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The anxiolytic effects of cannabidiol in chronically stressed mice are mediated by the endocannabinoid system: Role of neurogenesis and dendritic remodeling

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ABSTRACT

Repeated injections of cannabidiol (CBD), the major non-psychotomimetic compound present in the *Cannabis sativa* plant, attenuate the anxiogenic effects induced by Chronic Unpredictable Stress (CUS). The specific mechanisms remain to be fully understood but seem to involve adult hippocampal neurogenesis and recruitment of endocannabinoids. Here we investigated for the first time if the behavioral and pro-neurogenic effects of CBD administered concomitant the CUS procedure (14 days) are mediated by CB_1, CB_2 or $5HT_{1A}$ receptors, as well as CBD effects on dendritic remodeling and on intracellular/synaptic signaling (fatty acid amide hydrolase - FAAH, Akt, GSK3β and the synaptic proteins Synapsin Ia/b, mGluR1 and PSD95). After 14 days, CBD injections (30 mg/kg) induced anxiolytic responses in stressed animals in the elevated plus-maze and novelty suppressed feeding tests, that were blocked by pre-treatment with a CB_1 (AM251, 0.3 mg/kg) or CB_2 (AM630, 0.3 mg/kg), but not by a $5HT_{1A}$ (WAY100635, 0.05 mg/kg) receptor antagonist. Golgi staining and immunofluorescence revealed that these effects were associated with an increase in hippocampal neurogenesis and spine density in the dentate gyrus of the hippocampus. AM251 and AM630 abolished the effects of CBD on spines density. However, AM630 was more effective in attenuating the pro-neurogenic effects of CBD. CBD decreased FAAH and increased p-GSK3β expression in stressed animals, which was also attenuated by AM630. These results indicate that CBD prevents the behavioral effects caused by CUS probably due to a facilitation of endocannabinoid neurotransmission and consequent CB_1/CB_2 receptors activation, which could recruit intracellular/synaptic proteins involved in neurogenesis and dendritic remodeling.

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1. Introduction

Disruption of neuroplasticity mechanisms in limbic structures by chronic stress has been associated with the precipitation of anxiety and mood disorders symptoms (Post, 1992). Stress impairs neurogenesis and reduces dendritic complexity, as well as the expression of proteins involved in synaptogenesis and survival in the hippocampal formation (Duman and Duman, 2015; Samuels and Hen, 2011). In this sense, drugs that attenuate stress or restore these processes have potential therapeutic applications.

Cannabidiol (CBD) is the major non-psychotomimetic cannabinoid present in the *Cannabis sativa* plant. Clinical and pre-clinical studies indicate that this compound has anxiolytic, antidepressant, anticonvulsive, neuroprotective and antipsychotic properties (Campos et al., 2016; Fogaça et al., 2016). Although the mechanisms of this wide range of therapeutic actions are still not well understood, CBD effects can involve $5HT_{1A}$ and peroxisome proliferator-activated (PPARγ) receptors activation (Esposito et al., 2011; Russo et al., 2005). Moreover, even though CBD has low affinity for cannabinoid CB_1 and CB_2 receptors, it may enhance endocannabinoid (eCB) neurotransmission by interfering with the function of fatty acid amide hydrolase (FAAH), the enzyme responsible for the

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degradation of the eCB anandamide (Bisogno et al., 2001; Leweke et al., 2012; Watanabe et al., 1996).

Although several studies have explored the mechanisms of CBD action after acute administration on anxiety-related behaviors, the neuroplastic changes induced by repeated injections of the drug have not been well clarified. Recently, we showed that the anxiolytic effects produced by CBD in animals submitted to the Chronic Unpredictable Stress (CUS) model depend on the capacity of the adult hippocampus to generate new neurons (Campos et al., 2013b). Adult hippocampal neurogenesis reflects the property of neural progenitor cells located in the subgranular zone (SGZ) of the dentate gyrus to proliferate and migrate to the granular zone (GZ), where they can differentiate in to mature granule neurons (Kempermann, 2008). The different stages of neurogenesis can be detected by using specific marker, in combination with 5-bromo-2′-deoxyuridine (BrdU), a thymidine analogue that is incorporated into the DNA of newborn cells. For example, doublcortin (DCX) is a microtubule-associated protein present only in precursor cells and immature neurons that are in the differentiation/migration phase. On the other hand, NeuN is a nuclear protein only found in mature neurons (Kempermann, 2008). Ablation of specific population of precursor cells in the hippocampus (nestin positive or GFAP-positive cells) facilitates anxiety-like behaviors in mice (Revest et al., 2009; Campos et al., 2013b; Snyder et al., 2011). Moreover, intact adult hippocampal neurogenesis is involved in the anxiolytic effects of repeated antidepressant treatment in mice (Santarelli et al., 2003; David et al., 2009). Neurogenesis is proposed to play an important role in buffering stress response by activating the hippocampus-induced negative control of hypothalamic-pituitary-adrenal axis and influencing memory formation and learning (Kempermann, 2008; Snyder et al., 2011).

Modifications in hippocampal-dependent functions are frequently observed in psychiatric patients, a feature also reported in animal models based on the exposure to chronic stressors (David et al., 2010; Christian et al., 2014; Kang et al., 2016). Stress causes important changes in hippocampal function, and the number and the intensity of stressful life events are thought to be key contributing factors (but not the sole cause) for the precipitation of symptoms related to psychiatric conditions (Mundt et al., 2000; Slopen et al., 2011). The specific mechanism responsible for the effects of stress that promotes impaired hippocampal function is still unknown, but has been attributed to, among others, maladaptative plasticity changes related to neuronal atrophy or loss, decreased complexity and length of dendrites, as well as decreased spines density that has been reported in rodent models of chronic stress or glucocorticoid exposure (Bremner et al., 2002; Licznerski and Duman, 2013; Rajkowska, 2000; Rajkowska et al., 1999; Woolley et al., 1990).

Neurogenesis, synaptogenesis and neuronal survival are in part mediated by glycogen synthase kinase 3β (GSK3β) signaling. Several psychotropic drugs, including antidepressants, antipsychotics and mood stabilizers, increase the phosphorylation and consequent inactivation of GSK3β, mainly through Akt (O'Leary and Nolan, 2015). This process allows the transduction of factors such as β-catenin to migrate to the nucleus and induce the transcription of proteins involved in cytoskeleton organization, neuronal polarization and survival. GSK3β and synaptic proteins, for example the vesicular protein Synapsin Ia/b and Post-Synaptic Density 95 (PSD95), a membrane-associated guanylate cyclase that acts as NMDA-receptor scaffolding protein, are also involved in dendritic remodeling (Duman and Duman, 2015).

Despite current advances showing that CBD induces anxiolytic effects in different animal models and clinical studies, there have been no studies investigating the pharmacological mechanisms and intracellular pathways that underlie the effects of this drug on anxiety, neurogenesis, and dendritic remodeling in chronically stressed mice. The present work investigated for the first time if the anti-stress and pro-neurogenic responses to CBD depend on CB_1 and/or CB_2 receptor activation. Also, as CBD has a low affinity for CB_1/CB_2 receptors, we evaluated if CBD could inhibit FAAH expression in stressed animals, highlighting the hypothesis that its effects could be indirectly mediated by eCBs. Also, we investigated if CBD prevents the effects of stress on dendritic remodeling and synaptic proteins expression, as well as if CBD regulates intracellular pathways that are known to be involved in cytoskeleton organization and cell survival, including Akt/GSK3β, in the hippocampus.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (20–26 g, 8–9 weeks) from the Central Animal of the Medical School of Ribeirão Preto (FMRP-USP, Brazil) or Jackson Laboratories (New Haven, USA) were maintained in groups of five animals per cage in a temperature-controlled room (23 ± 2 °C) with a 12/12 h light-dark cycle (lights on: 6h30 a.m./lights off: 6h30 p.m.). Animals were maintained at the Department of Pharmacology Facility (FMRP-USP, Brazil) or the Ribicoff Facility (Connecticut Mental Health Center, USA). They received water and food *ad libitum* throughout the study period, except when food or water deprivation was used as stressors in the CUS or when animals were submitted to the Novelty Suppressed Feeding (NSF) test. Procedures were conducted in conformity with the Brazilian Society of Neuroscience and Behavior and the National Institute of Health (NIH) guidelines for the care and use of laboratory animals. The local Ethics Committees approved the experimental procedures. The total number of animals used in the present study was 354.

