Cannabidiol enhancement of serotonergic and glutamatergic signalling in a mouse model of depression induces fast and maintained antidepressant actions: implication of 5-HT$_{1A}$ receptors

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Abstract

Cannabidiol (CBD), the main non-psychomimetic component of marihuana, exhibits anxiolytic-like properties in many behavioural tests, although its potential for treating major depression has been poorly explored. Moreover, the mechanism of action of CBD remains unclear. Herein, we have evaluated CBD effects following acute and chronic administration in the olfactory bulbectomy mouse model of depression (OBX), and investigated the underlying mechanism. For this purpose**,** we conducted behavioural (open field and sucrose preference tests) and neurochemical (microdialysis and autoradiography of 5-HT_{1A} receptor functionality) studies following CBD treatment. We also assayed the pharmacological antagonism of CBD effects to dissect out the mechanism of action. Our results demonstrate that CBD exerts fast and maintained antidepressant-like effects as evidenced by the reversal of the OBX-induced hyperactivity and anhedonia. *In vivo* microdialysis revealed that CBD administration significantly enhanced serotonin and glutamate release in vmPFCx in a different manner depending on the emotional state and the duration of the treatment. The potentiating effect upon neurotransmitters release occurring immediately after the first injection of CBD might underlie the fast antidepressant-like actions in OBX mice. Both antidepressant-like effect and enhanced 5-HT/glutamate cortical release induced by CBD were prevented by 5-HT_{1A} receptor blockade. Moreover, adaptive changes in pre- and post-synaptic 5-HT_{1A} receptor functionality were also found after chronic CBD. In conclusion, our findings indicate that CBD could represent a novel fast antidepressant drug, via enhancing both serotonergic and glutamate cortical signalling through a 5-HT_{1A} receptor-dependent mechanism.

Highlights

- Cannabidiol exerts fast antidepressant-like actions in bulbectomized mice
- Cannabidiol enhances 5-HT and glutamate release in prefrontal cortex
- 5-HT_{1A} receptor mediates cannabidiol induced antidepressant-like effects
- 5-HT_{1A} receptor mediates serotonin and glutamate release induced by cannabidiol

Keywords: cannabidiol, antidepressant, glutamate, serotonin, olfactory bulbectomy, 5-HT_{1A} receptor

Abbreviations

Cannabidiol: CBD; ventromedial prefrontal cortex: vmPFCx; olfactory bulbectomy: OBX.

1. Introduction

Cannabinoid compounds have been used by different cultures to improve mood since ancient times. For this reason, the study of the endocannabinoid system and cannabinoid derivatives has gained a great interest in anxiety/depression research (Bambico et al., 2007; Hill and Gorzalka, 2005; McLaughlin et al., 2007; Shearman et al., 2003).

In this regard, cannabidiol (CBD), the main non-psychomimetic component of marihuana, has shown anxiolytic properties both in humans and rodents (Bergamaschi et al., 2011; Guimaraes et al., 1990) after acute or chronic administration (Campos and Guimaraes, 2008; Campos et al., 2013b; Resstel et al., 2009). Nevertheless, little is known about its potential for treating depression. It was proposed as a putative novel antidepressant as it displayed positive responses in the forced swimming test (FST) (El-Alfy et al., 2010; Zanelati et al., 2010) and also in the novelty suppressed feeding test (NSF) under chronic stress conditions (Campos et al., 2013b). Furthermore, CBD exerts a positive impact on some neuroplasticity markers of antidepressant effects, such as increased brain-derived neurotrophic factor levels (Magen et al., 2010). It also restores the impaired neuroproliferation of chronically stressed animals (Campos et al., 2013b), and presents anti-inflammatory and immunomodulatory effects (Esposito et al., 2011; Malfait et al., 2000).

The mechanism of action of CBD has been extensively scrutinized (McPartland et al., 2015). This multifaceted drug produces different pharmacological actions modulating several receptors in the central nervous system (CNS) (CB_1, CB_2, 5-HT_{1A}, TRPV1 and PPARγ receptors, among others) (Campos et al., 2013a;

Campos et al., 2013b; Casarotto et al., 2010; Costa et al., 2004; Do Monte et al., 2013; Esposito et al., 2011; Pazos et al., 2013; Soares Vde et al., 2010; Thomas et al., 2007). Given the crosstalk among systems involved in mood control, the ability of CBD to modulate some of them, could result advantageous for the treatment of such a complex disease as it is depression. Among all the above highlighted mechanisms, the CB_1 and 5-HT_{1A} receptors seem to be the most strongly implicated in CBD mood regulatory effects. CBD has been reported to act as an antagonist/inverse agonist of CB_1 receptors (Thomas et al., 2007) and also to increase anandamide (AEA) levels (Bisogno et al., 2001; Leweke et al., 2012). It has been also shown to exert a positive allosteric modulation of 5-HT_{1A} receptors rather than a direct agonism (Rock et al., 2012), a fact that could explain the unexpected key role of these serotonergic receptors in many CBD effects. Pharmacological approaches with selective receptor antagonists showed that the acute anxiolytic-like and panicolytic-like properties of CBD are predominantly mediated by 5-HT_{1A} receptors (Campos et al., 2013a; Campos and Guimaraes, 2008; Resstel et al., 2009; Soares Vde et al., 2010; Zanelati et al., 2010), whereas the anxiolytic-like effects induced by its chronic administration involving neurogenic actions, seem to be CB_1-receptor dependent (Campos et al., 2013b).

Classical antidepressants act through serotonergic potentiation whereas the effects of fast-acting agents seem to mediated by glutamatergic signalling (Du et al., 2006). However, there is scarce knowledge about the impact of CBD administration in serotonergic and glutamatergic pathways. In this regard, 5-HT_{1A} receptor is expressed in dorsal raphe nucleus (DRN) on 5-HTergic neurons and local GABAergic interneurons, where it controls 5-HT neuronal firing (Celada et

al., 2001). They are also located in cortical interneurons and pyramidal cells modulating neurotransmitters efflux (Santana et al., 2004). In addition, endocannabinoid system is also strongly implicated in the control of 5-HT and glutamate release at different locations (Bambico et al., 2007; Bisogno et al., 2001; Brown et al., 2003; McLaughlin et al., 2012; Mendiguren and Pineda, 2009; Navarrete and Araque, 2008). Thus, the study of CBD effects upon these neurotransmitters release pattern would shed light on the mechanistic basis of CBD behavioural actions.

Herein, we have evaluated the behavioural and neurochemical actions of CBD in the olfactory bulbectomy mouse model of depression (OBX) (Linge et al., 2013), since its efficacy as antidepressant under pathological conditions has not been proved yet. Firstly, we assayed the behavioural effects induced by acute and chronic administration of CBD, and in parallel we performed microdialysis studies to assess the CBD effects on the serotonin (5-HT) and glutamate release in the ventromedial prefrontal cortex (vmPFCx), a pivotal area for the behavioural outcome depending on the emotional status. In addition, the functionality ([^{35}S]GTPγS autoradiography) of brain 5-HT$_{1A}$ receptors following chronic administration of CBD was analysed, given their role in the mechanism of action of antidepressants. Finally, pharmacological antagonism studies were performed to determine the receptor implicated in CBD behavioural and neurochemical outcomes.

2. Material and Methods

2.1. Animals and OBX surgery

Experiments were conducted with 3 month old male C57BL6 mice weighing 25–30 g. All procedures were carried out with the previous approval of the Animal Care Committee of the University of Cantabria and according to the Spanish legislation (RD 53/2013) and the European Communities Council Directive (2010/63/UE) on “Protection of Animals Used in Experimental and Other Scientific Purposes. Animals were individually caged all throughout the study, housed in climate controlled rooms with 12 h light–12 h dark cycle, and provided with food and water *ad libitum*.

The OBX and sham-operation (SHAM) procedure was performed as previously described (Linge et al., 2013) (Supplementary experimental procedures). After a 4-week recovery period, treatments and experiments were performed using different sets of animals for each experimental procedure (Supplementary experimental procedures). All efforts were made to minimise animal suffering, to reduce the number of animals used, and to utilise alternatives to in vivo techniques, if available.

2.2. Drugs and treatments

(-)Cannabidiol, WAY100635 (WAY) and AM251 (AM) were dissolved in vehicle (VEH) (2% Tween 80®: 5% Propilenglycol®: saline).

Acute and chronic administration studies were conducted to investigate the behavioural (open field and sucrose preference tests) and neurochemical (microdialysis studies and 5-HT_{1A} receptor autoradiography) effects induced by

CBD (Supplementary experimental procedures). In the acute studies, the effect of CBD alone (50 mg/kg) or in combination with antagonists was assessed in the open field test and microdialysis studies (30 min post-administration; i.p.). Antagonists doses were those normally reported in the literature and devoid of any effect by themselves on the parameters analysed in the open field (WAY 0.3 mg/kg, AM251 0.3 mg/kg). In the chronic treatment, CBD was administered for 14 days following a drug regime (50 mg/kg/day for 3 days + 10 mg/kg/day until the end of treatment; i.p.) that was selected after preliminary assays. After 2 weeks CBD treatment, animals were sacrificed and brain samples collected and stored at -80°C for the autoradiographic studies.

2.3. Behavioural testing (Supplementary experimental procedures)

Open field test (OFT): we evaluated CBD effects on the OBX-induced hyperactivity (to assess antidepressant-like effects) and central ambulation (to assess anxiolytic-like effects) (Linge et al., 2013). Both the acute effects (30 min post-injection) and persistent actions (24 hours post-injection in the chronic study after 1, 3, 7 and 14 days of treatment) were evaluated.

Sucrose preference test: OBX-mice anhedonia was assessed in the sucrose preference test to check depressive-like behaviour and antidepressant-like actions. A choice of sucrose (1%) and water solutions were provided in the home cage and the consumption was quantified (Linge et al., 2013).

2.4. Microdialysis studies

Concentric dialysis probes were implanted in the vmPFCx. Microdialysis experiments were conducted 24 h after surgery in freely moving mice by continuously perfusing probes. After a 180 min stabilization period, six 20-min

fractions were collected to obtain basal values and another six samples after the i.p. administration of drugs. 5-HT and glutamate were determined by high-performance liquid chromatography (HPLC) (Supplementary experimental procedures).

