THE PHARMACOLOGICAL REDUCTION OF HIPPOCAMPAL NEUROGENESIS ATTENUATES THE PROTECTIVE EFFECTS OF CANNABIDIOL ON COCAINE VOLUNTARY INTAKE

Miguel Ángel Luján[1], Lídia Cantacorps[1], Olga Valverde[1, 2*]

1 Neurobiology of Behaviour Research Group (GReNeC - NeuroBio), Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain.

2 Neuroscience Research Programme. IMIM-Hospital del Mar Research Institute, Barcelona, Spain.

[*]Author for correspondence:

Olga Valverde, MD PhD

Neurobiology of Behaviour Research Group (GReNeC - NeuroBio),

Department of Experimental and Health Sciences,

Universitat Pompeu Fabra

Dr. Aiguader 88; Barcelona 08003

olga.valverde@upf.edu

ABSTRACT

The administration of cannabidiol has shown promising evidence in the treatment of some neuropsychiatric disorders, including cocaine addiction. However, little information is available as to the mechanisms by which cannabidiol reduces drug use and compulsive seeking. We investigated the role of adult hippocampal neurogenesis in reducing cocaine voluntary intake produced by repeated cannabidiol treatment in mice. Cocaine intake was modelled using the intravenous cocaine self-administration procedure in CD1 male mice. Cannabidiol (20 mg/kg) reduced cocaine self-administration behaviour acquisition and total cocaine intake and enhanced adult hippocampal neurogenesis. Our results show that a 6-day repeated temozolomide treatment (25 mg/kg/day), a chemotherapy drug that blocks hippocampal neurogenesis, prevented cannabidiol-induced increment in the early stages of neuronal maturation and differentiation, without altering the basal levels of BrdU/NeuN and doublecortin immunostaining. The reduction of total cocaine intake and operant behaviour acquisition observed following cannabidiol exposure was attenuated by temozolomide treatment. Our results also show a similar effect of temozolamide on a cannabidiol-induced improvement of novel object recognition memory, a task influenced by the pro-neurogenic effects of cannabidiol (10 and 20 mg/kg). The anxiolytic effects of cannabidiol (10 and 20 mg/kg), however, remained unaffected after its pro-neurogenic effects decreased. The present study confirms that adult hippocampal neurogenesis is one of the mechanisms by which cannabidiol lowers cocaine reinforcement and demonstrates the functional implication of adult hippocampal neurogenesis in cocaine voluntary consumption in mice. Such findings highlight the possible use of cannabidiol for developing new pharmacotherapies to manage cocaine use disorders.

KEYWORDS: Cannabidiol, cocaine, neurogenesis, reinforcement, self-administration, temozolomide.

ABBREVIATIONS:

5-HT1$_A$R: 5-hydroxytryptamine 1 A receptor

BrdU: 5-bromo-2′-deoxyuridine

CB1R: cannabinoid receptor type 1

CB2R: cannabinoid receptor type 2

CBD: cannabidiol

DG: dentate gyrus

EPM: elevated plus maze

FR1: fixed ratio 1

OR: object recognition

TMZ: temozolomide

INTRODUCTION

Cocaine addiction is a chronic, relapsing disease characterized by compulsive drug use and seeking, despite its harmful consequences (Volkow et al., 2016). Repeated cocaine use promotes neural plasticity processes producing aberrant motivation towards the drug and related stimuli, which may cause neurobiological alterations leading to drug addiction (Everitt et al., 2018). However, as there are no effective treatments thereof, it would be necessary to develop innovative therapeutic strategies (Czoty et al., 2016).

Experimental research of the endocannabinoid system has invigorated the study of potential medical applications involving cannabinoid derivatives. Cannabidiol (CBD), the second major constituent of the *Cannabis Sativa* plant, stands out as one of the most promising compounds to develop new pharmacological strategies for untreated diseases (Ligresti et al., 2016). CBD is a non-psychoactive cannabinoid (Viudez-Martínez et al., 2018) that has drawn interest from clinical and preclinical research as a new pharmacological tool in the treatment of substance use disorders (Ware 2018; Wenzel & Cheer 2018). CBD reduces the behavioural and molecular manifestations of maladaptive neuroplasticity underlying drug addiction (Zlebnik & Cheer 2016). For instance, CBD attenuates cue-induced heroin-seeking in rats (Ren et al., 2009) and humans (Hurd et al., 2015), prevents cue- and stress-induced reinstatement of cocaine seeking in rats (Gonzalez-Cuevas et al., 2018) and reduces ethanol consumption in mice (Viudez-Martínez et al., 2017). Notwithstanding the foregoing, only small steps have been taken towards a reasonable understanding of the neural mechanisms recruited by CBD in drug-consuming animals. A better understanding of such processes would help to improve CBD-based pharmacotherapies by adjusting treatment schedules and providing a coherent understanding of existing data.

We have previously demonstrated that CBD prevents the acquisition of a stable pattern of cocaine self-administration and reduces total cocaine intake in CD-1 male mice (Luján et al., 2018). Interestingly, we described how CBD induces adult hippocampal neurogenesis in cocaine-consuming mice. Neurogenesis is a complex mechanism that involves the generation and development of individual cells through the lifespan of several species (Aguilar-Redondo et al., 2015). It mainly occurs in two specific brain regions: the subventricular zone of the lateral ventricles and the subgranular zone in the hippocampal dentate gyrus (DG) (Ming & Song, 2011). New neurons are generated from neural progenitor cells and further maturation processes allow its functional integration into pre-existing circuits. Although still under intensive debate, it is hypothesized that newly-generated neurons can improve memory and learning function acting as encoding units and as active modifiers of mature neuron firing, synchronization and network oscillations (Ming & Song, 2011). Moreover, previous studies have proposed increased hippocampal neurogenic states as a protective trait influencing drug-induced neuroplasticity (See Chambers, 2013, for a review). In this way, additional studies also support the pro-neurogenic effects of CBD (Campos et al., 2013; Schiavon et al., 2016; Fogaça et al., 2018), and the protective effects of this phytocannabinoid on cocaine voluntary intake and seeking behaviour (Gonzalez-Cuevas et al., 2018).

In the present study, we have used the chemotherapy drug temozolomide (TMZ) to block neurogenesis at hippocampal level (Akers et al., 2014). TMZ is an alkylating agent currently used in the treatment of glioblastoma. It is known to reduce cellular proliferation by delivering a methyl group to purine bases of DNA, thus interfering with the duplication process (Tatar et al., 2013). We therefore evaluated the contribution of neurogenesis in the protective effects of CBD in mice exposed to the intravenous

cocaine self-administration paradigm. Additionally, we assessed the implication of CBD-induced neurogenesis in its anxiolytic and cognitive effects, as adaptations in such processes may also have consequences in terms of cocaine-related behaviour. Spontaneous locomotor activity, stereotype-like behaviour, body weight gain and hippocampal neuro-inflammation were also monitored to discard unspecific TMZ treatment effects.

Hence, considering the behavioural and neurobiological effects shown by CBD in the pre-clinical literature, we have hypothesized that (a) the pharmacological reduction of CBD's pro-neurogenic properties will block its anxiolytic and cognitive effects, and, more importantly, (b) the protective effects of CBD on cocaine voluntary intake will partially decrease in animals treated with TMZ.

METHODS

Animals

All animal care and experimental protocols were approved by the UPF/PRBB Animal Ethics Committee, in accordance with European Community Council guidelines (2016/63/EU). Male CD-1 mice (PND 41-44) were purchased from Charles River (Barcelona, Spain). The animals were maintained in a 12-h light–dark cycle, at a stable temperature (22ºC), with a supply of food and water *ad libitum.* Three different groups of mice were used for the locomotor activity (n = 6/group) (Fig. 1a), elevated plus maze (EPM) and object recognition (OR) tests (n = 12/group) (Fig. 1b), and self-administration (n = 10/group) experiments (Fig. 1c).

Drug administration protocols and experimental design

Cocaine HCl (0.75 mg/kg; Alcaliber S.A., Madrid, Spain) was dissolved in 0.9% NaCl. CBD (10 and 20 mg/kg) was provided by courtesy of Phytoplant Research S.L., Córdoba, Spain and freshly prepared in 2% Tween-80 in 0.9% NaCl. TMZ (Sigma, St. Louis, USA) was diluted in 0.9% NaCl and suspended under sonication for 15 s. CBD doses were selected based on previous studies from our laboratory (Luján et al., 2018), that were within the range of doses used in previous studies (Fogaça et al., 2018; Ren et al., 2009).