2.2. Drugs

BrdU (Sigma-Aldrich, USA, 100 mg/kg); CBD (THC Pharma, Germany; 30 mg/kg); CB_1 receptor antagonist AM251 (Tocris, USA; 0.3 mg/kg) and CB_2 receptor antagonist AM630 (Abcam Biochemicals, USA; 0.3 mg/kg); $5HT_{1A}$ receptor antagonist WAY100635 (Sigma-Aldrich, EUA; 0.05 and 0.1 mg/kg). All drugs were administered intraperitonially (i.p.). BrdU was dissolved in sterile saline; all other drugs were diluted in 2% Tween 80 in 0.9% sterile saline and the doses were chosen based on previous works (Campos et al., 2013b; Griebel et al., 2000; Ichikawa and Meltzer, 1999, 2000).

2.3. Overall experimental design

Four major independent experiments were conducted in which we evaluated: I. if AM251 (experiment 1) or AM630 (experiment 2) could block CBD effects on behavior, neurogenesis and protein expression in CUS animals; II. if WAY100635 could block the anxiolytic-like effect induced by CBD in CUS animals (experiment 3) and, III. if CBD effects in facilitating dendritic remodeling could be mediated by CB_1 and/or CB_2 receptors (experiment 4). For each experiment, animals were divided into 5 to 8 groups, consisting of non-stressed controls and animals submitted to CUS (14 days, stressors started at 3 p.m.). Concomitantly to the CUS procedure animals received daily injections (i.p., 14 days/once a day) of vehicle, AM251 (0.3 mg/kg), AM630 (0.3 mg/kg) or WAY100635 (0.05 mg/kg) followed, 10 min after, by vehicle or CBD (30 mg/kg). The injections were administered 2 h after the daily stressor (Fig. 1A and B). For immunofluorescence experiments (neurogenesis), animals were submitted to the same protocol as described above. In

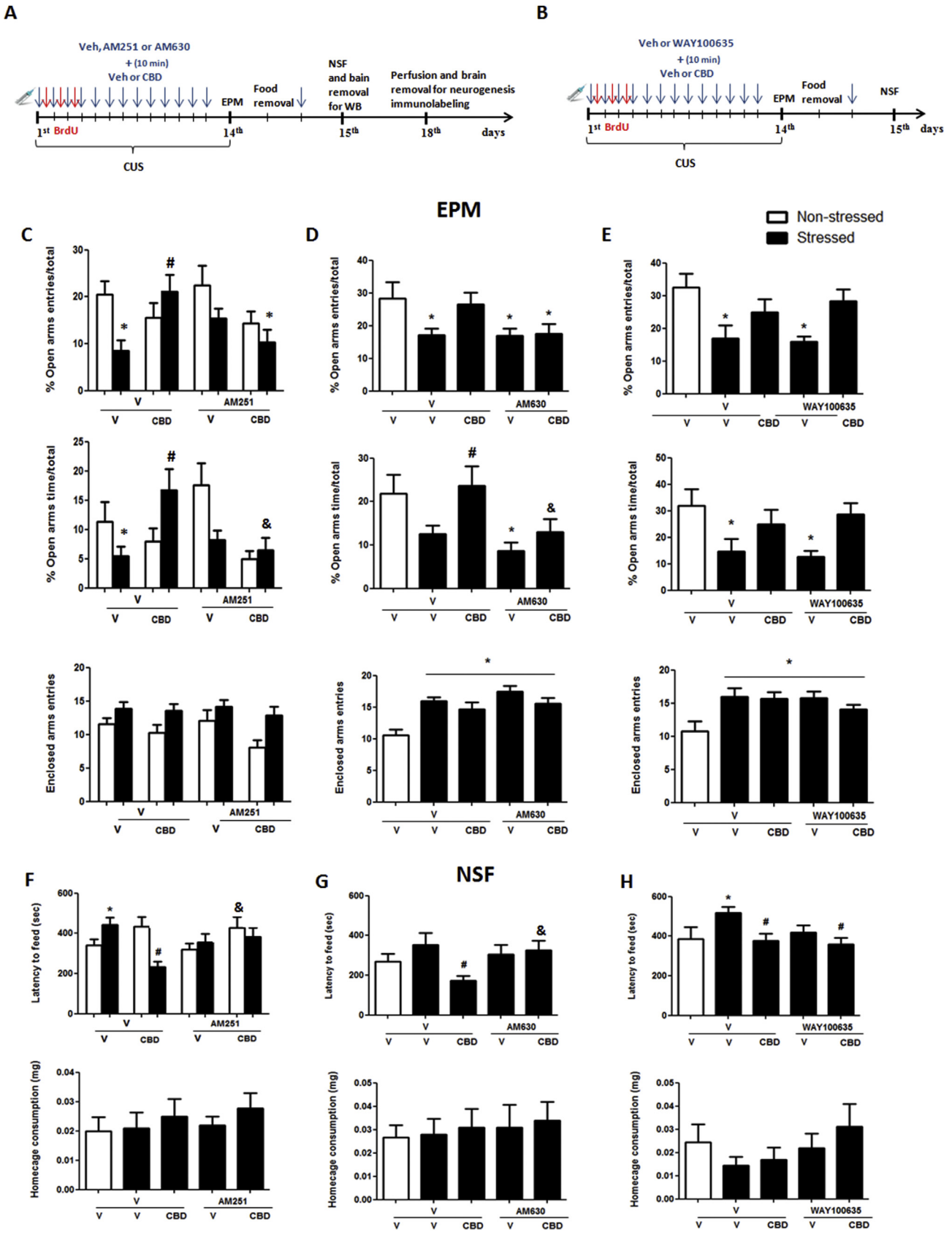


Fig. 1. The anxiolytic-like effect of CBD in chronically stressed mice is mediated by CB_1 and CB_2 receptors activation in the EPM and NSF tests. *(A)* Time-course of the experiments conducted with pre-administration of AM251 or AM630 and *(B)* WAY100635. Pre-treatment with the CB_1 receptor antagonist AM251 (*C* and *F)* or the CB_2 antagonist AM630 *(D* and *G)*, but not the $5HT_{1A}$ WAY100635 *(E* and *H)*, prevented the anxiolytic-like effect of CBD in stressed animals. No significant effect was found in homecage food consumption (*F, G* and *H*). Each bar represents the mean ± standard error of the mean (S.E.M.). One- *(D, E, G and H)* or Three-way ANOVA *(C and F)* followed by Duncan test. $^{*}p < 0.05$ compared to vehicle non-stressed group; #$p < 0.05$ compared to vehicle stressed group; & $p < 0.05$ compared to CBD stressed group. n = 8–13/group.

addition, mice received daily injections of BrdU (i.p., 100 mg/kg) for 3 consecutive days at the beginning of the CUS procedure; BrdU was administrated 10 min before the first drug injection.

On day 13th, mice were stressed 19 h before the EPM test (day 14). After the test, food was removed at 2 p.m. and the animals were treated 2 h later. The NSF test was performed 22 h after the last injection (day 15). Soon after the NSF, brains were removed and the hippocampus extracted to perform the western blotting (WB) analysis. For Golgi-Cox staining, brains were removed 24 h after the NSF (day 16, Fig. 4A). For immunolabeling, animals were perfused and had their brains removed on day 18, as at this time-point it is possible to detect neurons that migrated from the SGZ to the GZ and are in an initial maturation stage (Fig. 1A and B). In this case, from days 15th to 18th animals were not submitted to any procedure or treatment.

2.4. Chronic unpredictable stress

Mice were daily exposed to a sequence of randomized unpredictable stressors starting at the afternoon in a fixed time (3 p.m.) for 14 days (Fig. 1). Animals were submitted to a single stressor per day and 2 h after the drugs were administered for 14 days (Campos et al., 2013b).

The following stressors were used: cage or bedding alterations (sawdust removal, wet sawdust or inclined cage for 20 h), restraint stress (2 h), forced swimming (12 min), reversal of light/dark cycle (12/12 h), water or food deprivation (18 h).

