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RESEARCH PAPER

Involvement of the orexin/hypocretin system in the pharmacological effects induced by Δ⁹-tetrahydrocannabinol

Correspondence Rafael Maldonado and Fernando Berrendero, Laboratory of Neuropharmacology, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, PRBB, C/ Doctor Aiguader 88, 08003 Barcelona, Spain. E-mail: rafael.maldonado@upf.edu (R. Maldonado), fernando.berrendero@upf.edu (F. Berrendero)

África Flores, Marina Julià-Hernández, Rafael Maldonado and Fernando Berrendero

Laboratory of Neuropharmacology, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, PRBB, Barcelona, Spain

BACKGROUND AND PURPOSE

Anatomical, biochemical and pharmacological evidence suggest the existence of a crosstalk between the orexinergic and endocannabinoid systems. While the orexin receptor 1 (OX_1 receptor) modulates the reinforcing properties of cannabinoids, the participation of orexins in the acute pharmacological effects of Δ⁹-tetrahydrocannabinol (THC) remains unexplored.

EXPERIMENTAL APPROACH

We assessed the possible role of orexins in THC-induced hypolocomotion, hypothermia, antinociception, anxiolytic- and anxiogenic-like effects and memory impairment. Selective OX_1 and OX_2 receptor antagonists and OX_1 knockout (KO) mice as well as prepro-orexin (PPO) KO mice were used as pharmacological and genetic approaches. CB_1 receptor levels in control and PPO KO mice were evaluated by immunoblot analysis. The expression of c-Fos after THC treatment was analysed in several brain areas in wild-type mice and in mice lacking the PPO gene.

KEY RESULTS

The hypothermia, supraspinal antinociception and anxiolytic-like effects induced by THC were modulated by orexins through OX_2 receptor signalling. OX_1 receptors did not seem to be involved in these THC responses. No differences in CB_1 receptor levels were found between wild-type and PPO KO mice. THC-induced increase in c-Fos expression was reduced in the central amygdala, medial preoptic area and lateral septum in these mutant mice.

CONCLUSIONS AND IMPLICATIONS

Our results provide new findings to further clarify the interaction between orexins and cannabinoids. OX_1 and OX_2 receptors are differently implicated in the pharmacological effects of cannabinoids.

Abbreviations

DI, discrimination index; KO, knockout; MPE, maximum possible effect; PPO, prepro-orexin; THC, Δ⁹-tetrahydrocannabinol; WT, wild type

Tables of Links

TARGETS	
CB$_1$ receptor	OX$_1$ receptor
CB$_2$ receptor	OX$_2$ receptor

LIGANDS	
Δ^9-tetrahydrocannabinol (THC)	SB-334867
Orexin-A	TCS-OX2-29
Orexin-B	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Introduction

Δ^9-Tetrahydrocannabinol (THC), the main psychoactive constituent of *Cannabis sativa*, produces a wide spectrum of central and peripheral actions, including antinociception, hypothermia, immunomodulation, hypolocomotion, catalepsy, memory disruption, rewarding effects and emotional responses (Pertwee, 2008). The CNS responses to THC are mainly mediated by the cannabinoid receptor 1 (CB$_1$ receptor), which is a member of the family of GPCRs (Pertwee *et al.*, 2010). Thus, the antinociceptive, hypolocomotor and hypothermic effects of THC, as well as the somatic signs of THC withdrawal, were not observed in CB$_1$ knockout (KO) mice (Ledent *et al.*, 1999). The discovery of CB$_2$ receptors in neurons of the CNS (Van Sickle *et al.*, 2005) suggests that these receptors could also contribute to some central pharmacological effects of THC. Several heterologous systems, different from the endocannabinoid system, also participate in the behavioural responses induced by THC. Thus, dopamine (Sami *et al.*, 2015), endogenous opioids (Robledo *et al.*, 2008), monoamines (Viñals *et al.*, 2015), GABA (Radhakrishnan *et al.*, 2015), glutamate (Castaldo *et al.*, 2010), ACh (Goonawardena *et al.*, 2010), adenosine (Justinová *et al.*, 2014) and several neuropeptides (Verty *et al.*, 2004) have been shown to be involved in cannabinoid pharmacological responses.