2.5. [^{35}S]GTPγS autoradiography of 5-HT$_{1A}$ receptor functionality

[^{35}S]GTPγS autoradiography in coronal brain sections was carried out as previously described (Sim et al., 1995), using 10 μM (±)8-OH-DPAT for stimulated condition. Autoradiographic values of net agonist-stimulated [^{35}S]GTPγS binding were calculated by subtracting basal binding from agonist-stimulated binding. Data are expressed as percentage of agonist-stimulated binding over basal activity (100%) (Supplementary experimental procedures).

2.6. Data analysis

All the values are expressed as mean ± standard error of mean (S.E.M). For the behavioural and autoradiographic studies, the data were statistically analysed by one/two-way ANOVA (surgery and treatment as main factors), and for the microdialysis studies two-way repeated measures ANOVA were conducted to check the possible interaction among surgery, treatment and time. A *Student-Newman-Keuls* test was applied for the *post-hoc* analysis. GraphPad Prism 5.01 (San Diego, CA, USA) and Statistica 8 (Statsoft, Inc., Tulsa, USA) were used for the statistical analysis. A p value < 0.05 was considered significant.

3. Results

Different regimes of administration were initially assayed to choose the most appropriate. The first dose of CBD evaluated (10 mg/kg/day, i.p.) produced a significant reversal of OBX-induced hyperactivity after 2 weeks of treatment (Supplementary Fig. S1). As shown later (section 3.1.2; fig B.1), a higher dose of CBD (50 mg/kg/day, i.p.) induced sustained hyperactivity reversal from the first injection but it significantly decreased sucrose consumption in half of sham animals, and a trend was observed in OBX mice (Supplementary Fig. S2.a). This behavioural outcome was accompanied by a reduction in food intake (Supplementary Fig. S2.b) and body weight (Supplementary Fig. S2.c), reflecting an anorectic effect of CBD that would explain such decreased sucrose preference. Therefore, a regime combining both doses (50 mg/kg for 3 days + 10 mg/kg until the end of treatment) was chosen as appropriate for achieving fast onset of antidepressant-like actions, and avoiding the interference of the anorectic actions.

3.1. CBD induces immediate and maintained antidepressant-like effects in OBX mice

3.1.1. Acute antidepressant-like and anxiolytic-like effects of CBD in the open-field test

The acute effect of CBD (50 mg/kg; 30 min post-injection) was evaluated in the open field test. Regarding total distance travelled, two-way ANOVA revealed a significant interaction between surgery and treatment [$F(1,22) = 6.80$, $p < 0.05$]. *Post-hoc* comparison indicated that CBD significantly reversed OBX-induced

hyperactivity ($p < 0.01$; Fig. A.1). Acute CBD also increased central ambulation of OBX mice, as reflected in the higher percentage of distance travelled in the central zone ($p < 0.05$; Fig. A.2). CBD did not alter locomotor activity or central ambulation in sham animals.

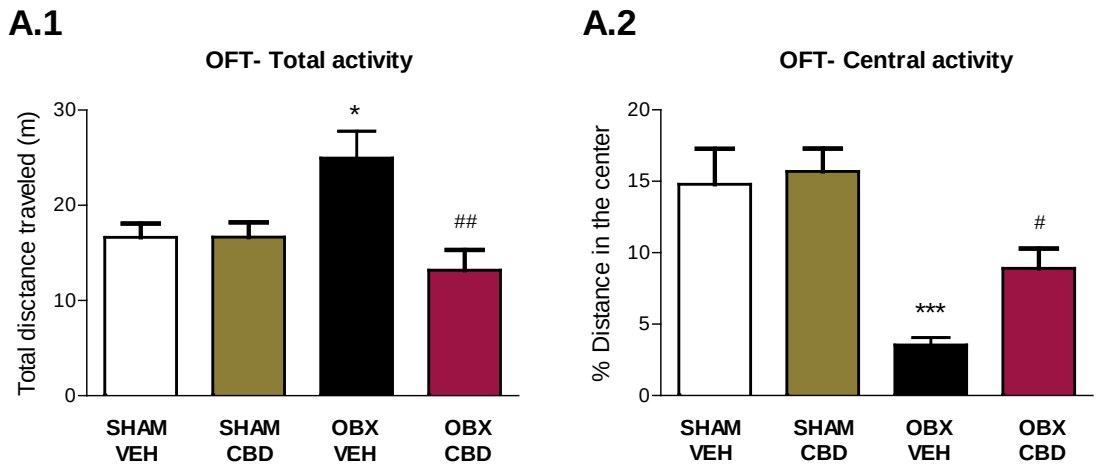


Figure A. ***Acute effect of CBD in the open field test*.** Acute CBD (50 mg/k; i.p.) significantly reversed both OBX-induced hyperactivity (A.1) and decreased central ambulation (A.2) 30 min *post*-injection and it was devoid of any behavioural effect in sham counterparts. Data represented as mean ± SEM, n= 6-7 mice per experimental group (*$p < 0.05$ and ***$p < 0.001$ *vs.* SHAM VEH; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs.* OBX VEH).

3.1.2. Time-course antidepressant-like effects of CBD administration

Open field test

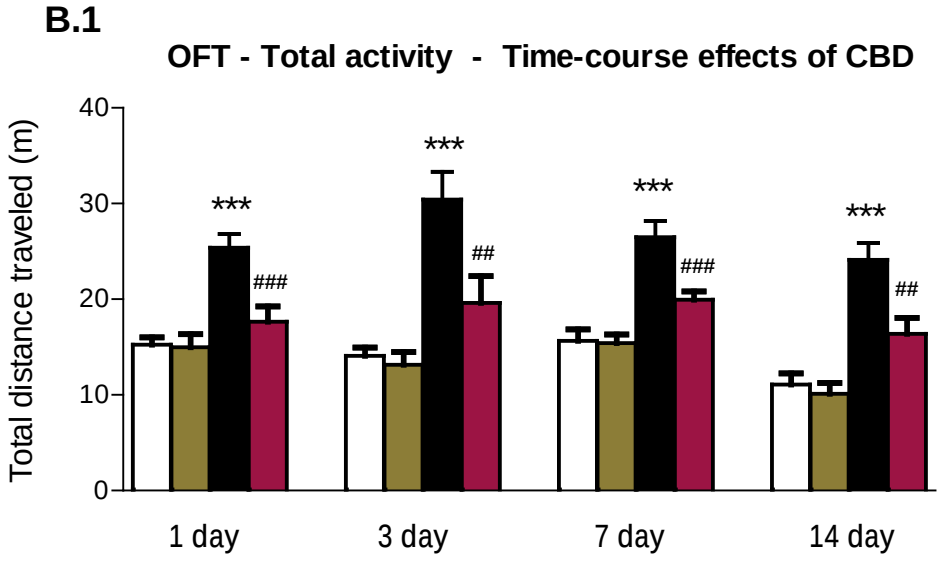
As shown in figure B.1, CBD reversal of OBX-hyperactivity was still present twenty-four hours after the first injection (OBX VEH *vs.* OBX CBD, $p < 0.001$). Moreover, this hyperactivity attenuation was still measured throughout the CBD treatment assessed 24h post 3 ($p < 0.01$), 7 ($p < 0.001$) and 14 ($p < 0.01$) days

of drug administration. Two-way ANOVA analysis revealed a significant interaction between surgery and treatment ([F(1,29) =7.48, $p < 0.05$] for day 1; [F(1,25) =5.20, $p < 0.05$] for day 3; [F(1,25) =7.29, $p < 0.05$] for day 7; and [F(1,25) =5.68, $p < 0.05$] for day 14). Interestingly, CBD did not alter sham animals locomotor activity.

Regarding central activity, the anxiolytic-like effect of CBD observed immediately after the first injection was not significantly preserved 24 hours later or thereafter (Supplementary Fig. S3).

Sucrose preference test

Two-way ANOVA analysis revealed a significant interaction between surgery and treatment ([F(1,27) = 4.30, $p < 0.05$] after 7 days of CBD treatment. As shown in figure B.2, OBX animals exhibited anhedonia, as reflected by a lower preference for sucrose solutions than their sham counterparts ($p < 0.01$). The sucrose intake of OBX-mice was totally restored after one week CBD treatment ($p < 0.01$) and two weeks (data not shown). At the same time points sham mice did not exhibit any alteration in the sucrose preference.



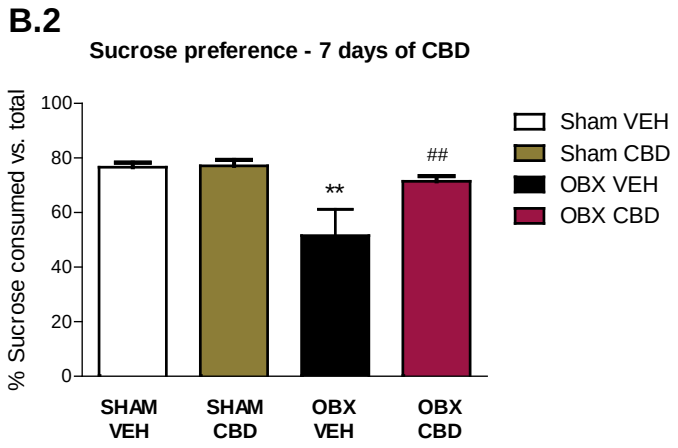


Figure B. Time-course antidepressant-like effects of CBD administration in the *open field* and *sucrose preference tests*. The effect of CBD upon the OBX-hyperactivity (B.1) was evidenced throughout the CBD treatment assessed 24h post 1, 3, 7 and 14 days of drug administration. Additionally, chronic CBD reversed OBX-induced anhedonia following 7 days of administration (B.2). Data represented as mean ± SEM of n= 7-9 mice per experimental group (**$p < 0.01$ and ***$p < 0.001$ *vs.* SHAM VEH; $^{\#\#}p < 0.01$ and $^{\#\#}p < 0.001$ *vs.* OBX VEH).

