitochondrial and glial structures to verify possible contamination during the purification process. Fourth, examining many genes simultaneously can lead to multiple comparisons error, which was only weakly corrected for in the present work. This may give false-positive results. However, it should be noted that some of the findings herein show both gene and protein expression changes, and others show a cluster of changes. Nevertheless, in future work, the statistical poser of the studies should be increased, and more rigorous multiple corrections should be employed.

4.7 FUTURE PERSPECTIVES

To confirm the CBD effect in BDNF-TrkB and VEGF signaling pathway, it will be interesting to conduct the stereotaxic surgery to insert the guide-cannula in the specific brain regions (PFC and DH) and do the pharmacological blockade using the inhibitor of TrkB and VEGF receptor. Moreover, it will be crucial to investigate the neurogenesis and neuroplasticity in both limbic brain regions (PFC and DH) through the morphological and image analysis. In special, for VEGF finding, the morphological studies estimating the hippocampal volume, microvessels length, estimation of astrocytes and microglia cell using the optical fractionator will be interesting to investigate the participation of VEGF in the CBD effect.

It will be essential to evaluate the mRNA and protein levels for the targets related to neurotrophic signaling, glutamatergic neurotransmission and synaptic proteins in PFC, DH, and VH of FSL rats at different time points after the treatment with CBD and KET to provide a deep understanding of the molecular mechanism involved in the behavioural response.

Furthermore, as already mentioned, it will be important to determine the levels of CBD and metabolites in the blood and rat brain to check whether there is a correlation between the immobility time presented in the test and CBD levels.

4.8 CONCLUSION

We show that a single injection with CBD or ketamine produces antidepressant-like effects in FSL rats submitted to FST without affecting the locomotor activity in the OFT. There was no correlation between the immobility time displayed in the FST and whole blood CBD levels.

Furthermore, we investigated the molecular and biochemical mechanisms involved on CBD and ketamine antidepressant effect. In contrast to our expectations, CBD and KET did not share a common molecular expression pattern in the genes and proteins examined.

Notably, we showed that FSL rats have several changes in the neurotrophic signaling, glutamatergic, neurotransmission, and synaptic proteins in the limbic brain regions (PFC, DH, and VH) compared to FRL rats. These findings reinforce that FSL is a valid genetic animal model to study the pathophysiology involved in depression and to investigate promising antidepressant compounds and their molecular mechanisms involved in the effect.

4.9 APPENDIX – SUPPLEMENTARY MATERIAL

The gene expression results from the following targets genes related to serotoninergic (5-HT1A; 5-HT1B; 5-HT2A; 5-HT2B; 5-HT2C; Sert (Slc6a4) and endocannabinoid (CB1; CB2; TRPV1; GPR55; FAAH; MAGL) neurotransmission and diverse gene (Opmr1; GAD65; GABAA) are presented in the following additional tables. The genes 5-HT2B, Sert (Slc6a4), Opmr1 (PFC, DH and VH), and CB2, TRPV1, GPR55 (DH and VH) were not amplified in the gene expression analysis.

4.9.1 Prefrontal cortex

The results found with CBD and S-ketamine acute injection on transcript levels related serotoninergic, endocannabinoid neurotransmission and diverse genes in prefrontal cortex of FSL rats were summarized in Table S1.

Table S1. Differences in strain and CBD and ketamine treatment of relative transcript levels in the prefrontal cortex of FSL and FRL rats. Values represent Mean ± SEM. Abbreviations that have not been used previously: n.s., not significant; S, Strain; T, treatment.

Simbol		**FRL-VEH**	**FSL-VEH**	**FSL-Ket**	**FSL-CBD**	**Statistical Analysis**
Serotoninergic Neurotransmission						
Htr1a	5-ht1a	1.197±0.139	1.191±0.139	1.004±0.191	1.283±0.174	S: (12)= 0.028; p= 0.977; n.s. T: F(2, 17)=0.696; p= 0.512; n.s.
Htr1b	5-ht1b	0.999 ± 0.023	0.878 ±0.021#	0.934±0.027	0.909±0.034	S: t(13)=3.783; p= 0,0023 T: F(2, 17)=0.949; p= 0.406; n.s.
Htr2a	5-ht2a	1.009±0.047	1.104±0.045	0.976±0.144	1.068±0.056	S: t(13)= 1.433; p=0.175; n.s. T: H(3)=1.188; p= 0.569; n.s.
Htr2c	5-ht2c	1.024±0.081	0.624 ± 0.066#	0.701±0.042	0.695±0.099	S: t(12)= 3.80; p=0.0025 T: F(2, 17)=0.384; p=0.687; n.s.
P11	P11	1.008±0.053	1.101 ± 0.08	1.083±0.084	1.096±0.11	S: t(14)= 0.963; p=0.352; n.s. T: F(2, 20)=0.011; p=0.988; n.s.
Endocannabinoid Neurotransmission						
Cnr1	CB1	1.003±0.018	0.93±0.047#	0.942±0.032	0.933±0.045	S: U= 14; p= 0.061; n.s. T: F(2, 20)=0.025; p= 0.974; n.s.
Cnr2	CB2	1.203±0.251	1.266±0.155	1.961±0.479	0.877±0.19	S: t(13)= 0.206; p= 0.839; n.s. T: H(3)=3.789; p= 0.152; n.s.
TRPV1	TRPV1	1.030 ± 0.089	0.737±0.102#	0.822±0.097	1.01±0.144*	S: t(14)= 2.159; p=

						0.048 T: F(2, 20)=4.393; p= 0.026; Fisher's LSD test: p= 0.0077
Gpr55	GPR55	1.019 ± 0.072	1.271±0.104	1.341±0.074	1.05± 0.193	S: t(13)= 1.929; p= 0.076; n.s. T: F(2, 19)=1.286; p= 0.2995); n.s.
Faah	FAAH	1.006±0.041	0.965±0.053	0.997±0.056	1.034±0.038	S: t(14)= 0.6173; p= 0.5492; n.s. T: F(2, 20)=0.4531; p= 0.642; n.s.
Magl	MAGL	1.011 ± 0.057	0.95±0.0.062	0.892±0.035	0.924 ± 0.047	S: t(14)= 0.7243; p=0.4808; n.s. T: F(2, 20)=0.3491; p=0.709; n.s.
Diverse Genes						
Gabrd	$GABA_A$	1.036 ± 0.098	0.985±0.114	1.053±0.129	1.074 ± 0.186	S: t(14)=0.34; p=0.738; n.s. T: F(2, 20)=0.107; p=0.899; n.s.
Gad2	GAD65	1.0 ± 0.035	0.98±0.027	0.938±0.024	0.971 ± 0.045	S: t(13)=0.437; p=0.669; n.s. T: F(2, 20)=0.42; p=0.663; n.s.

4.9.2 Dorsal hippocampus

The results found with CBD and S-ketamine acute injection on transcript levels related serotoninergic, endocannabinoid neurotransmission and diverse genes in dorsal hippocampus of FSL rats were summarized in Table S2.

Table S2. Differences in strain and CBD and ketamine treatment of relative transcript levels in the dorsal hippocampus of FSL and FRL rats. Values represent Mean ± SEM. Abbreviations that have not been used previously: n.s., not significant; S, Strain; T, treatment.

Simbol		FRL-VEH	FSL-VEH	FSL-Ket	FSL-CBD	Statistical Analysis
Serotoninergic Neurotransmission						
Htr1a	5-ht1a	1.106±0.201	1.341±0.092	1.371±0.208	1.334±0.206	S: t(14)= 1.059; p=0.307; n.s. T: F(2, 20)=0.012; p=0.987; n.s.
Htr1b	5-ht1b	1.01 ± 0.052	1.090 ±0.019	0.826±0.107*	1.087±0.038	S: U=21.5; p=0.2887; n.s. T: H(3)=6.984; p= 0.0243; Dunn's test: p= 0.047
Htr2a	5-ht2a	1.016±0.079	1.594±0.111[#]	1.524±0.14	1.623±0.1	S: t(13)= 4.110; p= 0.001 T: F(2, 19)=0.171; p=0.8444; n.s.
Htr2c	5-ht2c	1.114±0.22	0.407 ± 0.097[#]	0.434±0.048	0.556±0.123	S: U=6; p= 0.009 T: F(2, 19)=0.723; p=0.4984; n.s.
P11	P11	1.026±0.088	0.945 ± 0.077	0.99±0.051	0.924±0.064	S: t(14)= 0.693; p=0.499; n.s. T: F(2, 20)=0.261; p=0.773; n.s.
Endocannabinoid Neurotransmission						
Cnr1	CB1	1.006±0.044	0.987±0.04	1.001±0.029	1.005±0.013	S: t(14)=0.313; p=0.758; n.s. T: H(3)=0.274; p=0.879; n.s.
Faah	FAAH	1.006±0.046	0.9±0.02	0.92±0.032	0.908±0.07	S: U= 16; p=0.103; n.s. T: H(3)=0.826; p=0.661; n.s.
Magl	MAGL	1.016 ± 0.07	0.807±0.03[#]	0.792±0.039	0.79±0.054	S: U= 11.5; p=0.03 T: F(2, 20)=0.053; p=0.948; n.s.

Diverse Genes						
Gabrd	$GABA_A$	1.028 ± 0.092	0.965±0.109	1.12±0.109	1.116 ± 0.229	S: t(14)=0.438; p=0.6678; n.s. T: F(2, 20)=0.344; p=0.7127; n.s.
Gad2	GAD65	1.0 ± 0.035	0.98±0.027	0.938±0.024	0.971 ± 0.045	S: t(14)=1.202; p=0.249; n.s. T: F(2, 19)=0.033; p=0.967; n.s.

4.9.3 ***Ventral hippocampu***s

The results found with CBD and S-ketamine acute injection on transcript levels related serotoninergic, endocannabinoid neurotransmission and diverse genes in ventral hippocampus of FSL rats were summarized in Table S3.

Table S3. Differences in strain and CBD and ketamine treatment of relative transcript levels in the ventral hippocampus of FSL and FRL rats. Values represent Mean ± SEM. Abbreviations that have not been used previously: n.s., not significant; S, Strain; T, treatment.

Simbol		FRL-VEH	FSL-VEH	FSL-Ket	FSL-CBD	Statistical Analysis
Serotoninergic Neurotransmission						
Htr1a	5-ht1a	1.054±0.127	1.098±0.117	1.133±0.112	1.272±0.164	S: t(14)= 0.253; p=0.804; n.s. T: F(2, 19)=0.464; p=0.635; n.s.
Htr1b	5-ht1b	1.004 ± 0.03	1.041 ±0.02	0.947±0.053	1.050±0.057	S: t(13)=1.03; p=0.322; n.s. T: H(3)=1.824; p= 0.402; n.s.
Htr2a	5-ht2a	1.021±0.08	1.355±0.085[#]	1.219±0.043	1.13±0.064	S: t(14)= 2.852; p= 0.012 T: F(2, 19)=2.8; p= 0.086; n.s.
Htr2c	5-ht2c	1.023±0.085	0.685 ± 0.093[#]	0.821±0.148	0.767±0.127	S: t(15)=2.648; p= 0.018 T: F(2, 21)=0.331; p=0.722; n.s.
P11	P11	1.056±0.123	0.968 ± 0.081	1.079±0.046	0.812±0.049	S: t(15)= 0.604; p=0.554; n.s. T: F(2, 19)=3.449; p=0.053; n.s.
Endocannabinoid Neurotransmission						
Cnr1	CB1	1.013±0.059	0.987±0.07	0.894±0.045	0.947±0.045	S: t(15)=0.259; p=0.799; n.s. T: F(2, 21)=0.679; p=0.518; n.s.
Faah	FAAH	1.018±0.069	0.779±0.027[#]	0.831±0.038	0.804±0.038	S: U= 6; p=0.0023. T: F(2, 21)=0.612; p=0.552; n.s.
Magl	MAGL	1.025 ± 0.08	0.648±0.032[#]	0.75±0.048	0.674±0.057	S: U=3; p=0.0009

						T: F (2, 20)=1.36; p=0.279; n.s.
Diverse Genes						
Gabrd	$GABA_A$	1.093 ± 0.163	1.069±0.157	1.179±0.177	0.82 ± 0.099	S: t(15)= 0.104; p= 0.918; n.s. T: F(2, 21)=1.325; p=0.287; n.s.
Gad2	GAD65	1.001 ± 0.029	0.952±0.014	0.89±0.052	0.926 ± 0.028	S: t(14)= 1.465; p=0.165; n.s. T:H(3)=1.827; p=0.401; n.s.

