



The anxiolytic effect of cannabidiol on chronically stressed mice depends on hippocampal neurogenesis: involvement of the endocannabinoid system

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Abstract

Cannabidiol (CBD), the main non-psychotomimetic component of the plant *Cannabis sativa*, exerts therapeutically promising effects on human mental health such as inhibition of psychosis, anxiety and depression. However, the mechanistic bases of CBD action are unclear. Here we investigate the potential involvement of hippocampal neurogenesis in the anxiolytic effect of CBD in mice subjected to 14 d chronic unpredictable stress (CUS). Repeated administration of CBD (30 mg/kg i.p., 2 h after each daily stressor) increased hippocampal progenitor proliferation and neurogenesis in wild-type mice. Ganciclovir administration to GFAP-thymidine kinase (GFAP-TK) transgenic mice, which express thymidine kinase in adult neural progenitor cells, abrogated CBD-induced hippocampal neurogenesis. CBD administration prevented the anxiogenic effect of CUS in wild type but not in GFAP-TK mice as evidenced in the novelty suppressed feeding test and the elevated plus maze. This anxiolytic effect of CBD involved the participation of the CB₁ cannabinoid receptor, as CBD administration increased hippocampal anandamide levels and administration of the CB₁-selective antagonist AM251 prevented CBD actions. Studies conducted with hippocampal progenitor cells in culture showed that CBD promotes progenitor proliferation and cell cycle progression and mimics the proliferative effect of CB₁ and CB₂ cannabinoid receptor activation. Moreover, antagonists of these two receptors or endocannabinoid depletion by fatty acid amide hydrolase overexpression prevented CBD-induced cell proliferation. These findings support that the anxiolytic effect of chronic CBD administration in stressed mice depends on its proneurogenic action in the adult hippocampus by facilitating endocannabinoid-mediated signalling.

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Introduction

Cannabidiol (CBD), the main non-psychotomimetic cannabinoid derived from the plant *Cannabis sativa*, possesses a wide therapeutic potential (Izzo et al., 2009). In the context of psychiatric disorders, CBD administration has been shown to exert antipsychotic and anxiolytic effects in humans (Bergamaschi et al., 2011; Leweke

et al., 2012) as well as in several animal models (Guimaraes et al., 1990; Zuardi et al., 1995; Casarotto et al., 2010). However, the study of CBD actions has been mostly restricted to its acute effect, whereas its efficacy after chronic administration is largely unknown. Moreover, despite the growing interest in this compound, its mechanism of action is still unclear and numerous receptors have been proposed to mediate its different responses (Izzo et al., 2009). Thus, for example, CBD may regulate, directly or indirectly, the activity of peroxisome proliferator-activated receptor γ (PPAR γ), serotonin 5-HT_{1A} receptor, adenosine transporter, some members of the TRPV family and metabotropic CB₁ and

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CB₂ cannabinoid receptors. CB₁ and CB₂ receptors constitute the main molecular targets of psychotomimetic plant-derived cannabinoids such as Δ^9 -tetrahydrocannabinol (THC) and they are physiologically engaged by endogenous ligands, namely, the endocannabinoids (eCBs) 2-arachidonoylglycerol and anandamide (Heifets and Castillo, 2009). Although CBD administration has been found to exert some of its actions independently of CB₁/CB₂ receptors (Pertwee et al., 2010), in other studies CBD has been reported to regulate these receptors (Thomas et al., 2007; Bitencourt et al., 2008; Casarotto et al., 2010; Castillo et al., 2010) or to mimic eCB actions by impairing anandamide hydrolysis or re-uptake, thus facilitating eCB-mediated neuromodulation (Bisogno et al., 2001). In agreement, the antipsychotic actions of CBD have been proposed to be mediated by increased anandamide levels (Leweke et al., 2012).

Increasing evidence shows that adult hippocampal neurogenesis is associated with the regulation of cognitive and emotional functions and impaired neurogenesis has been implicated in psychiatric disorders such as anxiety and depression (David et al., 2010). Adult hippocampal neurogenesis is required to buffer stress and endocrine responses (Snyder et al., 2011) and attenuation of hippocampal neurogenesis promotes anxiety-related behaviours (Revest et al., 2009). Thus, factors that decrease neurogenesis impair learning and certain forms of memory and facilitate the appearance of stress-related disorders (David et al., 2010; Deng et al., 2010). On the other hand, proneurogenic stimuli such as enriched environment, running, social interaction and some antidepressant drugs exert antidepressant and anxiolytic actions (van Praag et al., 1999; Santarelli et al., 2003; Schloesser et al., 2010). Blockade of adult neurogenesis prevents some of the behavioural effects of antidepressants (Santarelli et al., 2003; David et al., 2009), although increased neurogenesis alone is not sufficient to reproduce the behavioural actions of antidepressant or anxiolytic drugs (Sahay et al., 2011). Altogether, these findings have led to the proposal that promoting adult neurogenesis may be a novel therapeutic strategy to palliate anxiety and mood disorders (Surget et al., 2011). In recent years the eCB system has been implicated in the regulation of adult neurogenesis (Galve-Roperh et al., 2009). Thus, CB₁ receptors enhance basal and excitotoxicity-induced hippocampal neural progenitor cell proliferation (Aguado et al., 2005, 2007) and chronic CB₁ receptor activation exerts a proliferative and proneurogenic action linked to anxiolytic and antidepressant-like effects (Jiang et al., 2005). In addition, CB₂ receptors also promote neural progenitor proliferation (Palazuelos et al., 2006; Goncalves et al., 2008), although the consequences of this CB₂ receptor-evoked progenitor expansion in neurogenesis and the regulation of depression and anxiety are as yet unknown.

On the basis of this background, the present work was undertaken to investigate: (i) the potential therapeutic

effect of chronic CBD administration in anxiety; (ii) whether this behavioural effect of CBD relies on hippocampal neurogenesis; (iii) the molecular mechanism of CBD anxiolytic action.

Method and materials

Materials

The following materials were kindly provided: CBD by THC-PHARM (Germany); SR141716 and SR144528 by Sanofi Aventis (France); the HiB5 cell line by Z. Kokaia (Lund Stem Cell Center, Sweden).