3.2. <u>Differential effects of acute and chronic CBD upon 5-HT and glutamate release in ventro-medial prefrontal cortex</u>

In order to explore the underpinning mechanism of CBD fast antidepressant-like actions, we firstly evaluated the acute effect of a single dose of CBD (50 mg /kg; i.p.) upon the release of 5-HT and glutamate in the ventromedial prefrontal cortex (vmPFCx) of sham and OBX-mice. Then, the effect of a challenge dose of CBD after a 14 days treatment was also assessed in sham and OBX mice, to analyse the possible adaptive changes induced by its chronic administration.

3.2.1. Acute CBD effects

In vivo microdialysis studies showed that acute administration of 50 mg/kg CBD prompted a significant increase in extracellular 5-HT contents in the vmPFCx of OBX-mice ($p < 0.05$ *vs.* OBX VEH), but not in sham counterparts (Fig. C.1). In addition, CBD increased extracellular glutamate levels in both sham ($p < 0.01$) and OBX ($p < 0.01$) animals (Fig. C.2).

3.2.2. Chronic effects of CBD

Microdialysis studies after chronic CBD administration demonstrated alterations in the pattern of neurotransmitters release, compared to the effects of a single CBD injection. After chronic administration, a CBD challenge dose induced a significant augmentation of extracellular 5-HT in all CBD-treated animals (SHAM CBD $p < 0.01$; OBX CBD $p < 0.05$) *vs.* vehicle-treated groups (Fig. C.3). However, the glutamate release was only increased in OBX-mice ($p < 0.01$; Fig C.4) since the sham counterparts treated with chronic CBD did not retain the response observed after acute CBD administration.

The analysis of the absolute basal neurotransmitter levels after chronic CBD administration revealed a significant elevation in glutamate contents in all CBD treated mice (Fig. C.6), more pronounced in the sham group (OBX CBD *vs.* OBX VEH $p < 0.05$, SHAM CBD *vs.* SHAM VEH $p < 0.01$). No significant differences were obtained in the 5-HT absolute basal levels analysis (Fig. C.5).

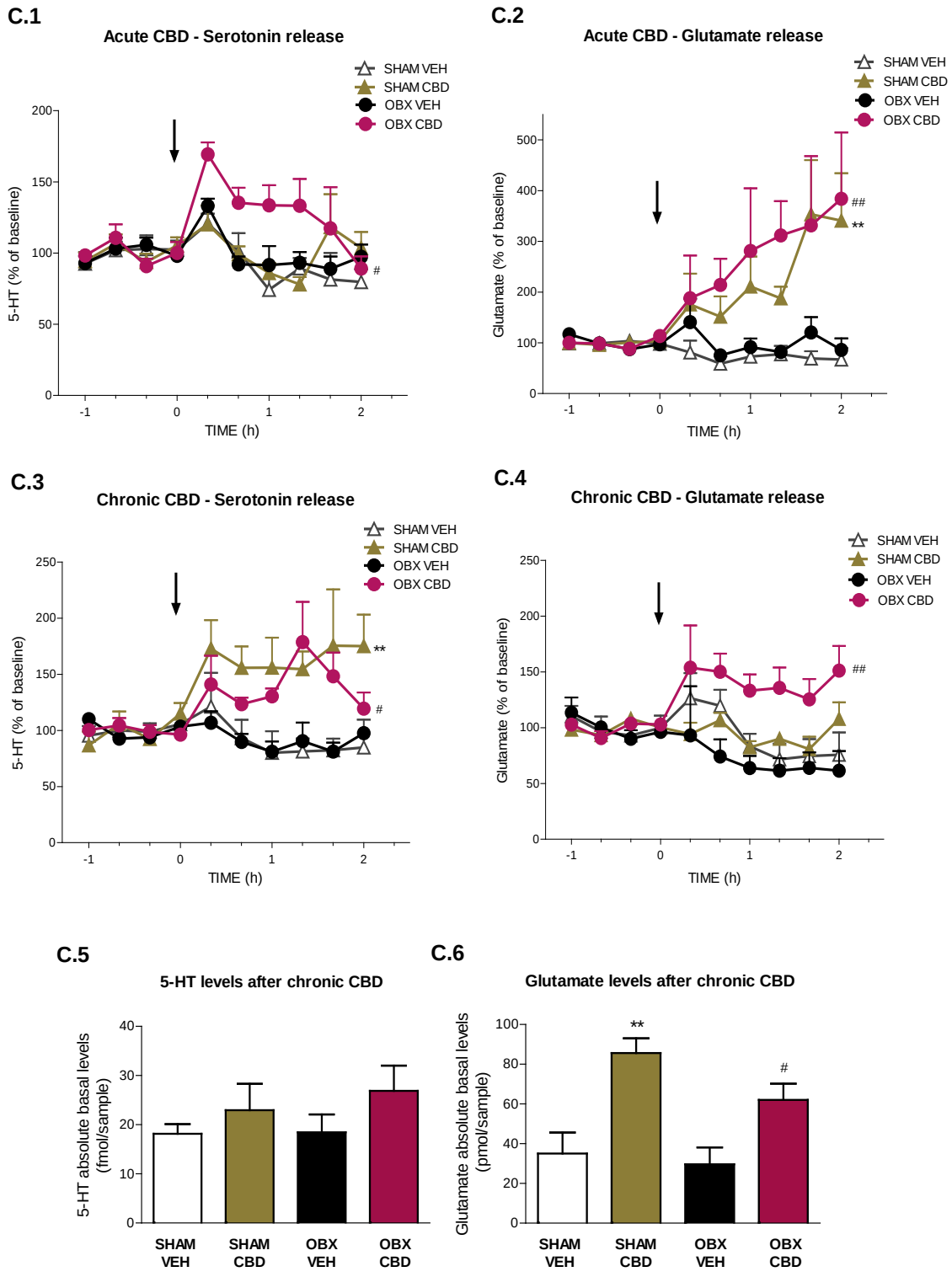


Figure C. Differential effects of acute and chronic CBD upon 5-HT and glutamate release in ventro-medial prefrontal cortex of OBX and sham mice. Acute CBD (50 mg/kg; i.p.) increased extracellular 5-HT in the vmPFCx of OBX-mice but not in sham counterparts (C.1), and increased glutamate levels in both groups (C.2). Following chronic administration, a challenge dose of CBD increased extracellular 5-HT in sham and OBX mice (C.3), whereas it induced an

increase of glutamate efflux only in OBX mice (C.4). Chronic CBD did not produce any significant change in the absolute basal levels of 5-HT (C.5) though it did increase glutamate absolute basal levels in both sham- and OBX-treated mice (C.6). Data represented as mean ± SEM, n=5-7 animals per experimental group (**$p < 0.01$ *vs.* SHAM VEH; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs.* OBX VEH).

3.3. Chronic CBD induces differential adaptive changes on 5-HT$_{1A}$ receptor functionality in OBX and sham mice

As shown in figure D, densitometric analysis revealed a decrease in (±)8-(OH)-DPAT-induced stimulation of [^{35}S]GTPγS binding in the DRN of OBX mice (-22 ± 4 % *vs.* SHAM VEH, $p < 0.05$), that was normalized after chronic CBD treatment (OBX CBD *vs.* OBX VEH, $p < 0.05$). A significant interaction between surgery and treatment was found in DRN [$F(1,23) = 5.55$, $p < 0.05$].

A decrease in the stimulated [^{35}S]GTPγS binding in OBX mice was also observed in postsynaptic areas such as amygdala (AMY: -30 ± 2 %, $p < 0.01$) and CA1-CA2 fields of the hippocampus (CA1-CA2: -89 ± 14 %, $p < 0.05$) compared to SHAM VEH group. Chronic CBD administration significantly reversed (±)8-(OH)-DPAT-induced stimulation of [^{35}S]GTPγS binding in both structures in OBX mice (OBX CBD *vs*. OBX VEH, $p < 0.05$ for both areas). Two-way ANOVA analysis revealed a significant interaction between surgery and treatment in CA1-CA2 [$F(1,29) = 5.37$, $p < 0.05$].

In mPFCx, no changes were detected between sham and OBX mice. However, CBD administration induced an increase in the stimulated [^{35}S]GTPγS binding in

both sham (SHAM CBD: + 42 ± 12 %, $p < 0.01$ *vs*. SHAM VEH) and OBX (OBX CBD: + 40 ± 7, $p < 0.01$ *vs*. OBX VEH) mice.

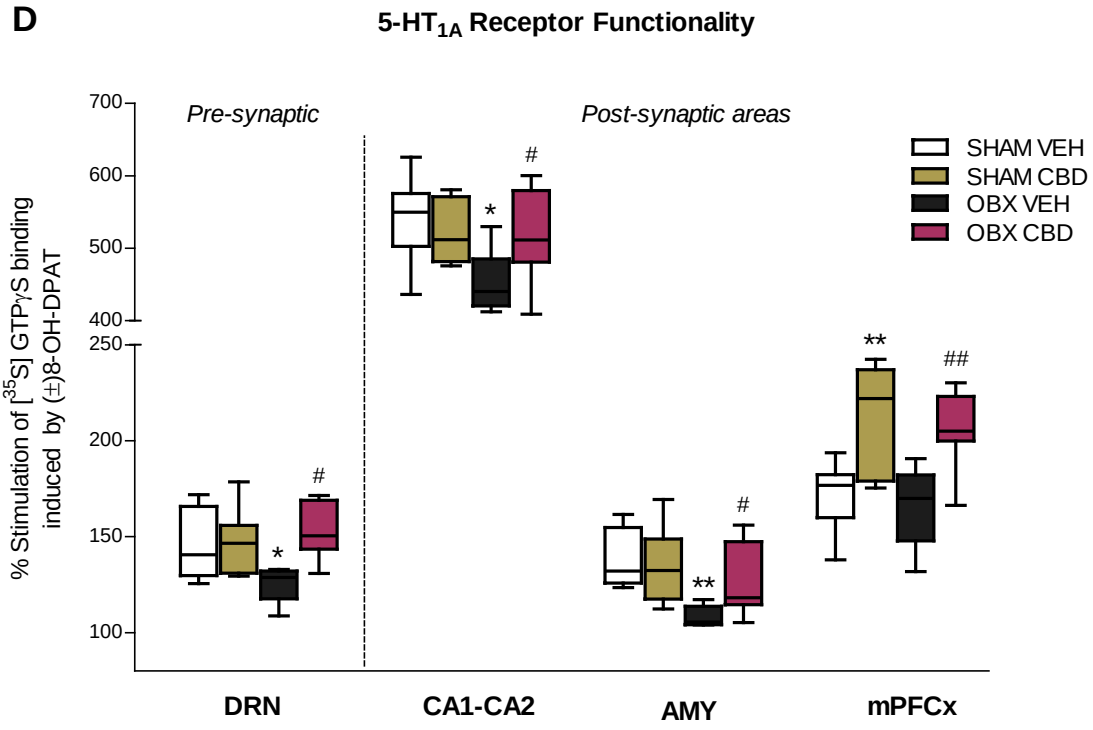


Figure D. Box and whiskers plot of 5-HT$_{1A}$ receptors functionality in different brain areas after chronic CBD administration. A decreased (±)8-(OH)-DPAT stimulated [^{35}S]GTPγS binding was measured in DRN, CA1-CA2, and AMY in OBX mice compared with sham animals. This impaired 5-HT$_{1A}$ receptor functionality in OBX-mice was restored after chronic CBD administration. A higher (±)8-(OH)-DPAT stimulated [^{35}S]GTPγS binding was detected in the mPFCx in all CBD-treated mice. Results are expressed as percentage of [^{35}S]GTPγS binding stimulation over basal values, as mean ± minimum/maximum of n= 6-9 mice per experimental group (*$p < 0.05$ and **$p < 0.01$ *vs.* SHAM VEH; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs.* OBX VEH).