4.10 Attachment

Certificate of approval of the Danish Animal Experiments Inspectorate.

Sag nr.: **2016−15−0201−01105**
(udfyldes af Dyreforsøgstilsynet)

Dyreforsøgstilsynet
Digital indberetning og ansøgning

Maternal infektion og psykiatrisk sygdom - Gregers Wegener

Status for ansøgning: Godkendt (23. december 2016)

Den samlede ansøgning indeholder:

Afsnit A

Afsnit B

Oprettede Afsnit C−skemaer:

C 1

Oprettede Afsnit D−Skemaer:

Oprettede Afsnit E−Skemaer:

Oprettede Afsnit F−Skemaer:

Oprettede Afsnit G−Skemaer:

G 1

G 2

G 3

G 4

G 5

Skemaer for onkologiske undersøgelser:

Bilag:	**Beskrivelse:**

DYREFORSØGSTILSYNET
Stationsparken 31
2600 Glostrup
Telefon: 7227 6900
E–mail: dyreforsoegstilsynet@fvst.dk
Hjemmeside: www.dyreforsoegstilsynet.dk

ANSØGNING OM
TILLADELSE TIL AT FORETAGE DYREFORSØG
i medfør af lov om dyreforsøg, jfr. lovbekendtgørelse nr. 726 af 9. september 1993 som ændret ved lov nr. 1081 af 20. december 1995

Maternal infektion og psykiatrisk sygdom

Sag nr.: **2016–15–0201–01105**
(udfyldes af Dyreforsøgstilsynet)

Afsnit A: OPLYSNINGER OM ANSØGEREN

Ansøgeren:	Navn: Gregers Wegener	
	Stilling: Professor, dr.med	
Arbejdssted:	Institution/virksomhed: Translational Neuropsychiatry Unit (TNU)	
	Adresse: Skovagervej 2	
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	E–mail: wegener@dadlnet.dk	
Ansøgerens uddannelse:	Art:	Årstal:
	dr.med, Århus Universitet	2012
	Ph.d. i Medicin	2001
	Cand. med	1997
Vigtigste tidligere ansættelser og supplerende uddannelser, herunder erfaring med dyreforsøg	Arbejdsstedets EAN–nummer: 5798002761689 SE–nummer: DK31006686 CVR–nummer: 29190925 TIDLIGERE ANSÆTTELSER: Institut for Psykiatrisk Grundforskning, Afd. For Biologisk Psykiatri: 1997–2000 Forskellige sygehuse i Århus Amt: 2001–2002 Center for Psykiatrisk Forskning: 2002–dd. TIDLIGERE ERFARINGER MED DYREFORSØG Jeg har arbejdet med dyreforsøg på rotter siden 1994 under supervision af Laborator, dr.med Klaus Thomsen. Jeg har siden 2002 haft selvstændig tilladelse til dyreforsøg	
Deltagelse i kursus i forsøgsdyrskundskab:	☒ Ja ☐ Nej — År: 1998 — Sted: Odense	Varighed: 1 uge
Tidligere meddelt tilladelse til dyreforsøg:	☒ Ja ☐ Nej — Seneste dato: Oktober 2012	J.nr.: 2012–15–2934–00254

Sag nr.: **2016−15−0201−01105**
(udfyldes af Dyreforsøgstilsynet)

Afsnit B: OPLYSNINGER OM FORSØGSSTED

Forsøgssted:	Institution/virksomhed Translational Neuropsychiatry Unit (TNU), Århus Universitet Adresse Skovagervej 2 Postnummer og By 8240 Risskov Telefon 78471112 Fax 78471108 E−mail wegener@dadlnet.dk
Hvor påtænkes dyrene opstaldet ?	På ovennævnte adresse. Enheden har en moderne indrettet dyrestald (fra 2012)
Hvilket personale skal passe dyrene, og hvilken baggrund har dette herfor ? (kontaktperson kan evt. anføres)	Alle der skal passe dyrene har gennemgået obligatorisk uddannelse. Følgende personer, alle ansatte pr 1/8 2016, vil indgå i pasningen: PRIMÆRE Dyrepasser Tessa Rasmussen Dyrepasser Stine Dhiin SEKUNDÆRE Bioanalytiker Per Mikkelsen Studerende/post.docs der har §2 kursus Undertegnede
Kort beskrivelse af, i hvilket omfang andre deltager i forsøgenes udførelse ?	Der vil der være et skiftende antal medicin−/speciale− eller ph.d.−studerende, som deltager i forsøgenes udførelse under min vejledning. Dog skal det fremhæves, at disse personer ikke vil deltage aktivt i forsøgsaktiviteterne før tilfredsstillende resultat fra §2 dyreforsøgskursus er opnået.
Hvilke foranstaltninger er der truffet vedrørende veterinær rådgivning og behandling ?	Der er truffet aftale med Dyrlæge, ph.d. Birgitte Kousholt, Institut for Klinisk Medicin, ved Aarhus Universitet om regelmæssigt besøg og veterinær rådgivning.
Dato 23. december 2016	Ansøgerens underskrift

DYREFORSØGSTILSYNET

Sag nr.: **2016−15−0201−01105**

(udfyldes af Dyreforsøgstilsynet)

Afsnit C−skema nr. C 1

Der skal anvendes et sæt afsnit C−skema for hver forsøgstype/forsøgsrække

Beskriv forsøget generelt: (Denne del af ansøgningen skal kunne forstås af ikke fagfolk og skal omfatte: A) En beskrivelse af forsøgets formål. B) En redegørelse for, at tilsvarende viden kun kan opnås ved anvendelse af levende dyr (B1) og ikke kan opnås ved mindre belastende undersøgelser (B2) eller ved anvendelse af færre dyr (B3). C) En kort redegørelse for antagelsen om, at forsøget er til væsentlig gavn. D) En beskrivelse af samtlige indgreb, der ønskes foretaget på dyrene, herunder en udførlig beskrivelse af den belastning, dyrene herved udsættes for.)

A) Formålet med disse undersøgelser er at studere neurobiologien og effekten af maternal infektion på udviklingen af psykiatrisk sygdom i transgene og selektivt fremavlede dyremodeller der er sårbare for herpesvirusinfektion, eller i genetiske dyremodeller for depression (FSL/FRL samt Sprague Dawley som kontrol). Mere specifikt, så vil vi søge at afdække hvad der sker i dyreadfærd og i hjernens gensammensætning/struktur i dyr der bliver inficeret med herpesvirus eller har lav grad af kronisk inflammation under eller lige efter moderens graviditet, eller efter faderen har haft en infektion. Vi vil endvidere studere om disse afkom vil være mere sårbare såfremt de i voksenlivet bliver udsat for en mild subkronisk stress. Efter aflivning (se nedenfor) vil hjernerne og vævet blive udtaget og analyseret med mikroskopi og molekylærbiologiske metoder

B1) Hjernens struktur og opbygning er uhyre kompleks, og funktionen afhænger af både interne neurobiologiske faktorer og eksterne miljømæssige faktorer. Hjernens korrekte neurobiologiske funktion er afhængig af samspillet mellem nerveceller, støtteceller og deres indbyrdes forbindelser. Det er desværre ikke muligt at genskabe en lignende forsøgsopstilling der kan belyse hele funktionelle psykiatriske problemstillinger uden anvendelse af forsøgsdyr.

B2) Da genetiske faktorer og miljø spiller er betydelig rolle ved udviklingen af den menneskelige sygdom er det ikke muligt at opnå indsigt i de involverede mekanismer uden anvendelse af metoder som der her søges om tilladelse til.

B3) Variansen i adfærdsundersøgelserne er stor, og derfor er det nødvendigt at anvende typisk 6−12 dyr per forsøgsgruppe. Dette antal søges minimeret ved anvendelsen af standardiserede tests og styrkeberegninger inden forsøg opstartes. Studierne suppleres sideløbende med celle forsøg for at minimere brug af forsøgsdyr.

C) De foreslåede forsøg anses at være til gavn for forståelsen af behandlingen af og opståelsen af psykiatriske og metaboliske lidelser (især depression) og samspillet med infektionssygdomme. Vi vil forsøge at få besvaret grundlæggende spørgsmål vedrørende mulige behandlinger. Disse problemstillinger er ikke tidligere belyst. Eksempelvis er depression en særdeles invaliderende sygdom med store lidelser for den enkelte patient og pårørende, og en tilstrand der belaster samfundet med store årlige udgifter (EURO 110 mia/år i Europa (European Journal of Neurology 2012, 19: 155‘62)). Der findes nogen behandling, men alle kendte behandlinger har en latenstid på 2” uger før de virker, og er kun effektive i 60—0% af de syge. Udvikling af nye stoffer er vanskelig, da forståelsen af sygdommens biologi er ikke klar. Modellerne der her søges om tilladelse til at anvende er transgene modeller særlig egnet til at belyse samspillet mellem maternel infektion med herpesvirus og miljømæssigefaktorer. Modellerne har vist sig at være velegnet til studiet af de immunologiske mekanismer, hvorved vi forventer at kunne studere samspillet med psykisk sygdom med en minimal mængde af dyr.

D) Dyrene, som opstaldes i grupper med fri adgang til føde og vand. Gravide dyr og/eller fader påføres enten Herpes infektion, PolyIC eller Lipopolysaccaride−pellet (LPS), der kan inducere influenzalignende symptomer. Afkommet vil først gennemgå adfdærdsundersøgelse for at klarlægge om infektion af forældrene og eventuel stress i voksenlivet medfører psykiske deficits (se nedenfor). Såfremt vi finder mulige psykiatriske defecits, kan der (med anvendelse af nye dyr således at samme adfærdstest ikke gentages på de samme dyr) blive behandlet med kendt eller ny mulig psykiatrisk medicin der påvirker immunsystemet, serotonin, noradrenalin eller glutamatomsætningen i hjernen. Behandlingen med lægemiddel kan foregå fra en enkelt dag til op til sædvanligvis fire uger. I et sådsant behandlingsforløb vil dyrene blive adfærdsmæssigt bedømt, ved hjælp af standardiserede undersøgelsesmetoder til screening af mentale faktorer hos gnavere, dvs adfærdsopstillinger der er målrettet til at skelne om dyrets adfærd er psykotisk, depressiv, angstlignende eller om dyret har en påvirket indlæring. Efter endt undersøgelse og aflivning, vil dyrenes organer blive genstand for detailundersøgelse. Belastningsvurdering: Induktion af herpesvisusinfektion, LPS eller PolyIC medfører forbigående sygdomsadfærd (1−2 dage) uden øvrige forandringer hos moderen/faderen. Injektion medfører en let, kortvarigt smerte. De injicerede antidepressive lægemidler forventes ikke at give væsentligt ubehag. I det omfang effekten af disse tidligere er undersøgt i gnavere, er der ikke observeret væsentlige bivirkninger i de doser, der planlægges anvendt i dette studium. Adfærdsunderøgelserne medfører kortvarig udsættelse for nye ukendte situationer. Dette kan virke forbigående stressende for dyrene. I tidligere studier er det vist at stresshormonerne hurtigt er tilbage ved udgangspunktet efter endt undersøgelse.