Prior to any behavioural manipulation, animals were treated with two consecutive cycles of TMZ, as reported by Niibori et al. (2012), Akers et al. (2014) and Castilla-Ortega et al. (2016). Each cycle involved the intraperitoneal (i.p.) administration of 25 mg/kg TMZ once a day for three consecutive days (Fig. 1a). Between the two TMZ cycles, there was a period of 4 days with no treatment. CBD treatment began 4 days after the last TMZ injection. The mice were injected with CBD (10 or 20 mg/kg, i.p.) or vehicle for ten consecutive days, and underwent behavioural testing (EPM and OR) 2 days later (Fig. 1b). For the cocaine self-administration studies, mice received CBD (20 mg/kg, i.p.) during the acquisition phase of the self-administration procedure, as previously reported (Luján et al. 2018). Mice were treated with CBD immediately before being placed in the operant chambers for each session (Fig. 1c). Once the experiments had concluded, all the mice were treated with 0.9% NaCl v/v BrdU 100 mg/kg (MERCK; New York, USA) three times within the same day, 24h after receiving the last CBD injection.

Body weight determination

To discard the unspecific effects of TMZ treatment in the behavioural procedures, we monitored body weight gain in a group of mice treated with TMZ. From the outset of TMZ treatment, corporal weight was monitored for 5 days per week, for three weeks.

Spontaneous locomotor activity

Locomotor activity was evaluated by placing the mice individually in the actimeter boxes (24 ×24 × 24 cm) (LE881 IR, Panlab s.l.u., Barcelona, Spain) in a low-luminosity room, as reported by López-Arnau et al. (2017). Animals treated with TMZ (n = 6/group) were placed in the actimeter boxes for 60 min. Horizontal locomotor activity, spontaneous stereotype-like behaviour and rearing were monitored using SedaCom 2.0 software (#76-0406, Panlab s.l.u., Barcelona, Spain).

Elevated plus maze test

The EPM test was performed as reported (Gracia-Rubio et al., 2016), and carried out 2 days after the last CBD (10 and 20 mg/kg) administration (10-day treatment). Each mouse (n = 12/group) was placed in the centre of the maze for 5 min. The number of entries and time spent in the open arms were recorded using Smart Software (#SCR_002852, Panlab s.l.u., Barcelona, Spain). Dependent measures were calculated and graphed following the formula $\frac{open\ arms\ time/entries}{open\ arms\ time/entries + closed\ arms\ time/entries} x\ 100$.

Novel object recognition task

The OR test was used to evaluate the effects of the CBD treatment on recognition memory, a hippocampal-dependent task. Additionally, CBD treatment was combined with TMZ treatment to unveil the participation of CBD-induced adult hippocampal neurogenesis in the task. The procedure was performed as described by Cantacorps et al. (2017) and commenced 24 h after the EPM test. The retention trial (5 min) took place 72 h later and objects A and B were simultaneously placed in the open-field. The

recognition index (%) was defined as $\frac{t_{novel\ object}}{(t_{novel\ object} + t_{familiar\ object})} \times 100$, with *"t"* as the time each mouse had spent exploring an object (recorded using Smart Software, Panlab s.l.u.). Upon completion of the OR retention trial, animals were perfused for brain tissue extraction and immunohistochemistry analysis.

Determination of adult hippocampal neurogenesis by BrdU/NeuN and DCX immunolabelling

In the present work, adult hippocampal neurogenesis changes were outlined in basis of the early stages of neuronal differentiation and maturation, since this period was shown especially sensible to the pro-neurogenic effects of CBD (Luján et al., 2018). Early stages of maturation of BrdU+ cells into neurons were assessed by double fluorescence immunohistochemistry against BrdU and the mature neuron marker Neuronal Nuclei (NeuN) antigen (Castilla-Ortega et al., 2017; Luján et al., 2018). In the case of the self-administering animals, we also determined neuronal differentiation by double fluorescence immunohistochemistry against doublecortin (DCX) (Francis et al., 1999). As in Luján et al. (2018), mice were anaesthetized with pentobarbital (500 mg/kg, i.p.) and perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS). 7 days before, mice had been treated three times with BrdU 100 mg/kg (MERCK) within a 24-h period. A mouse brain atlas (Paxinos and Franklin, 2004) was used to identify the anatomical location of the DG (a minimum of 3 coronal sections per animal, evaluated bilaterally). Floating brain sections (30 μm-thick) were incubated in 3% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) for 1h. Finally, BrdU-NeuN (n = 4/group) was analysed by incubating sections overnight with the corresponding primary antibody (BrdU, monoclonal rat 1:300, #AB_298940, Abcam; DCX, monoclonal rabbit 1:2000, #AB_732011, Abcam and NeuN, monoclonal

mouse 1:1000, #AB_2298772, MERCK). Samples were then incubated for 2h with a fluorescent secondary antibody, namely goat anti-rat IgG Alexa Fluor 488 (1:500; #AB_2534074, ThermoFisher, Barcelona, Spain), goat anti-mouse IgG Alexa Fluor 555 (1:500; #AB_2535846, ThermoFisher) and goat anti-rabbit IgG Alexa Fluor 488 (1:500; #AB_2630356, Abcam). Labelled images of the region of interest were obtained bilaterally using sequential laser scanning confocal microscopy (Leica SP2 and Zeiss LSM510). BrdU/NeuN+ and DCX cells were quantified as the mean number of labelled body cells in each hemisphere per brain slice. For each mouse, 3-4 slides were analysed bilaterally, resulting in a total of 6-8 DG images per subject. BrdU/NeuN+ and DCX cells were manually counted using ImageJ software (#SCR_003070, NIH, Bethesda, MD, USA) by an observer who was blind to treatment. Double labelling for BrdU/NeuN+ cells was determined by colour superposition. DCX positive cases were considered when the perisomal and the proximal part of the axonal growth cone regions were clearly marked by DCX.

Determination of microglial activation by Iba1 immunolabelling

To investigate the long-term impact of TMZ treatment on microglial activation, we evaluated the activation of the microglial cellular population in the hippocampal DG three weeks after TMZ administration. The activation of microglia was assessed using the Iba1 marker. Iba1 is a macrophages/microglial-specific calcium-binding protein involved in membrane ruffling and phagocytosis in activated microglia (Ohsawa et al., 2000). The immunodetection of Iba1+ cells was followed in accordance with the BrdU/NeuN immunohistochemistry protocol. For Iba1 targeting, we used an Iba1, monoclonal rabbit primary antibody (#AB_839504, 1:1000; Wako Pure Chemicals, Oaza Ogohara, Japan). Goat anti-rabbit IgG DyLight 680 (#AB_1057609, 1:5000; Rockland, Limerick, USA) was used as the secondary antibody. Iba1+ cells were

automatically identified using the Image-based Tool for Counting Nuclei (UCSB, Santa Barbara, USA) ImageJ plug-in (NIH, Bethesda, MD, USA). For these images, a complementary NeuN staining was followed to allow the identification of the subgranular zone/inner granule cell layer of the DG (Figure 3). Thereafter, the area was manually outlined using Adobe Photoshop Illustrator software (#SCR_014199, San Jose, USA).

Cocaine operant self-administration

Cocaine operant self-administration experiments were conducted as previously described by Soria et al. (2008), López-Arnau et al. (2017) and Luján et al. (2018). The combination of TMZ and CBD treatments yielded four experimental groups: saline-vehicle, TMZ-vehicle, saline-CBD and TMZ-CBD (n = 10/group). Mice were trained to self-administer cocaine (0.75 mg/kg/infusion) daily (2h) for 10 consecutive days under fixed ratio 1 (FR1). Surgical implantation of the catheter into the jugular vein was performed following anaesthetization with a mixture of Ketamine hydrochloride (100 mg/kg; Imalgène1000, Lyon, France) and Xylazine hydrochloride (20 mg/kg; Sigma Chemical Co., Madrid, Spain), injected in a volume of 0.15 mL/10 g body weight, i.p (Tourino et al, 2012). Mice were housed individually and allowed to recover for at least 3 days.

Acquisition of operant cocaine consumption. Active and inactive nose-poke holes were assigned randomly. Cocaine was delivered in a 20 µl injection over 2s via a syringe mounted on a microinfusion pump (PHM-100A, Med-Associates, Georgia, VT, USA) connected to the mouse's intravenous catheter. All FR1 sessions began with a cocaine priming infusion. When mice responded at the active hole, the stimulus lights lit up for 4s. Each infusion was followed by a 15s time-out period. Mice were considered to have acquired stable self-administration behaviour when the following criteria were met in 2

consecutive FR1 sessions: a) 80% stability in reinforcements (the number of infusions on each day deviated by < 20% from the mean number of infusions over the 2 consecutive days); b) ≥ 65% of infusions were received at the active hole; and c) a minimum of 5 infusions.