3.4. 5-HT$_{1A}$ receptor plays a key role in the behavioural and neurochemical effects of CBD

3.4.1. Fast effects of CBD in the OFT are mediated by 5-HT$_{1A}$ receptors

Selective antagonists of either 5-HT$_{1A}$ or CB$_1$-receptors were used to investigate the neurochemical mechanisms underlying acute effects of CBD in the OBX-induced behaviour. Antagonists of 5-HT$_{1A}$ (WAY100635, 0.3 mg/kg; i.p.) and CB$_1$ (AM251, 0.3 mg/kg; i.p.) receptors were coadministered with CBD (50 mg/kg; i.p.) 30 min before the OFT session. Antagonists doses were those normally reported in the literature and devoid of any effect by themselves on the parameters analysed in the open field. Interestingly, WAY100635 was able to prevent the CBD-induced reversal of OBX-hyperactivity (OBX CBD *vs.* OBX CBD+WAY, $p < 0.01$) (Fig. E.1). It also inhibited the beneficial effect of CBD on central ambulation scores in these subjects (OBX CBD *vs.* OBX CBD+WAY, $p < 0.05$) (Fig. E.2), not altering sham mice activity (data not shown). By contrast, AM251 did not counteract nor mimic the behavioural effects of CBD in OBX animals (Fig. E.1 and 2).

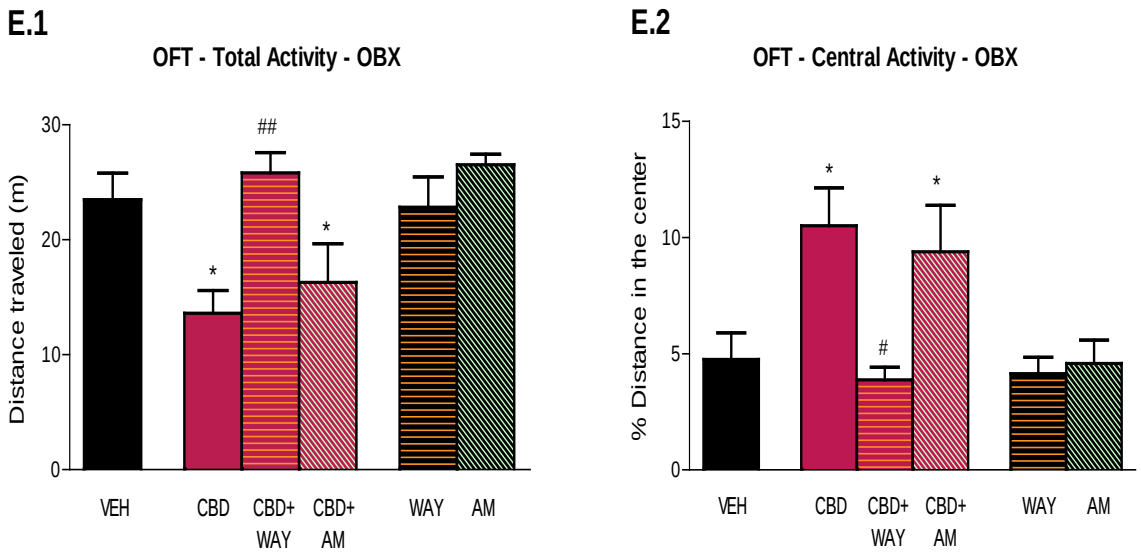


Figure E. Behavioural effects of acute CBD in OBX mice were prevented by 5-HT$_{1A}$ receptor blockade. In the *open field test,* WAY100635 (0.3 mg/kg; i.p) prevented both the reversal of OBX-hyperactivity (E.1) and the increase of central activity (E.2) induced by CBD (50 mg/kg). By contrast the selective CB_1 receptor antagonist AM251 (0.3 mg/kg; i.p.) did not counteract any of these effects. Data represented as mean ± SEM of n=5-7 animals per experimental group (*p < 0.05 *vs.* vehicle-treated group; $^{\#}p$ < 0.05 and $^{\#\#}p$ < 0.01 *vs.* CBD-treated group).

3.4.2. 5-HT$_{1A}$ receptor blockade prevented the increase in 5-HT and glutamate cortical release induced by CBD

Since the 5-HT$_{1A}$ receptor blockade abolished the behavioural actions of acute CBD in the open field, we studied the effect of WAY100635 upon the CBD-induced release of 5-HT and glutamate.

Administration of WAY100635 (0.3 mg/kg) prevented the 5-HT outflow induced by acute CBD administration in OBX animals (p < 0.05 *vs.* OBX CBD) (Fig. F.1). In sham animals, where CBD alone did not induce any change in 5-HT release, the blockade of 5-HT$_{1A}$ receptors resulted in a significant increase of 5-HT efflux (p < 0.01 *vs.* SHAM CBD) (Fig. F.2). Additionally, WAY100635 blocked the

increase of glutamate release induced by acute CBD in both OBX (Fig. F.3; $p < 0.05$) and sham (Fig. F.4; $p < 0.01$) mice. No significant alterations were measured upon 5-HT and glutamate levels when WAY100635 was administered alone to sham and OBX mice (Supplementary Fig. S4).

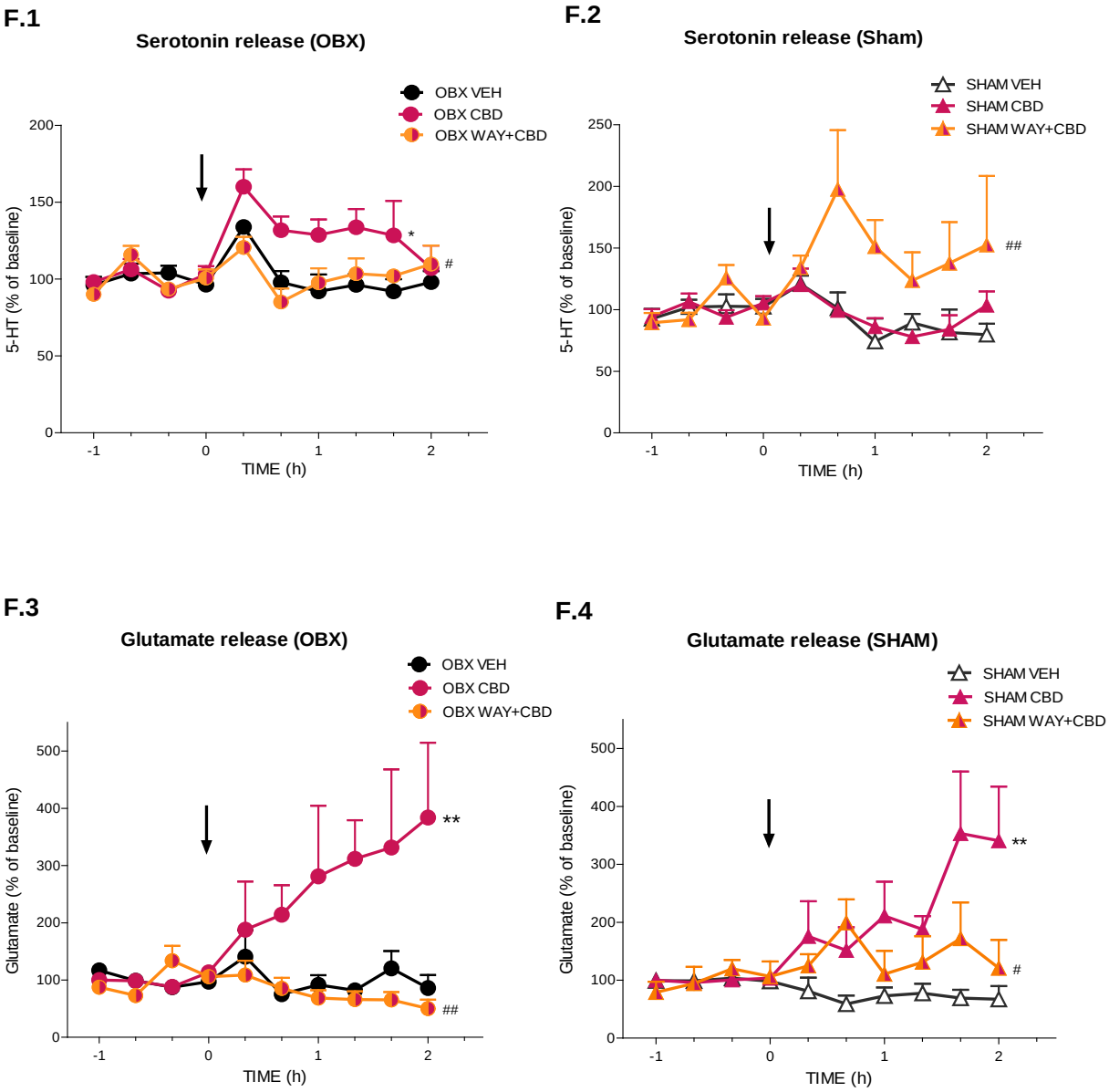


Figure F. Neurochemical effects of acute CBD were prevented by 5-HT$_{1A}$ receptor blockade. In microdialysis studies, cortical 5-HT outflow induced by CBD in OBX animals was prevented by WAY100635 coadministration (F.1). In sham mice, the coadministration of WAY100635 and CBD

resulted in an increased 5-HT release in vmPFCx (F.2). CBD-induced cortical glutamate release was also abolished by WAY100635 in both OBX (F.3) and sham mice (F.4). Data represented as mean ± SEM of n= 5-7 animals per experimental group (*$p < 0.05$ and **$p < 0.01$ *vs.* respective vehicle-treated group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs.* respective CBD-treated group).