Orexin-A and orexin-B (also known as hypocretin-1 and hypocretin-2) are hypothalamic neuropeptides produced by the proteolysis of a common precursor, prepro-orexin (PPO) (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998). The pharmacological effects of orexins are mediated by the activation of two different GPCRs (OX$_1$ and OX$_2$ receptors) (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998). Orexin-expressing neurons represent a small population exclusively located in the lateral hypothalamus, the perifornical area and the dorsomedial hypothalamus, but they have extensive projections throughout the brain (Peyron *et al.*, 1998). Consistent with this, the orexin system modulates diverse physiological processes such as sleep/wakefulness cycle (Kilduff and Peyron, 2000), feeding behaviour (Sakurai *et al.*, 1998), reward processing (Plaza-Zabala *et al.*, 2012a) and emotional responses (Sakurai, 2014).

Findings are emerging suggesting the existence of a crosstalk between the orexinergic and the endocannabinoid systems (Flores *et al.*, 2013). Anatomical studies show an overlapping distribution of CB$_1$ and orexin receptors in several brain areas (Marcus *et al.*, 2001; Mackie, 2005), and the existence of heterodimers between CB$_1$ and OX$_1$ receptors has also been demonstrated (Jäntti *et al.*, 2014). Moreover,

activation of the OX$_1$ receptor leads to the production of 2-arachidonoylglycerol, suggesting that endocannabinoids could contribute to orexin effects (Ho *et al.*, 2011). The involvement of the endocannabinoid system in some physiological functions of orexins, such as food intake (Crespo *et al.*, 2008; Cristino *et al.*, 2013) and nociception (Ho *et al.*, 2011), has been established. However, few studies have evaluated whether orexins participate in the pharmacological effects of cannabinoids, although a crucial role for the OX$_1$ receptor in the reinforcing properties of the synthetic cannabinoid WIN55,212-2 has been recently shown (Flores *et al.*, 2014a).

The objective of this study was to elucidate the possible involvement of orexins in the acute pharmacological effects induced by THC and the orexin receptor subtypes responsible for these effects. To achieve this, selective OX$_1$ and OX$_2$ antagonists and OX$_1$ KO mice as well as PPO KO mice were used as pharmacological and genetic approaches respectively. In addition, we investigated the brain areas responsible for these effects by analysing c-Fos expression using immunofluorescence techniques.

Methods

Animals

Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). Experiments were performed in male C57BL/6J mice (Charles River, L'Arbresle, France), in male PPO KO and OX$_1$ KO mice and their wild-type (WT) C57BL/6J littermates (12–16 weeks old). Generation of mice with a deletion of the PPO gene has been previously described (Chemelli *et al.*, 1999). These mice do not produce either the orexin-A or the orexin-B peptide and have been extensively characterized at the behavioural level. PPO KO mice were backcrossed for at least nine generations into C57BL/6J and genotyped, as described elsewhere (Chemelli *et al.*, 1999). OX$_1$ KO (Jackson Laboratories, Sacramento, CA, USA) mice were crossed at least seven generations to C57BL/6J mice and genotyped as previously described (Flores *et al.*, 2014a). Mice were housed from two to four per cage in a temperature (21 ± 1°C)-controlled and humidity (55 ± 10%)-controlled room with a 12 h/12 h light/dark cycle (light on between 08:00 and 20:00). Food and water were available *ad libitum*. For the immunoblot study, mice were quickly killed by cervical dislocation, and brains were immediately removed

in order to avoid tissue degradation. For the immunofluorescence study, mice were deeply anaesthetized with ketamine/xylazine (100/10 mg·kg^{-1}, i.p.) and then transcardially perfused with cold 4% paraformaldehyde. Animal procedures were carried out in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethical committee (CEEA-IMAS-UPF).