Statistical analysis

We analysed the results of the EPM, OR tests and BrdU/NeuN and Iba1 immunostaining using two-way ANOVA with the factors defined as *CBD* (with levels VEH, CBD 10 mg/kg and CBD 20 mg/kg) and *TMZ* (with levels SAL and TMZ). We used three-way ANOVA to analyse the acquisition phase of the self-administration experiment with factors defined as *CBD*, *TMZ* and *session*. Two-way ANOVA was calculated to evaluate the area under the curve and total infusions of the FR1 phase. Previously, we used the non-parametric Fisher exact test to compare acquisition ratios of self-administration behaviour.

Animals were randomly assigned to an experimental group. During the behavioural manipulations and data interpretation, researchers were blind to the treatment each animal had received. The exact group size for the individual experiments is shown in the corresponding figure legends. Data were expressed as mean ± SEM. The α-level of statistical significance was set at $p < 0.05$. When required, ANOVAs were followed by Tukey's post-hoc tests (GraphPad Prism 7, #SCR_002798, La Jolla, USA) only if F reached the level of significance ($p < 0.05$), dependent measures followed a normal distribution (Shapiro-Will's test) and no significant variance in homogeneity was observed (Bartlett's test).

RESULTS

TMZ does not affect body weight or locomotor activity

One-way ANOVA showed no effect of TMZ on body weight (*TMZ* factor; $F_{1,14} = 1.573$, *n.s.*) (Fig. 2a). Locomotor activity was also examined in mice treated with TMZ (Fig. 2b-g). Two-way ANOVAs discarded any significant effect of TMZ on basal locomotion (*TMZ* factor; $F_{1,10} = 2.47$, *n.s.*) (Fig. 2b), vertical exploration (*TMZ* factor; $F_{1,10} = 2.94$, *n.s.*) (Fig. 2d) or spontaneous stereotype-like behaviour (*TMZ* factor; $F_{1,10} = 0.62$, *n.s.*) (Fig. 2f) during a 60 min locomotor activity test. No interactions were found between factors. Additional examinations (Student's *t* tests) of the values accumulated throughout the session confirmed the lack of effect of TMZ on locomotion activity ($t_{10} = 1.57$, *n.s.*) (Fig. 2c), vertical exploration ($t_{10} = 1.71$, *n.s.*) (Fig. 2e) and spontaneous stereotype-like behaviour ($t_{10} = 0.63$, *n.s.*) (Fig. 2g).

Unlike CBD, TMZ does not modulate microglial activation in the DG

Data were analysed as a function of two factors: *TMZ* (SAL/TMZ) and *CBD* (VEH/CBD10/CBD20) treatments, resulting in 6 experimental groups (SAL-VEH, TMZ-VEH, SAL-CBD10, TMZ-CBD10, SAL-CBD20 and TMZ-CBD20). Two-way ANOVA showed no effects of TMZ on the number of Iba1+ cells in the DG ($F_{1,25} = 0.02$, $p > 0.05$), thus ruling out the possibility of a proinflammatory effect of TMZ that could mask any of the behavioural differences observed (Fig. 3). Interestingly, a significant effect of the *CBD* factor ($F_{2,25} = 7.39$, $p < 0.01$) showed that CBD reduced Iba1 immunostaining in the DG of both TMZ- and vehicle-treated animals, thus reflecting its anti-inflammatory properties. The interaction between both factors ($F_{2,25} = 5.14$, $p < 0.05$) indicated that the anti-inflammatory effects of CBD were not present to the same extent on the TMZ- and vehicle-treated mice. Thus, a significant difference

was observed between SAL-VEH and TMZ-CBD10 groups (Tukey, $p < 0.05$). No other post-hoc effects were observed between the TMZ- and SAL-treated groups (Tukey, *n.s.*).

TMZ treatment prevents the pro-neurogenic effect of CBD in the DG

After combined treatment with CBD (10 and 20 mg/kg) and TMZ, a two-way ANOVA analysis revealed that TMZ treatment reduced the number of BrdU/NeuN + cells in the DG (*TMZ* factor; $F_{1,25} = 19.23$, $p < 0.001$). Although no significant *CBD* effect ($F_{2,25} = 2.59$, *n.s.*) was appreciated, an examination of the interaction ($F_{2,25} = 7.5$, $p < 0.01$) yielded additional implications of the treatments. Subsequent post-hoc comparisons showed that SAL-CBD-treated (10 mg/kg) mice had more newborn hippocampal neurons in the DG than SAL-VEH-treated animals (Tukey, $p < 0.01$) (Fig. 4a), suggesting a pro-neurogenic profile of CBD. Importantly, this effect was blocked by TMZ treatment (SAL-CBD10 vs. TMZ-CBD10; Tukey, $p < 0.01$). Furthermore, TMZ did not reduce BrdU/NeuN+ cells in the DG of vehicle-treated animals (SAL-VEH vs. TMZ-VEH; Tukey, *n.s.*), suggesting that TMZ only blocked CBD-induced neuronal early maturation, since the basal levels of BrdU incorporation remaining unaltered in control groups.

TMZ prevents recognition memory improvement induced by CBD treatment

Two-way ANOVA analysis revealed a tendency for *CBD* main effect ($F_{2,65} = 2.88$, $p = 0.06$) and a significant *TMZ* effect ($F_{1,65} = 41.39$, $p < 0.001$) with interaction between both factors ($F_{2,65} = 4.25$, $p < 0.05$). Subsequent Tukey's post-hoc analysis of the interaction showed that CBD treated mice not receiving TMZ presented higher recognition indexes (Tukey; SAL-VEH vs SAL-CBD10 and SAL-CBD20, $p < 0.05$) (Fig. 4b). This cognitive effect disappeared in CBD treated mice receiving TMZ

(Tukey; TMZ-VEH vs TMZ-CBD10 and TMZ-CBD 20, *n.s.*) (Fig. 4b). Importantly, TMZ did not reduce object recognition index in control (VEH) mice (Tukey; SAL-VEH vs TMZ-VEH, *n.s.*) (Fig. 4b).

Anxiolytic effects of CBD remain unaltered after the TMZ treatment

Two-way ANOVA analysis of the percentage of time spent in the open arms of the maze supported the anxiolytic profile of the *CBD* ($F_{2,65} = 8.30$, $p < 0.001$). TMZ did not affect the anxiety-like responses ($F_{1,65} = 0.56$, *n.s.*) and no interaction between factors was found ($F_{2,65} = 0.12$, *n.s.*), thus indicating that the pharmacological reduction of adult hippocampal neurogenesis did not alter the anxiolytic effects of CBD (Fig. 4c). CBD factor main effects were further examined using Tukey post-hoc comparisons (Wei et al, 2012). Compared to the VEH group, CBD 10 and 20 mg/kg groups spent more time exploring the open arms (Tukey; $p < 0.01$). Two-way ANOVA analysis revealed that the percentage of entries in the open arm was affected by CBD treatment ($F_{2,65} = 3.14$, $p < 0.05$), without effects of the TMZ treatment ($F_{2,65} = 1.22$, *n.s.*) or interaction between factors ($F_{1,65} = 0.13$, *n.s.*). Post-hoc analysis showed that CBD 20 mg/kg group of mice performed more open arm entries compared to VEH group (Tukey; $p < 0.05$) (Fig. 4d). Two-way ANOVA analysis of the total arm-entries, as a locomotor response control variable, discarded any effects of CBD ($F_{2,65} = 0.47$, *n.s.*), TMZ ($F_{1,65} = 1.51$, *n.s.*) and their interaction ($F_{2,65} = 0.02$, *n.s.*), thus ruling out an unspecific locomotor activity affectation during testing (data not shown).

TMZ blocks the CBD-induced reduction of cocaine intake in the self-administration paradigm

Differences in reinforced nosepoke responding during cocaine self-administration were assessed following a three-way ANOVA analysis. *CBD* and *TMZ* treatments were

defined as between-subject factors whereas *day of training* was considered the within-subject factor. CBD treatment significantly reduced cocaine intake throughout the 10 days of FR1 reinforcement schedule ($F_{1,36} = 13.74$, $p < 0.001$) (Fig. 5a). On the contrary, TMZ treatment did not affect cocaine intake ($F_{1,36} = 0.43$, *n.s.*). Overall, cocaine-reinforced nosepokes increased as a day-of-training function ($F_{9,9} = 10.98$, $p < 0.001$). Interestingly, a significant interaction between TMZ and CBD factors was found ($F_{1,36} = 4.39$, $p < 0.05$), thus indicating that the effects of CBD were modulated by TMZ. A thorough Tukey's post-hoc examination of this interaction confirmed the reduction of cocaine intake produced by CBD in control mice (SAL-VEH vs. SAL-CBD; Tukey, $p < 0.001$). No differences were induced by CBD in TMZ-treated mice (TMZ-VEH vs. TMZ-CBD; Tukey, *n.s.*), thus indicating that the pro-neurogenic effects of CBD are necessary to produce its protective effects on cocaine intake. No interaction between the three factors (*CBD x TMZ x day of training*) was found ($F_{9,324} = 1.19$, *n.s.*).