4. Discussion

In this paper we demonstrate for the first time that CBD exerts rapid antidepressant-like effects as evidenced by the reversal of OBX-induced hyperactivity immediately after the first injection. Moreover, its efficacy is maintained and improved with the repeated administration, as the anhedonia was completely relieved after one week of treatment. The dose adjustment appears to be particularly important for the fast antidepressant effects. Hence, we found that 10 mg/kg of CBD exerts antidepressant-like actions after two weeks of treatment. Nevertheless, when a higher dose is administered at the beginning of the treatment (50 mg/kg), the reversal of OBX-hyperactivity is evident from the first injection and the anti-anhedonic effect appears after just one week administration.

Regarding anxiety-related behaviours, CBD also exhibited acute anxiolytic-like effects in OBX mice, in agreement with other authors' reports (Guimaraes et al., 1990; Moreira et al., 2006; Resstel et al., 2006; Casarotto et al., 2010). However, we did not detect any anxiolytic effect of CBD administration in sham-control mice, and neither anxiogenic-like effects (ElBatsh et al., 2012; Fogaca et al., 2014). This discrepancy could be due to methodological differences related with housing conditions (Linge, Pazos et al. 2013), drug administration regime and dosage (acute *vs* chronic), and contextual issues influencing behavioural

outcomes. Collectively, our findings demonstrate the antidepressant efficacy of CBD in OBX mice, an animal model of depression with comorbid anxiety –*face validity*– and extensively used for the preclinical research of antidepressant and anxiolytic effects of drugs –*predictive validity*– (Song and Leonard, 2005). Even more, CBD does not only show an earlier onset of action compared to classic antidepressants (Rodriguez-Gaztelumendi et al., 2009), but also triggers an immediate antidepressant-like effect, similarly to that reported for ketamine (Li et al., 2010; Maeng et al., 2008).

In our study, the initial high dose of CBD (50 mg/kg) reduced sucrose preference in some sham animals in parallel with a decline in food consumption and body weight. It is possible that CBD acting as a CB_1 receptor antagonist (Thomas et al., 2007) could promote anorectic effects as reported for CB_1 antagonist/inverse agonists like Rimonabant or AM251 (Colombo et al., 1998; Shearman et al., 2003). Therefore, we assumed that this initial decrease in sucrose consumption is likely caused by an alteration of the appetite rather than to an emotional detriment, since the parallel behavioural assessment revealed an improved emotional response of OBX mice and no decline in sham animals after CBD administration. All the above behavioural findings reinforce the need of adequate pharmacological strategies to optimize the benefits of CBD treatment (McCarberg and Barkin, 2007).

In order to analyse the concurrent neurochemical events that may account for the behavioural benefits of CBD, microdialysis studies in vmPFCx were performed after acute and chronic administration. PFCx is a key area in the maladaptive behavioural regulation (Davidson, 2002), specifically exhibited by depressed individuals and a typical feature of OBX mice (Fitzgerald et al., 2008; Song and

Leonard, 2005). Interestingly, acute CBD induced an increase in cortical 5-HT levels only in OBX animals, a finding that could explain the differential behavioural effects of CBD under physiological or pathological conditions. We found a decreased functionality of somatodendritic 5-HT_{1A} receptors in the DRN of OBX animals similarly to that described for depressed suicide patients (Savitz et al., 2009). Consequently, this lower inhibitory tone onto the DRN firing would drive an increased 5-HT efflux in the projection areas (Casanovas et al., 1999; Celada et al., 2001), when CBD is administered acutely to OBX mice but not to sham counterparts. Accordingly, we detected an increased cortical 5-HT release in sham mice after CBD only when 5-HT_{1A} receptors were blocked by an antagonist administration.

5-HT augmentation in mPFCx has been described after chronic (Gardier et al., 1996) but not acute (Beyer et al., 2002) administration of antidepressants, and it has been pointed as the main underpinning mechanism for their behavioural actions, together with adaptive changes in the serotonergic system. Following chronic CBD administration a challenge dose induced 5-HT efflux in the vmPFCx although in this case in both OBX and sham animals, likely indicating the occurrence of adaptive changes in serotonergic system. In this sense, chronic CBD promoted an increase in postsynaptic 5-HT_{1A} receptors functionality in mPFCx, not only in OBX but also in sham mice, as it occurs with SSRIs (Matsuda, 2013). Concomitantly, we expected to find a decreased functionality of somatodendritic DRN 5-HT_{1A} receptors of sham animals, that justified the increased 5-HT efflux induced by chronic CBD, but we did not observe any alteration. However, it should be noted that somatodendritic 5-HT_{1A} receptors desensitization is not always detectable by [^{35}S]GTPγS binding techniques (Gi-

proteins coupling to the receptor) but with other methodological approaches (e.g. 5-HT_{1A} receptor-mediated GIRK currents electrophysiology) (Rossi et al., 2006). In any case, we did not find behavioural changes associated to the increased 5-HT efflux induced by chronic CBD observed in sham mice, though other predictive paradigms may provide valuable information. On the other hand, OBX animals exhibited impaired functionality of 5-HT_{1A} receptors in limbic brain areas (i.e. amygdala and hippocampus) that were restored after chronic CBD treatment. This effect might be associated to the increased 5-HT efflux and improvement of behavioural deficits, suggesting a crucial role of these receptors in the pharmacodynamics of CBD.

Microdialysis in vmPFCx revealed that acute CBD promoted a marked glutamate elevation in both sham and OBX mice. This facilitated glutamatergic neurotransmission has been associated with the fast antidepressant efficacy of ketamine (Maeng et al., 2008). Thus, it could be postulated that the increased glutamate efflux triggered by CBD from the first injection could underlie the fast antidepressant-like effects of CBD in OBX. In the chronic approach, the increase of glutamate efflux induced by CBD was observed only in OBX mice. It is noteworthy that after the chronic CBD treatment, basal levels of glutamate were elevated in both sham and OBX mice, though in a lower magnitude in the OBX group. This finding could explain the lack of relative glutamate increment in sham animals after a challenge dose of CBD. A minor basal glutamatergic tone of OBX animals after chronic CBD treatment compared with sham mice, could be related to a dysfunction in glutamatergic system previously described in OBX animals (Webster et al., 2000) and also in depressed patients (Hashimoto, 2009).

To gather more information about CBD mechanism of action in behavioural responses, we analysed the implication of the two main targets of this compound, 5-HT_{1A} and CB_1 receptors (Fernandez-Ruiz et al., 2013). Our findings revealed a crucial role of 5-HT_{1A} receptors in the behavioural effects of CBD, since WAY100635 but not AM251, prevented both the reversal of OBX-hyperactivity and the anxiolytic-like effects displayed by CBD. These findings are in good agreement with studies in which CBD decreased immobility in the FST (Zanelati et al., 2010) and prevented anxiety/panic responses (Campos et al., 2013a; Campos and Guimaraes, 2008; Fogaca et al., 2014; Soares Vde et al., 2010) in a 5-HT_{1A} receptor-dependent manner. However, it has been also described that some acute and chronic anxiolytic-like effects of CBD are mediated by CB_1 receptors (Campos et al., 2013b; Casarotto et al., 2010; Do Monte et al., 2013). As anxiety is a complex syndrome affected by different brain processes (Davidson, 2002; Kheirbek et al., 2012), these two receptors could be implicated in the anxiety outcome at different levels. Nevertheless, the behavioural antidepressant-like effects of CBD were neither prevented nor mimicked by AM251, suggesting that the endocannabinoid system modulation is not contributing to CBD fast antidepressant-like effects. Accordingly, we postulated that CBD could induce 5-HT/glutamate release in vmPFCx through a 5-HT_{1A} receptor-dependent mechanism, responsible of its fast antidepressant-like effects. Microdialysis studies confirmed our hypothesis since not only 5-HT but also glutamate increase induced by CBD were prevented by WAY100635.

Altogether, our findings demonstrate a 5-HT_{1A}-dependent enhancement of glutamatergic and serotonergic neurotransmission in OBX mice immediately after the first injection of CBD could lie behind its fast antidepressant-like effects.

Likewise, the sustained increase in prefrontocortical glutamate contents, together with serotonergic increase and the adaptive changes in 5-HT_{1A} receptor functionality, might drive the consolidation and improvement of the antidepressant-like effects of chronic CBD (including anti-anhedonic actions).

Although further investigation is still required to fully elucidate how CBD acts on 5-HT_{1A} receptors to induce the boost of 5-HT and glutamate, we are proposing a putative neurochemical mechanism (Fig. G). Both serotonergic and glutamatergic potentiation, through the allosteric modulation of the 5-HT_{1A} receptor (Rock et al., 2012), might underlie fast antidepressant-like effects of CBD in the OBX model of depression. In prefrontal cortex, CBD would potentiate the inhibitory function of 5-HT_{1A} receptors upon GABAergic interneurons (Santana et al., 2004), favouring glutamate signalling in postsynaptic areas (Llado-Pelfort et al., 2012). This enhanced glutamatergic transmission, through pyramidal descending projections to DRN, might stimulate the neuronal firing of serotonergic neurons, driving to a 5-HT increase in mPFCx (Celada et al., 2001). In DRN, CBD could reduce the inhibitory effect GABAergic interneurons upon the discharge rate of serotonergic neurons, contributing to the increased cortical serotonergic output. The concurrent activation of somatodendritic 5-HT_{1A} receptors is known to inhibit the firing rate of DRN 5-HT neurons. The decreased functionality of these presynaptic 5-HT_{1A} receptors in DRN of OBX mice might explain the higher 5-HT release in mPFCx after CBD due to a lower inhibitory feedback. Finally, increased serotonin efflux in mPFCx of OBX mice after CBD might also modulate pyramidal neurons activity through the activation of 5-HT_{1A} and 5-HT_{2A} membrane receptors.