Animal procedures

Animal procedures were performed according to the European Union (86/609/EU) and Brazilian guidelines for the use of laboratory animals. Mice (3 months old) were housed (five per cage) with food and water available *ad libitum* and maintained in a temperature-controlled environment on a 12 h light/12 h dark cycle (lights on 07:00 hours). Procedures were designed to minimize the number of animals used and their suffering. In order to evaluate the effect of chronic CBD treatment in neurogenesis the groups received, at the beginning of the chronic unpredictable stress (CUS) paradigm, daily i.p. injections of 5-bromo-2'-deoxyuridine (BrdU; 100 mg/kg) during 3 consecutive days. Hemizygous male mice expressing thymidine kinase under the control of the glial fibrillary acidic protein (GFAP) promoter (code B6.Cg-Tg (GFAPtk) 7.1Mvs/J), as well as their corresponding wild-type (WT-C57BL/6J) littermates, were purchased from The Jackson Laboratory (USA). Experiments of depletion of astroglial neural progenitors and hippocampal proliferation were performed in GFAP-TK mice as previously described (Palazuelos et al., 2009). Ganciclovir (GCV; 100 mg/kg, Roche Farma, Spain) was administered by i.p. injection daily (7 d) starting 5 d before the beginning of the chronic stress procedures. GFAP-TK transgenic and WT mice, subjected (or not) to the CUS paradigm for 14 d, received daily i.p. injections of CBD (30 mg/kg) or vehicle (150 μ l PBS supplemented with 0.5 mg defatted bovine serum albumin and 4% dimethylsulfoxide) 2 h after the daily stressor. The CBD dose used was the same that induced acute antidepressant-like effects in mice (Zanelati et al., 2010). On days 14 and 15 the mice were subjected to the elevated plus-maze (EPM) and novelty suppressed feeding (NSF), respectively, to evaluate anxiety-like behaviours. The EPM and NSF tests were performed just before and 24 h after the last CBD injection, respectively. This 24-h interval between drug administration and test performance is sufficient, according to the pharmacokinetics of CBD in mice, to ensure complete drug elimination before the behavioural test (Deiana et al., 2012). In some experiments AM251 (1.0 mg/kg, Tocris- Bristol, UK) was administered i.p. 10 min prior to CBD (30 mg/kg) injection

(Casarotto et al., 2010). Mouse brains were perfused after behavioural analyses and processed for immunofluorescence analysis.

Chronic unpredictable stress

WT and GFAP-TK mice were subjected, during the light period of the cycle, to a variant of the chronic mild stress paradigm (Santarelli et al., 2003) for 14 d. Different mild stressors were used randomly: bedding alterations (sawdust removal, substitution of sawdust by 5-mm deep water for 4 h); 2 h restraint stress session; 10 min forced swimming; reversal of light/dark cycle; four light/dark successive alterations in 24 h (30 min of duration).

NSF test

The NSF behaviour test was performed in a 5 min test session as previously described (Santarelli et al., 2003). The apparatus consisted of an acrylic box (40 × 40 × 30 cm) with the floor covered by 2 cm of sawdust. Twenty-four hours before the test, all animals were food deprived. On the day of the test a single regular chow pellet was placed in a white platform located in the middle of the box. Each animal was placed in one of the apparatus corners and the latency to start to eat in the new environment was measured. The stopwatch was immediately stopped when the mouse bit the chow, using its forepaws sitting on its haunches. After the test all animals were returned to their home cages and the amount of food consumed in 5 min was measured. Basal feeding latency of control mice differed in separate CUS experiments (Figs. 3, 4). As these experiments were performed in different laboratories (Brazil and Spain), it is conceivable that differences in animal housing, environment and handling conditions are responsible for this.

EPM test

The EPM apparatus composed two open arms (30 × 7 × 0.25 cm), as opposed to two enclosed arms (30 × 7 × 15 cm), was elevated 60 cm from the floor and was made of dark grey plastic. At the beginning of the test, each mouse was placed in the central area of the apparatus with its head facing an enclosed arm. The test duration was 5 min and was performed in a sound attenuated and temperature-controlled (25 ± 1 °C) room, illuminated by three 40-W fluorescent bulbs placed 4 m above the apparatus. The Anymaze software (Stoelting Co., USA) was employed for behavioural analysis. It detects the position of the animal in the maze and calculates the number of entries and time spent in open and enclosed arms. Enclosed-arm entries were considered as an indicator of locomotor activity, whereas percentage of time spent in open arms and percentage open-arm entries were used as measures of anxiety. In the experiment performed with GFAP-TK mice (Fig. 3) only data from stressed mice were analysed due to a recording problem during the EPM procedure.

eCB quantification

Tissue samples, stored at –80 °C until the moment of analysis, were weighed and homogenized in an ice-cold glass dounce-homogenizer in a mixture 2:1:1 (v:v:v) of chloroform:methanol:Tris HCl 50 mM (pH 7.5). The organic and aqueous layers were separated by centrifugation (4500 g, 2 min) and the organic layer transferred to a clean vial and dried under a stream of argon. This fraction was reconstituted in 50 µl acetonitrile and analysed by high-pressure liquid chromatography coupled to mass spectrometry (LC-MS). LC-MS analysis was performed using an Agilent 1200LC-MSD VL instrument (Agilent Technologies, USA). LC separation was achieved with a Zorbax Eclipse Plus C18 column (5, 4.6 × 50 mm; Agilent Technologies) together with a guard column (5, 4.6 × 12.5 mm). The gradient elution mobile phases consisted of A (95:5 water:acetonitrile) and B (95:5 acetonitrile:water), with 0.1% formic acid as the solvent modifier. The gradient (flow rate of 0.5 ml/min) started at 0% B (for 5 min), increased linearly to 100% B over the course of 45 min and decreased to 0% B for 10 min before equilibrating for 5 min with an isocratic gradient of 0% B. MS analysis was performed with an electrospray ionization source. The capillary voltage was set to 3.0 kV and the fragmentor voltage was set at 70 V. The drying gas temperature was 350 °C, the drying gas flow rate was 10 l/min and the nebulizer pressure was 20 × psi. LC-MS measurements were made by selected ion monitoring in positive mode. Fractions were quantified by measuring the area under the peak and normalized using d8-AEA, d8-2-AG or d5-PEA (Cayman Chemical Company, USA) as internal standards. Absolute AEA, 2-AG and palmitoylethanolamide (PEA) levels were estimated by comparison with their respective deuterated standards. eCB levels were referred to tissue weight.

Microscopy

Adult coronal free floating brain sections (30 × µm) or fixed cell cultures were processed as described (Palazuelos et al., 2009). Briefly, after 1 h blockade with PBS supplemented with 0.25% Triton X-100 and 5% goat serum, brain sections were incubated overnight at 4 °C with the rat monoclonal anti-BrdU (Abcam, UK) or rabbit polyclonal anti-doublecortin (Santa Cruz Biotechnology, USA) primary antibodies, followed by incubation for 1 h at room temperature with the appropriate highly cross-adsorbed secondary antibodies (Invitrogen, USA). Doublecortin immunoreactivity was detected by the avidin–biotin immunoperoxidase method (Vectastain ABC kit; Vector Lab, USA) and the product of the reaction was revealed by adding the chromogen 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical, USA). Confocal fluorescence images were acquired by using Leica TCS-SP2 software (Wetzlar, Germany) and SP2 microscope with two passes by Kalman filter and a 1024 × 1024 collection box. *In vivo*, BrdU- and doublecortin-positive

cells were quantified in the subgranular zone of the hippocampus in a minimum of five coronal sections per animal. A 1-in-10 series of hippocampal sections located between 1.3 and 2.5 mm posterior to bregma were analysed and positive cells were normalized to the dentate gyrus area determined with X10 objective. The absolute number of positive cells was calculated considering the total hippocampal volume as determined by the sum of the areas of the sampled sections multiplied by the distances between them. Doublecortin immunoreactivity was quantified using a computerized image analysis system (ImagePro software).