Additional evaluations of the behavioural phenotype observed in the cocaine self-administration paradigm supported the implication of neurogenesis in the protective effects of CBD. Chi-square analysis of the acquisition ratios showed that groups differed in the percentage of animals reaching a stable pattern of cocaine self-administration ($\chi^2 = 18.1$, $p < 0.001$). In animals that did not receive TMZ treatment, CBD alone reduced the acquisition criteria by 60 % (Fig. 5b). In the TMZ-CBD group, however, only a 10 % reduction was observed. An assessment of the total cocaine intake throughout the 10 days of self-administration confirmed previous analyses. Therefore, two-way ANOVA analysis showed a significant effect of *CBD* ($F_{1,36} = 17.58$, $p < 0.001$). The *TMZ* factor was found to be insignificant ($F_{1,36} = 0.51$, *n.s.*). Once again, the interaction between both factors resulted in a significant effect ($F_{1,36} = 4.47$, $p < 0.05$). Tukey's post-hoc comparisons revealed that the protective effects of CBD (SAL-

VEH vs. SAL-CBD; Tukey, $p < 0.001$) were absent in TMZ-treated mice (TMZ-VEH vs. TMZ-CBD; Tukey, *n.s.*) (Fig. 5c). To discard unspecific instrumental learning deficiencies in TMZ-treated mice, we followed a two-way ANOVA analysis of the discrimination index between active and inactive nosepoke holes throughout self-administration (Fig. 5d). No between-group differences were observed (*TMZ;* $F_{1,36} = 0.64$, *n.s.*), (*CBD;* $F_{1,36} = 0.39$, *n.s.*), (*TMZ x CBD;* $F_{1,36} = 0.05$, *n.s.*).

TMZ prevents the pro-neurogenic effects of CBD in cocaine-consuming mice

Two-way ANOVA analysis of the number of NeuN/BrdU+ cells in the DG showed that CBD independently induced an increment in the number of NeuN/BrdU+ cells in cocaine-consuming mice ($F_{1,13} = 47.87$, $p < 0.001$), thus confirming our previous studies (Luján et al., 2018). TMZ yielded a significant effect on NeuN/BrdU + cells ($F_{1,13} = 43.22$, $p < 0.001$). A significant interaction between factors ($F_{1,13} = 43.22$, $p < 0.001$) allowed us to confirm that TMZ prevented the pro-neurogenic effects of CBD (Tukey, $p < 0.001$) (Fig. 5e). Crucially, TMZ alone did not impair basal levels of adult hippocampal neurogenesis (SAL-VEH vs. TMZ-VEH; Tukey, *n.s.*), implying that it only buffered the CBD-induced increments of NeuN/BrdU+ cells. Two-way ANOVA analysis of the number of DCX cells in the DG showed that CBD also increased neuronal differentiation in cocaine-consuming mice ($F_{1,12} = 4.75$, $p < 0.05$). TMZ treatment prevented the increase of neuronal differentiation ($F_{1,12} = 5.25$, $p < 0.05$) and a significant interaction between factors was also reported ($F_{1,12} = 5.05$, $p < 0.05$). Tukey post-hoc comparisons revealed that TMZ treatment specifically prevented neuronal differentiation in CBD-treated mice (Tukey, $p < 0.05$) (Fig. 5g), without altering basal levels of neuronal differentiation (SAL-VEH vs. TMZ-VEH; Tukey, *n.s.*).

DISCUSSION

In our study, we demonstrate that TMZ treatment dampened the protective effects of CBD after reducing adult hippocampal neurogenesis. TMZ/CBD-treated mice reached a stable pattern of cocaine self-administration and consumed cocaine to a similar extent compared to control groups. The pro-neurogenic effects of CBD elicited the improvement of recognition memory task and TMZ reversed this effect on memory. However, the anxiolytic effects of CBD remained unaltered after the TMZ treatment.

TMZ treatment can have cytotoxic effects when extensively administered (Tatar et al., 2013). Nonetheless, previous studies have found solutions for using TMZ as a neurogenesis-suppression agent without compromising neuroimmune responses or general behaviour (Niibori et al., 2012; Akers et al., 2014; Castilla-Ortega et al., 2016). Therefore, an intermittent, brief treatment schedule, with a dose of 25 mg/kg, was described as the most suitable strategy to inhibit DNA replication and only affect mitosis in cell populations with low proliferation profiles (i.e., neuronal precursor cells). Here, different behavioural measures were assessed to discard unspecific, cytotoxic effects of TMZ treatment. Firstly, we showed that no differences in body weight gain were observed after TMZ administration. We also ruled out any effect of TMZ on spontaneous locomotion and stereotyping. Additionally, we showed that TMZ did not have any consequences in terms of nosepoke hole stimuli discrimination, a possibility raised by the participation of hippocampal neurogenesis in stimuli discrimination of operant tasks (Johnston et al., 2016). We also confirm that TMZ treatment was not able

to produce substantial changes in hippocampal Iba1 cell population, thus discarding its ability to produce neuroinflammatory effects. Moreover, basal levels of adult hippocampal neurogenesis observed in control mice remained unaltered after TMZ treatment. That is to say, the treatment was mild enough to exclusively block the neuronal early maturation/differentiation increases produced by CBD. This outcome allowed us to associate the observed behavioural changes with the blockade of the pro-neurogenic effects of CBD, without having to deal with alterations in control mice.

CBD's capacity to reduce the behavioural and molecular manifestations of maladaptive neuroplasticity underlying cocaine abuse has recently been detailed. Indeed, Mahmud et al. (2016) reported that a single CBD (5 or 10 mg/kg) injection, once rats had acquired operant learning, was unable to modulate cocaine breaking points or seeking reinstatement. However, recent investigations have characterized a wide range of protective CBD effects on cocaine consumption acquisition, compulsive pursuing (Luján et al., 2018) and relapse (Gonzalez-Cuevas et al., 2018) in rodents. A comparison between such studies points to the importance of prolonging CBD treatments. This statement is supported by the fact that Gonzalez-Cuevas et al. (2018) and Luján et al. (2018), albeit using different CBD doses, extended their treatments for at least 7 days, in contrast to the more acute treatment of Mahmud et al. (2016), and observed similar protective effects of CBD. Despite compelling behavioural evidence, no studies have directly addressed the mechanisms needed to explain the effects of CBD on cocaine intake reduction. Recent studies have reported that CBD modulates dopamine release in the nucleus accumbens (Renard et al., 2016) through a mechanism involving the activation of 5-hydroxytryptamine 1A receptors (5-$HT1_AR$) (Norris et al., 2016). As a result, CBD-treated animals showed a reduction in amphetamine-induced locomotor sensitization. This finding, however, would need to be clarified using other

psychostimulant drugs, as CBD's inability to modulate cocaine-induced locomotor sensitization has also been reported (Luján et al., 2018). In addition, there is evidence pointing to the pro-neurogenic effects of CBD (Schiavon et al., 2016; Campos et al., 2017). Specifically, CBD treatment increased adult hippocampal neurogenesis and reduced cocaine intake in an operant self-administration paradigm (Luján et al. 2018). In this sense, upregulation of neuronal maturation/differentiation in the DG could be a relevant adaptive process providing protection against drug-taking escalation and compulsive pursuing (Chambers 2013). The relation between neurogenesis and drug reinforcement, although previously described (Noonan et al., 2010; Mandyam & Koob 2012; Deschaux et al., 2014; Bayer et al., 2015; Castilla-Ortega et al. 2016; Deroche-Gamonet et al., 2018), is still poorly understood. Thus far, the proposed mechanism involves new-born neurons that could explain the protective effect of CBD driven by adult hippocampal neurogenesis. The hypothesis states that young DG neurons (1-3 weeks old) promote the growth of dendritic trees receiving entorhinal inputs and modulate axonal projections to the CA3 layer, where information is integrated for output via the CA1 and subiculum layers to the nucleus accumbens and prefrontal cortex (Castilla-Ortega et al., 2017). This newborn neuron-driven modulation of hippocampal outputs may determine changes in the prefrontal cortical-striatal network function underpinning impulsive behaviour (Chambers 2013). Cortical-striatal network circuitry is a crucial feature of the adaptive inhibitory control processes responsible for reward delay and goal-directed behaviour (Everitt & Robbins 2016). It is noteworthy that CBD has been shown to specifically improve corticostriatal connectivity in healthy humans (Grimm et al., 2018). Within this theoretical framework, drugs of abuse would decrease the newborn neuronal integration of hippocampal processing and prefrontal cortical-striatal function would decline. Pro-neurogenic agents, such as CBD, increase

cell counts of young neurons that may improve hippocampal function, thus leading to a more optimal control of mesolimbic dopaminergic excitability and fronto-striatal coordinated activity. The present study links CBD-induced reduction of cocaine consumption to neural early maturation/differentiation increase and supports a protective effect of this mechanism.