Skyldes forsøgene krav fra myndighed, som led i godkendelse af stof eller produkt? ☐ Ja ☒ Nej

− hvis "Ja", beskriv nærmere:

Beskriv forsøgets type, art og forløb, herunder de planlagte indgreb og påvirkning af organfunktioner:
(Der vedlægges evt. forsøgsprotokol. Der angives og begrundes, hvorvidt de givne oplysninger skal hemmeligholdes.)

DYR

Både C57/Bl6 og relevante immundefekte genmodificerede mus (primært C57BL/6−Tmem173gt (avlstilladelse 2013−15−2935−00039) og C57/BL6−Mb2ldl (avlstilladelse 2013−15−2935−00039)) eller FSL/FRL rotter får enten Herpes infektion (200 ul, 9 dage efter parring) eller stoffer som dsDNA (9 og 12 dage efter parring) eller PolyIC (200 ul, 9 dage efter parring) til at inducere influenzalignende symptomer. En udvalgt population vil få en kontinuerlig lav dosis LPS forud for parringen (slow−release pellets). Behandlingen administreres i bughulen (i.p. injektion eller eventuelt ved indoperation af slow−release pellet i bughulen (som vil foregå under fuld bedøvelse med anvendelse af Hypnorm/Dormicum anæstesi og smertestillende efterfølgende som anført nedenfor). Belastning for dyrene: Til anæstesi bruges Isoflurangas eller hypnorm/dormicum, som giver en meget kortvarig belastning for dyrene og en hurtig opvågning. En klinisk sygdomsmanifestation vil vise sig som lettere ubehag, meget lignende forkølelse hos mennesker. De behandlede dyr og afkom vil blive vejet og observeret regelmæssigt og ved uventet vægttab på mere end 20% eller uventet lidelse vil dyrene blive aflivet. Belastningsgraden af disse behandlinger formodes at være moderat. I enkelte dyr kan der blive behov for at udtage blodprøver fra en halevene/fra tungen i forsøget, eller at bedømme metabolisk status (max en gang per dyr) ved hjælp af en oral glucose tolerance test. Dette gøres uden bedøvelse, og da det foretages af uddannet personale er vurderingen, at det ikke er forbundet med væsentlig ekstra lidelse eller smerte; men er dog et ubehag for dyret, der skønnes primært at bestå i at blive håndteret.

STRESSPROTOKOL

For at undersøge om infektion eller infektion−lignende behandling hos forældre leder til øget sårbarhed overfor stress hos afkom, ansøger vi om mulighed for at udføre på afkommet en op til 10 dages sub−kronisk stress protokol samt mulighed for at fodre med high−fat diet. Denne stress protokol indeholder elementerne fodchok (dag 1), restraint stress (dag 3), vanddeprivering (dag 5), fjernelse af skjul og berigning (dag 7), samt gentagne burskift (dag 9). Fodchok: dyret placeres i et konditioneringskammer i 3 minutter, hvorefter det modtager 3 milde fodchok (0.3 mA), som hver varer 1 sec. Disse 3 fodchok bliver givet med 3 minutters mellemrum og sessionen ender med en 3 min periode hvor ingen fodchok administreres. Restraint stress: dyret placeres i en lille transparent beholder (ca 3 cm i diameter og 11 cm lang) i 45 minutter. I beholderen er boret lufthuller og dyrene flyttes tilbage i deres hjembur så snart sessionen er slut. Vanddeprivering: dyrenes vandflaske fjernes i 16 timer (over natten). I denne periode har dyrene stadig fri adgang til mad. Fjernelse af skjul og berigning: dyrenes skjul samt berigning i form af bidepind og redemateriale fjernes i 16 timer (over natten). Gentagne burskift: 5 gange på et døgn overføres dyrene til et nyt bur. I de nye bure er hver gang adgang til skjul, berigning, vand og mad som vanligt. Belatningsgraden for stress proceduren formodes at være moderat. Tidligst en dag efter dyrene har gennemgået stressproceduren (samt kontroldyr, der ikke gennemgår stress) bliver de testet for symptomer på depression, angst og skizofreni. Eventuel behandling med lægemiddel kan finde sted under stresprotokollen eller efter denne.

EVENTUEL FARMAKOLOGISK BEHANDLING

Dyrene indgives et antidepressivt eller immunaktivt lægemiddel (antiinflammatorisk, tricyklisk, SSRI multimodal, NMDA receptor antagonist, mGlu receptor antagonist, NOS inhibitor eller neuropeptider (NPY og NPS), som påvirker signaltransmissionen i hjernen) peroralt, intranasalt, eller parenteralt. Injektioner gives subcutant eller intraperitonealt, sædvanligvis som en akut administration én til tre gange i kort tid (sædvanligvis en time) før forsøgets udførelse. Belastningen ved en fre enkelt injektioner formodes at være lille. Hvert dyr vil maksimalt få tre injektioner.

I nogle forsøg gives kronisk administration en gang dagligt i en periode på op til 4 uger, da det er kendt fra humane studier at behandling med psykotrope stoffer ofte har forsinket indsættende virkning (sædvanligvis mellem 2 og 4 uger). Belastningen ved kronisk administration er større end ved akut administration, og vurderes at være let moderat. Hvert dyr vil maksimalt få 28 injektioner over en periode på 4 uger.

I få eksperimenter kan intrakraniel eller kontinuerlig administration af lægemidler eller LPS være nødvendig, idet steady state kan være vigtig og visse lægemidler ikke passerer blod hjerne barrieren. Ved denne procedure indlægges enten en lille pellet/pumpe i bughulen (ca 5x5x5 mm) gennem en en−2 cm lille incision der efterfølgende sutureres, eller stereotaksisk, under generel bedøvelse (Hypnorm+Dormicum), en guidekanyle via craniotomi i specifikke områder af hjernen. I kraniet indsættes 2 små skruer der tjener til fixation af guidekanylen, som efterfølgende fixeres til craniet ved hjælp af dental acrylic (GC Fuji Plus). Dyret smertestilles de første 3 døgn med rimadyl (første dosis gives sammen med bedøvelsen). I hele perioden observeres dyret for tegn på infektion. Skulle disse optræde gives enten antibiotisk terapi, eller såfremt det er lidelsesvoldende for dyret, aflives dyret. Generne ved denne procedure formodes at være af middel sværhedsgrad.

Efter 5 dage og op til 3 uger efter operationen kan intrakraniel administration af lægemidler foretages. Proceduren foretages højest tre gange, men sædvanligvis kun en gang ½‘ time før adfærdstest. I guidekanylen indsættes en injektionskanyle, der er koblet til en ultrafin sprøjte (Hamilton). Der indgives 5 µl medicinopløsning ved administration i hjerneventriklerne, og 1 µl ved administration i et specifikt hjerneområde. Belastningen ved disse procedurer formodes at være af middel sværhedsgrad.

ADFÆRDSTEST

Med henblik på evaluering af effekten af evt behandling, ønskes dyrenes adfærd undersøgt i nedenstående etablerede screeningsprotokoller for psykisk sygdom. Adfærdsundersøgelserne medfører regelmæssig håndtering, og dermed kortvarig udsættelse for fremmede omgivelser. Der udvises stor omhyggelighed for at afholde en passende temperatur i

forbindelse med adfærdsundersøgelserne. Undersøgelserne for adfærdsændringer skønnes således ikke at være forbundet med væsentlig gene for dyrene. De adfærdstests vi ønsker at benytte os af, er som følger:

SKIZOFRENI:

LATENT INHIBITION: Dette er en test for selective attention disruption set i skizofreni. Testen består af 2 faser; pre−exposure på dag 1 og konditionering på dag 2. Under pre−exposure bliver dyrene udsat for 100 eksponeringer til en 5 sekunders tone (85 dB) (=to−be conditioned stimulus, CS) i en shuttle boks med et inter−stimulus interval på 25−55 sekunder. En kontrolgruppe bliver placeret i samme boks i samme tidsrum, men uden eksponeringer til lyden. På konditioneringsdagen bliver alle dyr placeret i shuttle boksen og bliver eksponeret for 50 avoidance trials, med et inter−stimulus interval på 25−55 sekunder. Konditioneringsfasen starter med præsentation af CS (85 dB lyd). Hvis dyret bevæger sig til den anden halvdel af boksen indenfor 5 sekunder slutter CS og unconditioned stimulus (UCS, 0.3 mA fodshok) bliver undgået. Avoidance failure (altså hvor dyret ikke bevæger sig til den anden side af testboksen) leder til præsentation af UCS sammen med CS. UCS varer max 2 sekunder, men ophører forinden hvis dyret bevæger sig til den anden side af boksen. Ideen er, at ved intakt Latent Inhibition, vil det tage længere tid for pre−exposure gruppen at lære associationen imellem CS og UCS i forhold til non−pre−eksponerede dyr. Dette fænomen er hæmmet i skizofrene individer, hvorfor reduceret latent inhibition bruges som indikation for skizofreni−lignende adfærd i dyr. Dyr der gennemgår denne test gennemgår aldrig Fear conditioning. Belastningsgraden formodes at være moderat.

PREPULSE INHIBITION: Prepulse inhibition er en test for sensorymotor gating ændringer set under skizofreni. Dyret placeres i et testkammer hvor det bliver præsenteret for høje lyde (40 ms, 120 dB) enten alene eller direkte følgene en prepulse (20 ms, 65−80dB). Størrelsen af startle med eller uden prepulse registres. Testen varer cirka 25 min og indeholder max 80 præsentationer af lyd. Belastningen formodes at være moderat.

KOGNITION:

CONDITIONED FEAR: Dette er en test for kognition. Undersøgelserne er typisk inddelt i tre på hinanden følgende dage: 1. Dyrene placeres i et særligt forsøgskammer, dertil indrettet med gittergulv. Efter et kort stykke tid, sædvanligvis 2 minutter, vil dyrene høre en hyletone (ca 75 dB) i normalt 30 sekunder. Hyletonen følges umiddelbart af et kortvarigt stød i gitteret, sædvanligvis 1 sekund, 0.5 mA. Proceduren gentages i alt 3 gange. Det elektriske stød er ubehageligt for dyrene, men ufarligt. 2. På dag 2 placeres dyrene i kammeret i op til 10 minutter. Der er ingen lyd og intet stød. 3. På dag 3 placeres dyrene i kammeret, dog således at gittergulvet er fjernet. Efter 2 minutter påsættes hyletone. Denne tone er til stede i resten af forsøget, op til 6 minutter for at kunne klarlægge extinctionfænomen. Dyr der gennemgår denne test gennemgår aldrig latent inhibition. Belastningsgraden formodes at være moderat, og en hyletone på 75 dB i forventes ikke at ændre dette jf forskningen i gener vdr støj, hvor 85 dB er tilladte værdier for støjbelastning gennem 8 timer (Arbejdstilsynet, At−vejledning D.6.1−4).

OBJECT RECOGNITION: Dette er en hippocampus uafhængig test for kognition. Undersøgelserne er typisk inddelt i to faser gennemført over en eller to dage. 1. Dyrene placeres i en firkantet arena på 50x50 cm under normal belysning. I arenaen er der to ens objekter som dyret har mulighed for at gøre sig bekendt med i løbet af 2−5 minutter. 2. Efter en periode sættes dyret igen ned i arenaen, denne gang med to objekter, hvoraf det ene er identisk med den første dag, og det andet nyt. I løbet af 2−5 minutter registreres tiden som dyret bruger ved det nye objekt (måler evnen til at huske det kendte og mindre interessante objekt). Belastningsgraden formodes at være lille.