Experimental design

The observer was blind to treatment in all the experiments. The allocation of animals to the different experimental groups was randomly assessed, as well as the order in which they were treated and evaluated. Effects of THC on locomotion, body temperature and nociception were evaluated in series and in this specified order ($n = 10$–11 in WT/PPO KO and $n = 8$–9 in WT/OX$_1$ KO experiments, $n = 13$–14 in VEH/SB-334867 and $n = 11$–12 in VEH/TCS-OX2-29 experiments). Independent sets of animals were employed to evaluate anxiogenic-like ($n = 7$–8 in WT/PPO KO and WT/OX$_1$ KO experiments and $n = 9$–12 in VEH/SB-334867 and VEH/TCS-OX2-29 experiments), anxiolytic-like ($n = 14$–15 in WT/PPO KO and $n = 7$–8 in WT/OX$_1$ KO experiments, $n = 10$–12 in VEH/SB-334867 and $n = 16$–18 in VEH/TCS-OX2-29 experiments) and amnesic-like effects of THC ($n = 7$–9 in all experiments). Biochemical studies included six to nine animals per group. A lower number of animals was employed in some experiments with WT/KO mice, due to reduced interindividual variability and limited availability.

Locomotor activity

Changes in horizontal locomotor activity after acute THC injection (5 and 10 mg·kg^{-1}) were evaluated by using individual locomotor activity boxes (9 × 20 × 11 cm, Imetronic, Pessac, France) in a low luminosity room, as previously reported (Berrendero *et al.*, 2005). Mice were placed in the locomotor cages 20 min after THC administration, and activity was recorded as the number of horizontal photocell counts for 15 min.

Rectal temperature

Rectal temperature before and after acute THC injection (5 and 10 mg·kg^{-1}) was measured in each mouse by placing 3 cm of an electronic thermocouple flexible rectal probe (Panlab, Madrid, Spain) in the mice rectum. Data are expressed as the difference between basal temperature and temperature recorded 35 min after THC or vehicle injection.

Nociception

The spinal reflex was determined with the tail immersion test, by using water at 50 ± 0.5°C as the nociceptive stimulus, immediately after rectal temperature measurement. Mice were maintained in a cylinder, and their tails were immersed in the heated water, as previously reported (Berrendero *et al.*, 2005). The latency to a rapid flick of the tail was taken as the endpoint, with a cut-off latency of 5 s to prevent tissue damage. Next, the integrated supraspinal response was tested with the hot plate test in a glass cylinder (16 cm high and 16 cm diameter) used to keep the mice on the heated surface of the plate, which was kept at a temperature of 52 ± 0.5°C

(Columbus Instruments, Columbus, OH, USA). The nociceptive threshold evaluated was the jumping response, with a 240 s cut-off (Berrendero *et al.*, 2005). Data are expressed as percentage of the maximum possible effect (MPE), using the following equation: (MPE %) = (individual latency − mean control latency)/(cut-off time − mean control latency) × 100.

Elevated plus maze

The elevated plus maze was used to test the anxiety-like effects induced by acute THC administration. THC effects on anxiety are biphasic; low doses of THC induce anxiolytic-like effects and high doses anxiogenic-like responses (Berrendero and Maldonado, 2002; Valjent *et al.*, 2002). The apparatus consisted of four arms (16 × 5 cm) set in cross from a neutral central square (5 × 5 cm) (Imetronic). Two opposite arms were delimited by vertical walls (closed arms), and the two other opposite arms had unprotected edges (open arms), as previously reported (Balerio *et al.*, 2005). Each mouse was placed in the central neutral area facing one of the open arms and observed during 5 min. Results are expressed as the percentage of time spent in the open arms. Anxiolytic-like behaviour was evaluated 30 min after THC injection at 0.3 mg·kg^{-1}. Anxiogenic-like effects were evaluated 5 h after THC administration at 5 mg·kg^{-1} in order to avoid the hypolocomotor effects induced by this cannabinoid.