2.5. Elevated plus-maze (EPM) test

The EPM was located in a sound attenuated and temperature controlled room (23 °C), which had an incandescent light (60 lux) placed 1.3 m away from the maze. On the 14th day, approximately 20 h after the previous injection, the mouse was placed on the central platform with the head facing one of the enclosed arms. The test lasted for 5 min and was video-recorded. The percentage of open arms entries (Peo), the time spent in these arms (Pto) and the number of enclosed arms entries were analyzed by Anymaze Software (version 4.5, Stoelting, Wood Dale-USA). Animals were only considered to enter an arm when 90% of their bodies were inside the region. All EPM tests were performed in the morning (8 a.m.–12 p.m.).

2.6. Novelty suppressed feeding (NSF) test

On the 15th day, 22 h after the last injection, animals were submitted to the NSF test during the afternoon (Campos et al., 2013b). Mice were 24-h food deprived and exposed, up to 10 min, to a cage (40 × 40 × 20 cm) containing approximately 2 cm of sawdust covering the floor and a central platform with a pellet of food illuminated by a bright light. Water was available *ad libitum* during the 24-h food deprivation. The latency to start eating in this new environment was measured. It was considered that the animals were eating if they remained at least 10 s feeding on the central platform. Soon after, as a control to assure that drugs or CUS did not alter food intake, the animals were placed in a familiar homecage, and the amount of food consumed in 10 min was measured.

2.7. Immunofluorescence and immunohistochemistry

On day 18th, the mice were anesthetized with 5% chloral hydrate (10 mL/kg) and perfused with 10% paraformaldehyde. Brains were removed and kept in formalin solution for 24 h and placed in a 30% sucrose solution during 30 h. They were cut into 30-μm thick sections in a cryostat (Cryocut, 1800, Leica, Heerbrugg-Switzerland) to obtain slices containing the hippocampus. To BrdU/NeuN immunofluorescence, the sections were firstly incubated in an HCl solution (2N, 37 °C, 30 min) and washed with boric acid (0.1 M, pH = 8.9) and PBS. To doublecortin (DCX) immunohistochemistry, sections were incubated in citrate buffer (70 °C, 30 min) and washed with PBS. For all experiments, sections were incubated in a 1% bovine serum albumin (BSA) for 2 h and overnight with the primary antibodies (goat anti-doublecortin 1:300 Santa Cruz Biotechnology; rat anti-BrdU 1:100 Abcam; mouse anti-NeuN 1:1000 Millipore). Then the sections were incubated with specific secondary antibodies for 2 h (Santa Cruz Biotechnology: anti-goat HRP, kit Vecstatin. Life Technologies: AlexaFluor® 488 goat anti-rat and AlexaFluor® 647 goat anti-mouse 1:1000). DCX slices were stained using diaminobenzidin (10 min). After the immunolabeling procedures, the sections were positioned in slices containing Fluoromout or Permount (Sigma-Aldrich) and coverslip.

For immunohistochemistry, DCX + cells were analyzed by light microscopy (Olympia, 20 and 40 ×), in which we measured the total number of DCX cells present in the SGZ of the dentate gyrus and the number of neurons that were outside the SGZ and considered to be migrating to the GZ. For this, we traced the dentate gyrus area from the SGZ using a Software (Image ProPlus) and all neurons that did not have their soma completely inside the SGZ were considered in a migration phase (Fig. 2E). For immunofluorescence, BrdU/NeuN$^+$ cells were measured obtaining z-stacks images sequence in the confocal microscope (Leica TSE-SPE, objectives 20 and 40 ×. Gain: 820; Offset: −2.5). In all experiments, 8 to 12 dentate gyrus slices (without distinguishing between right and left) were analyzed per animal, distributed over defined plans according to the Paxinos and Franklin Atlas [2007 (Plans 42–49)]. Results were expressed by dividing the total number of positive cells for the total sum of the dentate gyrus areas analyzed (number of positive cells/mm^2). An experimenter that was blind to the treatment groups performed all analysis.

2.8. Western blotting

Soon after the NSF test, the animals were sacrificed and the hippocampus removed. Tissues were macerated to protein extraction with a lysis buffer (NaCl 137 mM, Tris-HCl 20 mM pH = 7.6, 10% glycerol, NaF 100 mM, $NaVO_3$ 10 mM, 0.1% Triton-X-100) and protease inhibitor cocktail (Sigma-Aldrich). The samples were centrifuged (12000 rpm, 10 min, 4 °C) and the supernatant preserved. For the experiments performed using the synaptosome fraction (synaptic proteins), a group of animals had their hippocampus homogenized in a solution containing sucrose 0.32 M, Hepes 20 mM, EDTA 1 mM, NaF 5 mM, $NaVO_3$ 1 mM and protease inhibitor cocktail. The homogenate was centrifuged (2800 rpm, 10 min, 4 °C) and the supernatant discharged. The pellets containing synaptosomes were resuspended in a lysis buffer (NaCl 150 mM, Tris-HCl 50 mM pH = 7.4, 1% Triton-X-100, 0.1% SDS, EDTA 2 mM, $NaVO_3$ 1 mM, NaF 5 mM). Total proteins were quantified using Bradford or Pierce methods. Homogenates were submitted to a SDS-PAGE protein separation in 8–16% bis-acrilamide gels and transferred to a PVDF membrane (Bio-Rad). The membranes were incubated in 3% BSA for 2 h. Soon after, they were incubated overnight (4 °C) with the primary antibodies (Santa Cruz Biotechnology: goat anti-FAAH 1:500, goat anti-Synapsin Ia/b 1:1000, rabbit anti α1a-tubulin 1:3000, rabbit anti-β-actin 1:3000. Cell Signaling: rabbit anti-phospho-Akt Ser473 1:500, rabbit anti-phospho-Akt Thr308 1:500, rabbit anti-Akt 1:1000; rabbit anti-phospho GSK3β Ser9 1:1000, mouse anti-GSK3β 1:1000, rabbit anti-PSD95 1:1000, rabbit anti-mGluR1 1:1000) and with the specific secondary antibodies for 1 h (anti-mouse, anti-goat or anti-rabbit HRP 1:1000, Santa Cruz