Although the processes by which neural proliferation and maturation adaptations modulate mesocorticolimbic signalling are not fully known, there is evidence that may contribute to elucidating such a molecular mechanism. In this sense, Campos et al. (2013) demonstrated that CBD 30 mg/kg increased adult hippocampal neurogenesis *in vitro* by a mechanism involving the participation of CB1 and CB2 receptors, but not 5-$HT1_AR$. Afterwards, Fogaça et al. (2018) showed that both CB1 and CB2 receptors were differentially involved in the pro-neurogenic effects of CBD *in vivo* and that CBD anxiolytic and pro-neurogenic effects were accompanied by a decrease in hippocampal FAAH expression. We recently showed a hippocampal CB1R upregulation, increased neural maturation/differentiation and a reduction of cocaine intake induced by CBD in mice (Luján et al., 2018). In this previous study, the CBD-induced enhancement of CB1R expression activated CREB/MAPK pathways and increased BDNF expression, common downstream pathways linking hippocampal CB1R activation and adult hippocampal neurogenesis (Ortega-Martínez, 2015).

We were also interested to know the implications of the blockade of CBD pro-neurogenic effects in recognition memory and anxiety-like responses. Such processes may indirectly regulate drug intake escalation and excessive reinforcement (Ersche et al., 2012). Our results show that the blockade of CBD's pro-neurogenic effects did not modify its anxiolytic effects, thus suggesting that the CBD-induced neurogenesis and the anxiety-like effects concur by independent mechanisms. In this sense, Fogaça et al.

(2018), however, showed that CBD (30 mg/kg, 14 days) anxiolytic effects in the EPM test were accompanied by enhanced pro-neurogenic states in mice previously exposed to chronic unpredictable stress. Nevertheless, when CBD was injected to animals not exposed to chronic stress, neither anxiolytic nor pro-neurogenic effects were observed, in accordance with our previous study (Lujan et al., 2018), in which a lack of CBD (30 mg/kg, 10 days) anxiolytic effects was reported. CBD anxiolytic effects have consistently been related to its 5-$HT1_AR$ agonistic activity (Campos et al., 2012; Marinho et al., 2015). As in Campos et al. (2013), our results do not support the idea of CBD reducing anxiety states through hippocampal neuronal differentiation. We also ruled out the possibility that cocaine-intake attenuation was attributable to low-anxiety states due to CBD effects, as previously proposed (Craige et al., 2013). We have investigated the implication of CBD-induced neurogenesis in the cognitive effects as shown in the OR task. More specifically, a blockade of the pro-neurogenic effects of CBD attenuated performance in the OR test, a kind of declarative memory that is closely related to hippocampal function and adult neurogenesis (Aimoneet et al., 2011; Johnston et al., 2016). We postulate that the OR outcome here observed may be a behavioural measure of an improved cognitive function (Batalla et al., 2014) that may underlie CBD-induced protective effects on cocaine intake.

Little information is available as to the complex pharmacology of CBD, and more detailed studies are needed with a view to fully understanding the different processes by which this phytocannabinoid interacts with reinforcement neural systems. Although our research has demonstrated the participation of neural maturation/differentiation in the effects of CBD, other factors should also be considered in order to explain the observed variance. Thus, neurogenesis represents a complex mechanism that encompasses more sub-processes that the ones reported here. As such, the participation of increased

proliferation and survival in the behavioural changes observed remains an open question. In this case, further studies with extended periods of time after BrdU injections and specific neuronal proliferation markers (i.e. Ki67) are required. Moreover, it should be considered that the BrdU injection schedule (after CBD exposure) does not allow us to differentiate between the accumulative effects of the repeated treatment and the acute effects of the last CBD injection. Last, although neurogenesis reduction was sufficient in modulating the protective outcomes of CBD, it does not seem plausible that this was the only mechanism engaged by CBD to reduce cocaine intake. In particular, a closer examination of the 5-$HT1_AR$-induced modulation of dopaminergic signalling in the nucleus accumbens (as in Renard et al., 2016 and Norris et al., 2016) of cocaine-consuming mice would be required.

A recent report by Sorrells et al., (2018) noted that adult hippocampal neurogenesis was not present in human samples when measured by methods different from C^{14} labelling. Such a finding serves to question the existence of this neural mechanism in adult humans. However, the existence of newborn functioning neurons in the hippocampus of adult experimental animals is undeniable and an understanding of such a process continues to be an important hallmark in brain learning and memory function research. At present, however promising our results may be, they do not sufficiently support a therapeutical application of CBD in cocaine-abstinent individuals. The methodologies followed throughout our study provide evidence for a protective CBD mechanism in subjects already using the drug. This feature of CBD treatment could be especially useful for future agonist replacement therapies (Mariani et al., 2012), in which CBD could help to reduce the inherent reinforcing potential of the replacement compound.

In conclusion, the present results demonstrate the crucial participation of neural maturation and differentiation in the CBD-induced reduction of cocaine voluntary

intake. Our study expands upon the available evidence supporting CBD's capacity to modulate cocaine-related operant behaviours. Our research has also shown the participation of CBD's pro-neurogenic effects in the recognition memory improvements observed. Finally, our study presents solid evidence as to the functional implication of adult hippocampal neurogenesis in the reinforcing potential of cocaine. Given the capability of CBD to both enhance hippocampal neurogenesis and reduce cocaine voluntary intake, we would propose the compound as an effective experimental tool to assess the implication of the hippocampal newborn neuron function in the neurobiology of cocaine addiction.

ACKNOWLEDGEMENTS

This work was supported by Ministerio de Economía y Competitividad (grant number SAF2016-75966-R-FEDER), by the European Union's Horizon 2020 research and innovation programme 2014–2020 under grant agreement no 634143. M.A.L. received FPU grant (15/02492) from the Ministerio de Educacion, Cultura y Deporte. L.C. received FPI grant (BES-2014-070657) from the Ministerio de Economía y Competitividad. The Department of Experimental and Health Sciences (UPF) is an "Unidad de Excelencia María de Maeztu" funded by the MINECO (Ref. MDM-2014-0370). The authors thank Gerald-Patrick Fannon for his English proofreading and editing of the manuscript, and Phytoplant Research S.L. for providing CBD compound. The authors declare no conflicts of interest.

AUTHORS CONTRIBUTION

M.A.L. and O.V. were responsible for the study concept and design. M.A.L. and L.C carried out the experimental studies. M.A.L. and O.V. drafted the manuscript and

participated in interpreting the findings. All authors critically reviewed the content and approved the final version for publication.

REFERENCES

Aguilar-Arredondo A, Arias C & Zepeda A (2015) Evaluating the functional state of adult-born neurons in the adult dentate gyrus of the hippocampus: From birth to functional integration. *Rev Neurosci* 26:269–279.

Aimone JB, Deng W & Gage FH (2011) Resolving New Memories: A Critical Look at the Dentate Gyrus, Adult Neurogenesis, and Pattern Separation. *Neuron* 70:589–596.

Akers KG, Martinez-Canabal A, Restivo L, Yiu AP, De Cristofaro A, Hsiang H-L, Wheeler AL, Guskjolen A, Niibori Y, Shoji H, Ohira K, Richards BA, Miyakawa T, Josselyn SA & Frankland PW (2014) Hippocampal Neurogenesis Regulates Forgetting During Adulthood and Infancy. *Science* 344:598–602.

Batalla A, Crippa JA, Busatto GF, Guimaraes FS, Zuardi AW, Valverde O, Atakan Z, McGuire PK, Bhattacharyya S & Martín-Santos R (2014) Neuroimaging studies of acute effects of THC and CBD in humans and animals: a systematic review. *Curr Pharm Des* 20:2168–85.

Bayer R, Franke H, Ficker C, Richter M, Lessig R, Büttner A & Weber M (2015) Alterations of neuronal precursor cells in stages of human adult neurogenesis in heroin addicts. *Drug Alcohol Depend* 156:139–149.

Campos AC, Fogaça M V, Scarante FF, Joca SRL, Sales AJ, Gomes F V, Sonego AB, Rodrigues NS, Galve-Roperh I & Guimarães FS (2017) Plastic and

Neuroprotective Mechanisms Involved in the Therapeutic Effects of Cannabidiol in Psychiatric Disorders. *Front Pharmacol* 8:269.