Novel object recognition test

The object recognition test was used to evaluate the amnesic-like effects of THC, which was performed in a V-shaped maze. Mice were habituated for 9 min in the maze. After 24 h, mice were put back into the maze for 9 min (training) where two identical objects were presented, and the time spent by the mice exploring each object was recorded. Twenty-four hours later, mice were again placed in the maze for another 9 min (test), one of the familiar objects was replaced by a novel object and the total time spent exploring each of the two objects (novel and familiar) was computed. A discrimination index (DI) was calculated as the difference between the time spent exploring the novel (*TN*) and the familiar object (*TF*) divided by the total exploration time: $DI = [(TN - TF)/(TN + TF)]$. A high DI is considered to reflect greater memory retention for the familiar object. OX$_1$ and OX$_2$ antagonists were injected immediately after the training session, 30 min before THC (10 mg·kg^{-1}).

Immunoblot analysis

To rule out a possible modification of CB$_1$ receptor levels in mice lacking the PPO gene, tissues from different brain regions (hippocampus, striatum, amygdala and hypothalamus) from PPO KO mice and WT littermates were analysed in basal conditions by immunoblotting. Frozen tissue processing to obtain total solubilized fractions and Western blot analysis was performed as previously described (Ozaita *et al.*, 2007). The antibodies used for immunoblotting were anti-CB$_1$ (guinea pig, 1:1000, Frontier Science,Madison, WI, USA) and anti-GAPDH (mouse, 1:10 000, Santa Cruz Biotechnology, Dallas, TX, USA). Bound primary antibodies were detected respectively with horseradish peroxidase-conjugated rec-Protein A (1:5000, Zymed Laboratories Inc., San Francisco, CA, USA) and anti-mouse antibody (1:20 000; Cell Signaling Technologies, Danvers,

MA, USA) and visualized by enhanced chemiluminescence detection (West-Femto-SuperSignaling, Pierce, Rockford, IL, USA). The OD of the immunoreactive bands was quantified after acquisition on a ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA) controlled by The Quantity One software v4.6.3 (Bio-Rad). For quantitative purposes, the OD values for the protein of interest were normalized to the detection of the housekeeping protein GAPDH as loading control in the same sample and expressed as a percentage of the mean value of WT group.

Immunofluorescence studies

c-Fos expression studies were performed in several brain areas previously reported to become activated after acute THC administration (Valjent *et al.*, 2002; Allen *et al.*, 2003). The anatomical coordinates of the mouse brain atlas (Paxinos and Franklin, 2001) were used as a guide to identify the brain regions under study. The coordinates relative to bregma were as follows: core and shell subregions of the nucleus accumbens, from 1.54 to 0.98 mm; dorsal striatum, from 0.86 to 0.26 mm; lateral septum, from 1.10 to 0.38 mm; medial preoptic area of the hypothalamus, from 0.26 to −0.22 mm; paraventricular nucleus of the hypothalamus, from −0.58 to −1.06 mm; paraventricular nucleus of the thalamus, from −0.94 to −1.46 mm; central amygdala, from −1.46 to −1.94 mm; and periaqueductal grey, from −3.80 to −4.24 mm. Tissue preparation and immunofluorescence were performed as previously reported (Plaza-Zabala *et al.*, 2012b). Briefly, 2 h after THC (10 mg·kg^{-1}) injection, mice were perfused with cold 4% paraformaldehyde. The brains were extracted, postfixed and then cryoprotected in a solution of 30% sucrose at 4°C. Coronal frozen sections were made at 30 μm on a freezing microtome and stored in a 5% sucrose solution. Free-floating slices were blocked in a solution containing 3% normal goat serum and 0.3% Triton X-100 in 0.1 M PB (NGS-T-PB) and incubated in the same solution with the primary antibody to c-Fos (rabbit, 1:500, sc-72, Santa Cruz Biotechnology). The next day, sections were incubated with the secondary antibody to rabbit (1:500, