Early-life stress induces emotional and molecular alterations in female mice which is partially reversed by cannabidiol

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ABSTRACT

Gender is considered as a pivotal determinant of mental health. Indeed, several psychiatric disorders such as anxiety and depression are more common and persistent in women than in men. In the past two decades, impaired brain energy metabolism has been highlighted as a risk factor for the development of these psychiatric disorders. However, comprehensive behavioural and neurobiological studies in brain regions relevant to anxiety and depression symptomatology are scarce. In the present study, we summarize findings describing cannabidiol effects on anxiety and depression in maternally separated female mice, as a well-established rodent model of early-life stress associated with many mental disorders. Our results indicate that cannabidiol could prevent anxiolytic- and depressive-related behaviour in early-life stressed female mice. Additionally, maternal separation with early weaning caused long-term changes in brain oxidative metabolism in both nucleus accumbens and amygdalar complex measured by cytochrome c oxidase

quantitative histochemistry. However, cannabidiol treatment could not revert brain oxidative metabolism impairment. Moreover, we identified hyperphosphorylation of mTOR and ERK 1/2 proteins in the amygdala but not in the striatum, that could also reflect altered brain intracellular signalling related with to bioenergetic impairment. Altogether, our study supports the hypothesis that MSEW induces profound long-lasting molecular changes in mTOR signalling and brain energy metabolism related to depressive-like and anxiety-like behaviours in female mice, ameliorated partially by CBD administration.

HIGHLIGHTS

- CBD shows antidepressant and anxiolytic effects in maternally separated (MSEW) female mice
- Maternal separation reduces cytochrome c oxidase activity in the amygdalar complex
- MSEW induces hyperphosphorylation of mTOR and ERK1/2 in the amygdalar complex

Abbreviation List

ACB	Nucleus accumbens
ACBc	Nucleus accumbens core
ACBs	Nucleus accumbens shell
BLA	Basolateral amygdalar nucleus
BST	Bed nuclei of the stria terminalis
CBD	Cannabidiol
CCO	Cytochrome c oxidase
CEA	Central amygdalar nucleus
COA	Cortical amygdalar area
EDTA	Ethylenediaminetetraacetic acid
EPM	Elevated plus maze
ERK1/2	Extracellular signal-regulated protein kinase 1/2
LA	Lateral amygdalar nucleus
MEA	Medial amygdalar nucleus
MSEW	Maternal separation with early weaning
mTOR	Mammalian target of rapamycin
PD	Postpartum day
p-ERK1/2	Phosphorylated extracellular signal-regulated protein kinase 1/2
p-mTOR	Phosphorylated mammalian target of rapamycin

PVH	Paraventricular hypothalamic nucleus
SN	Standard nest
STRd	Dorsal striatum region

Keywords: cannabidiol, mTOR, ERK 1/2, maternal separation, early life stress, cytochrome c oxidase

1. INTRODUCTION

Major depressive disorder is globally affecting over 300 million people, equivalent up to 4% of the world's population, being more common among females (5.1%) than males (3.6%) (World Health Organization, 2017). This disorder is considered as a leading cause of disability worldwide (World Health Organization, 2017), which is becoming an enormous burden cost for the national health system estimated to be between 3-4% of Gross National Product in developed countries (Jong-Wook, 2003). One of the major predictors of adulthood major depressive disorder is early-life stress, since depressive patients reported significantly higher rates of childhood adversity, including parental neglect, sexual and physical abuse among others than non-depressive individuals (Saleh et al., 2017). Notably, women and men are differentially affected on adult mood disorders by early-life stress depending on the adversities time course. Indeed, postnatal stress trajectory predicts female depression and anxiety symptoms in contrast to males, who are more affected by early pregnancy stress (Herbison et al., 2017).

In the past few decades, mitochondrial dysfunction has gradually become more prominent in the psychiatric disorders research, since it is considered a risk factor for depression pathogenesis (Kasahara and Kato, 2018). Further, it is known that some mental disorders, such as major depression, could be a prodromal stage in some patients with mitochondrial diseases (Anglin et al., 2012; Mancuso et al., 2013). In actual fact, there are some variants in mitochondrial DNA encoding structural subunits of the mitochondrial oxidative phosphorylation, such as complexes I, II and IV, that are implicated in human depression (Kasahara and Kato, 2018), including cytochrome c oxidase activity. Mitochondrial activity is involved in cerebral energy demand, production and protection against reactive oxygen species and apoptosis throughout metabolism of different molecules such as lipids, steroids and proteins (Bansal and Kuhad, 2016; Picard et al., 2018; Wallace, 1999). Therefore, mammalian target of rapamycin (mTOR) signalling pathway mediates a wide range of processes related to the regulation of several energy-demanding cellular functions or energy metabolism (Haissaguerre et al., 2014; Kolar et al., 2021), spanning from protein and lipid synthesis to mitochondrial activity and cytoskeleton dynamics (Haissaguerre et al., 2014). Likewise, mTOR inhibits mitophagy and autophagy processes (Kim et al., 2011), which can ensure normal mitochondria turnover. Accordingly, mitochondria damage is related to hyperphosphorylation of both mTOR (Bordi et al., 2019) and extracellular signal-

regulated protein kinase 1/2 (ERK 1/2) proteins (Duncan et al., 2018; Feng et al., 2016), leading to the accumulation of mitochondrial damage and deficits in the activation of proapoptotic downstream cascades (Bordi et al., 2019). Thus, mitochondrial dynamics might have a pivotal role in the attenuated neurotransmitter synthesis and neuroplasticity present in depressive processes (Allen et al., 2018; Bansal and Kuhad, 2016).

In our laboratory, we use a well-established mouse model of early-life stress known as maternal separation with early weaning (MSEW), (George et al., 2010), which recapitulates the main phenotype observed in depression, such as anhedonia (Gracia-Rubio et al., 2016), social interaction disruption (Portero-Tresserra et al., 2018), despair-like behaviour (Gracia-Rubio et al., 2016) and anxiety-like behaviours (Martín-Sánchez et al., 2021). Moreover, animals exposed to MSEW exhibit a higher vulnerability to drug consumption (Castro-Zavala et al., 2020; Portero-Tresserra et al., 2018) as well as alterations in glutamatergic (Castro-Zavala et al., 2020), serotonergic (Gracia-Rubio et al., 2016) and endocannabinoid systems (Martín-Sánchez et al., 2021; Portero-Tresserra et al., 2018). Despite the fact that we observed a sexual dimorphism in several outcomes related to MSEW, depressive-like behaviour and cocaine seeking in the present model (Castro-Zavala et al., 2021), there are no previous reports studying possible brain metabolic capacity alterations in MSEW females, since most of the studies have been developed only in male mice. Indeed, sex and gender studies represent an important framework since women show higher vulnerability to depressive stages than males (World Health Organization, 2017). Accordingly, we have previously reported sex-specific altered brain CCO activity, changes in brain monoamine turnover rate, and increased pro-inflammatory cytokines in several brain regions in rats after early life stress by maternal separation (González-Pardo et al., 2020) and in congenitally helpless rats, a genetic model of depressive-like behaviour (Shumake et al., 2004). Altogether, these results suggest a close relationship between the mitochondrial alterations and depressive-like behaviour, identifying possible shared specific related pathways.

Among the proposed antidepressant treatments by clinical and preclinical studies, cannabidiol (CBD) has emerged as a novel therapeutic candidate because of its rapid-acting antidepressant (Gáll et al., 2020; Linge et al., 2016; Sales et al., 2019; Silote et al., 2019) and anxiolytic-like effects (Campos et al., 2013; Fogaça et al., 2018). CBD is one of the most abundant molecules of the *Cannabis sativa* plant, and there is a great interest in its potential medical use due to its non-intoxicating and non-psychotomimetic properties (Ligresti et al., 2016). Moreover, CBD is a multi-target compound that exerts

its effects within the central nervous system interacting with 5-hydroxytryptamine 1A (5-HT1A), transient potential vanilloid 1 (TRPV1), G-protein 55 (GPR55) and peroxisome proliferator-activated gamma (PPARγ) receptors, as well as antagonizing adenosine reuptake (Luján and Valverde, 2020), among others. Additionally, CBD could also interfere with the endocannabinoid system, acting as a negative allosteric modulator of cannabinoid receptor type 1 and 2 (CB1 and CB2) at physiologically relevant concentrations (Laprairie et al., 2015; Tham et al., 2018). Accordingly, CBD may exert anti-inflammatory (Esposito et al., 2011; Magen et al., 2010) and pro-neurogenic (Campos et al., 2013; Esposito et al., 2011; Fogaça et al., 2018; Luján et al., 2020) effects that may converge to mitigate depressive-related behaviours in rodent models.

Importantly, there are no previous studies that pay attention to the antidepressant role of CBD in an early-life stress model in female mice, because most of the studies on the antidepressant and anxiolytic effects of CBD were carried out in male rodents. Therefore, we hypothesized that MSEW could promote changes in mitochondrial metabolism that could explain some of the behavioural and molecular alterations in the development of the litters that has not been previously studied. Moreover, would like to identify whether sub-chronic CBD treatment during adulthood could reverse a possible abnormal brain mitochondrial capacity as well as the behavioural alterations. To that end, we performed the MSEW procedure in female mice and we analysed the regional CCO activity by quantitative histochemistry. Western blot analysis of the mTOR and ERK phosphorylated proteins were carried out as markers of mitochondrial damage, in both amygdala and striatum, brain regions involved in emotion regulation.

2. METHODS

2.1. Animals

CD1 male and female mice purchased from Charles River (France) were used as breeders. All animals were maintained at 21 ± 1 °C, humidity 55 ± 10% and a 12:12h light:dark cycle, with lights on at 07:30h. Water and food were available *ad libitum*. Cages were cleaned weekly except during postpartum days when dams were left undisturbed until postpartum day (PD) 10. All procedures were carried out in accordance with national (BOE-2013-1337) and EU (Directive 2010-63EU) guidelines regulating animal research and were approved by the local ethics committee (CEEA-PRBB).

2.2. Rearing conditions

The maternal separation protocol has been previously conducted in our laboratory (Figures 1A, 3A, 4A, 5A) (Martín-Sánchez et al., 2021; Castro-Zavala et al., 2020; Portero-Tresserra et al., 2018). On arrival, breeding pairs were established and housed in Plexiglas cages (36.9 x 15.6 x 13.2 cm) for ten days. Each male mouse was removed when pregnancy was confirmed and then, female mice were housed individually. Pregnant females were daily checked at 9 and 17h for parturition. The date of delivery was assigned as a PD 0 for each litter. Litters were randomly assigned to MSEW or SN group. In MSEW group, mothers were separated to another cage from the offspring for 4 h per day on PD2-5 (09:30–13:30h) and 8 h per day on PD6-16 (09:30–17:30 h). The offspring remained in their home cages with a heating blanket (32–34 °C) for thermoregulation and they were early weaned at PD17. Meanwhile, offspring of SN group remained undisturbed with their mothers until the weaning day (PD21). After that, mice were housed in groups of 4-5 animals of the same sex. Male littermates were dismissed for the experiment and were used for other studies.

2.3. Drugs

Female mice were treated daily for 10 consecutive days (PD50-60), with CBD (20 mg/k) or vehicle by intraperitoneal (i.p) injection, following Luján et al., (2018) (PD50-60). Five days after the last injection, mice underwent behavioural tests (Figure 1A and 4A) or Western blot analysis (Figure 5A and 6A). CBD was dissolved in the vehicle solution consisting of ethanol/cremophor EL (*Kelliphor*; Sigma-Aldrich, Darmstadt, Germany)/distilled water (1:1:18). The volume of injections was 0.1 mL per 10 g of mouse body weight. CBD was generously provided by Phytoplant Research S.L. (Córdoba, Spain; purity >98%).

2.4. Experiment 1: Anxiolytic and antidepressant effects of CBD in MSEW female mice

2.4.1. Elevated plus maze

After 5 days of the last CBD/vehicle injection (n=10 per group), on PD65, elevated plus maze (EPM) test was performed using a black maze elevated 30 cm above the ground (Gracia-Rubio et al., 2016; Martín-Sánchez et al., 2021). Each mouse was placed in the centre of the maze and was allowed to freely explore for 5 min. The software SMART

187 2.5 (Panlab s.l.u., Barcelona, Spain) automatically reported the time spent and the number
188 of entries in the open arms, as well as the travelled distance.

189 *2.4.2. Tail suspension test*

190 On PD66, each mouse was suspended individually 50 cm above a benchtop. Mice
191 were individually video-recorded and an observer, blind to the experimental conditions,
192 evaluated the percentage of time the animal was immobile for 6 min (Gracia-Rubio et al.,
193 2016).

194 *2.4.3. Cytochrome c oxidase quantitative histochemistry*

195 All female mice used in Experiment 1 were euthanized on PD67 and brains were
196 quickly removed, frozen in cooled isopentane, and stored at -40 ºC for histochemistry.
197 Later, brain tissue was preserved in dry ice and processed (Department of Psychology,
198 University of Oviedo, Spain). Then, series of 30 µm-thick coronal brain sections were
199 obtained from each subject with a cryostat microtome at -20 ºC and processed for
200 cytochrome c oxidase (CCO) quantitative histochemistry, following the method by
201 Gonzalez-Lima and Jones (1994).

202 *2.4.4. Data analysis: Cytochrome c oxidase activity and functional brain networks*

203 Mean CCO activity values were measured by optical densitometry and converted
204 to CCO activity units. To that end, we used brain homogenate standards of previously
205 assessed enzymatic activity by spectrophotometry for each subject in the following brain
206 regions of interest according to mouse brain atlas (Allen Institute for Brain Science,
207 version 2, 2011; http://atlas.brain-map.org/): cortical amygdalar area (COA), lateral (LA),
208 basolateral (BLA), medial (MEA) and central (CEA) amygdalar nuclei, dorsal striatum
209 region (STRd), nucleus accumbens core (ACBc) and shell (ACBs) areas, paraventricular
210 hypothalamic nucleus (PVH) and bed nuclei of the stria terminalis (BST).

211 Functional connectivity was assessed by calculating separate pair-wise Pearson's
212 correlations of CCO activity across all regions of interest for each group. A 'jackknife'
213 statistical procedure was used to correct against the type I statistical errors caused by
214 multiple comparison analysis and small sample sizes. The 'jackknife' procedure is
215 performed by removing one subject from the dataset and calculating the correlation
216 analysis with n-1 subjects. The CCO activity data for an individual is then included again

in the group dataset, and the next individual is removed. Correlations and significance levels are then re-calculated and this procedure is repeated until all individuals have been removed once. Only correlations that remain significant ($p<0.01$) across all possible combinations are valid. This comment has been included in the materials and method section. Interregional correlations of CCO activity represent the degree to which the neuronal synaptic activity between two brain regions is related to one another, or how they vary together (covariance). For covariance-based analyses to be useful in neuroimaging, they must make a significant contribution to the understanding of the data sets beyond what could be derived from analysis of mean regional activity. A high covariance or interregional correlation between areas X and Y means that if region X increases its activity, so too will Y (in the case of a positive covariance). In case of negative covariances, if region X increases its activity, Y will decreases it (McLntosh and Gonzalez-Lima, 1994).