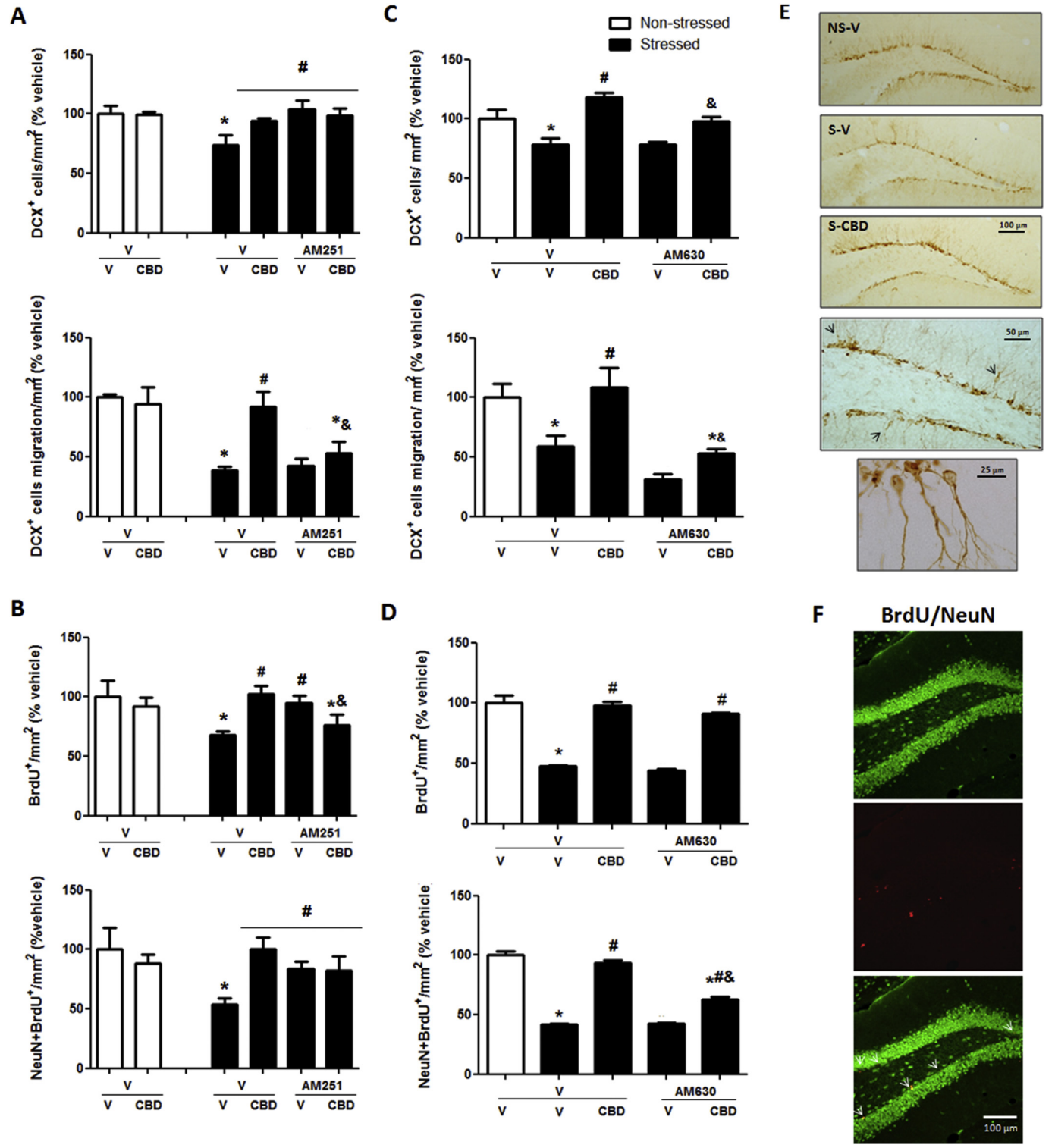


Fig. 2. The pro-neurogenic effect of CBD in chronically stressed mice is differently mediated by CB_1 and CB_2 receptors. Pre-treatment with the CB_2 antagonist AM630 (*C* and *D*), but not the CB_1 antagonist AM251 (*A* and *B*), prevented CBD effect in increasing the number of DCX^+ and $BrdU/NeuN^+$ neurons. Both antagonists abolished CBD effect in increasing DCX^+ cells migration from the subgranular zone (SGZ) to the granular zone (GZ) of dentate gyrus (*A* and *C*). AM251 *(B)*, but not AM630 *(D)*, prevented CBD effect in increasing the number of $BrdU^+$ cells. *(E)* Shows a representative photomicrography of DCX^+ neurons in the dentate gyrus of non-stressed vehicle (NS-V), stressed vehicle (S–V) and stressed CBD groups (S-CBD, light microscope, 20 ×). The black arrows indicate cells that migrated from the SGZ to the GZ (40 ×). *(F)* Indicates a representative photomicrography of $BrdU^+$, $NeuN^+$ and $BrdU/NeuN^+$ cells (confocal microscope, 20 ×). The white arrows indicate double stained cells ($BrdU/NeuN^+$). Each bar represents the mean ± standard error of the mean (S.E.M.). One-way ANOVA followed by Duncan test. *p < 0.05 compared to vehicle non-stressed group; #p < 0.05 compared to vehicle stressed group; & p < 0.05 compared to CBD stressed group. n = 4–6/group.

Biotechnologies) and revealed with a chemiluminescent reagent (ECL, GE Healthcare) or a colorimetric kit (4CN, PelkinElmer). Unsaturated, normalized and preserved bands had their density quantified using the Software Image Studio Digits (3.1) or ImageLab (BioRad). Results were expressed as percentage of the non-stressed vehicle.

2.9. Golgi-Cox method

On the 16th day, 24 h after the NSF test, animals were sacrificed by cervical displacement, and their brains were removed. The Golgi-Cox procedure was performed using the FD Rapid Golgi Stain Kit (FD Neurotechnologies, USA) accordingly to the manufacturer recommendations. At the end of the process, brains were rapidly frozen in isopentane and dry ice and cut into 100-μm thick sections in a cryostat (Cryocut, 1800, Leica, Heerbrugg- Switzerland) to obtain slices containing the hippocampus. The sections were transferred to gelatinized slides and two days after drying at room temperature they were stained and submitted to alcohol-induced dehydration. Finally, the slides were covered with Permount and coverslips.

Golgi-Cox impregnated neurons located in the dentate gyrus were analyzed using a light microscope (Zeiss, 60 × or Olympia, 100 ×). Neurons were traced by Neurolucida software (MBF Biosciences), and an experimenter blind to experimental conditions measured the total size of the dendritic tree, the number of branches and the number of dendritic spines in 10 μm-branches classified as secondary and tertiary. Six neurons per animal were analyzed using the following criteria: the neurons were relatively isolated, displayed a defined cell body and a complete dendritic tree evidenced by well-defined endings, and presented intact primary, secondary and tertiary branches.

2.10. Statistical analysis

The results were analyzed by Student's t-test, one-, two-, or three-way ANOVA, as appropriate, followed by the Duncan test. For two- or three-way ANOVA factors were treatment 1 and/or treatment 2 and stress. The post-hoc analysis was only performed in case of significant interactions between factors. Results were expressed as means ± standard errors, and the significance level was set at $p \leq 0.05$. For all analysis, we used the SPSS Software (Version 20.0).

3. Results

3.1. Anxiolytic effects of CBD in CUS mice are mediated by CB_1 and CB_2, but not $5HT_{1A}$ receptor activation

First, to evaluate if the anti-stress effects induced by CBD could be mediated by CB_1, CB_2 and/or $5HT_{1A}$ receptors, we submitted the animals to CUS (14 days) and injected daily vehicle or the antagonists (AM251 – experiment 1, AM630 – experiment 2 or WAY100635 – experiment 3) followed, 10 min later, by vehicle or CBD. At the end of the procedure, the animals were submitted to the EPM test and, 24 later, to the NSF. The experimental design is summarized in Fig. 1A and B. Repeated CBD administration prevented the anxiogenic effects of CUS measured in the EPM and in the NSF test (Fig. 1, Three-way ANOVA followed by Duncan test, $p < 0.05$.*1C*: Peo: T1*T2*Stress, $F_{7,95} = 2.39$, $p < 0.05$. Pto: T1*T2, $F_{7,95} = 8.00$, $p < 0.05$; T2*Stress, $F_{7,95} = 10.93$, $p < 0.05$.*1F*: T1*T2*Stress, $F_{7,70} = 4.32$, $p < 0.05$. One-way ANOVA followed by Duncan test, $p < 0.05$, *1D:* Peo: $F_{4,47} = 3.09$, $p < 0.05$. Pto: $F_{4,47} = 3.70$, $p < 0.05$.*1E:* Peo: $F_{4,43} = 4.50$, $p < 0.05$. Pto: $F_{4,43} = 2.92$, $p < 0.05$.*1F*: $F_{4,44} = 2.67$, $p < 0.05$.*1H*: $F_{4,45} = 2.48$, $p < 0.05$). The anxiolytic effects of CBD were prevented by pre-administration of the CB_1 and CB_2 selective antagonists, AM251 and AM630, respectively, but not by the 5-HT1A receptor antagonist WAY100635 in the EPM and NSF. The lower dose of WAY100635 chosen to perform the experiments (0.05 mg/kg) was based on previous dose-response studies, in which the higher (0.1 mg/kg) produced anxiolytic-like effects in the EPM (Figure S1A, One-way ANOVA followed by Duncan test. Pea: $F_{3,26} = 4.05$, $p < 0.05$*)* and tended to increase food intake in the NSF (Figure S1B: $F_{3,26} = 2.72$, $p = 0.09$). CUS increased enclosed arm exploration in comparison to control animals, suggesting a stress-induced hyperlocomotion (One-way ANOVA, $p < 0.05$. Fig. 1D: $F_{3,26} = 3.54$, $p < 0.05$.*1E*: $F_{4,47} = 8.88$, $p < 0.05$). This effect, however, could not be confirmed when total number of crossings and distance travelled in the maze was evaluated (Table S1, $p > 0.05$). None of the treatments altered homecage food consumption (Fig. 1F, G and 1H, $p > 0.05$). CBD did not induce behavioral responses in non-stressed animals ($p > 0.05$). Stress tended to increase corticosterone levels in comparison to non-stressed animals (Figure S2, t-Student test, $t_9 = 2.16$, $p = 0.058$).

3.2. Neurogenic effects of CBD in CUS mice are mediated differentially by CB_1 and CB_2 receptors

To evaluate if the pro-neurogenic effect induced by CBD could be mediated by the eCB system, the animals that were submitted to the procedure described above (experiment 1 and experiment 2) were sacrificed and perfused three days after the NSF test. Soon after, their brains were removed to perform immunostaining for DCX and BrdU/NeuN (Fig. 1A and B). CUS decreased the total number of DCX^+ cells, the number of cells that migrated from the SGZ to the GZ of the dentate gyrus, the total number of $BrdU^+$ cells, and double-labeled BrdU/$NeuN^+$ neurons (Fig. 2, One-way ANOVA followed by Duncan test, $p < 0.05$. Total DCX^+: *2A*: $F_{5,22} = 3.40$, $p < 0.05$; *2C*: $F_{4,22} = 12.96$, $p < 0.05$. DCX^+ cells migration: *2A*: $F_{5,22} = 7.71$, $p < 0.05$; *2C*: $F_{4,22} = 7.48$, $p < 0.05$. $BrdU^+$: *2B*: $F_{5,21} = 3.12$, $p < 0.05$; *2D*: $F_{4,23} = 76.90$, $p < 0.05$. BrdU/$NeuN^+$: *2B*: $F_{5,21} = 3.19$, $p < 0.05$; *2D*: $F_{4,23} = 157.54$, $p < 0.05$). CBD prevented all of these effects. AM251 treatment alone increased the number of DCX^+ and $BrdU^+$ cells but, when combined with CBD, attenuated its effects on cell migration and $BrdU^+$ cell number. AM630 treatment alone did not induce any effect on neurogenesis but attenuated the effects of CBD on the number of DCX^+ cells, cell migration and the number of double-labeled BrdU/$NeuN^+$ neurons, but not on total $BrdU^+$ cell number. CBD did not induce any effect in non-stressed animals.

3.3. Repeated CBD administration alters FAAH and GSK3β expression in CUS mice via CB_2 receptors activation

To evaluate if chronic treatment with CBD could produce alterations in FAAH expression and/or p-GSK3β, at the end of the last behavioral test of experiments 1 and 2 (NSF, Fig. 1A and B), animals were sacrificed, and their hippocampi were removed to perform WB analysis. The CUS procedure did not induce any effect on FAAH expression (Fig. 3A, $p > 0.05$) or GSK-3β phosphorylation (Fig. 3B and C, $p > 0.05$) in comparison to non-stressed controls. However, CBD decreased FAAH expression in the hippocampus of stressed animals in comparison to stressed vehicle (Fig. 3A, One-way ANOVA followed by Duncan test, $F_{2,38} = 3.88$, $p < 0.05$) and increased phospho-GSK3β levels in comparison to stressed and non-stressed vehicle animals (Fig. 3B, Two-way ANOVA followed by Duncan test, $F_{3,42} = 4.63$, $p < 0.05$). The latter effect was abolished by pre-treatment with AM630 but not AM251 (Fig. 3C, One-way ANOVA followed by Duncan test, $F_{4,45} = 5.03$, $p < 0.05$). No

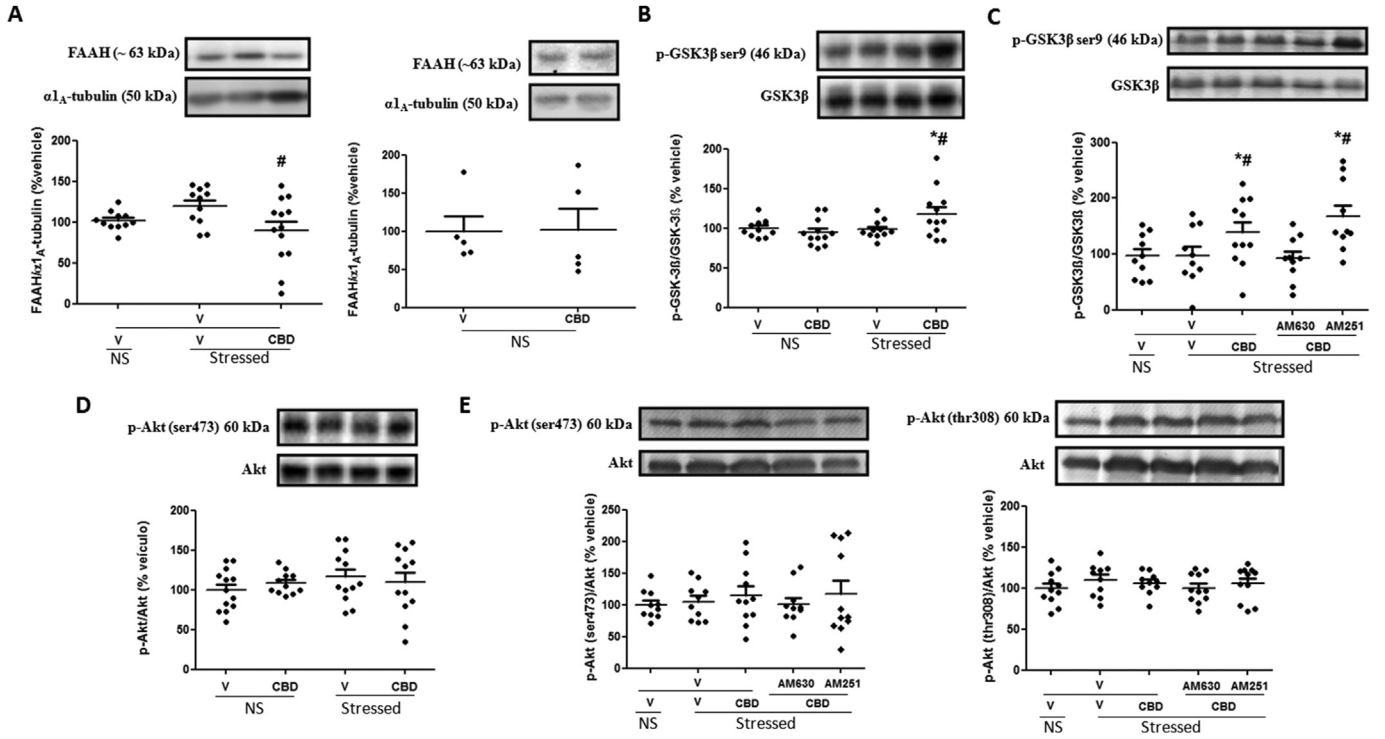


Fig. 3. CBD decreases FAAH expression and increases p-GSK-3β through CB_2 receptors. CBD decreased FAAH expression in comparison to stressed animals *(A)* and increased p-GSK3β (*B*) that was prevented by AM630 but not AM251 *(C)*. CBD did not alter p-Akt (*D* and *E*) and did not alter FAAH, p-GSK3β or p-Akt in non-stressed (NS) animals. Student's t-test *(A)*, One- *(A, C and E)* or Two-way ANOVA *(B and D)* followed by Duncan test. *p < 0.05 compared to vehicle non-stressed group; #p < 0.05 compared to vehicle stressed group; & p < 0.05 compared to CBD stressed group. n = 10–13/group.

significant effect was found for Akt phosphorylated at Ser473 or Thr308 sites (One-way ANOVA followed by Duncan test, $p > 0.05$. p-Akt ser473: $F_{4,47} = 0.36$, $p > 0.05$; p-Akt thr308: $F_{4,48} = 0.70$, $p > 0.05$). CBD did not alter FAAH, p-GSK3β or p-Akt in non-stressed animals ($p > 0.05$).

3.4. CBD prevents the decrease in dendritic spines density in stressed animals via CB_1 and CB_2 receptors activation and increases synaptic protein expression

In an independent experiment (experiment 4) we evaluated if CBD could prevent the alterations induced by CUS on synaptogenesis. For this, animals were submitted to CUS (14 days) and daily treated with vehicle, AM251 or AM630 followed, 10 min later, by vehicle or CBD. At the end of the procedure, the animals were submitted to the EPM and, 24 h later, to the NSF test (results not shown). Soon after, the animals had their brain removed to perform WB for synaptic proteins. On the next day, another group of animals had their brains removed to perform the Golgi-Cox method (Fig. 4A). Chronic CBD administration prevented the stress-induced decrease in the number of dendritic spines in secondary and tertiary branches, the total dendritic length and the total number of dendritic branches in dentate gyrus granule cells of the hippocampus (Fig. 4, Two-way ANOVA followed by Duncan test, Stress*Treatment, $p < 0.05$.*4B:* secondary branches: $F_{3,12} = 14.01$, $p < 0.05$; tertiary branches: $F_{3,12} = 16.41$, $p < 0.05$.*4C*: dendritic length: $F_{3,1\ 2} = 4.57$, $p < 0.05$; number of branches: $F_{3,12} = 15.84$, $p < 0.05$). Pre-treatment with AM251 or AM630 prevented the effects of CBD on dendritic spine density in secondary (Fig. 4D, One-way ANOVA followed by Duncan test, $F_{6,21} = 12.42$, $p < 0.05$) and tertiary branches (One-way ANOVA followed by Duncan test, $F_{6,21} = 13.63$, $p < 0.05$). Chronic CBD administration did not affect non-stressed animals. We also examined levels of synaptic proteins by Western Blot in synaptoneurosome preparation, and found that chronic CBD prevented the stress-induced decrease in Synapsin Ia/b and increased PSD95 expression in comparison to stressed animals (Two-way ANOVA followed by Duncan test, Stress*Treatment, $p < 0.05$.*4E*: $F_{3,46} = 8.62$, $p < 0.05$; *4F*: $F_{3,46} = 6.39$, $p < 0.05$). No significant difference was found for mGluR1 expression (Fig. 4G, $p > 0.05$). In all cases, CBD did not induce any alteration in animals that were not submitted to CUS ($p > 0.05$).

4. Discussion

This study aimed to elucidate the mechanisms by which repeated injections of CBD induce anxiolytic effects in mice submitted to CUS. In addition to its anxiolytic responses, repeated CBD prevented the stress-induced decrease in hippocampal neurogenesis, dendritic remodeling and the expression of synaptic proteins in the hippocampus. Furthermore, CBD decreased FAAH expression and increased p-GSK3β. Selective CB_1 and CB_2 receptor antagonists abolished the behavioral responses and the increase in spine density induced by CBD, whereas the pro-neurogenic effects were dependent to a greater extent on CB_2 receptors. These receptors also were shown to modulate the increase in p-GSK3β. The results suggest that the anti-stress effects observed after repeated CBD administration are differentially and complementary mediated by CB_1 and CB_2 receptors.

CBD shows great potential for the treatment of neuropsychiatric disorders. Recently, CBD has been approved in several countries for the treatment of drug-resistant epilepsy, mainly in children (Leo et al., 2016), and for the management of multiple sclerosis spasticity (Barnes, 2006). Acute injections of the drug systemically or into several limbic structures produce anxiolytic effects in different

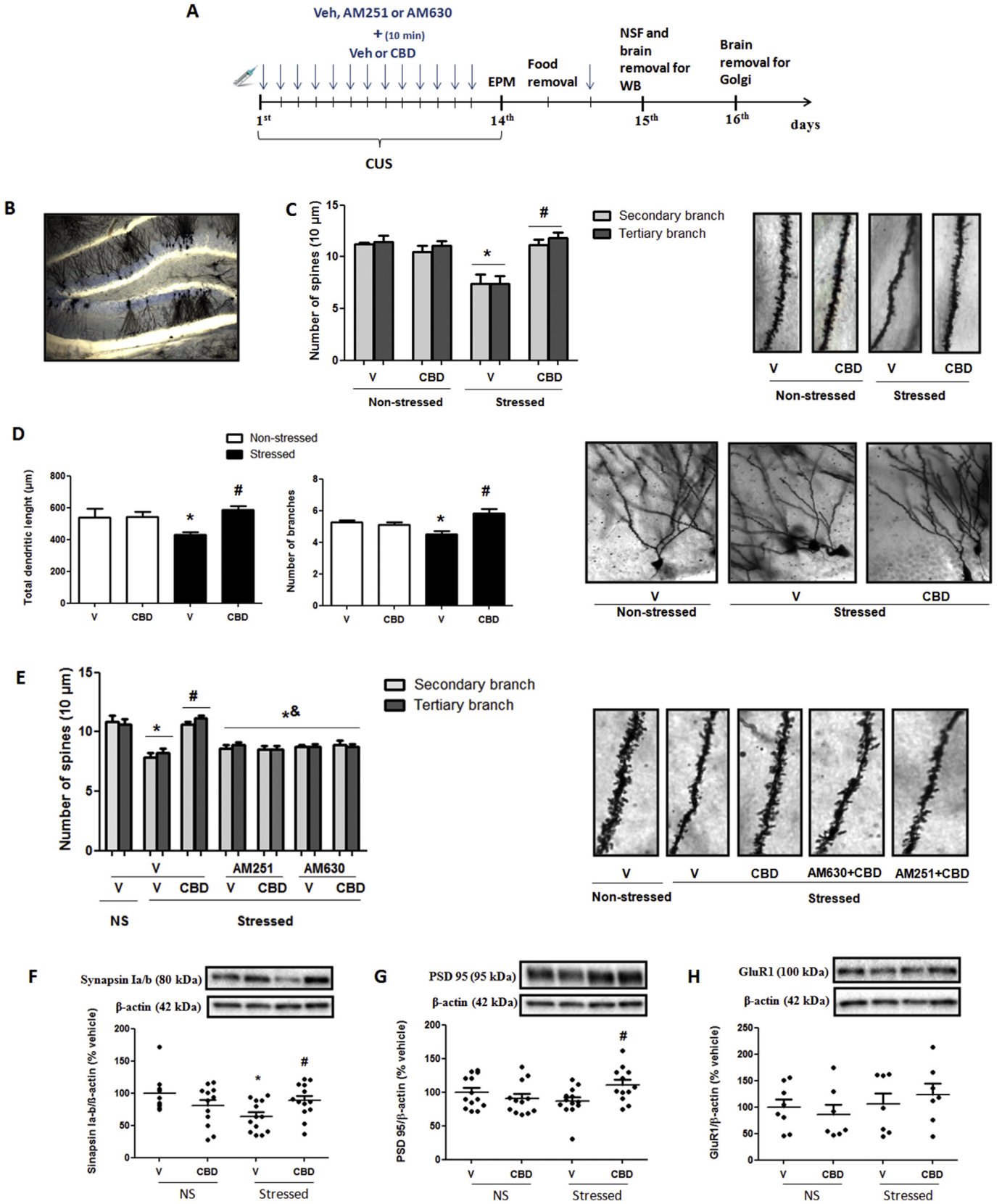


Fig. 4. CBD promotes dendritic remodeling through CB_1 and/or CB_2 receptors and increases synaptic proteins expression. *(A)* Time-course of the experiments. *(B)* Golgi staining neurons in the dentate gyrus of the hippocampus of a non-stressed vehicle animal. CBD increased the number of spines (*C*), total dendritic length and number of branches *(D)* in comparison to stressed vehicle group (n = 4/group). CBD effect on spines density was abolished by pre-treatment with AM251 and AM630 (*E*), n = 4/group. CBD reversed the stress-induced decrease in Synapsin Ia/b *(F)* and increased PSD95 expression in hippocampal synaptoneurosomes of stressed animals *(G)*, n = 12–13/group. The drug did not induce any effect on mGluR1 expression *(H)*, n = 7–8/group. Each bar represents the mean ± standard error of the mean (S.E.M). One- *(E)* or Two-way ANOVA *(C, D, F, G and H)* followed by Duncan test. *p < 0.05 compared to vehicle non-stressed group; #p < 0.05 compared to vehicle stressed group; & p < 0.05 compared to CBD stressed group.

animal models (Campos and Guimaraes, 2008; Fogaca et al., 2014; Guimaraes et al., 1990; Resstel et al., 2006). In chronic treatment studies, the drug induces antidepressant- and reduction in panic-like responses (Campos et al., 2013a; Schiavon et al., 2016), but failed to alter anxiety-related behaviors evaluated in the EPM and light-dark box tests (O'Brien et al., 2013; Schiavon et al., 2016). In the present work, the neuroplasticity and anxiolytic effects of CBD were only observed in animals submitted to CUS. Therefore, after repeated treatment the drug is more likely to prevent anxiety behaviors caused by chronic stress exposure suggesting that, under this condition, CBD induces anti-stress rather than anxiolytic effects. As CBD did not induce behavioral responses in non-stressed animals, we did not include non-stressed groups treated with antagonists and/or CBD in subsequent mechanistic experiments. Thus, for most experiments the combination of antagonists and CBD was only performed in animals submitted to CUS.

Several studies have been conducted trying to elucidate CBD mechanisms of action. For example, pre-treatment with WAY100635, a $5HT_{1A}$ receptor antagonist, prevented the anxiolytic effects induced by CBD injected systemically and into brain areas such as the periaqueductal grey matter and medial prefrontal cortex in different animal models, including predator threat stress, EPM and contextual conditioning fear (Campos et al., 2012; Campos and Guimaraes, 2008; Fogaca et al., 2014). Based on these and other findings, CBD has been proposed to be a $5HT_{1A}$ receptor positive allosteric modulator (Rock et al., 2012). Therefore, we first hypothesized that the anti-stress activities of the drug would depend on $5HT_{1A}$ receptors. However, in the current study WAY100635 did not attenuate CBD mediated behavioral responses. The chosen dose of WAY100635 to perform the experiments (0.05 mg/kg) was based on a previous dose response-curve, in which the higher dose (0.1 mg/kg) induced anxiolytic-like effects in the EPM test. Similar results were also found in previous studies (Griebel et al., 1999, 2000). Although we did not perform binding assays, it is likely that at this dose WAY100635 effectively blocks $5HT_{1A}$ receptors in the behavioral experiments. The same dose, for example, has been shown to abolish dopamine release induced by antipsychotics and anticonvulsant mood stabilizers in different brain areas, including the prefrontal cortex, striatum and nucleus accumbens (Ichikawa and Meltzer, 1999, 2000).

In the EPM test, the CUS procedure increased the enclosed arms entries in comparison to non-stressed animals, suggesting a stress-induced hyperlocomotion, which could interfere with the interpretation of the results. However, we did not find any difference in other parameters analyzed, such as the total number of crossings and total distance travelled in the maze. Also, there was no difference between the stressed vehicle and stressed CBD groups in the number of enclosed arm entries, suggesting that the anxiolytic-like response of the drug was not related to locomotor effects. Moreover, the anxiogenic-like effect of stress was confirmed using another anxiety-related test, the NSF.

We have previously shown that repeated administration of CBD (30 mg/kg, dose based on a previous dose-response curve) prevents the anxiogenic effects of CUS by facilitating adult hippocampal neurogenesis (Campos et al., 2013b). In this study the anxiolytic effects of CBD were blocked when neurogenesis was inhibited in transgenic GFAP-thymidine-kinase mice (Campos et al., 2013b). We also found that the proliferative effects of CBD in cultured hippocampal embryonic cells (HIB5) were inhibited by incubation with CB_1 or CB_2-receptor antagonists, but not WAY100635. Overexpression of FAAH also prevented the neurogenic effects of CBD (Campos et al., 2013b). Together, these *in vitro* results indicate that the pro-neurogenic effect of CBD depends on facilitation of the eCB system through activation of CB_1/CB_2 receptors. The present study extended these findings showing that the behavioral and pro-neurogenic CBD effects are also mediated by the eCB system *in vivo*. Moreover, here we investigated cell signaling pathways that could be related to these responses as well as the effect of CBD on dendritic remodeling. The results also demonstrated that the individual involvement of CB_1 and CB_2 receptors depends on the stage analyzed. AM630, but not AM251, prevented CBD effects on the number of DCX^+ and $BrdU/NeuN^+$ neurons, whereas both drugs abolished the increase in DCX^+ neuronal migration. On the other hand, AM251, but not AM630, attenuated CBD increase of the total number of $BrdU^+$ cells. Since BrdU is a synthetic nucleoside that can be incorporated into all replicating cells and not only neurons, we suggest that CB_1 receptors could mediate the proliferation and survival of other non-neural cells, such as glial cells. The results also indicate that CB_2 receptors are more involved in the migration, differentiation and initial survival of neurons. However, further studies are necessary to clarify the different roles of these two receptors on neurogenesis, distinguishing CBD effects on proliferation and late survival. Nevertheless, the results are consistent with the behavioral findings that both CB_1 and CB_2 receptors, but not $5HT_{1A}$, underlie the anti-stres effects of repeatedly administered CBD.

The lower doses chosen for AM251 and AM630 (0.3 mg/kg) were based on previous experiments from our group and others, in which a higher dose (1 mg/kg) tended to be anxiolytic or anxiogenic (Campos et al., 2013b; Garcia-Gutierrez and Manzanares, 2010; Komaki et al., 2015; Rodgers et al., 2005). In the present study, neither AM251 nor AM630 (0.3 mg/kg) altered anxiety-related behaviors when administered alone in animals submitted to CUS and were able to block CBD effects. However, chronic injections of AM251 increased neurogenesis in stressed animals. Other studies had also found that AM251 or SR141716A, another CB_1 antagonist/inverse agonist, facilitates neurogenesis, respectively, after 30 min (Hill et al., 2006) and 4 h or 3 days of administration (Jin et al., 2004; Rueda et al., 2002). The pro-neurogenic effect of SR141716A was absent in animals knockout to $TRPV_1$, but not CB_1 receptors, suggesting that it could be indirectly mediated by the facilitation of eCBs action on $TRPV_1$ receptors (Jin et al., 2004). However, results regarding the effects of CB_1 antagonists on neurogenesis are contradictory in the literature, since it was also shown that sub-chronic administration of SR141716A decreases the formation of new neurons (Lee et al., 2009). These discrepancies are probably due to different animal species used, as well as distinct time-points and neuronal markers employed. For example, AM251 increased cell proliferation after 1 and 24 h of treatment but decreased cell maturation when analyzed 48 h and 7 days later (Wolf et al., 2010).

Previous studies report that a three-week exposure of rats to CUS can interfere with eCB signaling, including decreased CB_1 receptors, increased FAAH expression, and decreased anandamide levels in the hippocampus (Hill et al., 2008a, 2008b; Reich et al., 2009). In the present study, however, CUS did not induce an alteration of FAAH expression in comparison to non-stressed vehicle animals. The inconsistency found between our work and these previous studies could be due to the different species of rodents (mice versus rats, respectively) or the duration of CUS exposure (2 versus 3 weeks, respectively). On the other hand, the behavioral and pro-neurogenic effects of CBD were associated with a decrease in FAAH expression in the hippocampus of animals submitted to CUS, but not in non-stressed animals. CBD has a low affinity for CB_1/CB_2 receptors (Thomas et al., 2007) but *in vitro* evidence suggests that it can enhance the eCB neurotransmission by inhibiting FAAH enzyme activity (Bisogno et al., 2001; De Petrocellis et al., 2011; Watanabe et al., 1996). Accordingly, chronic administration of CBD increases anandamide levels but not 2-arachidonoylglycerol in the hippocampus of stressed animals

(Campos et al., 2013b) and in the plasma of schizophrenia patients (Leweke et al., 2012). Similarly, subchronic or chronic administration of the FAAH inhibitor URB597 prevents the behavioral effects of stress in animals submitted to CUS (Bortolato et al., 2007; Lomazzo et al., 2015). Additionally, single intracerebroventricular or i.p. injections of URB597 induces anxiolytic-like effects in mice submitted to social defeat stress (Rossi et al., 2010) and oral administration of the FAAH inhibitor, JNJ5003 (21 days), or FAAH deletion in genetically modified animals prevents the anxiogenic effect induced by chronic restraint stress (Hill et al., 2013). It is important to highlight, however, that most studies showing that CBD inhibits FAAH were conducted *in vitro* using enzymatic assays to evaluate the inhibition of [^{14}C]-anandamide hydrolysis (Bisogno et al., 2001; De Petrocellis et al., 2011; Watanabe et al., 1996). In our study we measured total FAAH expression by Western Blot, which does not provide a measure of enzyme activity, but suggests that chronic treatment decreases its expression. Future studies are needed to understand how CBD interacts with FAAH *in vivo* (*i.e.*, by inhibiting the catalytic site of FAAH and/or its expression).