T MAZE: Dette er en hippocampus afhængig test for kognition. Undersøgelserne er typisk inddelt i to faser gennemført over en eller to dage. 1. Dyrene placeres i en T formet arena under normal belysning. I arenaen er der to arme (toppen at 'T') som dyret har mulighed for at gøre sig bekendt med i løbet af 2−5 minutter, dog således at den ene arm ikke er tilgængelg på dag 1. Efter en periode sættes dyret igen ned i arenaen, denne gang med begge arme åbne. I løbet af 2−5 minutter registreres tiden som dyret bruger i den ukendte arm (måler evnen til at huske den kendte og mindre interessante arm). Belastningsgraden formodes at være lille.

DEPRESSION OG MANI:

OPEN FIELD TEST: Open Fjeld test er en motor−funktjons test. Dyrene placeres i et åbent kvadratisk felt (op til 1 x 1 m). Bevægelse registreres over et tidsrum (sædvanligvis 20 min, højst 1 time) og belastningen hermed er minimal.

SUKROSEPREFERENCETEST: Dyrene bliver i op til 12 timer præsenteret for et valg imellem 2 vandflasker hvor den ene indeholder vand og den anden en op til 5% sukroseopløsning. Mængden af sukroseindtag i forhold til vand registreres. Testen kan gentages dagligt i op til 14 dage, dog sædvanligvis kun 4 gange indenfor en 14 dages periode. Grunden til at denne test potentielt kan gentages op til 14 dage er, at dyrene skal trænes til et stabilt respons; sædvanligvis tager dette nogle få dage, men da vi ikke på forhånd ved præcis hvor længe denne habituering foregår ansøger vi om maksimalt 14 dage. Da mus er yderst territoriale dyr, vil det til tider være nødvendigt at enkelt−opstalde dyr, som skal gennemgå denne test, så vi ved hvor meget det enkelte dyr har drukket. Det er vores erfaring at enkeltopstaldning under hele forsøget er bedre end at enkeltopstalde dem imens testen foregår og sætte dem sammen igen efter endt sukrosetest, da dette leder til meget uro i buret. Pga enkeltopstaldningen vurderes denne test at være af moderat belastningsgrad.

PORSOLT'S SWIM TEST: Porsolt's Swim Test er en klassisk test for depressions−lignende adfærd. Dyrene placeres i en plastcylinder med en diameter på cirka 20 cm og vandtemperatur 20−26 grader. Dyrenes adfærd bliver optaget og sidenhen kvantificeret. Testen består af en 15 min habitueringssession fulgt af en 6 min test, 24 timer efter habitueringssessionen. Alternativt udføres testen som 2 1 min sessioner med 18 grader vand. De samme dyr bliver dog aldrig udsat for begge disse protokoller. Efter seancerne bliver dyret taget op, tørret grundigt og sat tilbage i sit hjemmebur under varmelampe. Belastningen formodes at være moderat.

TAIL SUSPENSION TEST: En klassisk test for depressions−lignende adfærd (kun hos mus). Dyrene ophænges i halen på

en særlig designet krog ved hjælp af et lille stykke plaster i 6 minutter. Dyrenes adfærd bliver optaget og sidenhen kvantificeret som immobil eller kæmpende. Efter seancerne bliver dyret sat tilbage i sit hjemmebur under varmelampe. Belastningen formodes at være moderat.

STIMULANT−INDUCERET HYPERAKTIVITET Stimulant challenge er en model for mani. Testen foregår i den same type boks, som Open Field testen og foregår ved at en lav−dosis amfetamin (max 5 mg/kg og normalt 2.5mg/kg) eller MK−801 (0.15 mg/kg i.p) injiceres i.p. i dyret 15−30 minutter før testen. En sådan challenge vil lede til hyperlokomotion, hvilket minder om manisk adfærd hos mennesker. Belastningsgaden af dosering med amfetamin og MK−801 formodes at være let og hvert dyr gennemgår normalt 1 og max 2 challenges. Ved 2 challenges vil der være 24 timer imellem trials (dag 1: habituering, dag 2: test). Dyrene er på forhånd blevet habitueret til testboksene et døgn tidligere, for at undgå novelty−induceret hyperlokomotor aktivitet. Denne habituering foregår normalt uden farmakologisk behandling, men det kan være nødvendigt at indgive amfetamin også på denne trial (hvis dette bliver nødvendigt får musene i alt 2 amfetamin behandlinger; en på habitueringsdagen og en 24 timer senere på testdagen). Dyrene er sædvanligvis i forsøgsboksene i 20 min og højst 90 min

ANGST:

ELEVATED PLUS MAZE: Elevated plus maze er en test for angst. En plus maze er en plusformet hævet platform hvor 2 af armene i plusset har mørke vægge (lukkede arme) mens de andre 2 arme er helt åbne uden vægge (åbne arme). Dyret placeres i midten af krydset og har derefter lov til frit at undersøge labyrinten i 5 minutter. Tid i hhv åbne og lukkede arme registreres. Belastningen formodes at være let, idet musen musen aldrig 'tvinges' ud i en lys arm; en mere angst mus vil simpelthen opholde sig i en lukket arm hvor den føler sig tryg.

LIGHT/DARK COMPARTMENT BOX: Dette er en test for angst. En arena (25x50 cm) bestående af en mørk (overdækket) del og en lys del − adskildt af en væg med en lille åbning imellem. Dyret placeres i midten af arenaen og har derefter lov til frit at undersøge den i 5 minutter. Tid i hhv lyse og mørke områder registreres. Belastningen formodes at være let, idet musen musen aldrig 'tvinges' ud i en lys arm; en mere angst mus vil opholde sig i en lukket arm hvor den føler sig tryg.

ANTAL ADFÆRDSTEST PER DYR: Dyrene udsættes ikke for alle ovennævnte adfærdstest. Sædvanligvis vil hvert dyr alene gennemgå de tests, der beskriver den modalitet der undersøges (depression/mani, angst, kognition eller psykose hver for sig). Den samlede varighed af undersøgelserne som dyrene kan udsættes for vil højest være to fire uger for de testopstilling der varer længst tid (kognition). De øvrige test vil finde sted over dage. Det er vurderingen at den samlede belastning som hvert dyr udsættes for ikke vil overstige 'moderat' på,grund af adfærdstestenes kortvarige natur (minutter) og separation i tid hvor forsøget finder sted (der foretages ikke flere belastende tests den samme dag).

AFLIVNING AF DYRENE VED FORSØGETS AFSLUTNING Det vil foregå ved enten dekapitering med henblik på hurtigt at isolere hjernevæv til frysning, eller ved transcardiel perfusion. Sidste indebærer at dyret aflives med en injektion af pentobarbital+lidokain, hvorefter en kanyle føres ind i venstre hjertekammer. Gennem kanylen skylles der med først iskoldt saltvand, dernæst formalinvæske. Denne væske fikserer hjernevævet, og muliggør senere mikroskopi. Belastningen ved denne procedure indebærer fastholden i forbindelse med dekapitering, og injektion af aflivelsesmiddel før perfusionen. Der ansøges om tilladelse til at aflive dyrene op til fire uger efter endt behandling. Grunden til dette er at der er klinisk evidens for at behandling med psykoaktive stoffer, inklusive antidepressiva, inducerer langvarige ændringer. Vi er interesserede i at beskrive forskellen mellem de relativt kortvarige effekter af behandling med de mere langvarige effekter. Efter behandlingerne skitseret ovenfor observeres dyrene nøje indtil aflivning, og der opstaldes i dyrestalden med vanlig pleje og tilsyn.

Belastningsgraden:

Ubehag Moderat	**− beskriv:** Behandlingsprocedurerne kan være ubehagelige, og forbundet med middel gener som skitseret ovenfor. Visse adfærdsundersøgelser kan være forbundet med forbigående ubehag (fx udsættelse for vand og elektrisk strøm). Alle test er ufarlige. Blodprøvetagning fra halevene indebærer at der lægges et lille snit i halen, hvorfra blod samles med kapillærrør, og blodprøve fra tungen indebærer at der kortvarigt prikkes et lille hul i en af venerne på tungens underside, hvorfra blodet opsamles. Dyret kan få forbigående ømhed ved disse procedurer. Dette er ufarligt. Det kan opfattes ubehageligt for dyrene at blive isoleret
Lidelse Moderat	**− beskriv:** i.p. injektioner, samt infektion er forbundet med moderat lidelse.
Smerte Let	**− beskriv:** i.p. injektioner er forbundet med let smerte.
Påvirket bevægelsesfrihed ☐ Ja ☒ Nej	**− hvis "Ja", beskriv:**
Påføres varigt mén ☐ Ja ☒ Nej	**− hvis "Ja", beskriv:**

Belastningens varighed Længerevarende **Varighed** 3 Måneder **Aflives i bedøvelsen (uden på noget tidspunkt at være vågnet op efter forsøgets indledning):** ☐ Ja ☒ Nej
Beskriv den anvendte anæstesi for hver dyreart: Isofluran eller Hypnorm/Dormicum
Beskriv den påtænkte smertebehandling og anden lindrende behandling for hver dyreart: Der gives ikke operation til hovedparten af dyrene, og de tænkte adfærdsundersøgelser er ikke invasive. Dyr der undergår operationsprocedurer få smertestillende medicin. Hvis dyret påføres smerte anvendes NSAID (rimadyl) i 3 dage. Behandlingen startes samtidig med bedøvelsen ved den operative procedure.
Angiv de velfærdsmæssige kriterier (humane endpoints) for afbrydelse af forsøget for hver dyreart: Dyrene tilses dagligt og burene skiftes efter behov, sædvanligvis 2 gange per uge. Såfremt der opstår uforudsete lidelsesvoldende komplikationer aflives dyrene omgående. Lidelsesvoldende komplikationer defineres som: tydeligt tab af legemsvægt (op til 20% af udgangsvægten), manglende selvhygiejne (pleje og renhed af pels og øjenomgivelser) og manglende aktivitet (dvs manglende anvendelse af hele opstaldningsburet, hvis det tX udelukkende opholdes sig i et hjørne).
Angiv aflivningsmetoden for hver dyreart: Dyrene aflives (bedøvelse og/eller dekapitering) så snart forsøgets formål er opnået eller tidligere såfremt der opstår uforudsete lidelsesvoldende komplikationer. Undertiden afsluttes et forsøg med at hjernen perfusionsfikseres efter en overdosis pentobarbitai og efter at ale reflekser er fraværende.
Beskriv pasningen af og tilsyn med dyrene, herunder særlige foranstaltninger ved tilsyn: Dyrene vii blive tilset minimum 1 gang dagligt og passet af personale med specialuddannelse i overensstemmelse med §3 i Bekendtgerelsen om kvalifikationskrav til personer, der beskæftiger sig med forsøgsdyr. Nyligt doserede dyr og dyr med særlige behov vil blive intensivt overvåget af kvalificeret personale jf. nævnte bekendtgørelse § 2. Herudover er der truffet aftale med dyrlæge om regelmæssigt besøg og veterinær rådgivning. Dyrene opstaldes sædvanligvis i sociale grupper, medmindre særlige forsøgsmæssige hensyn taler derfor (eksempelvis ved detailregistrering af foder/væskeindtag). Der anvendes scantainer eller filtertop ved opstaldning af immumdefekte dyr.
Dyreart og antal pr. år: (Der ønskes en redegørelse for valget af dyreart. Såfremt der påtænkes anvendt genetisk modificerede dyr, skal der indgives særskilt ansøgning herom i henhold til lov om kloning og genmodificiering af dyr, mv. Ansøgeren skal i så fald anvende et G−skema for hver variant. Såfremt der påtænkes anvendt klonede dyr, skal indgives særskilt ansøgning herom i henhold til lov om kloning og genmodificering af dyr mv. Ansøgeren skal i så fald anvende et F−skema for hver gruppe af klonede dyr.) Der ønskes anvendt op til 600 mus og 800 rotter per år. Musene er transgene dyr særlig egnede til studiet af infektion, og rotterne er disponeret til depressiv fænotype, og særlig egnet til st studere modtageligeden for infektion.
Ønsket varighed af tilladelsen til det beskrevne forsøg angivet i antal år: 5 **− begrund:** Projektet involverer et antal ph.d projekter, og forventes at kunne gennemføres i løbet af 5 år.