2.5. Experiment 2: Implication of MSEW on emotional memory

2.5.1. Passive avoidance test

In a different cohort, mice underwent a passive avoidance test five days after the last CBD/vehicle injection (PD65; n=10 per group; Figure 3A), following Saavedra et al., (2013). The experiment was conducted in a two-chambers apparatus: a weakly and brightly lit chambers (2–5 and 160 lx, respectively; size: 19x19x27cm) (Panlab s.l.u., Barcelona, Spain). The dark compartment had a stainless-steel grid floor for shock delivery. On the acquisition trial, each mouse was placed into the bright compartment. A sliding door between both compartments was opened after 30s, and the latency to enter into dark chamber was recorded for up to 90s. When the animal crossed into dark compartment, the door was closed and the mouse received a foot shock (0.5mA, 3s). Then, the mouse was immediately removed from the apparatus. 24h later, mice were placed into the brightly lit compartment, and the procedure was repeated without the foot shock (retention trial). Latency to enter the dark chamber was evaluated with a cut-off of 300s.

2.6. Experiment 3: mTOR-ERK 1/2 signalling pathways and mitochondrial damage in MSEW females

2.6.1. Western blot assay

Animals were sacrificed by cervical dislocation five days after the last CBD/vehicle injection (Figure 4 and 5; n=6/group) on PD65 to evaluate the expression of mTOR, phosphorylated mTOR (p-mTOR), ERK1/2 and phosphorylated ERK1/2 (p-ERK1/2) in striatum and amygdala from SN and MSEW groups. Brains were immediately removed from the skull and placed in a cold plaque, extracting the amygdala and striatum. Brain samples were dissected and were immediately stored at −80 °C until the western blot assay was carried out. Samples were homogenised in cold lysis buffer, supplemented with protease inhibitor (Complete ULTRA Tablets Mini EASYpack, Roche, Mannheim, Germany) and phosphatase inhibitor (PhosSTOP EASYpack, Roche, Mannheim, Germany). Equal amount of protein (16 μg) for each sample were mixed with loading buffer (153 mM TRIS pH = 6.8, 7.5% SDS, 40% glycerol, 5 mM EDTA, 12.5% 2-β-mercaptoethanol and 0.025% bromophenol blue) and loaded onto 10% polyacrylamide gels, and transferred to PVDF sheets (Immobilion-P, MERCK, Burlington, USA). Membranes were blocked with bovine serum albumin 5% for 1h at room temperature and then, an overnight incubation with primary antibodies (Table 1) was assessed at 4 °C. After that, membranes were incubated for 1 h with their respective secondary fluorescent antibodies: goat anti-rabbit (1:2500, Rockland, PA, USA) or goat anti-mouse (1:2500, Abcam, Cambridge, UK) at room temperature. Images were acquired on an Amersham Typhoon NIR Plus laser scanner and quantified using Image Studio Lite software v5.2 (LICOR, USA).

Antibody	Description	Host	Dilution	Company	Item number	MW (kDa)
ERK1/2	extracellular signal–regulated kinases 1/2	Mouse	1:1000	Abcam	ab54230	43, 41
mTOR	mammalian target of rapamycin	Rabbit	1:1000	Cell Signaling Technology	#2972	289
p-ERK1/2	phosphorylated extracellular signal–regulated kinases 1/2	Mouse	1:5000	Abcam	#ab50011	42, 44
p-mTOR (Ser2448)	phosphorylated mammalian target of rapamycin	Rabbit	1:1000	Cell Signaling Technology	#2971	289
Tub	beta III-Tubulin	Rabbit	1:2500	Abcam	#ab18207	55

Table 1. List of the primary antibodies used for western blot assay.

2.7. Statistical analysis

We first checked the data for normality (Kolmogorov-Smirnov's test) and homoscedasticity (Levene's test). Then, we carried out a two-way ANOVA (with *rearing* and *treatment* as variables) to assess differences between groups in EPM, TST and Western blot data. When applicable, pairwise comparisons were analysed with Bonferroni's correction. Then, we evaluated differences between groups using the non-parametric Log-rank test (percentage of females that entered dark compartment during retention trial). All the statistical analysis were performed using the software IBM SPSS Statistics 23.0.

Group differences in mean regional brain CCO activity were analysed for each region of interest by two-way (*rearing* × *treatment*) ANOVAs and post-hoc analyses were performed by Holm-Sidak method when interactions between variables were significant. Functional connectivity of CCO activity between brain regions for each experimental group was analysed by Pearson's product-moment pairwise correlations corrected for multiple comparisons type I error using a 'jack-knife' multiple interaction procedure as previously described (Cantacorps et al., 2018; Shao and Tu, 1995). The statistical analyses were carried out using SigmaPlot 12.5 (Systat Software Inc., Richmond, CA, USA). Statistical differences were found when $p < 0.05$.

3. RESULTS

3.1. Experiment 1: Anxiolytic and antidepressant effects of CBD

3.1.1. Experiment 1: Anxiolytic effect of CBD in both SN and MSEW mice

In order to evaluate differences in anxiety-related behaviour between MSEW and SN female mice after CBD treatment, mice underwent an EPM test. To do so, we measured three different variables: time in open arms, number of entries in open arms and travelled distance. The two-way ANOVA revealed a *treatment* effect in percentage of time spent in open arms ($F_{1,35}$= 17.50, $p < 0.001$; Figure 1B), percentage of entries in open arms ($F_{1,35}$= 15.24, $p < 0.001$; Figure 1C), percentage of time in closed arms ($F_{1,35}$= 17.50, $p < 0.001$; Figure 1D) and percentage of entries in closed arms ($F_{1,35}$= 14.89, $p < 0.001$; Figure 1E). The analysis showed that CBD (20mg/kg) increased all these variables in both SN and MSEW, having an anxiolytic role in female mice. However, we found neither *rearing* effect nor interaction between variables. When we analysed the travelled distance in the EPM, we did not find significant differences among groups (Figure 1F).

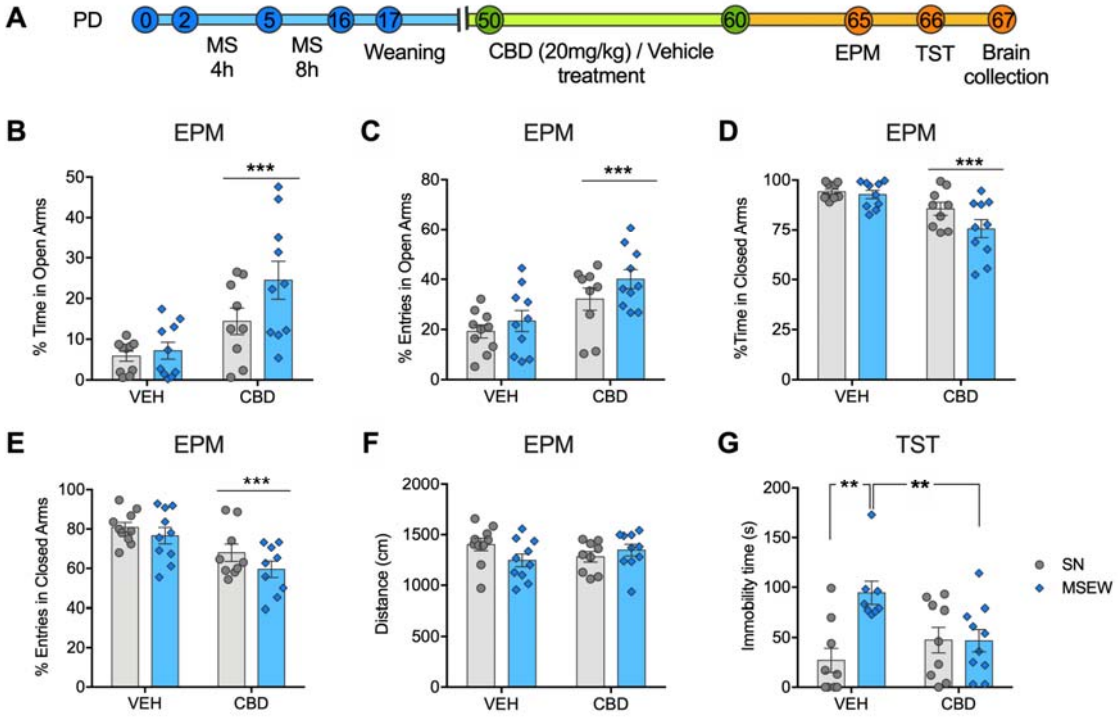

Figure 1. Anxiety-like and despair-like behaviours in MSEW and SN mice after CBD treatment. (A) Experimental timeline of the Experiment 1. Grey (standard nest, SN) and blue (maternal separation with early weaning, MSEW) bars represent (B) the percentage of time spent in open arms, (C) the percentage of entries in open arms, (D) the percentage of time in closed arms, (E) the percentage of entries in closed arms (F) travelled distance in the elevated plus maze (EPM; n= 10/group), and (G) the immobility time in tail suspension test (TST; SN-Vehicle n=9, MSEW-Vehicle n=8, SN-CBD n=10, MSEW-CBD n=10). CBD, cannabidiol; MS, maternal separation; PD, Postpartum day. $^{**}p < 0.01$; $^{***}p < 0.001$ for CBD- and Vehicle-treated groups comparisons. All data are represented as the mean ± SEM.

3.1.2. Experiment 1: Antidepressant effect of CBD in MSEW female mice

When we analysed the immobility time in TST as a despair-like behaviour measure, two-way ANOVA revealed a *rearing* effect ($F_{1,36}$=7.66, $p < 0.01$) and *treatment* × *rearing* interaction ($F_{1,36}$=7.96, $p < 0.01$; Figure 1G). The analysis indicated that MSEW female mice spent more time immobile than SN animals. The *post-hoc* analysis with Bonferroni's correction showed that MSEW-Vehicle animals show higher immobile time than SN-Vehicle control mice ($p < 0.01$). Moreover, the post-hoc comparison revealed that CBD reduced the immobility time only in MSEW females compared to its vehicle control group ($p < 0.01$), indicating an antidepressant role only in MSEW mice.

3.1.3. Experiment 1: Quantitative CCO activity

3.1.3.1.Mean CCO values

The effects of *rearing* and *treatment* on the metabolic brain regions are shown in Figure 2. Two-way ANOVAs revealed differences in the CCO activity due to *rearing* effects in the ACBs ($F_{1,34}$ = 5.24, $p < 0.05$; Figure 2A), ACBc ($F_{1,34}$ = 5.96, $p < 0.05$; Figure 2B), PVH ($F_{1,34}$ = 6.69, $p < 0.05$; Figure 2D), the BST ($F_{1,35}$ = 6.26, $p < 0.05$; Figure 2E), BLA ($F_{1,35}$ = 5.39, $p < 0.05$; Figure 2F) and LA ($F_{1,35}$ = 7.46, $p \leq 0.01$; Figure 2I). The analysis showed a significantly higher metabolic activity of SN groups in the ACBs, ACBc, BST, BLA and LA as compared to MSEW groups. On the other hand, it is worth mentioning that CBD *treatment* decreased metabolic capacity in the PVH to baseline levels in MSEW-Vehicle females ($F_{1,34}$ = 5.13, $p < 0.05$; Figure 2D). Finally, significant interaction between *rearing* × *treatment* was only found in the CEA ($F_{1,35}$ = 5.32, $p < 0.05$; Figure 2H). *Post-hoc* tests revealed that SN-CBD and MSEW-Vehicle females had significantly lower CCO activity as compared SN-Vehicle female mice in this structure cerebral. However, the CCO activity was equivalent in all four groups in the STRd, MEA and COA (Figure 2C, G and J).

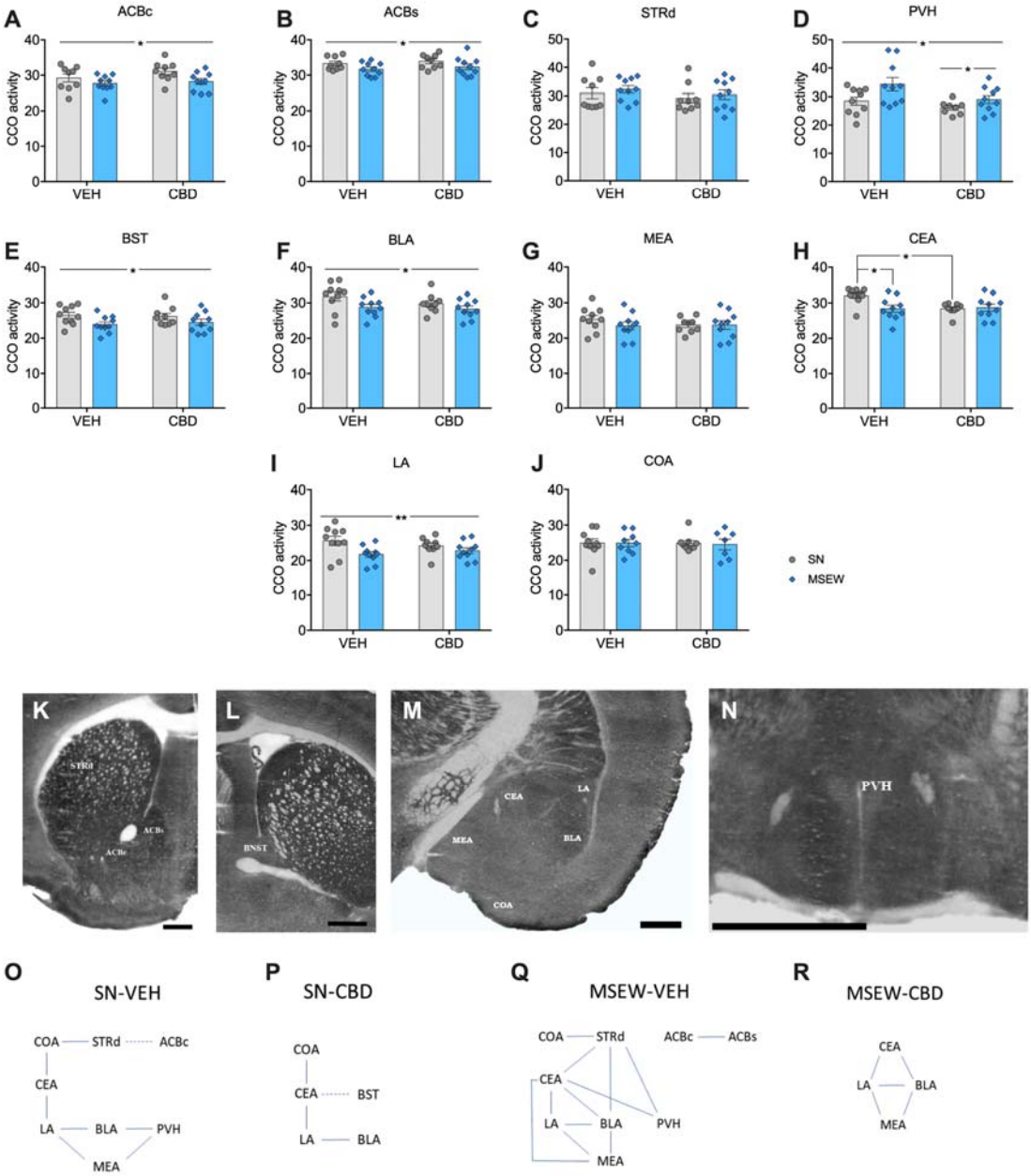

Figure 2. Mean CCO activity values of the different brain regions of interest in each experimental group. Data represent mean CCO activity values ± standard error (SEM) using rearing and CBD treatments as main factors (A-J). Two-way ANOVA: *Rearing* effect * $p < 0.05$; ** $p < 0.01$. Photomicrographs show sample mouse brain sections stained after CCO histochemistry (K, Scale bar ?µm; L, Scale bar ?µm; M, Scale bar ?µm; N, Scale bar ?µm). Pair-wise interregional activity correlations by experimental group. Schematic diagram showing significant interregional correlations in CCO activity in SN Vehicle- (O) and CBD-treated females (P) in comparison to MSEW Vehicle- (Q) and CBD-treated animals (R). Solid and dotted lines represent respectively highly positive and negative pair-wise Pearson's correlations ($r > 0.7$; $p \leq 0.01$). Abbreviations COA, cortical amygdalar area; LA, lateral; BLA basolateral; MEA, medial and CEA, central amygdalar nuclei; STRd, dorsal striatum region; ACBc and ACBs, nucleus accumbens core and

shell areas respectively; PVH paraventricular hypothalamic nucleus and BST, bed nuclei of the stria terminalis.

3.1.4. Experiment 1: Functional brain networks

Interregional correlation analysis of CCO activity revealed different significant functional connections among experimental groups. Specifically, the vehicle groups showed a more intricate network, with higher positive correlations (solid lines, $p \leq 0.01$; Figure 2O and Q) involving the amygdaloid complex, paraventricular hypothalamic nucleus and dorsal striatum. In addition, a high negative correlation (dotted line) was detected between the dorsal striatum and the ACBc ($p < 0.001$; Figure 2O) in the SN-Vehicle group, which disappeared in the Vehicle group after maternal separation (Figure 2Q). On the other hand, a modified pattern of functional connectivity was observed in CBD-treated groups (Figure 2P and R). In this regard, these groups showed increased functional connectivity between amygdala nuclei, independently of the rest of the brain regions. Only a high negative correlation was detected between the CEA and the BST ($p < 0.01$) in the SN-CBD group (Figure 2P).

3.2. Experiment 2: CBD treatment on emotional memory in MSEW and SN female mice

Since most of changes in the quantitative CCO activity and functional network in the Experiment 1 were found in the amygdala structure, we evaluated differences in emotional learning due to maternal separation and CBD treatment by carrying out the passive avoidance test. Since data of the Acquisition and Retention trials were not normally distributed (Figure 3B), we applied a non-parametric test (Log-Rank test) to evaluate possible differences among groups in the retention trial phase. The analysis of the percentage of animals that entered the dark compartment during retention trial using the Log-rank test shows no differential responses of the experimental groups ($\chi 2 = 3.891$ on df 3, $p > 0.05$; Figure 3C).

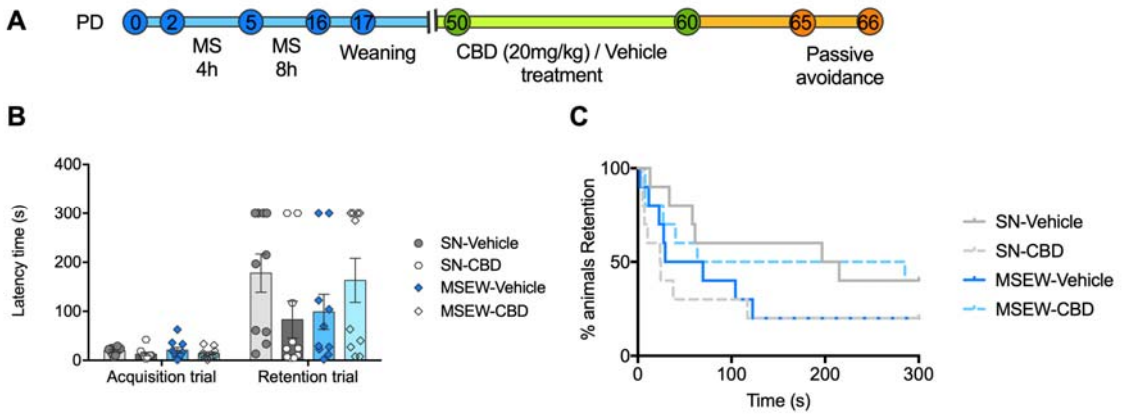

Figure 3. CBD treatment effects in passive avoidance test. (A) Schematic schedule of the Experiment 2. (B) Representation of the latency time in both acquisition and retention trials in the passive avoidance test (n=10/group). (C) Percentage of females that entered dark compartment during retention trial (Log-rank test). Cannabidiol (CBD); Maternal separation (MS) with early weaning (MSEW); standard nest (SN). All data are represented as the mean ± SEM.

3.3. Experiment 3: Early-life stress impact on p-ERK1/2 and p-mTOR expression

3.3.1. Experiment 3: CBD treatment does not alter either pERK1/2 or p-mTOR expression in the striatum of female mice

Since deficits in p-mTOR and p-ERK 1/2 signalling pathways have been reported in mitochondria metabolism and mitophagy processes (Bordi et al., 2019; Duncan et al., 2018; Iyer et al., 2014), we evaluated whether MSEW could promote alterations in ERK1/2 and mTOR expression. In the light of the results obtained in the Experiment 1, we investigated differences in the phosphorylation and expression levels of both extracellular signal–regulated kinases 1/2 (ERK1/2) and mammalian target of rapamycin (mTOR) related to rearing conditions in the striatum following a repeated CBD treatment (Figure 4A). The two-way ANOVA revealed no significant changes in ERK1/2, p-ERK1/2 and p-ERK1/2/ERK1/2 ratio (Figure 4B, C and D, respectively) in the striatum of female mice, irrespective of *rearing* and *treatment* conditions. Meanwhile, the expression of mTOR, p-mTOR and the p-mTOR/mTOR ratio (Figure 4E, F and G, respectively) did not show obvious changes between groups.