Chronic stress decreases hippocampal volume and induces neuronal atrophy in rodents and humans (Bianchi et al., 2003; Campbell et al., 2004; Magarinos et al., 1997; Soetanto et al., 2010). Antidepressant/anxiolytic drugs can restore dendritic synaptic plasticity (Duman and Duman, 2015), but the influence of CBD has not been investigated. The results of the current study show that chronic CBD prevented the stress-induced decrease in total dendritic length, number of branches and spine density of neurons located in the dentate gyrus of the hippocampus. The reduction in spine density caused by CUS was abolished by pre-treatment with either AM251 or AM630.

The involvement of the eCB system in dendritic remodeling has been described before. CB_1 knockout animals present anxiety-like behaviors similar to wild-type mice exposed to CUS for 21 days, an effect associated with a reduction in apical dendritic length and branches in mPFC neurons (Hill et al., 2011). Also, CB_1 deletion specifically in GABAergic hippocampal neurons reduces long-term potentiation (LTP) and dendritic length, number of branches and spines density, whereas an opposite effect is found in glutamatergic neurons (Monory et al., 2015). CB_2 knockout mice also show decreased LTP and spine density in the hippocampus (Li and Kim, 2016). Repeated treatment with a CB_2 agonist increases spine density in rodent hippocampal slices, an effect abolished by CB_2 antagonists or by deletion of these receptors in mutant mice (Li and Kim, 2016). Specifically in synaptoneurosomes, CBD increased synaptic proteins expression, as Synapsin Ia/b and PSD95. Genetic variations on synapsins and decreased PSD95 protein expression are observed in patients with major depression, bipolar disorder and schizophrenia (Cruceanu et al., 2012; Dyck et al., 2011). In this sense, drugs that can enhance the glutamatergic-mediated synaptic transmission by increasing vesicle formation and the expression of NMDA receptor-scaffolding proteins have been extensively studied (Duman and Duman, 2015).

Considering that CB_1/CB_2 activation recruits several intracellular pathways involved in neuroplasticity and survival, such as Akt and its downstream target GSK3β (Ozaita et al., 2007), we also investigated if repeated administered CBD could modulate these signaling proteins in stressed animals. GSK3β plays an important role in neuropsychiatric disorders and is a target of several psychotropic drugs (Duman and Aghajanian, 2014). GSK3β inhibitors reverse the hormonal, behavioral and cellular alterations observed in stressed animals (Silva et al., 2008). Hyperactivation of GSK3β enzyme activity induced by its mutation in knock in mice results in cognitive impairments, higher vulnerability to learned helplessness behavior, and decreased neural progenitor cells proliferation (Pardo et al., 2016). We showed for the first time that the pro-neurogenic and anxiolytic effects of CBD are associated with an increase in levels of GSK3β phosphorylation, which inhibits enzyme activity in the hippocampus. This effect was dependent on CB_2, but not CB_1 receptors. However, it is important to highlight that, similar to FAAH, stress did not alter the phosphorylation of GSK3β *per se*. Thus, in this case we cannot conclude that CBD reverses the effect of stress, but instead alters the expression of these proteins over basal levels in stressed animals.

Studies associating GSK3β and cannabinoids in psychiatric

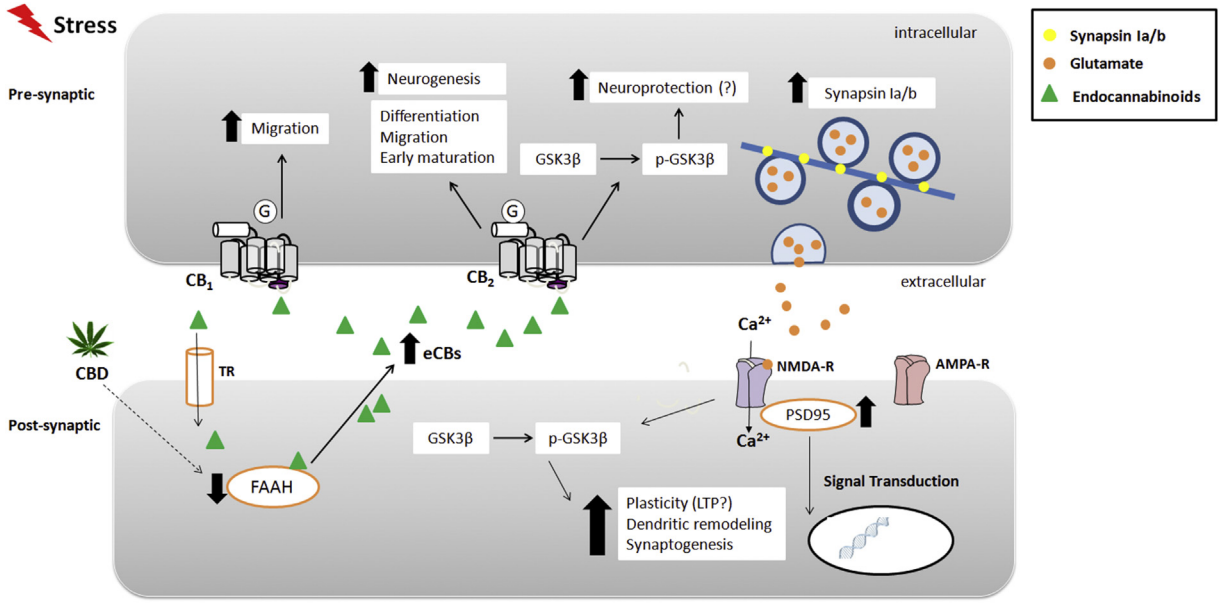


Fig. 5. Mechanisms of action proposed for CBD anti-stress effects. Under stress conditions, CBD may increase eCBs synaptic levels in the hippocampus. Activation of CB_2 receptors facilitates neurogenesis and the phosphorylation of GSK3β, which could occur either pre- or post-synaptically. Moreover, activation of both CB_1 and CB_2 receptors induces dendritic remodeling probably by increasing synaptic vesicles formation and the expression of NMDA-scaffolding proteins, enhancing glutamatergic-mediated neurotransmission and signal transduction of proteins involved in neuroplasticity.

disorders are uncommon in the literature. Similar to our results, Δ9-tetrahydrocannabinol (Δ9-THC), the psychotomimetic compound of *Cannabis sativa*, increased phospho-GSK3β in several limbic structures, including the hippocampus, which could contribute to its neuroprotective effect (Ozaita et al., 2007). Since we did not find any changes in levels of total or phospho-Akt, a known upstream kinase that phosphorylates GSK3β, we suggest that other kinases must be involved in this process, such as mitogen-activated protein kinases (MAPK), which is recruited after CB2 receptors activation (Dhopeshwarkar and Mackie, 2014) and can phosphorylate GSK3β (Gould et al., 2004). Also, as CBD promoted an increase in dendritic spine density and synaptic protein expression in CUS animals, the effect of the drug on GSK3β could be related to a facilitation of NMDA receptors activity, which can promote phosphorylation of GSK3β through protein kinase C (PKC) (Ortega et al., 2010) and regulation of protein phosphatases (Fig. 5) (Li and Jope, 2010). Although the present study provides the first evidence that the anxiolytic-like effects of CBD could be related to modulation of the GSK3β pathway, studies are needed to further investigate this finding.

Taken together, the results provide new insights into the mechanisms by which CBD prevents neuroplasticity and behavioral changes induced by chronic stress. The anti-stress effects produced by chronic CBD injections are associated with reduced expression of FAAH and are dependent on activation of CB_1 and CB_2 receptors. This activation, in turn, modulates significant intracellular signaling mechanisms involved in neuroplastic processes, such as neurogenesis and dendritic remodeling (Fig. 5). Importantly, the results provide further evidence that CBD could be a useful therapeutic approach for the treatment of anxiety-related disorders and identifies potential novel signaling pathways that could be targeted for future drug development.

Author disclosure

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2018.03.001.

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