Der kan medsendes en redegørelse med begrundelse for, hvorfor ansøgeren finder, at betingelserne i offentlighedslovens §§ 12 og 13 for at nægte aktindsigt er tilstede.

DYREFORSØGSTILSYNET Sag nr.: **2016‒15‒0201‒01105**

(udfyldes af Dyreforsøgstilsynet)

Afsnit G‒skema nr. G 1

Ansøgning om tilladelse til anvendelse af GENETISK MODIFICEREDE DYR, jfr. lov om kloning og genmodificering, i forbindelse med dyreforsøg.

Skemaet anvendes for forsøg, der er beskrevet nærmere i ansøgningens Afsnit C – skema nr.: **Afsnit C 1**

Dyreart Mus (Mus musculus)	**Genkode** B6.129 Ifnb1tm1Lky/J
Stammebetegnelse C57/Bl6	**Beskrevet genetisk modifikation** Nyindsættelse **Ved andet, beskriv**
Oprindelse/Leverandør Jackson lab	
Beskriv, hvorfor det skønnes nødvendigt eller formålstjenligt for forsøget at anvende genmodificerede dyr Det er for nyligt blevet vist, at mus producerer et type I interferon (IFN) respons på dag 2 efter infektion med herpes simplex virus (HSV). Derudover er mus med en defekt receptor for disse IFN (IFNAR) mere modtagelige over for infektion, hvilket understreger vigtigheden af type I IFN. Trods dette er der på nuværende tidspunkt ingen viden om hvilke celletyper, der producerer disse signalstoffer under HSV infektion. Vi vil gerne undersøge hvilke celletyper, der producerer type I IFN samt hvilke(n) signaleringsvej(e), der spiller en rolle for dette respons. For at finde frem til disse celler vil vi benytte interferon beta enhanced yellow fluorescent protein (IFNb EYFP) mus, hvis celler vil fluorescere med et gult protein, når genet for type I IFN aktiveres. Alle celler med kerner kan producere type I IFN, men vores hypotese er, at det er en bestemt gruppe af celletyper, der gør det ved HSV infektion. HSV infektion hos mennesker er en alvorlig seksuelt overført sygdom, der hos voksne bl.a. kan give smertefulde læsioner i genitalområdet, betændelse i endetarmen eller meningitis. Hos nyfødte kan HSV infektion give neonatal herpes simplex med et alvorligt forløb pga. et endnu ikke færdigudviklet immunforsvar, og som kan resultere i død. Projektet vil være med til at belyse en vigtig del af immunforsvaret mod HSV infektion og kan i sidste ende bidrage med relevant viden for udvikling af nye behandlingsmuligheder. Det vides ikke hvilke konsekvenser for centralnervesystemet og udviklingen af psykisk sygdom som disse ændringer medfører.	
Beskriv de molekylære og fysiologiske ændringer som forventes i homo‒ og heterozygoter (Herunder ønskes oplysning om nedsat levedygtighed, nedsat fertilitet, fosterdød, neonatal mortalitet og adfærdsændringer.) Modellen er blevet modificeret ved Ifnb1 genet. Der er blevet introduceret et internal ribosomal entry site (IRES) og enhanced yellow fluorescent protein (EYFP) lige efter Ifnb1 genets STOP codon, men ingen regulatoriske elementer er blevet modificeret. Når Ifnb1 bliver udtrykt, vil EYFP blive co translateret, og derfor bliver celler, der producerer type I IFN, gule, og de kan derefter detekteres med eks. flowcytometri eller immunohistokemi.	
Beskriv eventuelt fænotypiske ændringer som forekommer i homo/heterozygoter Homozygote mus er levedygtige, fertile og størrelsesmæssigt identiske med C57BL/6J mus. Musen udtrykker ingen fysisk eller adfærdsmæssig anormalitet. Eventuelle fænotypiske ændringer vil blive rapporteret.	
Specificer og beskriv sygelighed eller anden belastning af dyrenes velfærd fremkaldt af den genetiske modifikation Dyr i avl fremviser ingen øget sygelighed eller belastning ved opstaldning i patogenfrit miljø.	
Specificer og vurder den mulige ekstra belastning, som opstår på grund af, at det påtænkte forsøg, der beskrives i Afsnit C, skema nr. , udføres på genetisk modificerede dyr Der arbejdes med dyrene efter gældende regler og eventuelle syge dyr vil straks blive aflivet. Humane endpoints er præcis de samme som beskrevet for ikke genmodificerede dyr.	
Anfør de dyreværnsmæssige forholdsregler der agtes iværksat for at imødegå disse belastninger De bliver aflivet inden for 3 måneder efter de er påbegyndt adfærdstestning, men før, hvis de når endpoints for forsøgene.	

DYREFORSØGSTILSYNET

Sag nr.: **2016−15−0201−01105**

(udfyldes af Dyreforsøgstilsynet)

Afsnit G−skema nr. G 2

Ansøgning om tilladelse til anvendelse af GENETISK MODIFICEREDE DYR, jfr. lov om kloning og genmodificering, i forbindelse med dyreforsøg.

Skemaet anvendes for forsøg, der er beskrevet nærmere i ansøgningens Afsnit C – skema nr.: **Afsnit C 1**

Dyreart Mus (Mus musculus)	**Genkode** STING
Stammebetegnelse C57BL/6−Tmem173gt	**Beskrevet genetisk modifikation** Andet **Ved andet, beskriv** ENU induceret model

Oprindelse/Leverandør Taconic

Beskriv, hvorfor det skønnes nødvendigt eller formålstjenligt for forsøget at anvende genmodificerede dyr

Den transmembrane signalerings adapter (STING) er for nylig blevet vist impliceret i induktion af type I interferoner (IFN) som reaktion på "fremmede" syntetiske DNA og / eller RNA. Det er fornyelig vist, at mus der mangler STING, mangler type I IFN produktion efter infektion med bakterien, Listeria. Under herpes og influenza virus infektion bliver der produceret "fremmede"− virale DNA/RNA som bliver genkendt af cellens receptorer. Vores in vitro forsøg viser at STING er vigtigt for at producere type I IFN efter disse virale DNA/RNA bliver genkendt. Men de underliggende mekanismer er ukendt. Vi vil gerne undersøge rollen for STING under herpes og influenza infektion in vivo for at; 1. identificere gener, der er vigtige i det medfødte immunrespons, 2. identificere hvor i kroppen STING er vigtigt, 3. undersøge STINGs rolle i kroppes forsvar imod virus infektion (f.eks. aktivering af immunceller). Vi bruger STING−/−mus til forsøg for at vurdere vigtigheden af STING i immuneforsvarets genkendelse af virus og dermed bekæmpelse af sygdommen. Målet med infektions modellen er at undersøge infektionspatologien og immunologiske reaktioner i hjernen, lungerne og i rygmarven. Virusinfektioner giver ophav til mange sygdomme mod hvilke man i dag ikke har kurerende behandling for (f.eks. herpesvirus og influenza viruys). Der er derfor behov for mere viden om, hvordan kroppens forsvar mod virusinfektioner fungerer, hvilket kan bane vej for udvikling af ny antiviral behandling. Det vides ikke hvilke konsekvenser for centralnervesystemet og udviklingen af psykisk sygdom som disse ændringer medfører.

Beskriv de molekylære og fysiologiske ændringer som forventes i homo− og heterozygoter (Herunder ønskes oplysning om nedsat levedygtighed, nedsat fertilitet, fosterdød, neonatal mortalitet og adfærdsændringer.)

Modellen er en ENU induceret model, hvor C57BL/6 mus er blevet fodret med ENU i drikkevandet. ENU inducerer tilfældige mutationer i genomet. Det gav ophav til en stamme, som havde en punktmutation i genet sting, som er essentielt for medfødt immunrespons mod intracellulære mikroorganismer.(Infection and Immunity 79:688): Homozygote mus er levedygtige, fertile og størrelsesmæssigt identisk med en C57BL/6 mus. Musen udtrykker ingen fysisk eller adfærdsmæssig anormalitet.

Beskriv eventuelt fænotypiske ændringer som forekommer i homo/heterozygoter

Det er muligt at der vil ske en ændring i fænotypen i de homozygote dyrs immunsystem, som følge af manglende udtryk af sting. Evt. andre fænotypiske ændringer vil blive rapporteret.

Specificer og beskriv sygelighed eller anden belastning af dyrenes velfærd fremkaldt af den genetiske modifikation

Dyr i avl fremviser ingen øget sygelighed eller belastning ved opstaldning i patogen frit miljø.

Specificer og vurder den mulige ekstra belastning, som opstår på grund af, at det påtænkte forsøg, der beskrives i Afsnit C, skema nr. , udføres på genetisk modificerede dyr

Der arbejdes med dyrene efter gældende regler og eventuelle syge dyr vil straks blive aflivet. Endpoints er præcis de samme som for ikke genmodificerede dyr. STING mus kan have en øget modtagelighed overfor virale infektioner, da disse mus muligvis mangler type I IFN response. Belastnings graden er den samme for BL6C57 mus og STING□/□mus. Hvis STING□/□musene skulle udvikle sygdommen hurtigere end ikke genmodificeret dyr, vil de blive aflivet tidligere end ikke genmodificerede dyr, dvs. belastnings graden er den samme når forsøget afsluttes. Det forventes ikke at adfærdstestning vil påvirke sygelighed.

Anfør de dyreværnsmæssige forholdsregler der agtes iværksat for at imødegå disse belastninger
De bliver aflivet inden for 3 måneder efter de er påbegyndt adfærdstestning, men før, hvis de når endpoints for forsøgene.

DYREFORSØGSTILSYNET Sag nr.: **2016−15−0201−01105**

(udfyldes af Dyreforsøgstilsynet)

Afsnit G−skema nr. G 3

Ansøgning om tilladelse til anvendelse af GENETISK MODIFICEREDE DYR, jfr. lov om kloning og genmodificering, i forbindelse med dyreforsøg.