Neural progenitor cultures

The HiB5 hippocampal progenitor cell line was grown as described (Palazuelos et al., 2011) in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 1% penicillin/streptomycin and 10% (v/v) foetal calf serum. Cells were grown in polyornithine-coated plates. HiB5 cell cultures were incubated in 5% CO₂ at 33 °C, the proliferation-permissive temperature of the oncogenic tsA58 allele of the SV40 large T antigen. Incubation at 39 °C results in loss of proliferative capacity and neural differentiation. In some experiments, HiB5 cells were pretreated for 30 min with SR141716 or SR144528 (2 μM) and then incubated with CBD (at the indicated doses) or WIN 55,212-2 (25 nM, Sigma) for 16 h. Stock solutions were prepared in dimethylsulfoxide. No significant influence of vehicle on any of the variables measured was observed at the final concentration used (0.1%, v/v). Control incubations included the corresponding vehicle content.

Proliferation and cell cycle analyses

HiB5 cells were pretreated with SR141716 and SR144528 (2 μM) for 30 min, cultured in the continuous presence of CBD (100 nM) for 16 h and, subsequently, with BrdU (100 μg/ml) for 30 min followed by immunostaining. For flow cytometry analysis HiB5 cells were trypsinized, permeabilized and fixed in 1% (w/v) of bovine serum albumin and 30% ethanol-PBS and labelled with 5 μg/ml Hoechst 33342 (Invitrogen). Fluorescence intensity was analysed by using a LSR flow cytometer (Becton Dickinson, USA). Ten thousand cells per analysis were recorded. In some experiments, HiB5 cells were transiently transfected 1 d after plating with 1 μg pCIG2-FAAH-expressing vector or empty vector (Mulder et al., 2008) by using Lipofectamine 2000 following manufacturer's instructions (Invitrogen).

Data analysis

Data are presented as mean ± S.E.M. Significant differences between the groups were evaluated by *t* test, one, two or three-way analysis of variance (ANOVA) test, followed by Duncan's *post hoc* test. *p* values < 0.05 were considered significant.

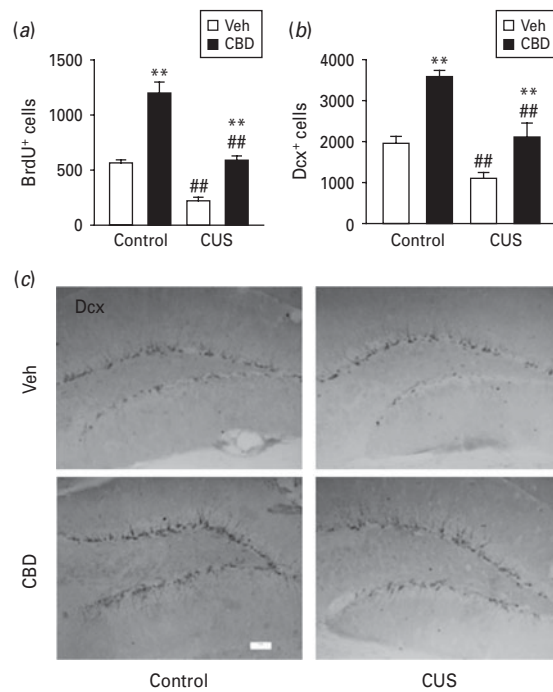


Fig. 1. Chronic unpredictable stress (CUS)-induced reduction of hippocampal neurogenesis is attenuated by cannabidiol (CBD) administration. (a) 5-bromo-2'-deoxyuridine (BrdU)-positive cells were quantified in the subgranular zone of the hippocampus in control non-stressed mice and in mice subjected to CUS that had received vehicle (Veh) or CBD (30 mg/kg; $n=5$ per group). (b) Doublecortin (Dcx) immunoreactivity was quantified in the same groups of animals. (c) Representative images of Dcx immunohistochemistry are shown. Bar size, 100 μm. ** $p < 0.01$ vs. the respective vehicle-treated mice; ## $p < 0.01$ vs. the respective non-stressed mice ($p < 0.05$. Two-way analysis of variance followed by Duncan's *post hoc* test).

Results

CBD increases adult hippocampal neurogenesis and exerts an anxiolytic effect in a CUS model

To investigate the mechanism by which CBD exerts its anxiolytic effects and, in particular, its relation to hippocampal neurogenesis, we first exposed WT mice to a CUS model and CBD or vehicle was administered i.p. at 30 mg/kg for 14 d. CUS inhibited adult hippocampal neurogenesis as determined by quantification of BrdU-positive and doublecortin-expressing cells (Fig. 1a–c). CBD administration promoted hippocampal proliferation in control mice and counteracted the inhibitory effect of CUS in cell proliferation ($F_{1,19}=154$, $p < 0.001$). Similarly, CUS induced a reduction of doublecortin+ cells that was reversed by CBD administration ($F_{1,19}=27$, $p < 0.001$). The increase in neurogenesis induced by CBD administration in WT mice was confirmed by a higher number of BrdU-positive newly born cells that expressed the mature neuronal marker NeuN (Fig. 2a,b; *t* test $t_6=9.6$, $p < 0.001$). To determine if CBD exerts its anxiolytic effect via hippocampal neurogenesis, we employed

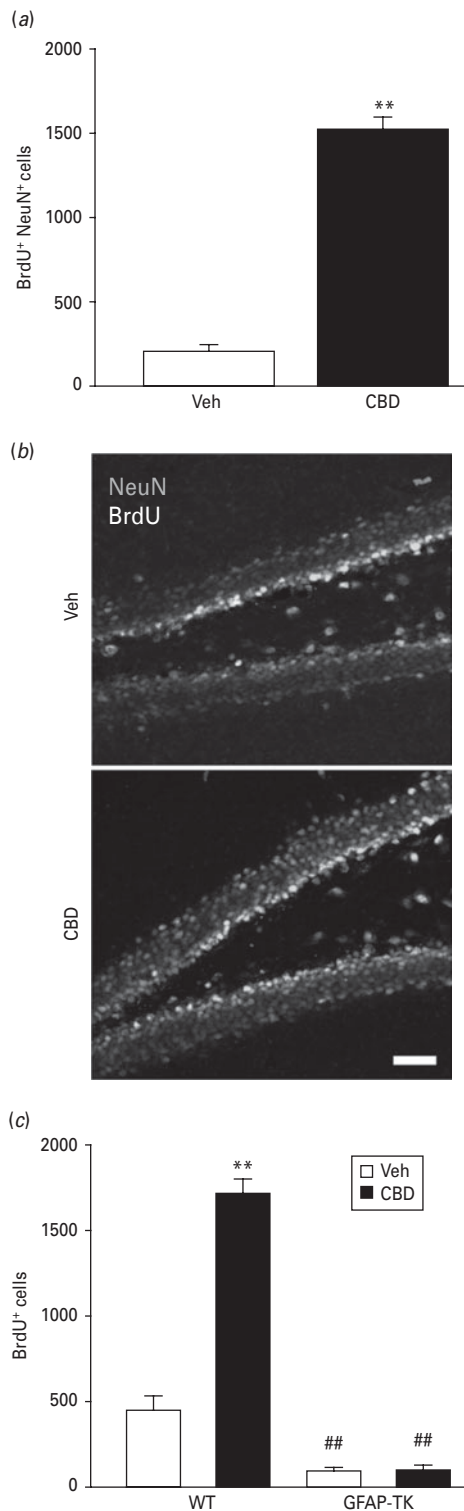


Fig. 2. Cannabidiol (CBD) administration exerts a proneurogenic effect. (a, b) Neurogenesis was determined by quantification of double-positive cells for 5-bromo-2'-deoxyuridine (BrdU) and NeuN in CBD- and vehicle (Veh)-treated wild-type (WT) mice. Representative immunofluorescence images are shown. Scale bar, 50 μ M (c) BrdU-positive cells were quantified in the hippocampus of non-stressed WT and glial fibrillary acidic protein thymidine kinase (GFAP-TK) transgenic mice after Veh or CBD administration (30 mg/kg) for 14 d. Mice also received ganciclovir (100 mg/kg) for 7 d ($n=9, 6, 8$ and 8 animals per