Although the herein proposed mechanism for CBD seems to be the most feasible accounting for our results, we do not discard the additional involvement of other receptors and/or the crosstalk among systems in the overall observed effects. In all, CBD is a multitarget drug that can modulate a variety of systems implicated in mood control and therefore, result in a great value from a clinical point of view.

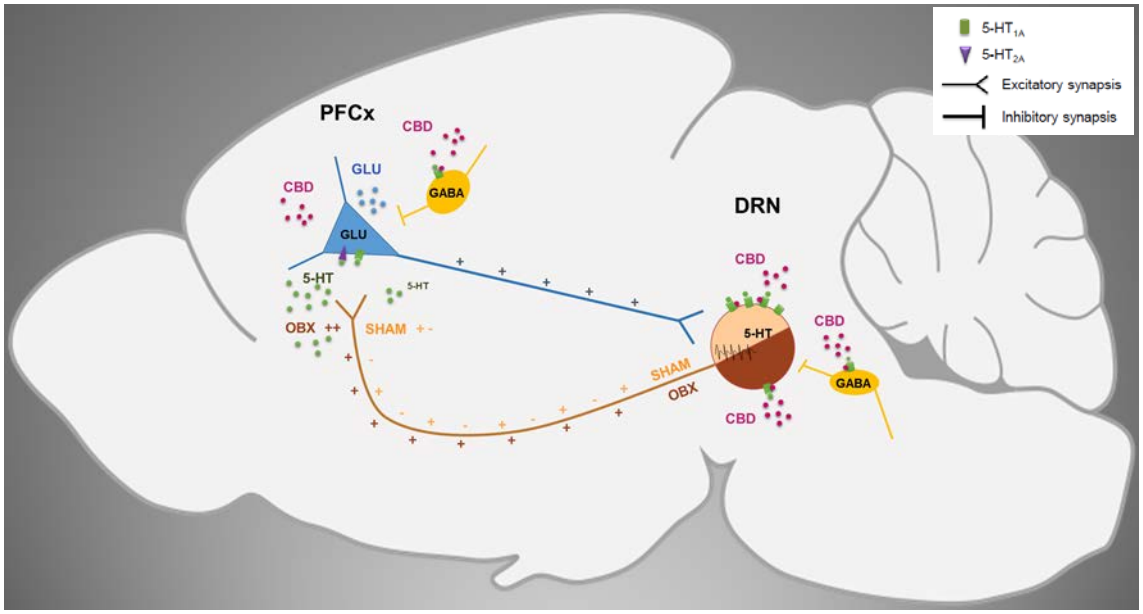


Figure G. ***Proposed neurochemical mechanism of action of CBD to induce fast antidepressant effects.*** In prefrontal cortex, CBD would potentiate the inhibitory function of 5-HT_{1A} receptors upon GABAergic interneurons, favouring glutamate signalling in postsynaptic areas, the stimulation of pyramidal descending projections to DRN, and therefore the neuronal firing of serotonergic neurons, and the 5-HT increase in mPFCx. In DRN, CBD would increase 5-HT neurons firing by reducing the inhibitory effect of GABAergic interneurons, but on the other it would decrease de 5-HT neuron firing potentiating the inhibitory control of local 5-HT_{1A} receptors. The decreased functionality of presynaptic somatodendritic 5-HT_{1A} receptors in OBX mice would explain the higher 5-HT release in PFCx.

Conclusions

This work evidences that CBD could represent a novel drug for treating depressive disorders in a very fast manner, acting by the enhancement of serotonergic and glutamatergic transmission through a 5-HT_{1A} receptors modulation. The fast onset of antidepressant action of CBD and the simultaneous anxiolytic effect would solve some of the main limitations of the current antidepressant therapies. Furthermore, the broad range for therapeutic dosage and the lack of psychotomimetic effects confers to this drug fundamental advantage for its use in clinical practice comparing to other fast-acting antidepressant alternatives. Finally, this novel strategy consisting in the dual potentiation of serotonergic and glutamatergic transmission could bring new light to the discovery of new fast and effective antidepressant therapies.

Author Contributors

The bulk of the experimental work, data analysis and interpretation, and the draft of the paper was carried out by Raquel Linge. Laura Jiménez-Sánchez equally contributed to the microdyalisis experiments performance and related data analysis and interpretation. Leticia Campa analysed microdyalisis samples in the HLPC. Fuencisla Pilar-Cuéllar and Rebeca Vidal participated in autoradiography and behavioural protocols development and in data interpretation. Angel Pazos contributed to the study design and data interpretation. Albert Adell supervised microdyalisis studies participating in the experimental design, data analysis and interpretation. Alvaro Diaz supervised the experimental work, participated in the

study design, data analysis and interpretation. All authors contributed to and approved the final version of the paper.

Disclosure

The authors declare no conflict of interest.

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Supplementary Experimental Procedures

Olfactory bulbectomy

The OB procedure was performed as previously described (Linge et al., 2013).Mice were anesthetized with isoflurane (2%; Schering Plough, United Kingdom) to perform the bilateral olfactory bulbectomy. In brief, the head was shaven and a midline sagital incision was made in the skin overlying the skull. A burr hole was drilled through which both olfactory bulbs were bilaterally aspired by a suction pump. Finally, the hole was filled with bone wax in order to avoid further bleeding. After bulbectomy/sham surgery, a four week period was awaited in order that mice recovered and the OBX syndrome was developed. At the end of the study, animals were sacrificed and the lesions were verified to discard frontal pole lesions and/or incomplete removal of olfactory bulbs. Sham operations were done in the same way, although the bulbs were left intact.

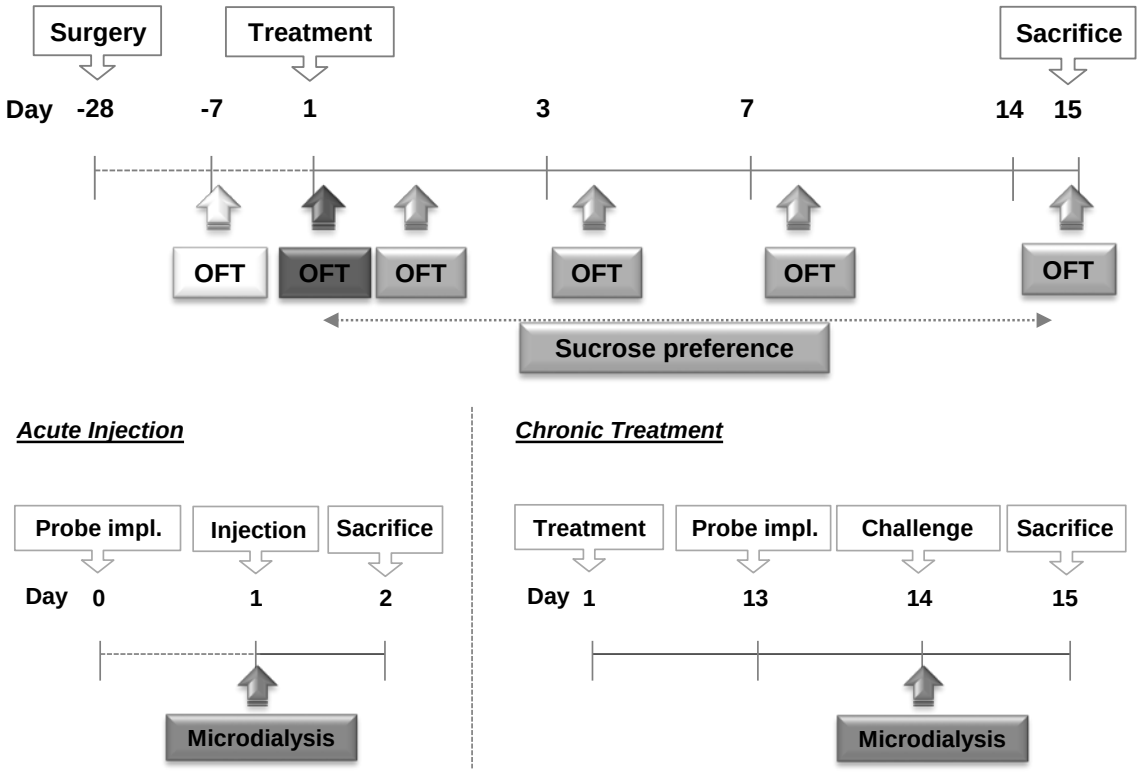
Experimental schedule and pharmacological treatments

All the drugs ((-)Cannabidiol, WAY100635 and AM251) were purchased from TOCRIS (Bristol, United Kingdom), and were administered intraperitoneally (i.p.) dissolved in vehicle (2% Tween80®: Propilenglycol® 5%: saline).

Acute behavioural effects of CBD alone (50 mg/kg) or in combination with antagonists were assessed in the open field (30 min post-administration; i.p). 5-HT_{1A}-receptor (WAY100635, 0.3 mg/kg) and CB_1-receptor (AM251, 0.3 mg/kg) antagonists were used. For the chronic studies, CBD or vehicle were administered daily during 14 days (i.p.; once a day) and open field sessions were

carried out 24 hours after the last injection, at different time-points along the chronic administration (after 1, 3, 7, and 14 days of treatment).

Different doses and regimes of administration were tested to choose the most appropriate: a) 10 mg/kg (Supplementary Fig.S1); b) 50 mg/kg (Supplementary Fig. S2); and c) a combination. Finally, the 50 mg/kg dose was selected as the initial dose for the first 3 days followed by a 10 mg/kg dose for the remaining time. At the end of the 2 week CBD treatment, animals were sacrificed and brain samples were collected and stored at -80°C for the autoradiographic studies.



In the microdialysis studies, the acute effects of CBD alone or in combination with WAY100635 (0.3 mg/kg) upon the release of 5-HT and glutamate in the medial prefrontal cortex were analysed. For chronic studies, the effect of a challenge dose of CBD was assessed after 2 weeks of treatment, in order to analyse the possible adaptive changes induced by its chronic administration.