Skemaet anvendes for forsøg, der er beskrevet nærmere i ansøgningens Afsnit C – skema nr.: **Afsnit C 1**

Dyreart Mus (Mus musculus)	**Genkode** Ifi204 (p204)
Stammebetegnelse C57BL/6J tm(Ifi204)KAF	**Beskrevet genetisk modifikation** Andet **Ved andet, beskriv** nedregulering
Oprindelse/Leverandør Taconic	

Beskriv, hvorfor det skønnes nødvendigt eller formålstjenligt for forsøget at anvende genmodificerede dyr

cGAS og p204 er for nylig blevet vist impliceret i induktion af type I interferoner (IFN) som reaktion på ”fremmede” syntetiske DNA. Det er fornyelig vist, at mus der mangler cGAS ellerp204, mangler type I IFN produktion efter infektion med Herpes virus. Under herpes− og influenza virus infektion bliver der produceret ”fremmede”□virale RNA og DNA som bliver genkendt af cellens receptorer. Vores in vitro forsøg viser at cGAS og p204 er vigtige for at producere type I IFN efter disse viralt DNA bliver genkendt. Men de underliggende mekanismer er ukendt. Vi vil gerne undersøge rollen for cGAS og p204 under virus infektion in vivo for at; 1. identificere gener, der er vigtige i det medfødte immunrespons, 2. identificere hvor i kroppen cGAS og p204 er vigtigt, 3. undersøge cGAS’s og p204s rolle i kroppes forsvar imod virus infektion (f.eks. aktivering af immunceller). Vi bruger cGAS−/− og p204−/− mus til forsøg for at vurdere vigtigheden af cGAS og p204 i immuneforsvarets genkendelse af herpes− og influenza A virus og dermed bekæmpelse af sygdommen. Målet med i.p, i.v, eller vaginal infektions modellerne er at undersøge infektionspatologien og immunologiske reaktioner kroppen. Virusinfektioner giver ophav til mange sygdomme mod hvilke man i dag ikke har kurerende behandling for (f.eks. herpes− eller influenza virus). Der er derfor behov for mere viden om, hvordan kroppens forsvar mod virusinfektioner fungerer, hvilket kan bane vej for udvikling af ny antiviral behandling. Endpoints er præcis de samme som beskrevet i C1−2 for ikke genmodificerede dyr. cGAS og p204 mus kan have en øget modtagelighed overfor virale infektioner, da disse mus muligvis mangler type I IFN response. Belastnings graden er den samme for BL6C57 mus og cGAS−/− og p204−/− mus. Hvis cGAS−/− og p204−/− musene skulle udvikle sygdommen hurtigere end ikke genmodificeret dyr, vil de blive aflivet tidligere end ikke genmodificerede dyr, dvs. belastnings graden er den samme når forsøget afsluttes.
Det vides ikke hvilke konsekvenser for centralnervesystemet og udviklingen af psykisk sygdom som disse ændringer medfører.

Beskriv de molekylære og fysiologiske ændringer som forventes i homo− og heterozygoter (Herunder ønskes oplysning om nedsat levedygtighed, nedsat fertilitet, fosterdød, neonatal mortalitet og adfærdsændringer.)

Modellen er lavet ved homolog rekombination hvor exon 2 og 3 af musens Ifi204 gen er udskiftet med neomycin resistance kassette. Ændringen betyder at musen ikke udtrykker Ifi204 genet. Homozygote mus er levedygtige, fertile og størrelsesmæssigt identisk med en C57BL/6 mus. Musen udtrykker ingen fysisk eller adfærdsmæssig anormalitet

Beskriv eventuelt fænotypiske ændringer som forekommer i homo/heterozygoter

Homozygote mus har muligvis en øget modtagelighed overfor infektioner. Ellers forventes ingen påvirkning af den normale tilstand af musene og modifikationen bør ikke påføre musene nogen belastning.

Specificer og beskriv sygelighed eller anden belastning af dyrenes velfærd fremkaldt af den genetiske modifikation

Dyr i avl fremviser ingen øget sygelighed eller belastning ved opstaldning i patogen frit miljø.

Specificer og vurder den mulige ekstra belastning, som opstår på grund af, at det påtænkte forsøg, der beskrives i Afsnit C, skema nr. , udføres på genetisk modificerede dyr

Der arbejdes med dyrene efter gældende regler og eventuelle syge dyr vil straks blive aflivet. Endpoints er præcis de samme som beskrevet for ikke genmodificerede dyr. p204□□mus kan have en øget modtagelighed overfor virale infektioner, da disse mus muligvis mangler type I IFN response. Belastnings graden er den samme for BL6C57 mus og p204□□mus. Hvis p204□□musene skulle udvikle sygdommen hurtigere end ikke genmodificeret dyr, vil de blive aflivet

DYREFORSØGSTILSYNET

Sag nr.: **2016−15−0201−01105**
(udfyldes af Dyreforsøgstilsynet)

Afsnit G−skema nr. G 4

Ansøgning om tilladelse til anvendelse af GENETISK MODIFICEREDE DYR, jfr. lov om kloning og genmodificering, i forbindelse med dyreforsøg.

Skemaet anvendes for forsøg, der er beskrevet nærmere i ansøgningens Afsnit C – skema nr.: **Afsnit C 1**

Dyreart Mus (Mus musculus)	**Genkode** Mb21d1 (cGAS er et synonym)
Stammebetegnelse C57BL/6−Mb21d1	**Beskrevet genetisk modifikation** Funktionsophævelse **Ved andet, beskriv**
Oprindelse/Leverandør Taconic	

Beskriv, hvorfor det skønnes nødvendigt eller formålstjenligt for forsøget at anvende genmodificerede dyr

cGAS og p204 er for nylig blevet vist impliceret i induktion af type I interferoner (IFN) som reaktion på "fremmede" syntetiske DNA. Det er fornyelig vist, at mus der mangler cGAS ellerp204, mangler type I IFN produktion efter infektion med Herpes virus. Under herpes og influenza virus infektion bliver der produceret "fremmede"□virale DNA som bliver genkendt af cellens receptorer. Vores in vitro forsøg viser at cGAS og p204 er vigtige for at producere type I IFN efter disse viralt RNA/DNA bliver genkendt. Men de underliggende mekanismer er ukendt. Vi vil gerne undersøge rollen for cGAS og p204 under virus infektion in vivo for at; 1. identificere gener, der er vigtige i det medfødte immunrespons, 2. identificere hvor i kroppen cGAS og p204 er vigtigt, 3. undersøge cGAS's og p204s rolle i kroppes forsvar imod virus infektion (f.eks. aktivering af immunceller). Vi bruger cGAS−/− og p204−/− mus til forsøg for at vurdere vigtigheden af cGAS og p204 i immuneforsvarets genkendelse af herpes og influenza virus og dermed bekæmpelse af sygdommen. Målet med i.p, i.v, eller vaginal infektions modellerne er at undersøge infektionspatologien og immunologiske reaktioner kroppen. Virusinfektioner giver ophav til mange sygdomme mod hvilke man i dag ikke har kurerende behandling for (f.eks. herpes− og influenzavirus). Der er derfor behov for mere viden om, hvordan kroppens forsvar mod virusinfektioner fungerer, hvilket kan bane vej for udvikling af ny antiviral behandling. Endpoints er præcis de samme som for ikke genmodificerede dyr. cGAS og p204 mus kan have en øget modtagelighed overfor virale infektioner, da disse mus muligvis mangler type I IFN response. Belastnings graden er den samme for BL6C57 mus og cGAS−/− og p204−/− mus. Hvis cGAS−/− og p204−/− musene skulle udvikle sygdommen hurtigere end ikke genmodificeret dyr, vil de blive aflivet tidligere end ikke genmodificerede dyr, dvs. belastnings graden er den samme når forsøget afsluttes.
Det vides ikke hvilke konsekvenser for centralnervesystemet og udviklingen af psykisk sygdom som disse ændringer medfører.

Beskriv de molekylære og fysiologiske ændringer som forventes i homo− og heterozygoter (Herunder ønskes oplysning om nedsat levedygtighed, nedsat fertilitet, fosterdød, neonatal mortalitet og adfærdsændringer.)

Mb21d1 genet koder for et cytosol DNA sensor cyklisk GMP−AMP−syntase, der aktiverer TMEM173 / STING vejen for type 1 interferon produktion. Disse mus bærer en knock−out−mutation for Mb21d1 genet, i hvilket exon 2 (der koder for det katalytiske domæne) er blevet udskåret.
For at generere denne knock−out stammen C57BL / 6NTac−Mb21d1tm1a (EUCOMM) Hmgu / IcsOrl mus blev avlet til mus, der udtrykker en nuklear lokaliseret FLPe rekombinase under kontrol af KAG, kylling beta−actin promotor og cytomegalovirus forstærker, (C57BL / 6− Tg (CAG−FLPe) 36Ito / ItoRbrc) til udskæring lacZ og neomycin kassetter, efterladende exon 2 floxet. De betingede muterede mus blev derefter avlet til B6.C−Tg (CMV−CRE) 1Cgn / J mus. De resulterende knock−out mus blev siden tilbagekrydset til C57BL / 6J mus for at fjerne Cre rekombinase transgenet. Homozygote mus er levedygtige, fertile og størrelsesmæssigt identisk med en C57BL/6 mus. Musen udtrykker ingen fysisk eller adfærdsmæssig anormalitet.

Beskriv eventuelt fænotypiske ændringer som forekommer i homo/heterozygoter

Det er muligt at der vil ske en ændring i fænotypen i de homozygote dyrs immunsystem, som følge af manglende udtryk af cGAS. Evt. andre fænotypiske ændringer vil blive rapporteret.

Specificer og beskriv sygelighed eller anden belastning af dyrenes velfærd fremkaldt af den genetiske modifikation

Homozygote mus har muligvis en øget modtagelighed overfor infektioner. Ellers forventes ingen påvirkning af den normale tilstand af musene og modifikationen bør ikke påføre musene nogen belastning.
Musene vil blive opstaldet i scantainer.

tidligere end ikke genmodificerede dyr, dvs. belastnings graden er den samme når forsøget afsluttes. Det forventes ikke at adfærdsundersøgelser vil påvirkle sygeligheden.
Anfør de dyreværnsmæssige forholdsregler der agtes iværksat for at imødegå disse belastninger De bliver aflivet inden for 3 måneder efter de er påbegyndt adfærdstestning, men før, hvis de når endpoints for forsøgene.

Specificer og vurder den mulige ekstra belastning, som opstår på grund af, at det påtænkte forsøg, der beskrives i Afsnit C, skema nr. , udføres på genetisk modificerede dyr
Der arbejdes med dyrene efter gældende regler og eventuelle syge dyr vil straks blive aflivet. Endpoints er præcis de samme som beskrevet for ikke genmodificerede dyr. cGAS□□mus kan have en øget modtagelighed overfor virale infektioner, da disse mus muligvis mangler type I IFN response. Belastnings graden er den samme for BL6C57 mus og cGAS□□mus. Hvis cGAS□□musene skulle udvikle sygdommen hurtigere end ikke genmodificeret dyr, vil de blive aflivet tidligere end ikke genmodificerede dyr, dvs. belastnings graden er den samme når forsøget afsluttes.
Anfør de dyreværnsmæssige forholdsregler der agtes iværksat for at imødegå disse belastninger
De bliver aflivet inden for 3 måneder efter de er påbegyndt adfærdstestning, men før, hvis de når endpoints for forsøgene.

DYREFORSØGSTILSYNET

Sag nr.: **2016−15−0201−01105**

(udfyldes af Dyreforsøgstilsynet)

Afsnit G−skema nr. G 5

Ansøgning om tilladelse til anvendelse af GENETISK MODIFICEREDE DYR, jfr. lov om kloning og genmodificering, i forbindelse med dyreforsøg.