Table 1. Home cage food consumption measured in transgenic GFAP-TK and WT littermates subjected or not to chronic unpredictable mild stress

Treatment	Genotype	Stress	Food (g)
CBD	WT	No	0.19 \pm 0.00
Vehicle	WT	No	0.21 \pm 0.01
CBD	GFAP-TK	No	0.20 \pm 0.00
Vehicle	GFAP-TK	No	0.18 \pm 0.00
CBD	WT	Yes	0.20 \pm 0.00
Vehicle	WT	Yes	0.20 \pm 0.00
CBD	GFAP-TK	Yes	0.17 \pm 0.02
Vehicle	GFAP-TK	Yes	0.20 \pm 0.05

GFAP-TK, Glial fibrillary acidic protein thymidine kinase; WT, wild type; CBD, cannabidiol.

Data are expressed as mean \pm s.e.m.

GFAP-TK transgenic mice and their WT littermates. This approach allowed us to investigate the consequence of blocking adult hippocampal progenitor cell proliferation by GCV administration (Garcia et al., 2004; Palazuelos et al., 2009) on CBD anxiolytic action. Thus, GCV administration to GFAP-TK mice blunted CBD-induced hippocampal progenitor cell proliferation as determined by BrdU-positive cell quantification (Fig. 2c; $F_{1,12}=20.58$, $p<0.001$).

In non-stressed WT mice, CBD administration did not change NSF, but, in mice subjected to CUS, CBD exerted an anxiolytic-like effect by decreasing the latency to eat in the novel environment (Fig. 3a; $F_{1,17}=47.32$, $p<0.01$) without changing food intake in the home cage (Table 1). Analysis of the EPM test in stressed animals showed that in WT mice CBD promoted an anxiolytic-like effect by increasing the percentage of entries and time spent in the open arms (Fig. 3b; $F_{1,13}=8.13$, $p<0.05$). Similar to the NSF results, in the EPM, CBD effects were also prevented by hippocampal cell proliferation ablation in GFAP-TK mice. No effect in the number of enclosed-arm entries was found (Fig. 3c). These results evidenced that repeated CBD administration exerts an anxiolytic-like effect in mice subjected to CUS and that this occurs in parallel with changes in hippocampal neurogenesis. Importantly, blockade of adult neurogenesis prevented the anxiolytic effect of CBD on the NSF and EPM tests, therefore supporting the requirement of hippocampal neurogenesis in CBD actions.

CBD promotes hippocampal neurogenesis via CB₁ cannabinoid receptors by increasing anandamide levels

Considering the diversity of molecular targets that have been proposed to mediate CBD actions (Izzo et al., 2009),

group, respectively). ** $p<0.01$ vs. the respective Veh-treated mice; ## $p<0.01$ vs. the respective WT mice ($p<0.05$ Two-way analysis of variance followed by Duncan's *post hoc* test).

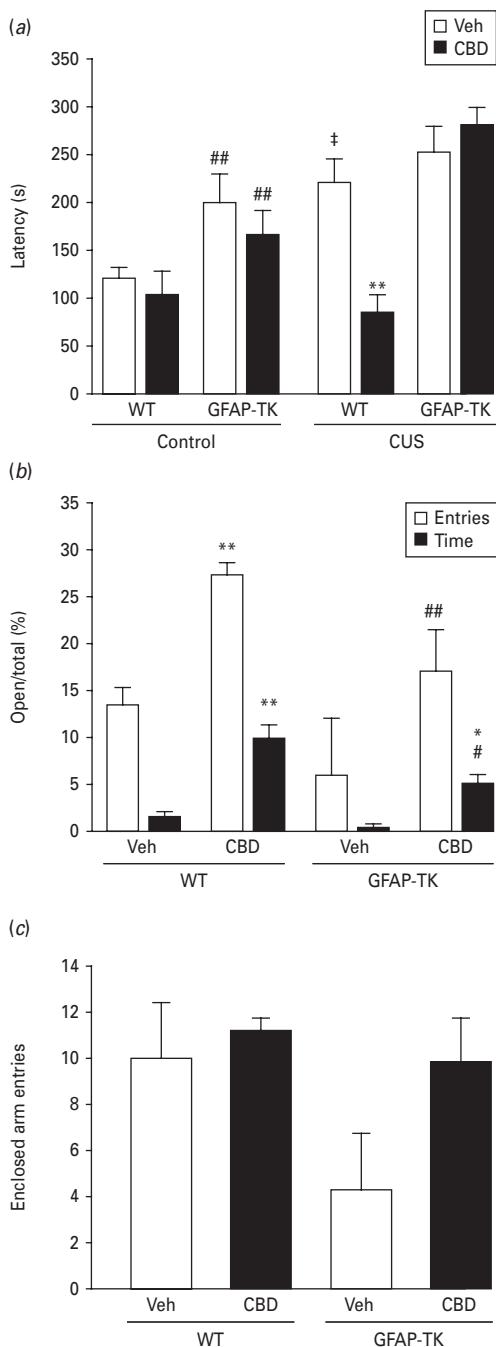


Fig. 3. Cannabidiol (CBD) administration exerts an anxiolytic effect in a chronic unpredictable stress (CUS) paradigm.

(a) Wild-type (WT) and glial fibrillary acidic protein thymidine kinase (GFAP-TK) transgenic mice were subjected to CUS or left undisturbed and time latency in the novelty suppression feeding test was determined after chronic administration of vehicle (Veh) or 30 mg/kg CBD ($n = 14, 12, 6, 7, 5, 4, 3$ and 4 animals per group, respectively). (b, c) The anxiolytic effect of CBD administration in mice subjected to CUS was also determined in the elevated plus-maze test. White bars represent the percentage of entries into the open arms; black bars represent the percentage of the time spent in the open arms ($n = 4, 3, 4$ and 4 animals per group, respectively). Entries in the enclosed arms were also quantified (c). * $p < 0.05$, ** $p < 0.01$ vs. the respective Veh-treated mice; # $p < 0.05$, ## $p < 0.01$ vs. the respective WT mice; ‡ $p < 0.01$ vs. the respective non-stressed mice (two-way analysis of variance followed by Duncan's *post hoc* test).