Behavioural testing

Open field test

The open field test was performed during the light phase and animals were transported to the experimental room at least 30 min before the start of each experiment to acclimatize. The open field apparatus was a brightly lit (350 lx) white wooden box (50 cm × 50 cm × 30 cm) with white floor and bright walls. Mice were released in the center of the apparatus for 5 min, and behaviour was video-tracked by a computerized system (Any-maze Video-Tracking software, Stoelting Co., U.S.A.). Total distance travelled and percentage of distance travelled in the centre (30 cm × 30 cm) was evaluated.

Sucrose preference test

Sucrose (1%; Scharlau Chemie S.A., Spain) was dissolved in drinking water and animals were trained to drink sucrose and water solutions in their home cage *ad libitum* during 2 days, measuring the total fluid consumption per day previous to the test. Then the amount of sucrose and water consumed was registered with a time interval of 24 hours. The percentage of sucrose consumed by each animal was calculated and the mean sucrose intake of each experimental group was compared.

Microdialysis studies

Concentric dialysis probes with a 4-mm membrane length were implanted under sodium pentobarbital anaesthesia (60 mg/kg i.p.) in the vmPFC (AP +2.2 mm, L ±0.2 mm, DV -3.4 mm; from bregma), according to Paxinos and Franklin (Paxinos

and Franklin, 2001). Microdialysis experiments were conducted 24 h after surgery in freely moving mice by continuously perfusing probes with artificial cerebrospinal fluid (125 mM NaCl, 2.5 mM KCl, 1.26 mM $CaCl_2$, 1.18 mM $MgCl_2$, and 1 µM citalopram) at a rate of 1.5 µl/min. After a 180 min stabilization period, dialysate samples of 30 µl were collected every 20 min, six 20-min fractions were collected to obtain basal values (expressed as concentration of neurotransmitter in 30 µl sample) and another six samples after the i.p. administration of drugs. 5-HT and glutamate were determined by HPLC as previously described (Lopez-Gil et al., 2007). At the completion of dialysis experiments, mice were sacrificed and a fast green solution was perfused through the dialysis probes to stain the surrounding tissue for subsequent histological examination. The amount of neurotransmitter release (% *vs.* basal) was determined at every time point for each animal and the mean of the experimental groups was compared for 5-HT and glutamate contents. The absolute basal levels of 5-HT (fmol/sample) and glutamate (pmol/sample) after the chronic approach were also compared among groups. At the completion of the experiments, mice were sacrificed and brain tissue was processed according to standard procedures (cresyl violet staining) to verify the correct placement of dialysis probe.

[^{35}S]GTPγS autoradiography of brain 5-HT$_{1A}$ receptors functionality

At the end of chronic CBD treatment and after behavioural evaluation, animals were left for a 24 hours wash-out period and then sacrificed. Brains were obtained and stored at -80°C. Coronal brain sections (20 µm) were cut at -20°C using a microtome cryostat and thaw-mounted in Superfrost®Plus slides (Thermo Fisher

Scientific, USA) and stored at -80°C until use. [^{35}S]GTPγS autoradiography was carried out as previously described (Sim et al., 1995). Slide-mounted sections were preincubated for 30 min at room temperature in buffer containing 50 mM Tris–HCl, 0.2 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM DL-dithiothreitol and 2 mM GDP (pH 7.7). Slides were subsequently incubated for 2 h in the same buffer containing adenosine deaminase (10 mU/ml) with [^{35}S]GTPγS (0.04 nM; Sigma-Aldrich, Missouri, USA). Consecutive sections were incubated in the absence (basal [^{35}S]GTPγS binding) and in the presence of 10 μM (±)8-OH-DPAT (Sigma-Aldrich, Missouri, USA) (5-HT_{1A} receptor mediated stimulation of [^{35}S]GTPγS binding); non-specific binding was determined in the absence of agonist and in the presence of guanosine-5-O-(3-thio)-triphosphate (GTPγS, 10 μM, Sigma-Aldrich, Missouri, USA). After the incubation, the sections were washed twice for 15 min in cold 50 mM Tris–HCl buffer (pH 7.4) at 4ºC, rinsed in distilled cold water and then dried under a cold air stream. These sections were exposed to autoradiographic films Biomax MR films (GE Healthcare, Madrid, Spain) together with [^{14}C] microscales (GE Healthcare, Madrid, Spain) at 4ºC for 2 days. Autoradiographic densities were determined by densitometry using Scion Image software (Scion Corporation, MD, USA). Density values were obtained and measured as nCi/g of tissue. Autoradiographic values of net agonist-stimulated [^{35}S]GTPγS binding were calculated by subtracting basal binding from agonist-stimulated binding and the data are expressed as percentage of agonist-stimulated binding over basal activity (100%).

References

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Lopez-Gil, X., Babot, Z., Amargos-Bosch, M., Sunol, C., Artigas, F., Adell, A., 2007. Clozapine and haloperidol differently suppress the MK-801-increased glutamatergic and serotonergic transmission in the medial prefrontal cortex of the rat. Neuropsychopharmacology 32, 2087-2097.

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Sim, L. J., Selley, D. E., Childers, S. R., 1995. In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5'-[gamma-[35S]thio]-triphosphate binding. Proc Natl Acad Sci U S A 92, 7242-7246.

Supplementary Figures

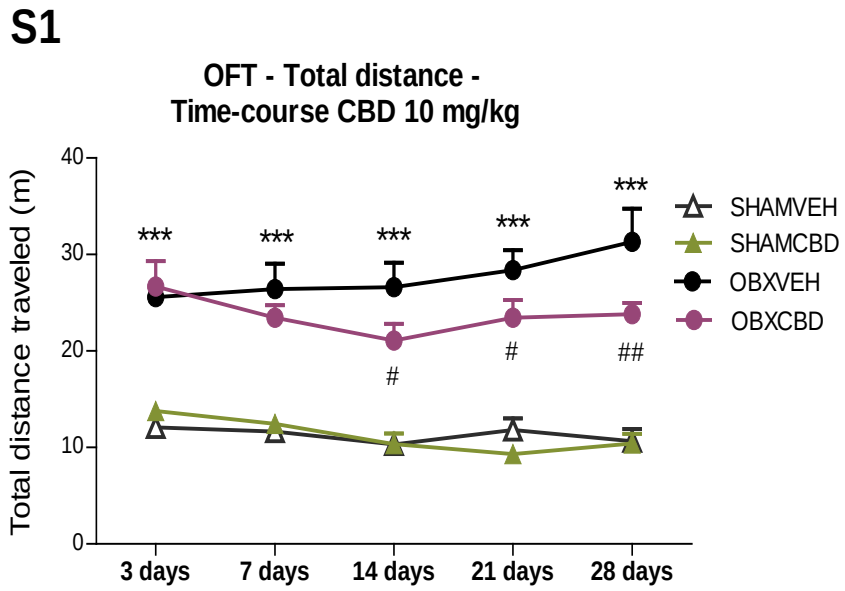


Figure S1: Behavioural effects of CBD (10 mg/kg/day) chronic administration in the *open field*. CBD exerted antidepressant effects in OBX mice as evidenced by the locomotor hyperactivity attenuation in the open field test. Data represented as mean ± SEM of n=10-12 animals per experimental group (***$p < 0.001$ *vs*. sham-vehicle; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs*. OBX-vehicle).