Campos AC, Moreira FA, Gomes FV, Del Bel EA & Guimarães FS (2012) Multiple mechanisms involved in the large-spectrum therapeutic potential of cannabidiol in psychiatric disorders. *Philos Trans R Soc Lond B Biol Sci* 367:3364–78.

Campos AC, Ortega Z, Palazuelos J, Fogaça M V., Aguiar DC, Díaz-Alonso J, Ortega-Gutiérrez S, Vázquez-Villa H, Moreira F A., Guzmán M, Galve-Roperh I & Guimarães FS (2013) The anxiolytic effect of cannabidiol on chronically stressed mice depends on hippocampal neurogenesis: involvement of the endocannabinoid system. *Int J Neuropsychopharmacol* 16:1407–1419.

Cantacorps L, Alfonso-Loeches S, Moscoso-Castro M, Cuitavi J, Gracia-Rubio I, López-Arnau R, Escubedo E, Guerri C & Valverde O (2017) Maternal alcohol binge drinking induces persistent neuroinflammation associated with myelin damage and behavioural dysfunctions in offspring mice. *Neuropharmacology* 123:368–384.

Castilla-Ortega E, Blanco E, Serrano A, Ladrón De Guevara-Miranda D, Pedraz M, Estivill-Torrús G, Pavón FJ, Rodríguez De Fonseca F & Santín LJ (2016) Pharmacological reduction of adult hippocampal neurogenesis modifies functional brain circuits in mice exposed to a cocaine conditioned place preference paradigm. *Addict Biol* 21:575–588.

Castilla-Ortega E, Ladrón de Guevara-Miranda D, Serrano A, Pavón FJ, Suárez J, Rodríguez de Fonseca F & Santín LJ (2017) The impact of cocaine on adult hippocampal neurogenesis: Potential neurobiological mechanisms and contributions to maladaptive cognition in cocaine addiction disorder. *Biochem*

Pharmacol 141:100–117.

Chambers RA (2013) Adult hippocampal neurogenesis in the pathogenesis of addiction and dual diagnosis disorders. *Drug Alcohol Depend* 130:1–12.

Craige CP, Enman NM & Unterwald EM (2013). Chapter Six - Stress, Anxiety, and Cocaine Abuse. In: *The Effects of Drug Abuse on the Human Nervous System*. Academic Press. pp. 135–167.

Czoty PW, Stoops WW & Rush CR (2016) Evaluation of the "Pipeline" for Development of Medications for Cocaine Use Disorder: A Review of Translational Preclinical, Human Laboratory, and Clinical Trial Research. *Pharmacol Rev* 68:533–562.

Deroche-Gamonet V, Revest J-M, Fiancette J-F, Balado E, Koehl M, Grosjean N, Abrous DN & Piazza P-V (2018) Depleting adult dentate gyrus neurogenesis increases cocaine-seeking behavior. *Mol Psychiatry*.

Deschaux O, Vendruscolo LF, Schlosburg JE, Diaz-Aguilar L, Yuan CJ, Sobieraj JC, George O, Koob GF & Mandyam CD (2014) Hippocampal neurogenesis protects against cocaine-primed relapse. *Addict Biol* 19:562–574.

Ersche KD, Turton AJ, Chamberlain SR, Müller U, Bullmore ET & Robbins TW (2012) Cognitive Dysfunction and Anxious-Impulsive Personality Traits Are Endophenotypes for Drug Dependence. *Am J Psychiatry* 169:926–936.

Everitt BJ, Giuliano C & Belin D (2018) Addictive behaviour in experimental animals: prospects for translation. *Philos Trans R Soc B Biol Sci* 373 (1742):20170027.

Everitt BJ & Robbins TW (2016) Drug Addiction: Updating Actions to Habits to Compulsions Ten Years On. *Annu Rev Psychol* 67:23–50.

Fogaça M V., Campos AC, Coelho LD, Duman RS & Guimarães FS (2018) The anxiolytic effects of cannabidiol in chronically stressed mice are mediated by the endocannabinoid system: Role of neurogenesis and dendritic remodeling. *Neuropharmacology* 135:22–33.

Francis F, Koulakoff A, Boucher D, Chafey P, Schaar B, Vinet M-C, Friocourt G, McDonnell N, Reiner O, Kahn A, McConnell SK, Berwald-Netter Y, Denoulet P & Chelly J (1999) Doublecortin Is a Developmentally Regulated, Microtubule-Associated Protein Expressed in Migrating and Differentiating Neurons. *Neuron* 23:247–256.

Gonzalez-Cuevas G, Martin-Fardon R, Kerr TM, Stouffer DG, Parsons LH, Hammell DC, Banks SL, Stinchcomb AL & Weiss F (2018) Unique treatment potential of cannabidiol for the prevention of relapse to drug use: preclinical proof of principle. *Neuropsychopharmacology* 10:2036-2045

Gracia-Rubio I, Moscoso-Castro M, Pozo OJ, Marcos J, Nadal R & Valverde O (2016) Maternal separation induces neuroinflammation and long-lasting emotional alterations in mice. *Prog Neuro-Psychopharmacology Biol Psychiatry* 65:104–117.

Grimm O, Löffler M, Kamping S, Hartmann A, Rohleder C, Leweke M & Flor H (2018) Probing the endocannabinoid system in healthy volunteers: Cannabidiol alters fronto-striatal resting-state connectivity. *Eur Neuropsychopharmacol* 28:841–849.

Hurd YL, Yoon M, Manini AF, Hernandez S, Olmedo R, Ostman M & Jutras-Aswad D (2015) Early Phase in the Development of Cannabidiol as a Treatment for Addiction: Opioid Relapse Takes Initial Center Stage. *Neurotherapeutics* 12:807–815.

Johnston ST, Shtrahman M, Parylak S, Gonçalves JT & Gage FH (2016) Paradox of pattern separation and adult neurogenesis: A dual role for new neurons balancing memory resolution and robustness. *Neurobiol Learn Mem* 129:60–68.

Ligresti A, De Petrocellis L & Di Marzo V (2016) From Phytocannabinoids to Cannabinoid Receptors and Endocannabinoids: Pleiotropic Physiological and Pathological Roles Through Complex Pharmacology. *Physiol Rev* 96:1593–1659.

López-Arnau R, Luján MÁ, Duart-Castells L, Pubill D, Camarasa J, Valverde O & Escubedo E (2017) Exposure of adolescent mice to 3,4-methylenedioxypyrovalerone increases the psychostimulant, rewarding and reinforcing effects of cocaine in adulthood. *Br J Pharmacol* 174:1161–1173.

Luján MÁ, Castro-Zavala A, Alegre-Zurano L & Valverde O (2018) Repeated Cannabidiol treatment reduces cocaine intake and modulates neural proliferation and CB1R expression in the mouse hippocampus. *Neuropharmacology* 143:163–175.

Mahmud A, Gallant S, Sedki F, DCunha T & Shalev U (2016) Effects of an acute cannabidiol treatment on cocaine self-administration and cue-induced cocaine seeking in male rats. *J Psychopharmacol*. 31:96-104.

Mandyam CD & Koob GF (2012) The addicted brain craves new neurons: putative role for adult-born progenitors in promoting recovery. *Trends Neurosci* 35:250–260.

Mariani JJ, Pavlicova M, Bisaga A, Nunes E V, Brooks DJ & Levin FR (2012) Extended-release mixed amphetamine salts and topiramate for cocaine dependence: a randomized controlled trial. *Biol Psychiatry* 72:950–956.

Marinho ALZ, Vila-Verde C, Fogaça MV & Guimarães FS (2015) Effects of intra-

infralimbic prefrontal cortex injections of cannabidiol in the modulation of emotional behaviors in rats: Contribution of 5HT1A receptors and stressful experiences. *Behav Brain Res* 286:49–56.

Ming G li & Song H (2011) Adult Neurogenesis in the Mammalian Brain: Significant Answers and Significant Questions. *Neuron* 70:687–702.

Niibori Y, Yu T-S, Epp JR, Akers KG, Josselyn SA & Frankland PW (2012) Suppression of adult neurogenesis impairs population coding of similar contexts in hippocampal CA3 region. *Nat Commun* 3:1253.

Noonan MA, Bulin SE, Fuller DC & Eisch AJ (2010) Reduction of Adult Hippocampal Neurogenesis Confers Vulnerability in an Animal Model of Cocaine Addiction. *J Neurosci* 30:304–315.