Skemaet anvendes for forsøg, der er beskrevet nærmere i ansøgningens Afsnit C – skema nr.: **Afsnit C 1**

Dyreart Mus (Mus musculus)	**Genkode** C57BL/6 IFNAR KO
Stammebetegnelse C57BL/6 IFNAR KO	**Beskrevet genetisk modifikation** Andet **Ved andet, beskriv** Ødelæggelse af genet for interferon Alpha/Beta receptor (IFNAR).
Oprindelse/Leverandør Taconic	
Beskriv, hvorfor det skønnes nødvendigt eller formålstjenligt for forsøget at anvende genmodificerede dyr Vi vil bruge IFNAR KO musene for at få en bedre forståelse af cytokin IFN I rolle i forbindelse med inflammation i hjernen. IFNAR ko musene mangler receptoren for Type1 interferon og derfor kan de ikke reagere på Type 1 interferon. Dette hjælper med at undersøge, hvilke specifik rolle Type 1 interferon spiller i de inflammatoriske processer i Centralnervesystemet, CNS Det vides ikke hvilke konsekvenser for centralnervesystemet og udviklingen af psykisk sygdom som disse ændringer medfører.	
Beskriv de molekylære og fysiologiske ændringer som forventes i homo− og heterozygoter (Herunder ønskes oplysning om nedsat levedygtighed, nedsat fertilitet, fosterdød, neonatal mortalitet og adfærdsændringer.) IFNAR genet er funktionsophævet ved insættelse af et neomycin gen. Ved hemizygote mus er mængden af IFNAR reduceret i forhold til baggrundstammen. Hos homozygote mus er der ingen udtrykkelse af IFNAR overhovedet. Denne helt eller delvise manglende udtrykkelse af IFNAR har ingen betydning for musens udvikling. De vil leve og yngle fuldstændigt paa samme niveau som baggrundstammen.	
Beskriv eventuelt fænotypiske ændringer som forekommer i homo/heterozygoter Der er ingen histopatologiske eller patofysiologiske effekter som følge af funktionsophævelse af genet for IFN I receptoren.	
Specificer og beskriv sygelighed eller anden belastning af dyrenes velfærd fremkaldt af den genetiske modifikation Dyr i avl fremviser ingen øget sygelighed eller belastning ved opstaldning i patogen−frit miljø.	
Specificer og vurder den mulige ekstra belastning, som opstår på grund af, at det påtænkte forsøg, der beskrives i Afsnit C, skema nr. , udføres på genetisk modificerede dyr IFNAR KO mus har muligvis en øget modtagelighed overfor virale infektioner. Der arbejdes med dyrene efter gældende regler og eventuelle syge dyr vil straks blive aflivet. Endpoints er de samme som beskrevet i C skema for ikke genmodificerede dyr, dvs. hvis musene skulle udvikle sygdommen hurtigere end ikke genmodificeret dyr, vil de blive aflivet tidligere end ikke genmodificerede dyr.	
Anfør de dyreværnsmæssige forholdsregler der agtes iværksat for at imødegå disse belastninger De bliver aflivet inden for 3 måneder efter de er påbegyndt adfærdstestning, men før, hvis de når endpoints for forsøgene.	

OVERALL DISCUSSION

5 OVERALL DISCUSSION

The present PhD project was divided in two main studies as following: **Study 1A and 1B:** To investigate the influence of sex, strain of mice (Swiss or C57BL/6) and specie of rodents (mice or Flinders Sensitive Line rats) on the antidepressant-like effect produced by CBD in animal models of depression; **Study 2:** To investigate a broadly the molecular mechanisms involved in the antidepressant-like effect induced by CBD, and also evaluate whether CBD shares molecular expression patterns in genes related to glutamatergic neurotransmission, via neurotrophic signaling and synaptic proteins with ketamine in the brain regions involved with the neurobiology of depression (PFC, DH and VH) of the Sensitive Line of Flinders rats.

- **The influence of the sex, strain, and specie of rodents in the CBD effect**

In Study 1, our findings indicate that sex, strain, species, and chosen time of the administration may interfere with the behavioural response produced by CBD in rodents exposed to animal models of depression. The study is the first to investigate the behavioural and temporal effects produced by CBD and KET in both genders of FSL rats. In Study 1A, we found that male Swiss mice, CBD and IMIP decreased the immobility time in the TST, which is suggestive of an antidepressant-like effect. On the other hand, in female Swiss mice and C57BL/6 mice (male and female), CBD did not change the behavioural responses elicited in the TST. In contrast, CBD did not affect the parameters analyzed on the EPM. In Study 1B, a single injection of CBD produced an antidepressant effect at 1 hour CBD in male FSL rats. It induced a bimodal effect in females, with a depressive effect at 1h post-injection and an antidepressant effect 2h after its administration. The antidepressant effect of ketamine was also observed at 1h post-injection, but not 2h later.

Our results are in accordance with previous studies in which CBD produced an antidepressant-like effect in both male mice and rats submitted to different behavioral tests, including the FST (SALES et al., 2018a, 2018b; ZANELATI et al., 2010), TST (EL-ALFY et al., 2010; SCHIAVON et al., 2016a), olfactory bulbectomy (LINGE et al., 2016) and CUMS (XU et al., 2019). Accumulating evidence revealed that the strain selected affects the baseline behaviour in the TST (LIU; GERSHENFELD, 2001; RIPOLL et al., 2003; TRULLAS; JACKSON; SKOLNICK, 1989; VAN DER HEYDEN; MOLEWIJK; OLIVIER, 1987; VÕIKAR et al., 2001), the antidepressant drugs response (LIU; GERSHENFELD, 2001; RIPOLL et al., 2003; VAN

DER HEYDEN; MOLEWIJK; OLIVIER, 1987), and neurochemical profile (DAVID et al., 2003), which could explain the strain differences observed in the curret study.

We found that CBD and IMIP did not change the immobility time in female Swiss and C57BL/6 mice submitted to TST in the present study. Previous studies demonstrated that sex is a crucial factor that influences the drug response in different rodent species submitted to diverse animal models of depression (DAVID et al., 2001; FERNÁNDEZ-GUASTI et al., 2017; FRANCESCHELLI et al., 2015; GÓMEZ et al., 2014; SIMPSON; KELLY, 2012; WRIGHT; KABBAJ, 2018). Despite that, only one study evaluated the CBD antidepressant effect in females of two genetic models of disease (WKY and FSL rats). The oral administration of CBD (food pellet; dose: 30 mg/kg) induced a hedonic and antidepressant-like effect in saccharine preference test and FST in female and male WKY rats (SHBIRO et al., 2019), conversely, CBD produced an antidepressant-like effect only in male FSL rats (SHBIRO et al., 2019).

In our study, CBD induced a bimodal effect in female rats, with a depressive effect at 1h post-injection and an antidepressant effect 2h after its administration. In males, the antidepressant effect was only observed at 1h post-injection. Corroborating with our findings with male FSL rats, previous studies reported the same antidepressant effect in male rats exposed to FST (DE MORAIS et al., 2018; RÉUS et al., 2011), LH (SALES et al., 2018b), and in a genetic model of disease, including WKY and FSL rats (SALES et al., 2018c; SHBIRO et al., 2019; SHOVAL et al., 2016). However, it is crucial to consider that the administration route influences the CBD effect. For example, earlier studies reported that i.p. injection with CBD produced its effect generally 1 hour post-injection (DE MORAIS et al., 2018; RÉUS et al., 2011; SALES et al., 2018c). On the other hand, through the oral route (food pellet), the antidepressant-like effect induced by CBD in FSL and WKY rats appears 2 hours later (SHBIRO et al., 2019; SHOVAL et al., 2016). Therefore, as described previously, the discrepancy in behavioural findings can be explained by the differences in the pharmacokinetic parameters (HUESTIS, 2007; MILLAR et al., 2018, 2020; TURNER et al., 2011).

Interestingly, this study investigated for the first time the dose-response curve with CBD and the treatment at different time-points (1 and 2 hours) in female FSL rats submitted to the FST and OFT. The findings revealed that CBD (30 mg/kg) produced an antidepressant-like effect only 2 hours after the treatment. In contrast, 1 hour later, the same dose had the opposite effect, a depressive-like effect. However, as mentioned previously, the oral administration with CBD did not change the saccharin preference and immobility in the FST of the females FSL rats, but only in

male rats (SHBIRO et al., 2019). It is important to remember that the time to reach the Cmax depends on the vehicle, administration route, and the animal species used (DEIANA et al., 2012b). Therefore, CBD effect will depend on the plasma and brain concentrations achieved and, consequently, it is modified by the time chosen for the injection. In this sense, variability in the concentration could explain the opposite results found in female FSL rats treated with the same CBD dose after different time-points.

Furthermore, we showed that a single dose of ketamine (15 and 20 mg/kg) produces an antidepressant-like effect in female FSL rats exposed to FST. Our finding strengthens previous results showing the efficacy of the treatment with KET in female rats, even though the effective doses vary compared to males (CARRIER et al., 2015; CHOU et al., 2018; RINCÓN-CORTÉS; GRACE, 2017; SARKAR; KABBAJ, 2017; TIZABI et al., 2012). Despite knowing the influence of the oestrous cycle on the behavioural effect (CARRIER; KABBAJ, 2013; SARKAR; KABBAJ, 2017), a prior study from our group reported that the oestrous cycle does not modulate the FST behaviour in female FSL rats (ESKELUND et al., 2016). For this reason, we did not investigate the effect of the cycle in the present study. In fact, additional investigation to understand the sex diferences in the ketamine antidepressant will be interesting.

- **The molecular mechanism involved on CBD effect in FSL rats.**

In the present study, we explore in-depth the molecular mechanism implicated in the rapid antidepressant-like effect produced by CBD and S-ketamine in male FSL rats in genes and proteins levels related to glutamatergic neurotransmission, neurotrophic signaling, and synaptic proteins in brain regions essentials in the depression neurobiology and treatment response: the PFC, the DH, and the VH.

In the PFC, we found that the acute administration with CBD elevated the levels of BDNF (protein), EAAT3 (gene), Nr2a (gene), and Nr2b (protein) in FSL rats. CBD is a multi-target drug that can modulate several of systems. It is known that CBD acts as an agonist of the 5-HT1A receptor (RUSSO et al., 2005). Our group demonstrated that acute administration of CBD induced antidepressant-like effect in FST, depending on the activation of 5-HT1A receptor (ZANELATI et al., 2010). In the same way, the direct administration of CBD into the ventromedial PFC (vmPFC) induced stress-coping behaviour in rats exposed to FST, which was abolished by pretreatment with the 5-HT1A receptor antagonist (WAY100635) (SARTIM; GUIMARÃES; JOCA,

2016). Furthermore, it was evidenced that the activation of 5-HT1A receptor induced a rapid antidepressant-like effect in rodents, associated with increase BDNF mRNA and protein levels in PFC (FUKUMOTO et al., 2017a), thus, suggesting that 5-HT1A activation regulates BDNF levels in the brain. In the present study, we found that CBD increases BDNF protein levels in the PFC of FSL rats. Consistent with our results, previous works revealed that the rapid antidepressant-like effect induced by CBD is accompanied by a rapid increased in the levels of BDNF in the PFC (SALES et al., 2018c; XU et al., 2019), which was prevented by the previous treatment with TrkB inhibitor, K252a (SALES et al., 2018b). Altogether, it is possible that CBD would act activating the 5-HT1A receptor, triggering BDNF release and consequent activation of TrkB receptors, which can lead to gene expression and synthesis of postsynaptic proteins involved in neurogenesis, neuronal survival, and synaptic plasticity.

Furthermore, we evidenced that CBD-treated FSL rats exhibited an upregulation in transcript levels of EAAT3 in the PFC compared to FSL vehicle-treated, but without affecting the protein levels. However, this glutamate transporter is important to remove the excess of glutamate from the synaptic cleft, preventing cell damage (BJØRN-YOSHIMOTO; UNDERHILL, 2016), andit is involved in the synaptic plasticity (JARZYLO; MAN, 2012; SCIMEMI; TIAN; DIAMOND, 2009). As discussed previously, t EAAT3 transporter reduced the recruitment of extrasynaptic Nr2b-containing NMDA receptor (SCIMEMI; TIAN; DIAMOND, 2009). Notably, the activation of Nr2b in the extrasynaptic NMDA receptor occurs when there is an excess of glutamate at the synaptic terminal, which leads to excitotoxicity and cell death, impairing the synaptic strengthening (AMIDFAR et al., 2019; HANSEN et al., 2018; LAI; ZHANG; WANG, 2014). Altogether, it is possible that CBD increased the EAAT3 expression in the PFC, which regulates the regional glutamate levels and decreased recruitment of extrasynaptic Nr2b-containing NMDA receptor, facilitating synaptic transmission and preventing its deleterious effects.