and the involvement of CB₁ cannabinoid receptors regulating hippocampal neurogenesis (Aguado et al., 2005, 2007), we sought to investigate the potential involvement of the eCB system in CBD-induced neurogenesis and anxiolytic action. WT mice, subjected to CUS and administered with CBD or vehicle, were co-treated with either the CB₁ antagonist AM251 (1 mg/kg i.p. for 14 d) or vehicle. The anxiolytic-like effect of CBD in mice subjected to CUS in the NSF test was abrogated by AM251 administration (Fig. 4a; $F_{1,66} = 7.3$, $p < 0.01$). In the EPM test, there was a significant stress \times drug treatment interaction ($F_{1,65} = 4.3$, $p < 0.05$). CUS decreased the percentage of open arm entries in control animals ($t_{15} = 2.39$, $p < 0.05$), an effect that was prevented by CBD. AM251 antagonized the increase in open-arm entries induced by CBD (Fig. 4b; $F_{1,65} = 4.3$, $p < 0.05$) in stressed animals, although on this occasion CBD administration did not increase the time spent in the open arms. No effect of CBD in the number of enclosed-arm entries was found (Fig. 4c) and there were no differences in non-stressed animals (Table 2). These results indicate that, although CBD is not believed to bind with high affinity to CB₁ receptors (Izzo et al., 2009), this receptor is involved in the CBD anxiolytic action observed here. We therefore evaluated if CBD modulates the eCB tone, as previously suggested, by inhibiting anandamide degradation (Bisogno et al., 2001; Leweke et al., 2012). Hippocampi from vehicle and CBD-treated mice were obtained and eCB levels were quantified by LC-MS. CBD-treated mice showed increased AEA levels, whereas 2AG and PEA were not affected (Fig. 4d).

CBD promotes neural progenitor proliferation via CB₁ and CB₂ cannabinoid receptors

To investigate the mechanism of action of CBD on neural progenitor cells, we used the HiB5 hippocampal progenitor cell line, which provides a good model to investigate the mechanism of action of cannabinoids as they express CB₁ and CB₂ cannabinoid receptors when cultured in proliferating conditions (Palazuelos et al., 2011). In addition, other potential mediators of CBD actions such as the vanilloid receptor TRPV1 and the serotonin 5-HT_{1A} receptor are also expressed in HiB5 cells (data not shown). Thus, HiB5 cells were exposed to increasing concentrations of CBD (50–500 nM) and quantification of BrdU-positive cells revealed that CBD promoted cell proliferation in a dose-dependent manner (Fig. 5a; $F_{5,17} = 25.6$; $p < 0.001$). In addition, treatment of HiB5 cells with the CB₁/CB₂ receptor-mixed agonist WIN 55,212-2 (25 nM) and the eCB degradation inhibitors JZL184 (100 nM) and URB597 (60 nM) promoted progenitor cell proliferation (Fig. 5b). Hippocampal progenitors were next exposed to CBD (100 nM) together with CB₁ or CB₂ receptor-selective antagonists (SR141716 and SR144528, respectively, both at 2 μ M) and CBD proliferative action was prevented (Fig. 5b,c). Based on previous reports (Campos and Guimaraes, 2008; Zanelati et al., 2010;

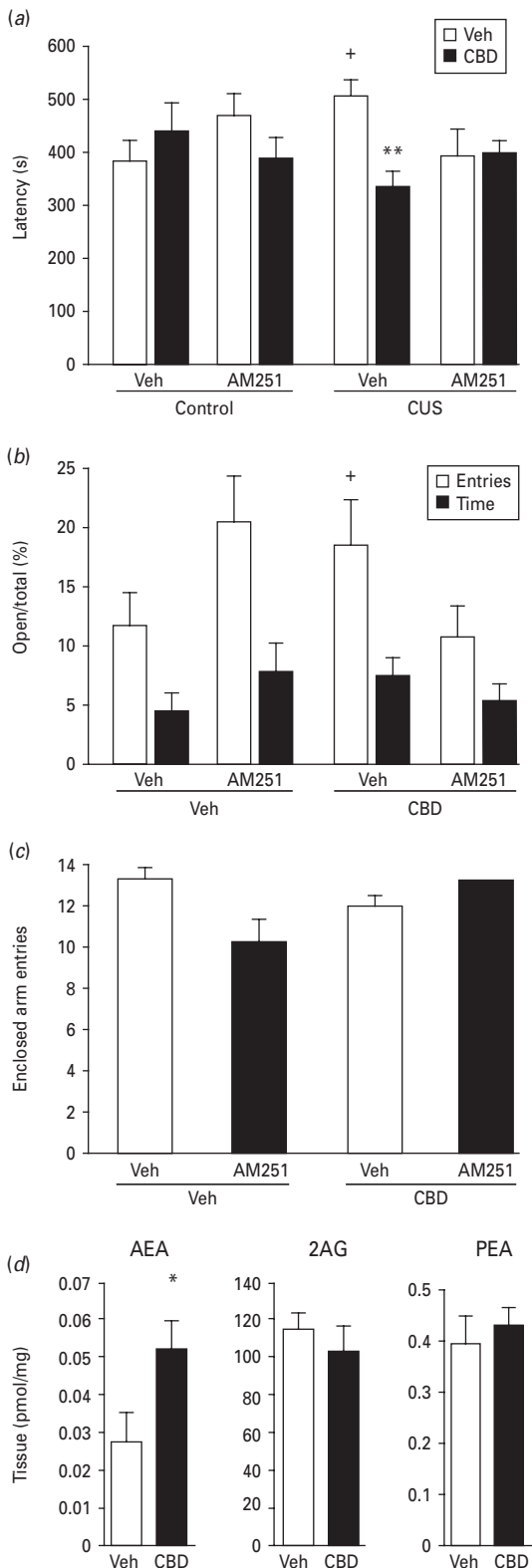


Fig. 4. The anxiolytic effect of cannabidiol (CBD) administration in chronic unpredictable stress (CUS) depends on CB₁ cannabinoid receptors. (a) Wild-type (WT) mice were subjected to CUS or left undisturbed and time latency in the novelty suppression feeding test was determined after chronic administration of vehicle (Veh) or 30 mg/kg CBD ($n=9, 8, 10$ and 10 animals per group, respectively). AM251 was

Table 2. Exploratory activity in the elevated plus maze of non-stressed mice treated for 14 d with vehicle or CBD (30 mg/kg.d) 10 min after AM251 (1 mg/kg.d) or vehicle

Treatment	Entries (%)	Time (%)	Enclosed entries
Vehicle	24.1 ± 4.1	10.3 ± 2.3	12.6 ± 0.9
CBD	20.3 ± 14.6	9.2 ± 4.5	10.6 ± 0.8
AM251	19.2 ± 13.5	9.0 ± 2.1	11.3 ± 1.0
AM251 + CBD	19.9 ± 8.6	6.3 ± 1.9	9.8 ± 1.2

CBD, Cannabidiol.

Data are expressed as mean ± S.E.M.

Gomes et al., 2011) we also determined the potential involvement of the 5-HT_{1A} receptor in CBD-induced progenitor cell proliferation. CBD treatment increased HiB5 cell number (Fig. 5d) and this effect was abrogated by the presence of the CB₁ and CB₂ antagonists, whereas the 5-HT_{1A}-selective antagonist WAY-100635 (2 μM) failed to prevent CBD-induced proliferation. The CB₂ antagonist alone exerted a paradoxical slight increase in cell proliferation. These results indicate that HiB5 cells express functional eCB receptors that can be activated indirectly by CBD and drive progenitor cell proliferation.