Norris C, Loureiro M, Kramar C, Zunder J, Renard J, Rushlow W & Laviolette SR (2016) Cannabidiol Modulates Fear Memory Formation Through Interactions with Serotonergic Transmission in the Mesolimbic System. *Neuropsychopharmacology* 41:2839–2850.

Ohsawa K, Imai Y, Kanazawa H, Sasaki Y & Kohsaka S (2000) Involvement of Iba1 in membrane ruffling and phagocytosis of macrophages/microglia. *J Cell Sci* 113:3073–3084.

Ortega-Martínez S (2015) A new perspective on the role of the CREB family of transcription factors in memory consolidation via adult hippocampal neurogenesis. *Front Mol Neurosci* 8:46.

Paxinos G & Franklin KBJ (2012) The Mouse Brain in Stereotaxic Coordinates 4th edn. *Elsevier Academic Press.*

Ren Y, Whittard J, Higuera-Matas A, Morris C V & Hurd YL (2009) Cannabidiol, a nonpsychotropic component of cannabis, inhibits cue-induced heroin seeking and normalizes discrete mesolimbic neuronal disturbances. *J Neurosci* 29:14764–14769.

Renard J, Loureiro M, Rosen LG, Zunder J, de Oliveira C, Schmid S, Rushlow WJ & Laviolette SR (2016) Cannabidiol Counteracts Amphetamine-Induced Neuronal and Behavioral Sensitization of the Mesolimbic Dopamine Pathway through a Novel mTOR/p70S6 Kinase Signaling Pathway. *J Neurosci* 36:5160–5169.

Schiavon AP, Bonato JM, Milani H, Guimarães FS & Weffort de Oliveira RM (2016) Influence of single and repeated cannabidiol administration on emotional behavior and markers of cell proliferation and neurogenesis in non-stressed mice. *Prog Neuro-Psychopharmacology Biol Psychiatry* 64:27–34.

Soria G, Barbano MF, Maldonado R & Valverde O (2008) A reliable method to study cue-, priming-, and stress-induced reinstatement of cocaine self-administration in mice. *Psychopharmacology (Berl)* 199:593–603.

Sorrells SF, Paredes MF, Cebrian-Silla A, Sandoval K, Qi D, Kelley KW, James D, Mayer S, Chang J, Auguste KI, Chang EF, Gutierrez AJ, Kriegstein AR, Mathern GW, Oldham MC, Huang EJ, Garcia-Verdugo JM, Yang Z & Alvarez-Buylla A (2018) Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. *Nature* 555:377-381.

Tatar Z, Thivat E, Planchat E, Gimbergues P, Gadea E, Abrial C & Durando X (2013) Temozolomide and unusual indications: Review of literature. *Cancer Treat Rev* 39:125–135.

Tourino C, Valjent E, Ruiz-Medina J, Herve D, Ledent C & Valverde O (2012) The

orphan receptor GPR3 modulates the early phases of cocaine reinforcement. *Br J Pharmacol* 167:892–904.

Viudez-Martínez A, García-Gutiérrez MS, Medrano-Relinque J, Navarrón CM, Navarrete F & Manzanares J (2018) Cannabidiol does not display drug abuse potential in mice behavior. *Acta Pharmacol Sin.*

Viudez-Martínez A, García-Gutiérrez MS, Navarrón CM, Morales-Calero MI, Navarrete F, Torres-Suárez AI & Manzanares J (2017) Cannabidiol reduces ethanol consumption, motivation and relapse in mice. *Addict Biol* 23:154-164.

Volkow ND, Koob GF & McLellan AT (2016) Neurobiologic Advances from the Brain Disease Model of Addiction. *N Engl J Med* 374:363–371.

Ware MA (2018) Medical Cannabis Research: Issues and Priorities. *Neuropsychopharmacology* 43:214–215.

Wei J, Carroll RJ, Harden KK & Wu G (2012) Comparisons of treatment means when factors do not interact in two-factorial studies. *Amino Acids* 42:2031–2035.

Wenzel JM & Cheer JF (2018) Endocannabinoid Regulation of Reward and Reinforcement through Interaction with Dopamine and Endogenous Opioid Signaling. *Neuropsychopharmacology* 43:103–115.

Zlebnik NE & Cheer JF (2016) Beyond the CB1 Receptor: Is Cannabidiol the Answer for Disorders of Motivation? *Annu Rev Neurosci*:1–17.

FIGURE LEGENDS

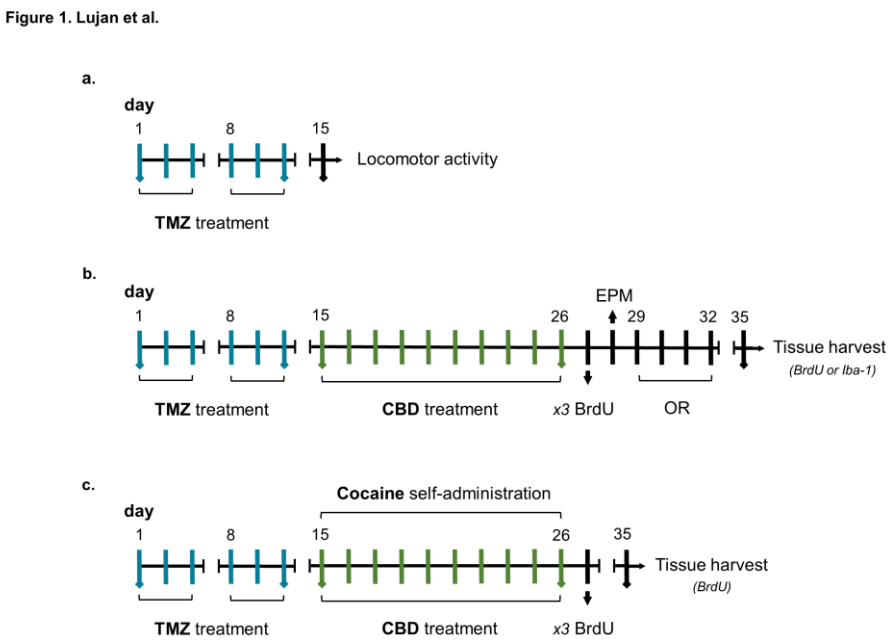

Figure 1. Schematic representation of TMZ and CBD treatments and subsequent behavioural experiments carried out with the three groups of animals. **(a)** Pharmacological and behavioural procedures carried out with the group of mice used to analyse spontaneous locomotor activity. **(b)** Combined treatment of TMZ and CBD in mice tested in the EPM and OR tests. BrdU and Iba1 immunostaining was assessed following behavioural experiments. **(c)** Combined treatment of TMZ and CBD in cocaine-consuming mice. BrdU immunostaining was analysed after behavioural procedures.

Figure 2. Lujan et al.

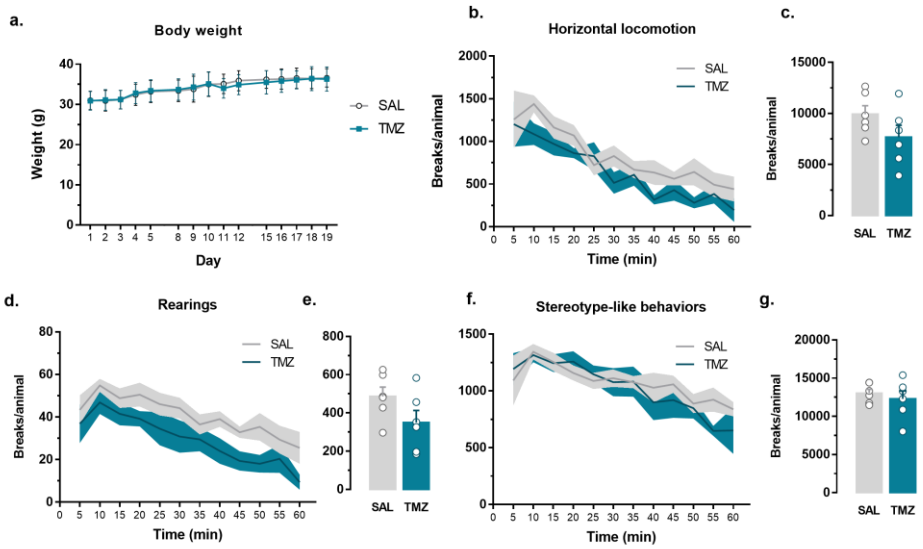

Figure 2. TMZ treatment did not show effects on body weight or spontaneous locomotor activity. **(a)** Body weight gain throughout the TMZ treatment (n = 36 animals/group). Lines (± SD) represent mean group body weight. **(b)** Time course of spontaneous horizontal locomotor activity for 60 min in the locomotor activity boxes (n = 6 animals/group). **(c)** Accumulated values of horizontal locomotor activity in the locomotor activity boxes (n = 6 animals/group). **(d)** Time course of number of rearings in the locomotor activity boxes (n = 6 animals/group). **(e)** Accumulated rearing values in the locomotor activity boxes (n = 6 animals/group). **(f)** Time course of stereotype-like behaviour for 60 min (n = 6 animals/group). **(g)** Accumulated values of stereotype-like behaviour in the open field test (n = 6 animals/group). Connecting lines represent mean of cumulative photobeam breaks per animal. SEM is represented as colour-specific surrounding area fill.