Accumulating evidence indicated that BDNF can modulate the NMDA receptor. In this way, acute exposure to BNDF selectively increases synaptically evoked NMDA currents dependent on the presence of Nr2b-containing receptors (KOLB; TRETTEL; LEVINE, 2005) and rapidly and reversibly elevated the amplitude and frequency of excitatory postsynaptic currents (EPSCs) dependent on the activation of NMDA receptor (MADARA; LEVINE, 2008; SONG et al., 1998). Our study found that CBD treatment upregulates Nr2a and Nr2b mRNA and protein expression in the PFC of FSL rats. Thereby, it suggests that CBD may increase BDNF levels that regulate the synaptic NMDA receptor opening and activation, promoting calcium influx, which activates kinases

and transcription factors including cyclic AMP response element-binding protein (CREB) responsible for elevating gene expression facilitating neuronal survival synaptic plasticity.

In contrast, a single injection of ketamine increases the levels of Nr2a (gene and protein), Nr2b (protein), and phospho GluR1(S831 protein) in PFC of FSL rats. Corroborating with our findings, earlier work demonstrated that ketamine increases the protein levels of Nr2b and GluR1 in mPFC, which was associated with a robust increase in LTP (BURGDORF et al., 2013). Moreover, ketamine increased Nr2b-specific currents in electrophysiological records (BURGDORF et al., 2013). Thus, the long-term changes in the synaptic transmission are oriented by the NMDA receptors that activate the AMPA receptors to induce the antidepressant-like actions induced by ketamine. Moreover, the AMPA receptor antagonist, NBQX, blocks the ketamine antidepressant response in the test (BURGDORF ET AL2013; MAENG ET AL., 2008; ZANOS ET AL., 2016). In the same way, the activation of AMPA receptor (ampakine drugs or AMPA potentiators) induce an antidepressant effect in different paradigms (ALT et al., 2006).

Furthermore, sortilin forms a complex with proBDNF (immature BDNF) and its receptor, p75NTR, the activation of such complex facilitates the apoptosis signaling pathway (EVANS et al., 2011; TENG et al., 2005). In the present study, we found that FSL rats treated with ketamine decrease the levels of sortilin in the PFC compared to FSL vehicle-treated group. Consistent with our findings, repeated treatment with fluoxetine (14 days) induced hedonic and antidepressant response and decreased the sortilin levels in the cortex of rodents submitted to CUMS (YANG et al., 2020). The present study suggests that ketamine decreases Sort1 (sortilin) levels in PFC, an effect associated with its behavioural response exhibited in the FST. This significant reduction in sortilin signaling pathway prevents the apoptosis and facilitates the neuronal survival and synaptic plasticity.

VEGF is a neurotrophin that has been implicated with neurogenesis, neuroprotection, and synaptic strength (BECERRIL-VILLANUEVA et al., 2019; JIN et al., 2002). We found that FSL ketamine-treated rats present lower VEGF transcript levels than FSL vehicle-treated rats in PFC. In contrast with our findings, it was reported that FSL ketamine-treated rats showed an increase total length of microvessels in the HPC and, potentially, might influence neuronal activity, synaptic plasticity and glial function (ARDALAN et al., 2016a, 2017b, 2020a), suggesting the involvement of VEGF in the ketamine effect. However, further investigations are needed to elucidate the participation of VEGF in the ketamine effect in the PFC.

Importantly, in the DH, we evidenced that both drug treatments (CBD and ketamine) decreased phospho GluR1 (S831) levels and elevated the levels of Nr2b of FSL rats. Additionally, CBD upregulates the VEGF mRNA expression and downregulates of Sort1 transcript. As discussed previously for PFC findings, the repeated administration with the monoaminergic antidepressant drug (YANG et al., 2020) and ECT (STELZHAMMER et al., 2013) decreases sortilin levels in the brain. The present finding suggests that CBD antidepressant effect requires a downregulation in sortilin levels in this limbic region to prevent apoptosis, which could facilitate neuronal survival, differentiation, and plasticity. In fact, it will be interesting to perform a pharmacological blockade of sortilin receptor to confirm the results and reinforce the role of this molecule in CBD effect.

In contrast to our PFC results, we found a downregulation of VEGF expression in DH of FSL compared to FRL rats, and the CBD injection increased the VEGF transcript levels. Previous work from our group revealed that FSL rats present a shorter length of microvessels in HPC, suggesting a possible VEGF deficit in this limbic region (ARDALAN et al., 2016b). The treatment with the conventional antidepressant drugs increased VEGF levels in this same brain region (CHEN et al., 2021; DEYAMA et al., 2019; GREENE et al., 2009; NOWACKA-CHMIELEWSKA et al., 2017; WARNER-SCHMIDT; DUMAN, 2007). Furthermore, a recent study revealed that treatment with CBD increases the blood flow in the healthy patients' hippocampus (BLOOMFIELD et al., 2020). In addition, CBD regulates the blood-brain barrier permeability associated with VEGF levels in human brain microvascular endothelial cells (HIND; ENGLAND; O'SULLIVAN, 2016), suggesting the participation of VEGF in CBD effect. Additionally, VEGF could modulate synaptic plasticity (TILLO et al., 2012). For example, adult hippocampal neuron cultures treated with VEGF increases LTP (KIM et al., 2008). Moreover, VEGF overexpression in rodents enhanced spatial memory formation in the HPC (CAO et al., 2004; LICHT et al., 2011). Thus, it suggests that CBD increases the VEGF release and consequent activation of its receptor, recruiting the second messenger and nuclear transcription factor, which leads to neuronal survival and synaptic strengthening in DH, leading to its behavioural response.

Furthermore, we found that FSL rats treated with CBD and ketamine reduced phospho GluR1 (S831) levels in the DH. Corroborating with our findings, a prior study revealed that single administration of ketamine reduced the levels of GluR1 phosphorylated in the HPC, comparatively, the acute injection with tricyclic antidepressant drug imipramine did not affect the phosphorylation

of this serine residue (MAENG et al., 2008). However, it is still unclear the role of phosphorylated GluR1 in depression neurobiology and treatment response. Thus, the findings suggest that the effect produced by both fast-acting antidepressants, ketamine and CBD, reduces GluR1 phosphorylation in the serine-831 residue of the AMPA receptor in DH. However, it warrants further investigations.

For Nr2b a subunit of NMDA receptor, we found that the injection with CBD and ketamine reduced the Nr2b protein levels in the DH compared to FSL vehicle-treated group. Supporting these findings, clinical studies have revealed that selective antagonism of Nr2b produces an antidepressant effect in patients diagnosed with depression and treatment-resistant depression (PRESKORN et al., 2008). Furthermore, preclinical studies revealed that selective antagonism of Nr2b produces a rapid antidepressant-like effect resembling ketamine (LI et al., 2010b; POLESZAK et al., 2016) and potentiates the antidepressant effect induced by imipramine, fluoxetine and escitalopram in rodents (POLESZAK et al., 2016). The reduced levels of Nr2b in DH are important for the antidepressant-like effect produce by CBD and ketamine in FSL rats.

Strikingly, CBD-treated rats have decreased the levels of mGluR5 (gene) in VH. In the same way, the pharmacological antagonism of metabotropic glutamatergic receptor mGluR5 with the drug MTEP ([(2- methyl-1,3-thiazol-4-yl)ethynyl]pyridine) (LEGUTKO et al., 2006; MOLINA-HERNÁNDEZ et al., 2006; PALUCHA et al., 2001; PAŁUCHA et al., 2005; PALUCHA; PILC, 2007; WIEROŃSKA et al., 2001) or negative modulator of this receptor (HIROSE et al., 2016; HUGHES et al., 2013) produce an antidepressant-like effect in rodents exposed to different tests. Likewise, the alternative therapy for depression, electroconvulsive therapy reduces the levels of mGluR5 mRNA in the dentate gyrus in the HPC (WATKINS; PEI; NEWBERRY, 1998). The antidepressant action induced by mGluR5 receptor antagonist is associated with the increment BDNF levels in the HPC (LEGUTKO et al., 2006). As mentioned previously, BDNF plays a critical role in LTP induction and increasing dendrite spines in the hippocampal neurons (VINCENTI et al., 2019). However, importantly, we did not find changes in the BDNF transcript and protein levels in the VH. Thus, the modulation of BDNF levels through mGluR5 receptors in the CBD effect is uncertain and warrants further investigations.

Furthermore, we found that CBD decreased phospho GluR1 (S831 and S845) levels and increased–GluR2 levels in the VH. Comparatively, ketamine only decreased protein levels of phospho GluR1 (S831) in the same brain region. Corroborating with our findings, a single administration of ketamine reduced the levels of GluR1 phosphorylated in the HPC (MAENG et al., 2008). Moreover, the chronic treatment with desipramine and paroxetine increased the membrane

expression of GluR2/3 in HPC (MARTINEZ-TURRILLAS; FRECHILLA; DEL RÍO, 2002). Thereby, the presented findings suggest that rapid antidepressant effect produced by ketamine depends on the reduction of the phosphorylation GluR1 on the serine-831 residue of the AMPA receptor in the VH to exert its effect. In contrast, the CBD effect decreases the phosphorylation of GluR1 (S831 and S845) and increases in the number of GluR2-containing AMPA receptors, suggesting that CBD may enhance the internalization of AMPA receptor and modulates the synaptic strengthening. However, further studies are needed to investigate this hypothesis.

OVERALL CONCLUSION

6 OVERALL CONCLUSION

In summary, our findings indicate that sex, strain, species, and chosen time of the administration may interfere with the behavioural response produced by CBD in rodents exposed to animal models of depression. In mice, CBD produced an antidepressant-like effect only in male Swiss mice in the TST. CBD did not significantly affect in female Swiss mice and both sexes of C57BL/6 mice in the test. However, in female FSL rats, CBD produced a dual effect, an antidepressant-like effect 2 hours after the injection, but at 1 hour, a depressive-like effect. In males FSL rats, CBD produced an antidepressant-like effect 1 hour after the injection and no effect at 2 hours. Besides, we confirm that KET has an antidepressant-like effect in female FSL rats. These findings point out that it is necessary to consider gender, strain, rodents species chosen, compound chemistry, exposure to a previous stressful condition, and behavioural test to plan the most appropriate experimental design when evaluating new potential drugs.

In addition, we investigated the molecular mechanisms involved on CBD and ketamine antidepressant effect of FSL rats in the limbic regions implicated with depression (PFC, DH and VH). Contrary to our expectations, CBD and KET did not share a common molecular expression pattern in the genes and proteins examined. For the PFC, CBD mainly modulates the BDNF and glutamatergic signaling pathway, while ketamine regulates the molecules associated with glutamatergic neurotransmission, VEGF and sortilin signaling pathways. However, for DH, CBD regulates the Sortilin, VEGF, glutamatergic systems, and ketamine regulated exclusively by glutamatergic neurotransmission. Our results suggest that CBD effect involved the restoration of glutamatergic dysfunction and facilitating the neurotrophic signaling pathway, which triggers neuronal survival and neuroplasticity. On the other hand, the effect of ketamine seems to involve only the restoration of normal glutamatergic function in the limbic brain areas. However, further investigations are necessary to elucidate the molecular mechanisms that participate in the behavioural response.

Notably, it was evidenced that FSL rats have several changes in the neurotrophic signaling, glutamatergic, neurotransmission, and synaptic proteins in the limbic brain regions (PFC, DH, and VH) compared to FRL rats. Thus, our findings reinforce that FSL is a validity genetic animal model to study the pathophysiology of depression and investigate promising antidepressant compounds and their molecular mechanisms involved in the effect.

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7 REFERENCES

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