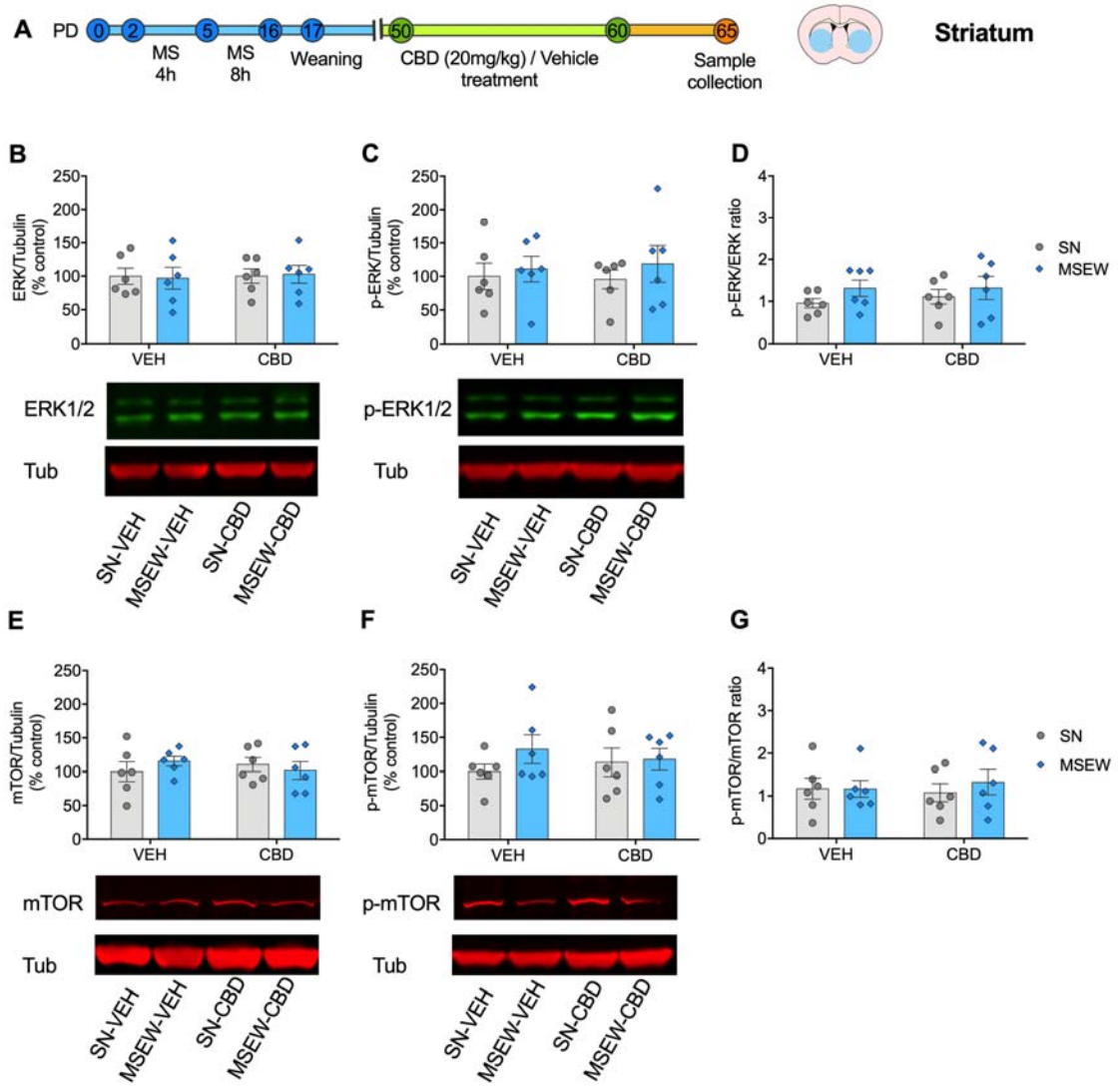

Figure 4. ERK1/2-mTOR pathway is not altered after CBD treatment (20mg/kg) in striatum of female mice. (A) Schematic timeline of Experiment 3. Mean fold change relative to the control of (B) ERK1/2, (C) p-ERK1/2, (D) p-ERK1/2/ ERK1/2 ratio, (E) mTOR, (F) p-mTOR and (G) p-mTOR/mTOR ratio in the striatum of standard nest (grey, SN) and maternally separated (blue, MSEW) female mice after CBD/vehicle treatment (n=6/group). All data are represented as the mean ± SEM.

3.3.2. Experiment 3: Hyperphosphorylation of ERK 1-2 and mTOR in amygdala of MSEW female mice

To clarify the impact of early-life stress in the expression of these proteins in the amygdala of females, the two-way ANOVA, with *rearing* and *treatment* as variables, showed a *rearing* effect in the expression of p-ERK1/2 ($F_{1,20} = 4.95$, $p < 0.05$; Figure 5C), p-mTOR ($F_{1,20} = 13.38$, $p < 0.001$; Figure 5F) and p-mTOR/mTOR ($F_{1,20} = 6.71$, $p < 0.05$; Figure 5G), indicating that MSEW females showed an increased level of the

phosphorylation of these proteins and the ratio p-mTOR/mTOR, regardless of their *treatment*.