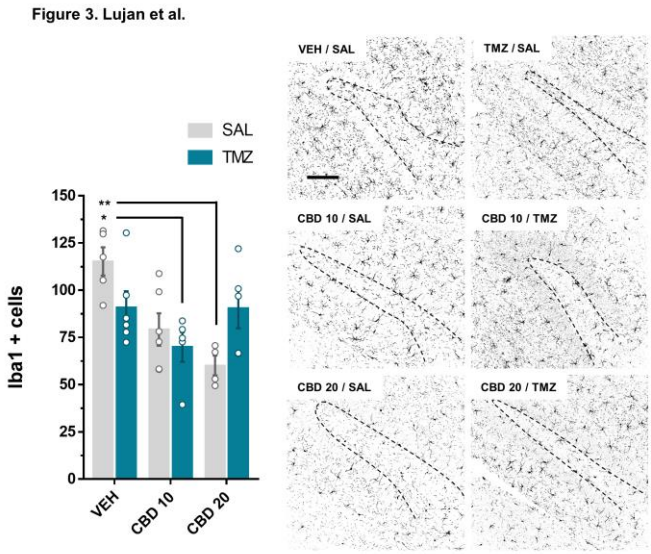

Figure 3. TMZ treatment did not induce neuro-inflammatory Iba1 activation in the DG, while CBD decreased it. Right panel summarizes data represented as means (± SEM) of Iba1 labelled cells (n = 4-5/group) (Tukey, * $p < 0.05$ ** $p < 0.01$ vs. indicated group). Left panel represents confocal sections of the DG, showing immunofluorescence for the microglial proliferation and activation marker Iba1 (black). Dashed lines indicate the subgranular cell layer of the dentate gyrus for better structure recognition. Scale bar = 150 µm (20x magnification).

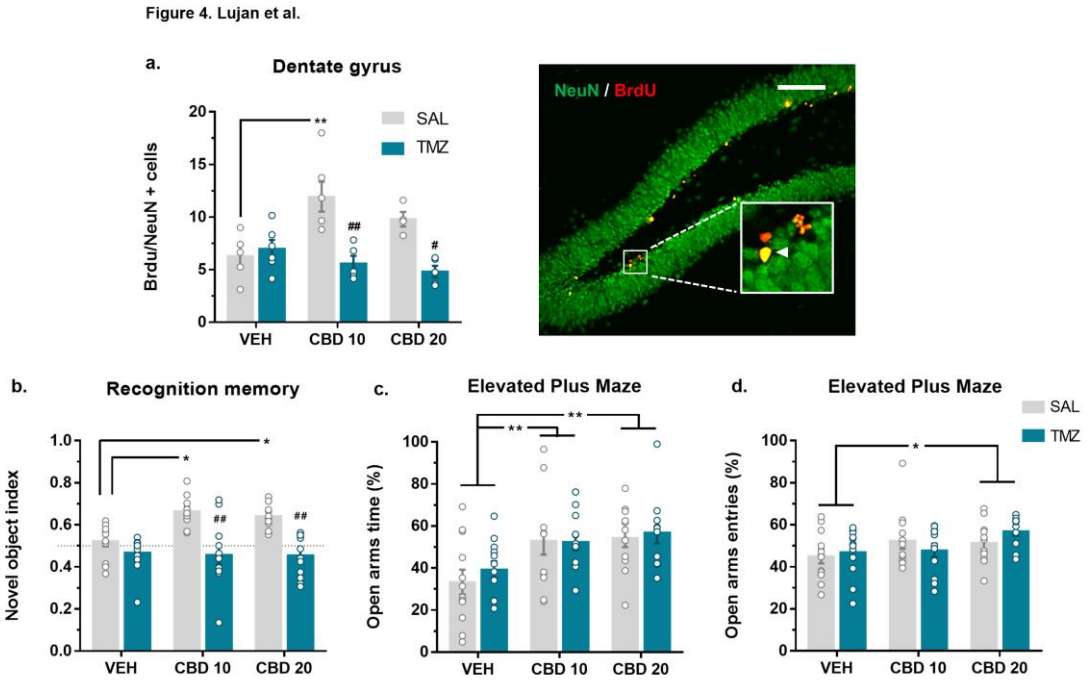

Figure 4. TMZ-induced blockade of CBD pro-neurogenic effects and behavioural consequences in the EPM and the OR tests. **(a)** Left panel summarizes data represented as means (± SEM) of BrdU/NeuN double-labelled cells (n = 5/group). Right panel represents representative confocal section of the DG, showing immunofluorescence for BrdU (red) and NeuN (green) in animals treated with TMZ and receiving CBD 10 and 20 mg/kg treatment. White arrow indicates a representative case of BrdU/NeuN double-labelled cell. Scale bar = 150 µm (20x magnification). **(b)** Discrimination index in the OR test shown during the retention trial (n = 12/group). **(c)** Percentage of time spent in the open arms (s) of the maze (n = 12/group). **(d)** Percentage of open arms entries (s) (n = 12/group). Tukey; * $p < 0.05$ ** $p < 0.01$ vs. indicated group; ## $p < 0.01$, # $p < 0.05$ vs. its respective SAL-pre-treated group.

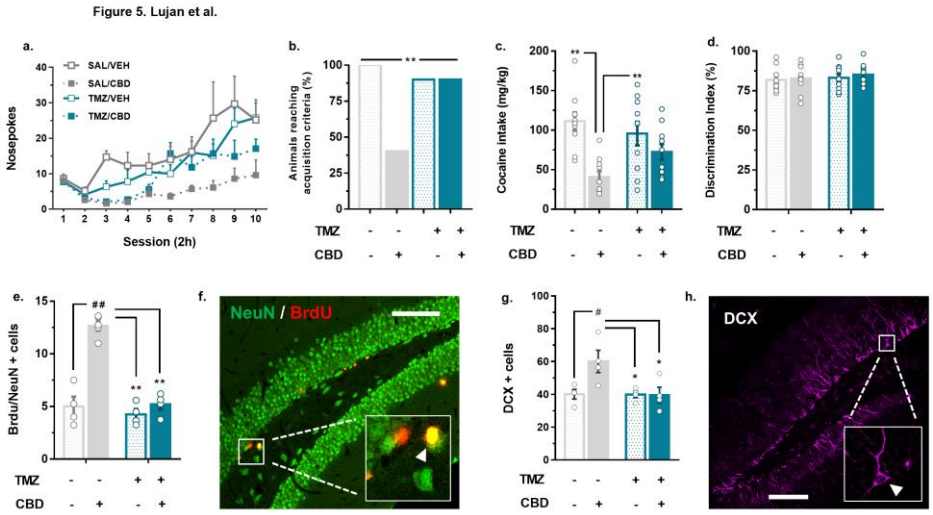

Figure 5. TMZ-induced blockade of CBD pro-neurogenic effects and reversed CBD effects on cocaine voluntary intake in the self-administration paradigm. **(a)** Nosepoke activations during cocaine self-administration sessions under an FR1 reinforcement schedule (n = 10/group). **(b)** Difference in stable self-administration acquisition ratios between treatments (Chi-square, ** $p < 0.001$). **(c)** Total cocaine intake (mg/kg) throughout the self-administration procedure (n = 10/group). **(d)** Mean daily discrimination index between active and inactive nosepoke holes (n = 10/group). **(e)** BrdU/NeuN double-labelled cells after cocaine self-administration (n = 4-5/group; means ± SEM). **(f)** Image panel represents representative confocal section of the DG, showing immunofluorescence for BrdU (red) and NeuN (green) in animals treated with TMZ and receiving CBD 20 mg/kg during cocaine self-administration. **(g)** Mean number (± SEM) of DCX cells after cocaine self-administration (n = 4/group). **(h)** Representative confocal section of the DG, showing immunofluorescence for DCX (magenta) in animals treated with TMZ and receiving CBD 20 mg/kg during cocaine

self-administration. White arrow indicates a representative case of DCX or BrdU/NeuN cell. Scale bar = 150 μm (20x magnification). Tukey; * $p < 0.05$ ** $p < 0.01$ vs. indicated group; # $p < 0.05$ ## $p < 0.01$ vs. indicated group.