As CBD does not bind with high affinity to CB₁ or CB₂ receptors, but CBD-induced hippocampal progenitor proliferation and anxiolytic-like effects were blocked by CB₁ receptor antagonism, we tested if CBD could act on neural progenitors by interfering with the activity of the eCB-degrading enzyme fatty acid amide hydrolase (FAAH; Bisogno et al., 2001). To assess this possibility, we overexpressed FAAH in progenitor cultures to deplete their eCB tone (Mulder et al., 2008). HiB5 cell proliferation was thus determined in cells transfected with pCIG2-FAAH or empty vector and subsequently exposed to CBD. Quantification of BrdU-positive cells revealed that overexpression of the FAAH enzyme prevented the proliferative effect of CBD (Fig 5e; $F_{1,15}=20.3$, $p<0.001$). In addition, the proliferative effect of CBD was evaluated by flow cytometry analysis of DNA content after Hoeschst 33342 staining. CBD treatment reduced the fraction of cells in the Go/G1 phase while increasing the fraction of cells in the S phase (Fig. 6a, b). This

administered (1 mg/kg) 10 min prior to CBD. (b, c) The anxiolytic effect of CBD administration in mice subjected to CUS was also determined in the elevated plus-maze test. White bars represent the percentage of entries into the open arms; black bars represent the percentage of the time spent in the open arms ($n=9, 9, 10$ and 10 animals per group, respectively). Entries in the enclosed arms were also quantified (c). (d) Endocannabinoid levels were determined in the hippocampus of mice treated chronically with Veh or CBD. PEA, Palmitoylethanolamide. * $p<0.05$, ** $p<0.01$ vs. the respective vehicle-treated mice; + $p<0.01$ vs. the respective non-stressed mice (analysis of variance followed by Duncan's *post hoc* test or Student's *t* test).

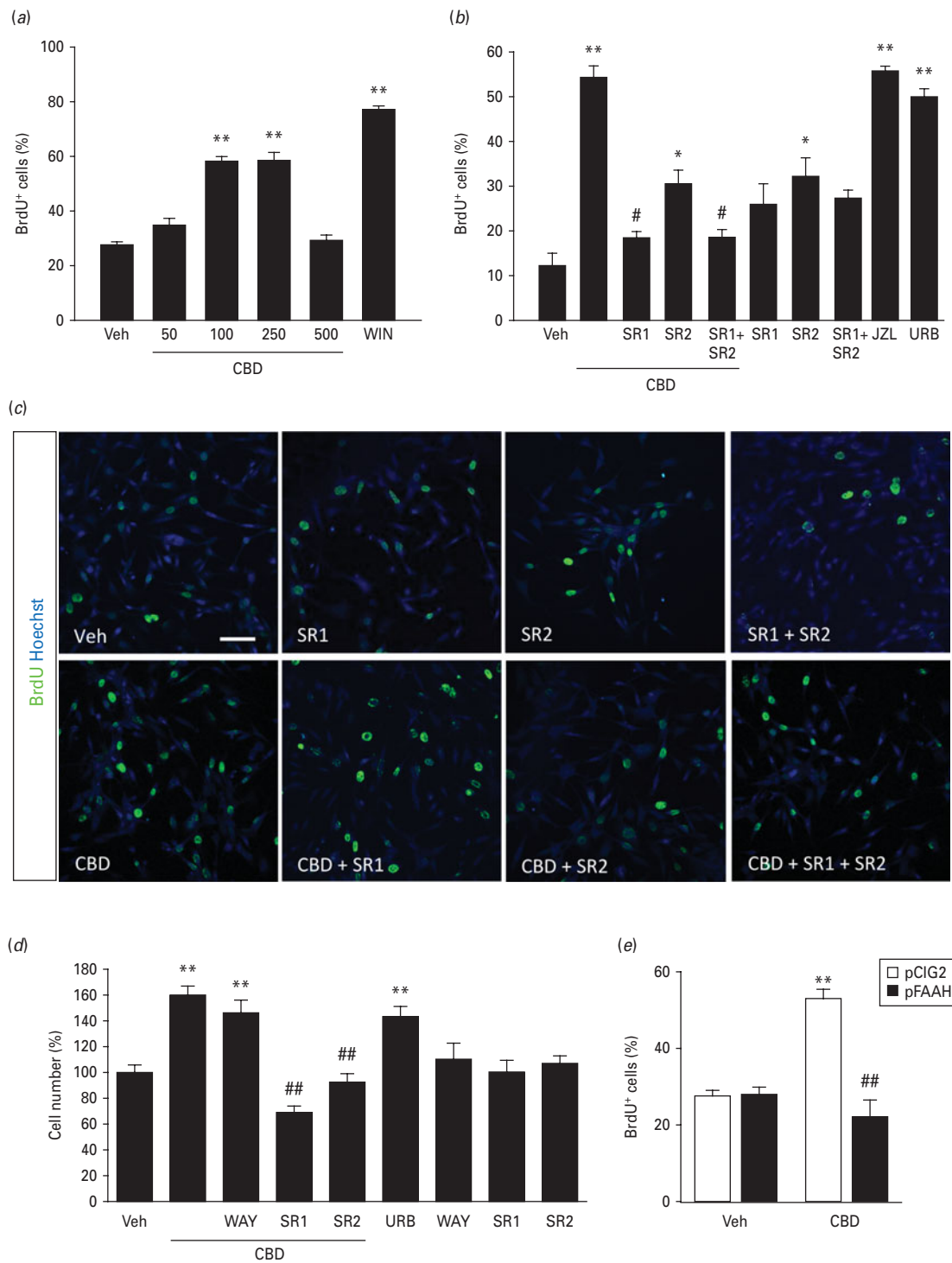


Fig. 5. Cannabidiol (CBD) promotes neural progenitor proliferation via CB₁ and CB₂ cannabinoid receptors. (a) Hippocampal HiB5 progenitors were treated with CBD at increasing concentrations (50, 100, 250 and 500 nM), WIN 55,212-2 (25 nM), URB597 (60 nM) or JZL184 (100 nM) for 18 h and 5-bromo-2'-deoxyuridine (BrdU)-positive cells were quantified after immunofluorescence and Hoechst 33342 counterstaining. Results are provided as percentage of total cells. (b, c) The proliferative effect of CBD (100 nM) was determined as above in the presence of the CB₁ and CB₂ receptor antagonists SR141716 (SR1) and SR144528 (SR2), either alone or together. Representative images are shown. Bar size 60 μ m. (d) Neural progenitors were treated with CBD (100 nM) for 48 h in the presence of SR1, SR2 or the 5-HT_{1A} antagonist WAY100235 (2 μ M) and the number of cells was quantified in each condition. (e) HiB5 cells were transfected with pCIG2-fatty acid amide hydrolase (FAAH) or empty vector, treated with CBD (100 nM) or vehicle (Veh) and 5-bromo-2'-deoxyuridine (BrdU)-positive cells were quantified. Analysis of variance followed by Duncan's *post-hoc* test, * $p < 0.05$, ** $p < 0.01$ vs. the respective Veh-treated cells. # $p < 0.05$, ## $p < 0.01$ vs. the respective CBD-treated (b, d) or Veh-treated pCIG2-transfected cells (e). Results correspond to three independent experiments.