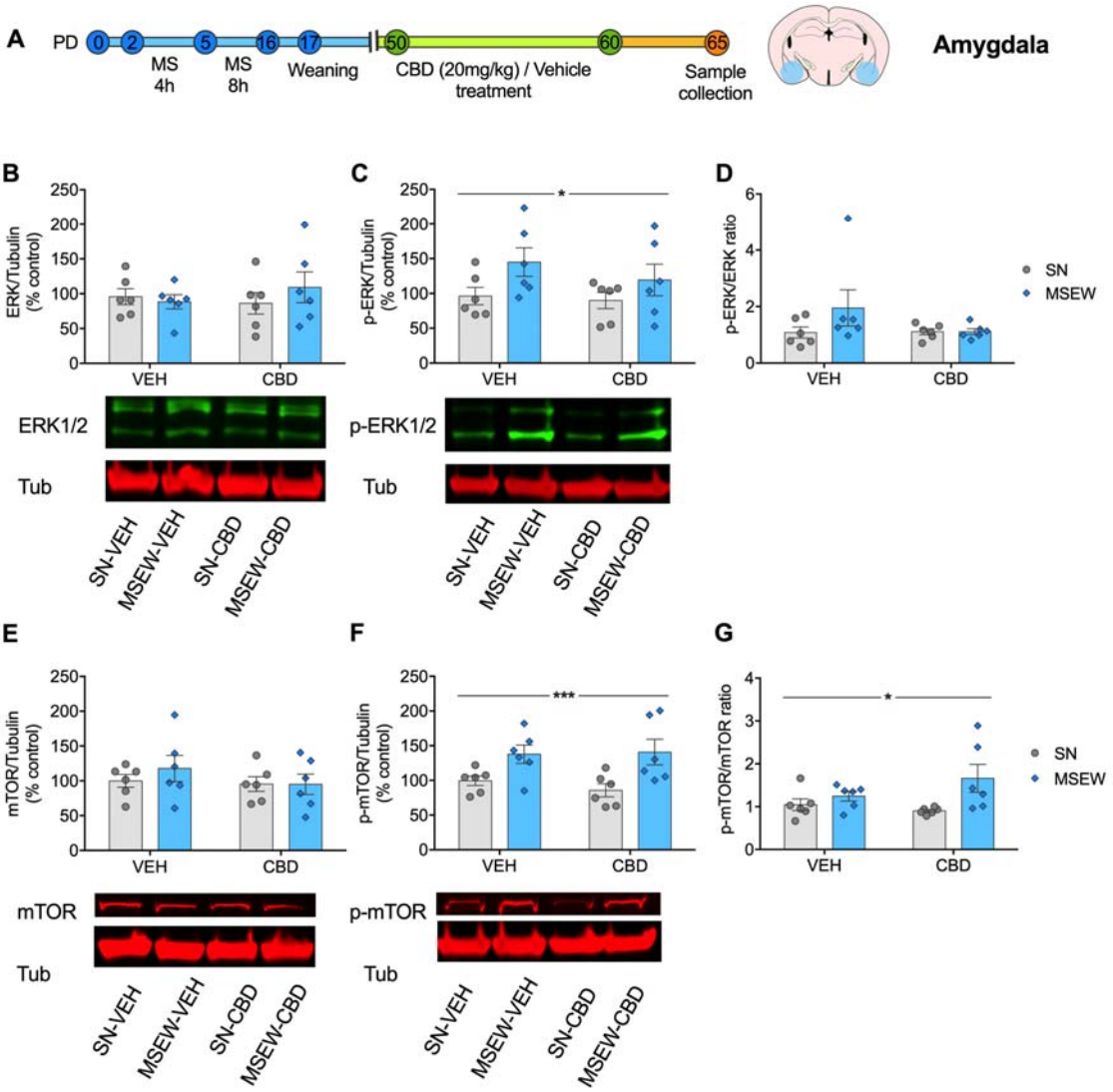

Figure 5. The phosphorylation of ERK1/2, phosphorylation of mTOR and ratio p-mTOR/ mTOR are increased in the amygdala of MSEW mice. (A) Schematic timeline of the Experiment 3. Mean fold change relative to the control of (B) ERK1/2, (C) p-ERK1/2, (D) p-ERK1/2/ ERK1/2 ratio, (E) mTOR, (F) p-mTOR and (G) p-mTOR/mTOR ratio in the striatum of standard nest (grey, SN) and maternally separated with early weaning (blue, MSEW) female mice after CBD/vehicle treatment (n=6/group) (two-way ANOVA, *rearing* effect $*p < 0.05$, $***p < 0.001$). All data are represented as the mean ± SEM.

4. DISCUSSION

The main aim of the present study was to elucidate whether MSEW promotes anxiety-related and depressive-related behaviours, the possible brain oxidative

alterations and molecular signalling related to early-life stress exposure in female mice. Moreover, we studied whether sub-chronic CBD treatment could reverse the mitochondrial damage ameliorating the cognitive impairments. The experiments were addressed to evaluate the long-lasting neuroplastic effects of the sub-chronic CBD treatment in maternally separated and control female mice. Therefore, we treated animals using one-daily injections CBD treatment (20mg/kg ip; for 10 days) and we left 5 days of wash-out, assuming that CBD has been metabolized and disappeared from the organism before the behavioural experiments based on previous reports (Deiana et al., 2012; Gonzalez-Cuevas et al., 2018). Thus, this is, to the best of our knowledge, the first time that behavioural and molecular implications of early-life stress and CBD sub-chronic treatment are evaluated in adult female mice.

4.1. CBD exerts anxiolytic and antidepressant effects in MSEW female mice

When we analysed anxiety-like behaviour, we observed that sub-chronic CBD (20 mg/kg) administration exerts anxiolytic-like effects in both SN and MSEW females, significantly increasing the percentage of time spent in the open arms and the percentage of entries in open arms, without alterations in motor activity in the EPM (Figure 1B, C and F). Previous studies shown that, although MSEW animals show lower locomotor activity during adolescence, this effect is diluted during adulthood (Gracia-Rubio et al., 2016) supporting our observations. A growing body of literature provides compelling evidence that CBD has anxiolytic effects in both stressed and non-stressed male mice (Campos et al., 2013; Fogaça et al., 2018; Luján et al., 2018; Sales et al., 2018). Our behavioural findings are in line with those obtained in mice after a chronic unpredictable stress paradigm and a 14-day CBD (30mg/kg) treatment (Campos et al., 2013; Fogaça et al., 2018) in males mediated by CB1/CB2 receptors (Fogaça et al., 2018), since CB1 and CB2 antagonism prevented the anxiolytic-like effects of CBD. By contrast, Schiavon et al., (2016) found that CBD only promoted anxiolytic-like responses after a single-low dose of CBD (3 mg/kg) in non-stressed male mice, without altering basal locomotor activity. These results agree with previous findings showing that single CBD administration could promote anxiolytic-like responses according to an inverted U-shaped dose-response curve, suggesting that low and high doses of CBD are ineffective in anxiety paradigms (Campos et al., 2012; Luján et al., 2018).

In addition to the anxiolytic-like effects, repeated CBD administration also demonstrated an antidepressant-like effect but only in early-life stressed females, since

it reduced immobility time in the TST (Figure 1F). Previously, acute (Sales et al., 2018) and chronic CBD treatments have been shown to exert antidepressant effects after chronic mild stress (Gáll et al., 2020; Xu et al., 2019), but also in basal conditions (Schiavon et al., 2016). Previous studies suggest that CBD antidepressant outcomes could be mediated by serotoninergic 5-HT_{1A} receptor activation (Linge et al., 2016), as well as the brain-derived neural factor-tropomyosin-receptor kinase B-mTOR (BDNF/Trkb/mTOR) pathway (Sartim et al., 2018).

4.2. Early-life stress and CBD modify CCO activity and brain connectivity pattern in female mice

Accordingly, analysis of CCO activity suggests that CBD treatment had regionally specific effects on brain oxidative capacity. As opposed to other methods to measure short-term (after several minutes) or stimulus-evoked neuronal activity like 18F-fluoro-2-deoxy-D-glucose (FDG) uptake or immediate early gene expression (like c-fos) techniques, CCO activity is a reliable index of long-term changes in enzyme levels after days of brain energy demands, also known as metabolic capacity (Gonzalez-Lima, 1992; Gonzalez-Lima and Cada, 1994; Papa et al., 1998). CCO activity mainly reflects neuronal synaptic activity since CCO is a rate-limiting enzyme in cellular respiration for ATP production, and ATP is mainly required to maintain neuronal electrophysiological properties, like the membrane resting potential after membrane depolarization or hyperpolarization induced by synaptic activity (Wong-Riley, 1989). In particular, the CEA and the PVH showed significantly lower CCO activity in CBD-treated groups as compared to their vehicle control groups. Since activation of the CEA is required for both anxiety and fear responses in rodents and humans (Canteras et al., 2010; Davis, 1998; Fox and Shackman, 2019; Gilpin et al., 2015; Izquierdo et al., 2016; Paré et al., 2004; Wilensky et al., 2006), the CCO activity results are in line with the behavioural results reported here, suggesting an anxiolytic-like effect of CBD in females.

CBD is a multi-target compound that seems to exert its pharmacological acting on different modulatory systems. Indeed, CBD acts as an allosteric modulator of CB1 (Laprairie et al., 2015; Tham et al., 2018) and CB2 receptors (Tham et al., 2018), but it also interacts with 5-HT1A (Sartim et al., 2016) receptors and TRPV receptors among others. However, the activation of 5-HT1A and CB1 receptors have been proposed as a putative main mechanism of action in the neuroprotective, anxiety-related responses and

oxidative brain outcomes (Campos et al., 2016; Luján and Valverde, 2020). Unfortunately, how CBD could inhibit brain oxidative metabolism is currently speculative. Despite the fact that mitochondrial CB1 receptor activation using exogenous cannabinoids and *in situ* endocannabinoid administration could decrease complex I enzymatic activity and respiration in neuronal mitochondria (Bénard et al., 2012), probably CBD is not exerting its function by directly acting on CB1 receptors.