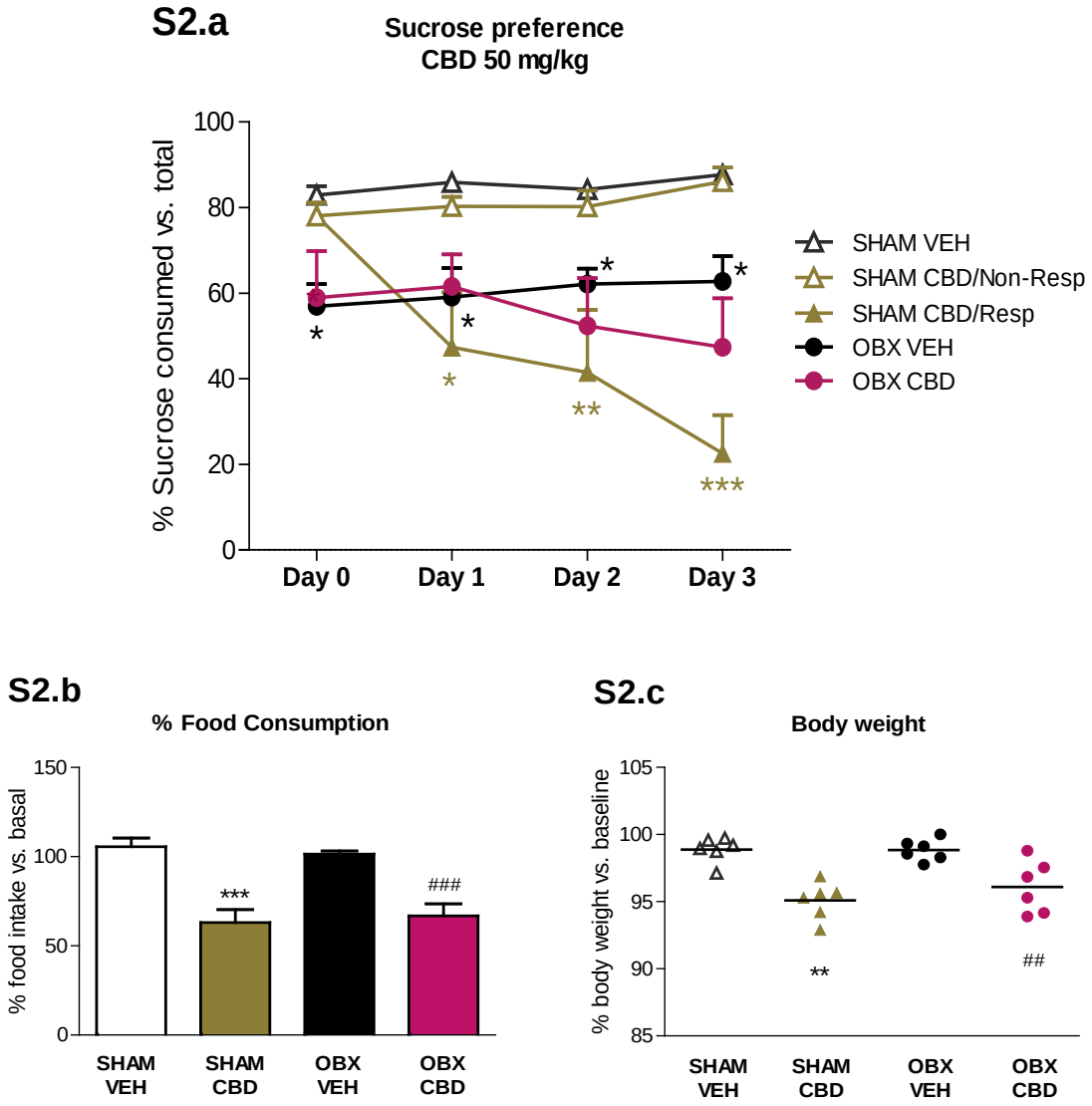


Figure S2. Effects of CBD (50 mg/kg/day) administration on sucrose preference, food consumption and body weight. CBD reduced sucrose intake (% of sucrose consumption over total liquid intake) in half of the sham mice and also enhanced the OBX induced anhedonia (S2.a). Such behavioural outcome was accompanied by a reduction in food intake (S2.b) and body weight (S2.c) of both sham and OBX mice treated with CBD. Results expressed as percentage of food consumption and body weight after VEH/CBD injection compared with basal values of each animal before drug treatment. Data represented as mean ± SEM, n=6 mice per experimental group (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ *vs.* sham-vehicle; ## $p < 0.01$ and ### $p < 0.001$ *vs.* OBX-vehicle).

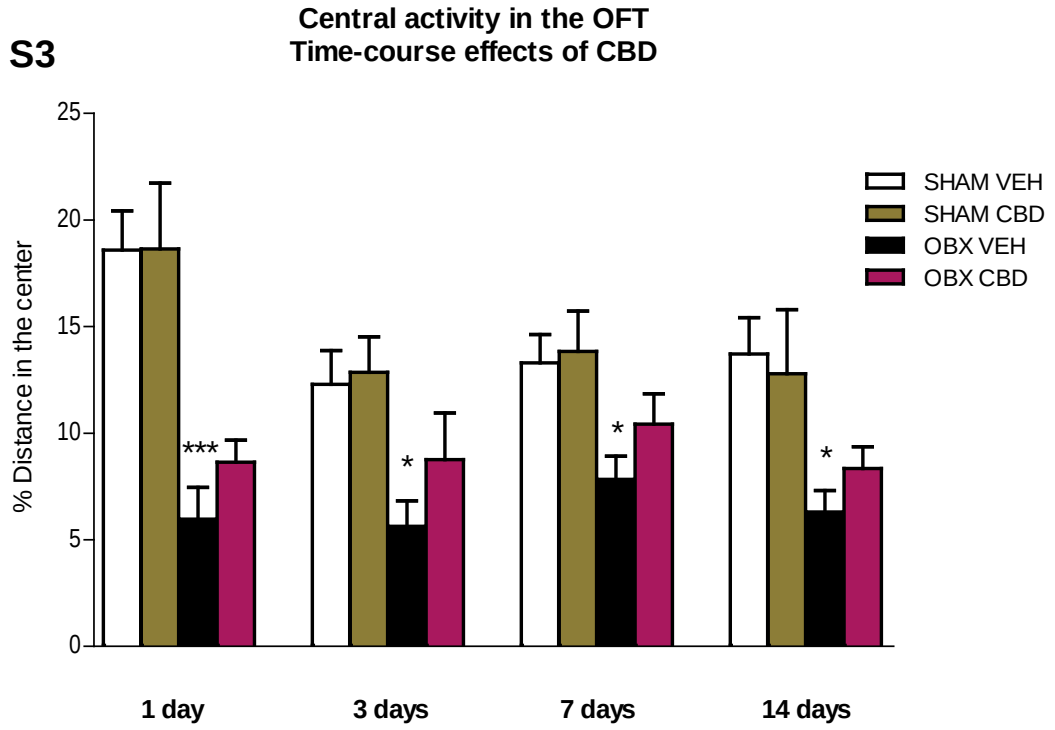


Figure S3. Effect of CBD chronic administration on central activity in the *open field test*. Non-significant changes in the central ambulation scores of sham and OBX mice were measured throughout the CBD treatment, assessed 24h post 1, 3, 7 and 14 days of drug administration. Data represented as mean ± SEM of n= 7-9 (*$p < 0.05$ and ***$p < 0.001$ *vs.* SHAM VEH).

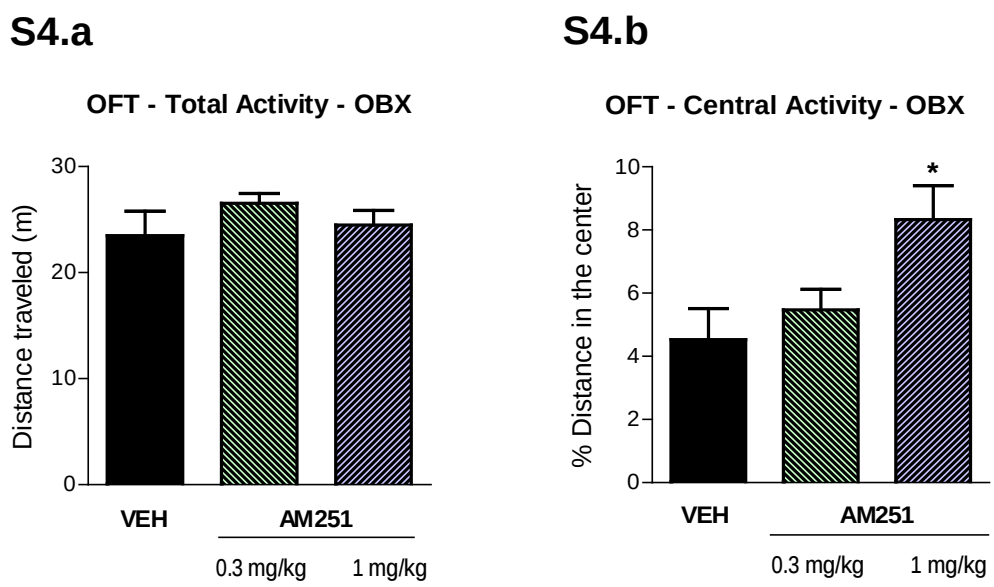
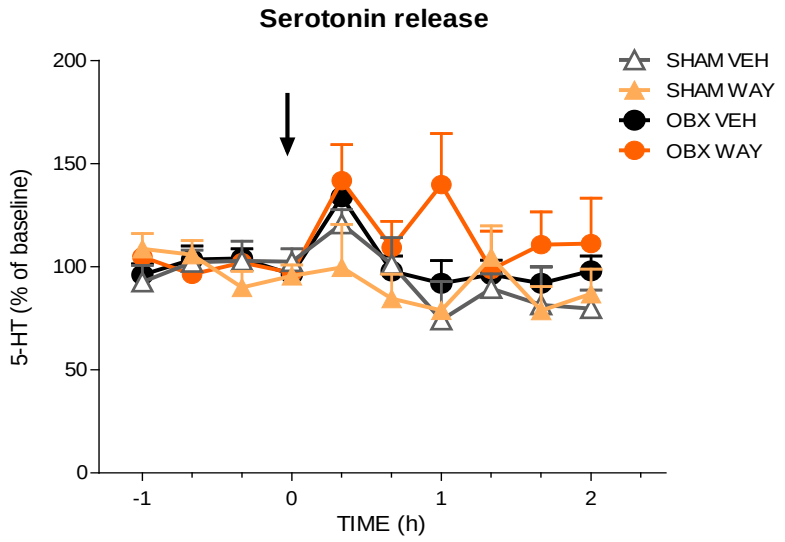


Figure S4. Effect of the CB_1-receptor antagonist AM251 in OBX mice in the open field test. AM251, 0.3 and 1 mg/kg were administered (i.p.) 30 min before the behavioural testing in the OFT. AM251 at 0.3 mg/kg and 1 mg/kg effects in the total (S4.a) and central activity (S4.b) of OBX mice in the OFT. Data represented as mean ± SEM of n=5-6 animals per experimental group.

S5.a



S5.b

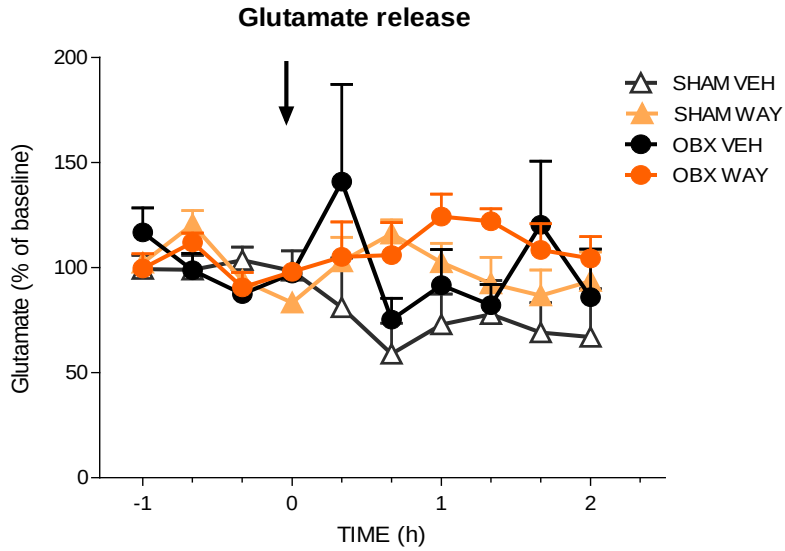


Figure S5. Effect of 5-HT$_{1A}$ receptor blockade on the cortical release of 5-HT and glutamate in sham and OBX mice. The administration of WAY100635 (0.3 mg/kg; i.p) alone did not induce any change in the extracellular levels of 5-HT (S4.a) and glutamate (S4.b) in the vmPFcx of sham and OBX animals. Data represented as mean ± SEM of n=5-7 animals per experimental group.