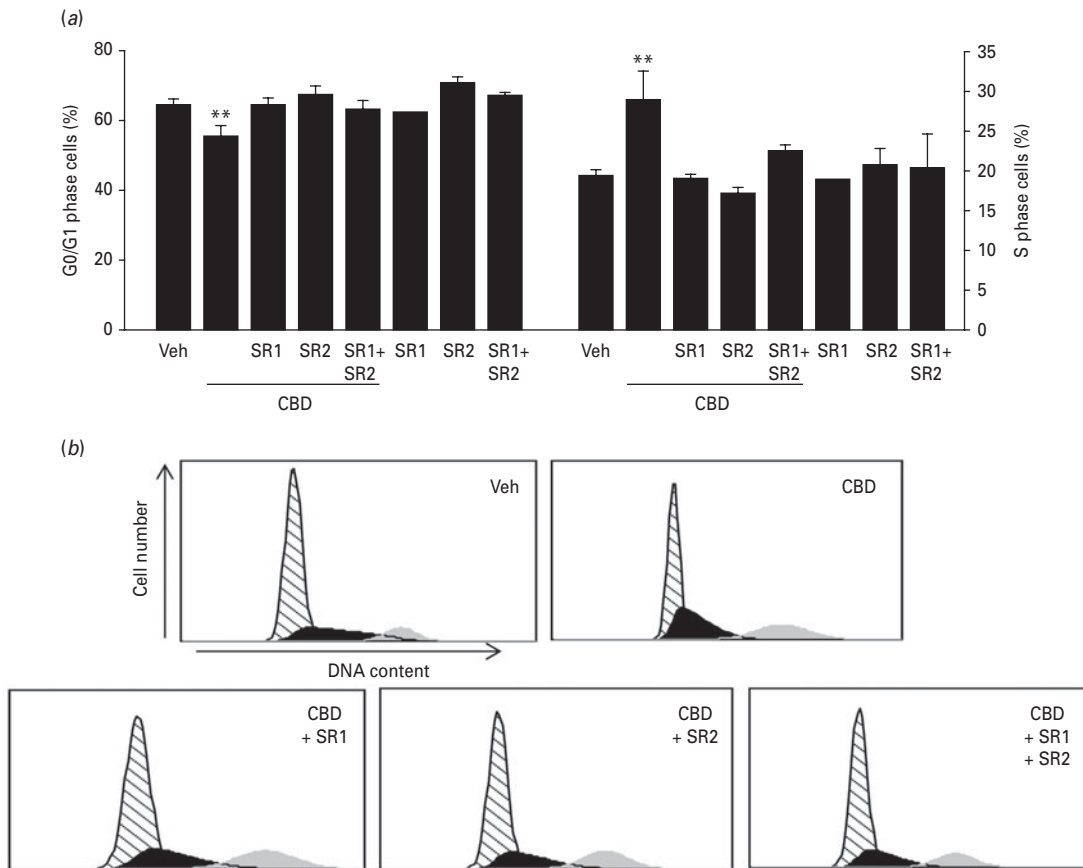


Fig. 6. Cannabidiol (CBD) promotes neural progenitor cell cycle progression at the G1/S transition in a CB₁ and CB₂ cannabinoid receptor-dependent manner. (a, b) HiB5 cells were treated with CBD (100 nM), alone or in the presence of SR141716 or SR144528 (SR1 or SR2, 2 μ M) and cell cycle analysis was performed after DNA content quantification by flow cytometry. (a) The relative fraction of cells in the G₀/G₁ and S phases is shown. (b) A representative DNA histogram of each condition is shown. Results correspond to three independent experiments. One-way analysis of variance followed by Duncan's *post hoc* test, ** $p < 0.01$ vs. vehicle (Veh)-treated cells.

G1-S phase progression was prevented by SR141716 and SR144528. Overall, these results show that eCBs promote hippocampal progenitor proliferation and this effect can be mimicked by CBD, whose action relies on CB receptor engagement.

Discussion

The results shown herein contribute to the elucidation of the cellular and molecular mechanisms involved in the anxiolytic effect of chronic CBD administration. Specifically, genetic ablation of proliferating progenitors in the adult mouse brain prevents CBD anxiolytic action, thus demonstrating the requirement of hippocampal neurogenesis. In addition, CBD drives hippocampal progenitor cell proliferation *in vitro*, an effect that is abrogated by pharmacological blockade of CB₁ and CB₂ cannabinoid receptors or by overexpression of the eCB-degrading FAAH enzyme. Taken together, our findings strongly support that chronic CBD administration exerts an anxiolytic and proneurogenic hippocampal action by increasing the eCB tone.

Behavioural actions of CBD and neurogenesis

CBD is a plant-derived cannabinoid of high interest owing to its anxiolytic, antipsychotic and antidepressive actions evidenced in human studies as well as in animal models (Izzo et al., 2009). For example, CBD is effective for the management of some symptoms of schizophrenia and psychosis with less adverse effects than other antipsychotics (Leweke et al., 2012) and is also effective in social anxiety disorder. The beneficial effects of CBD administration in psychiatric symptoms adds to its safe profile in humans and the existence of CBD-containing standardized medicines (e.g. Sativex) and well-defined administration routes (e.g. oral and oro-mucosal). However, the mechanism of CBD action is complex and remains obscure, as many targets have been shown to be candidates for its behavioural actions. CBD can facilitate eCB-mediated neuromodulation by decreasing anandamide hydrolysis or re-uptake (Bisogno et al., 2001) and, among others, some of the anxiolytic effects of CBD are mediated by CB₁ receptors (Casarotto et al., 2010). Other acute anxiolytic and antidepressant effects of CBD seem to depend on facilitation of 5-HT_{1A}

receptor-mediated neurotransmission (Campos and Guimaraes, 2008; Gomes et al., 2011).

The present study supports that the proliferative effects of CBD on hippocampal progenitors are mediated by CB₁ and CB₂ receptors secondary to an increased eCB tone resulting from the inhibition of anandamide deactivation. However, CBD failed to modify PEA levels, which may be attributed to intrinsic differences in stability between AEA and PEA, the differential contribution of other acylethanolamide degrading enzymes (e.g. *N*-acylethanolamine-hydrolyzing acid amidase) and their different bulk levels. Our findings are in agreement with a recent study reporting that CBD-induced hippocampal neurogenesis is absent in CB₁-receptor knockout animals (Wolf et al., 2010) and the similarity between the effects of CBD and eCB-degradation inhibitors. Thus, like CBD, anandamide- and 2-arachidonoylglycerol-degradation inhibitors promote hippocampal progenitor proliferation and neurogenesis (Aguado et al., 2007) and exert beneficial anxiolytic effects while being devoid of undesired CB₁ receptor-associated psychoactivity (Kathuria et al., 2003; Busquets-Garcia et al., 2011; Kinsey et al., 2011). CBD, as well as other cannabinoids, produce typically bell-shaped dose–response curves, as seen here *in vitro* in proliferative experiments. Higher CBD concentrations can activate TRPV1 receptors and this effect has been associated by the lack of anxiolytic action observed with these doses (Campos and Guimaraes, 2009).