Mitochondrial function as related to brain energy metabolism of limbic regions plays a pivotal role in stress responses, motivation, mood, and anxiety (Filiou and Sandi, 2019; Harro et al., 2011; Mällo et al., 2009; Matrov et al., 2019). In this regard, decreased oxidative metabolic capacity was found in the BLA, LA and BST of MSEW as compared to their SN. Accordingly, lower CCO activity levels in the amygdala nuclei and BST have been reported in congenitally-helpless rats, an animal model of depression (Shumake et al., 2003, 2002). Therefore, changes found in CCO activity in CEA of MSEW vehicle-treated animals would match the higher susceptibility to stress and increased depressive-like behaviour found in the TST. Conversely, MSEW CBD-treated and SN-Vehicle control group showed equivalent CCO activity in CEA, a matching result with antidepressant-like effects of CBD in female mice. This result agrees with the attenuation of the neuroendocrine stress response mediated by CBD (Viudez-Martínez et al., 2018).

Analysis of functional brain connectivity of CCO activity in the brain regions of interest showed that MSEW significantly altered interregional brain connectivity patterns. This interregional correlation of CCO activity can be interpreted as the degree to which the neuronal synaptic activity between two regions are related to one another, or how they vary together (covariance) (Auchter et al., 2020). In particular, MSEW females showed an increased connectivity of the PVH with both STRd and CEA as compared to the SN group. Given the important role of the PVH mediating the neuroendocrine stress response and amygdala (especially in CEA) after stress-inducing stimuli (Gray et al., 1989), our results suggest that MSEW induced the activation of a functional brain network related to an increased response to emotional stressors in MSEW animals, supporting anxiety- and depressive-related behaviours. Accordingly, increased connectivity of CCO activity between the ACBc and ACBs was previously associated with anxiety and increased freezing behaviour during contextual fear conditioning by our research group (González-Pardo et al., 2012).

On the contrary, CBD treatment clearly decrease the functional connectivity of amygdala nuclei with the rest of brain regions in both the MSEW and SN groups,

supporting the anxiolytic-like and antidepressant-like effects of CBD. Interestingly, the SN group treated with CBD showed negative pairwise correlations of CCO activity between the BST and the CEA, which are tightly interconnected and involved in anxiety and the organization of defensive responses (Fox and Shackman, 2019). Therefore, the inverse correlation found from the BST to the CEA would be directly related to the anxiolytic-like effects of CBD in female mice.

4.3. CBD does not alter emotional memory in female mice

Regarding to passive avoidance test results, our findings suggest that associative learning in MSEW females remains unaltered (Figure 3B and C), despite male mice show MSEW-related emotional memory impairments (Martín-Sánchez et al., 2021). Moreover, the sub-chronic CBD treatment administered shows no amnesic effect on contextual memory formation in female mice. Our results strongly contrast with previous studies that found that cannabinoids could be considered a potential treatment for post-traumatic stress disorders, since both WIN55,212-2 (Martín-Sánchez et al., 2021; Sbarski and Akirav, 2018) and CBD (Raymundi et al., 2020; Uhernik et al., 2018), interferes with emotional memories formation.

Nevertheless, it should be noticed that most of the studies using CBD chronic treatment were focused on male rodent's responses, so comparisons should be taken with caution. Indeed, sex-specific differential responses to CBD have become of particular interest recently (Kasten et al., 2019; Viudez-Martínez et al., 2020; Wanner et al., 2021). On the one hand, acute CBD treatment shows anxiety-related responses in a sex- and age-dependent manner (Kasten et al., 2019). On the other hand, Viudez-Martínez et al., (2020) have demonstrated sexual dimorphic responses to CBD treatment depending on the administration pattern, in several outcomes such as reducing alcohol intake. However, the present study is not addressed to assess any sex-differences due to the lack of a parallel male group, so further studies are required in this sense to evaluate possible sex-specific differences in the vulnerability to early-life stress and the responses to CBD chronic treatment.

4.4. Long-term hyperphosphorilation of mTOR and ERK 1/2 in the amygdala in early-life stressed females

Based on the behavioural and CCO activity results, we evaluated if MSEW induced changes in the mTOR-MAPK (ERK1/2) pathway in both striatum and amygdala, since the phosphorylation of both mTOR (Bordi et al., 2019) and ERK (Duncan et al., 2018; Feng et al., 2016) proteins are related to pro-apoptotic processes, mitochondrial damage, and depression (Chandran et al., 2013; Duman et al., 2012; Réus et al., 2014; Saeedi Saravi et al., 2017; Yamada and Jinno, 2019). Despite decreased mitochondrial CCO activity in the striatum of the MSEW female mice, we did not observe long-lasting effects on either the levels of ERK1/2, mTOR or their phosphorylated forms in the striatum of early-life stressed females. Our results are in line with those obtained by Chandran et al., (2013) because they found no changes in ERK or mTOR protein levels either in prefrontal cortex, hippocampus or dorsal raphe in rats after eight-weeks of unpredictable stress.

Meanwhile, we found several molecular alterations in the mTOR amygdalar pathways, indicating a greater stress-related sensitivity than other brain structures. Whereas MSEW females show an exacerbated expression of p-ERK1/2 and p-mTOR and an increased p-mTOR/mTOR ratio, Chandran et al., (2013) found decreased levels of phosphorylated mTOR and ERK1/2 only in the amygdala of stressed rats, supporting that the amygdala is a brain area highly sensitive to stress. The mTOR hyperactivation in the amygdalar complex of MSEW females agrees with the reduction of mitochondrial metabolism revealed by CCO activity. A plausible hypothesis could be that MSEW might trigger some mitochondrial-associated changes that may lead to the accumulation of mitochondrial damage revealed by a general decreased CCO metabolism of the BLA, CEA and LA. This mechanism has been already reported for explaining mTOR hyperactivation in the amygdalar complex in Down Syndrome (Bordi et al., 2019). In this sense, mTOR hyperactivation could indicate a negative modulation in the autophagy of mitochondria flux regulation in MSEW, leading to negative effects on mitochondrial replacement. We hypothesized that aberrant hyperactivation of mTOR amygdalar pathway of MSEW female could be reflecting an abnormal dysregulation of this pathway during development in response to early-life stress in a brain region-dependent manner, as also found in the frontal cortex and hippocampus of Down syndrome patients (Iyer et al., 2014; Perluigi et al., 2014).

4.5. Conclusion

The maternal separation model used here (MSEW) could be a reliable model that recapitulates some symptomatology-like of several mental disorders, since it induces depressive-like behaviour, as well as abnormal oxidative mitochondria metabolism as also occurs in some human mood and anxiety disorders. Our results indicate that CBD administration may improve behavioural cognitive impairment in MSEW females, since it shows antidepressant-like and anxiolytic effects and ameliorates the associative memories in MSEW females. However, we acknowledge as potential limitations that CBD does not revert the mitochondrial metabolic alterations that maternal neglect induces during early-life developmental stages. Probably, the neurodevelopmental changes promoted by MSEW are so deep and robust that a later CBD extended exposure window during adulthood is not enough to revert the underlying molecular alterations.

Acknowledgments

This work was supported by Ministerio de Economía y Competitividad (grants number PID2019-104077-RB-100 and PSI2017-83038-P), Ministerio de Sanidad, Asuntos Sociales e Igualdad (Retic-ISCIII-RD/16/0017/0010-FEDER and Plan Nacional Sobre Drogas (#2018/007). The Department of Experimental and Health Sciences (UPF) is a "Unidad de Excelencia María de Maeztu" funded by the AEI (CEX2018-000792-M).

The authors declare no conflict of interest.

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