The importance of the eCB system, and, in particular, of CB₁ receptors, in mood control and depressive behaviours has been investigated for decades (Hill et al., 2009). Plant-derived cannabinoids exert a wide variety of effects on depressive and anxiety behaviours (Izzo et al., 2009). Alterations of the eCB system such as changes in CB₁ receptor expression and eCB levels are associated with major depression and suicide commitment (Hungund et al., 2004). An emerging paradigm from cannabinoid research is that the eCB system constitutes an allostatic signalling system that contributes to cellular plasticity responses in adaptation to stress-induced alterations (Patel and Hillard, 2008). Indeed, stress induces an inhibitory effect on neurogenesis that can be partially reverted by engaging the eCB system (Hill et al., 2006; present report). The role of adult neurogenesis in the regulation of cognition and mood is the object of intense study since the initial discovery of the adult hippocampal neurogenic niche (David et al., 2010; Deng et al., 2010). Blockade of hippocampal neurogenesis prevents some of the beneficial effects of antidepressant drugs and stimuli, although its ablation is not sufficient to induce depression and anxiety. However, blockade of adult neurogenesis makes mice more susceptible to stress-induced depressive behaviours (Snyder et al., 2011). Pharmacological manipulation and inducible genetic expansion of adult neurogenesis can improve depressive or anxiety-related behavioural changes (Santarelli et al.,

2003; Sahay et al., 2011); likewise, proneurogenic stimuli such as environmental enrichment and running improve mood and cognition (Schloesser et al., 2010; Parihar et al., 2011). Thus, the emerging scenario indicates that adult hippocampal neurogenesis is involved in the plastic processes that allow for adaption to environmental changes (Dranovsky et al., 2011). Accordingly, by using transgenic GFAP-TK or hippocampus-irradiated mice, it has been demonstrated that inhibition of adult neurogenesis increases hypothalamic-pituitary-adrenal axis activity and glucocorticoid resistance (Snyder et al., 2011). The stress-induced anxiogenic response as determined in the NSF test is buffered by adult-born hippocampal neurons and, reciprocally, the neurogenic niche influences hippocampal progenitor cell fate (Dranovsky et al., 2011). Our results indicate that chronic CBD administration, by promoting neurogenesis, favours a similar anxiolytic response in stressed mice. The proneurogenic effect of CBD in non-stressed animals was not associated with behavioural changes in the NSF and EPM tests. This result agrees with previous reports showing that adult neurogenesis does not alter NSF behaviour under baseline conditions (Snyder et al., 2011), thus suggesting that hippocampal neurogenesis, rather than simply controlling emotional behaviours, favours adaptation and resilience to stress (Dranovsky et al., 2011; Snyder et al., 2011). Different from previous reports (Guimarães et al., 1990; Campos and Guimarães, 2008; Gomes et al., 2011), CBD did not induce any anxiolytic effect in non-stressed animals. In our study, however, the animals were tested 24 h after drug injection. This suggests that repeated CBD administration prevents the effects of CUS rather than induces an acute anxiolytic effect.

Role of cannabinoid receptors in proneurogenic stimuli

Antidepressive stimuli such as environmental enrichment and voluntary wheel running exert a proneurogenic action that has been shown to depend on the presence of functional CB₁ receptor signalling (Hill et al., 2010; Wolf et al., 2010). Voluntary running increased CB₁ receptor binding sites as well as anandamide levels in the hippocampus, but not in the prefrontal cortex, and administration of the CB₁ antagonist AM251 prevented running-induced proliferation (Hill et al., 2010). These results are in agreement with the ability of chronic CB₁ receptor activation to increase hippocampal progenitor proliferation and neurogenesis, which is associated with an anxiolytic/antidepressive cannabinoid action (Jiang et al., 2005). Aging-associated CA1 and CA3 neuronal loss and cognitive impairment are exacerbated in CB₁ receptor-deficient mice (Bilkei-Gorzo et al., 2005). Thus, similar to the beneficial anxiolytic action of chronic CBD in a CUS model, ageing-associated decline of neurogenesis can be partially prevented by the CB₁/CB₂ receptor-mixed agonist WIN 55,212-2, a beneficial action

that relies on the modulation of neuroinflammation (Marchalant et al., 2009a) and progenitor mobilization (Marchalant et al., 2009b). The role of CB₁ receptors in hippocampal neurogenesis, however, could be more complex, since spatially and locally restricted eCB signalling induction by CBD is proneurogenic, THC failed to promote or even inhibited adult neurogenesis (Wolf et al., 2010). This latter effect may be related to the spatial learning impairments caused by THC, an effect that is absent in animals treated with CBD (Fadda et al., 2004). In agreement with the hypothesis that the anxiolytic effect of repeated administration of CBD in the CUS model is mediated by the proneurogenic action of the CB₁ receptor, pharmacological blockade of this receptor blunted the behavioural effect of CBD.

CB₂ cannabinoid receptor agonists are also suitable candidates to promote neural progenitor proliferation (Palazuelos et al., 2006, 2011; Goncalves et al., 2008), although the consequences of this CB₂ receptor-evoked progenitor expansion in neurogenesis are as yet unknown. Ageing-associated decline of hippocampal and olfactory bulb neurogenesis can be prevented by the CB₂ receptor-selective agonist JWH-133 (Goncalves et al., 2008). Brain CB₂ receptors have recently been suggested to exert anxiolytic effects (Busquets-Garcia et al., 2011) and may be involved in social play behaviour by influencing post-natal neurogenesis in the amygdala (Krebs-Kraft et al., 2010). Thus, CBD can exert a dual action via CB₂ receptors acting directly in undifferentiated progenitor cells (Palazuelos et al., 2011) or indirectly through its immunosuppressive actions (Marchalant et al., 2009a). In addition, CBD administration protected from β -amyloid peptide-induced neuroinflammation and increased doublecortin-positive cells by a mechanism involving PPAR γ receptors (Esposito et al., 2011). Anxiolytic- or antidepressant-like effects of single CBD administration in several animal models are prevented by the 5-HT_{1A} receptor antagonist WAY-100635 (Campos and Guimarães, 2008; Zanelati et al., 2010; Gomes et al., 2011). In the present study, however, 5-HT_{1A} antagonism failed to prevent CBD-induced proliferation, whereas CB₁ or CB₂ antagonists completely abrogated this response. Thus, although a partial contribution of 5-HT_{1A} receptors cannot be ruled out, CBD-induced proliferation seems to be largely mediated via cannabinoid receptors.

In conclusion, it is likely that, at least in part, some of the distinctive psychoactive effects of plant-derived cannabinoids in anxiety and depression (Izzo et al., 2009) may be due to their different regulatory properties on adult neurogenesis. The therapeutic potential of non-psychoactive cannabinoids in anxiety and depression, and in particular the anxiolytic effect of CBD, opens the door for their use to manage psychiatric symptoms in disorders such as ageing, stress and neuroinflammation, in which the neurogenic niche is affected.

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Statement of Interest

None.

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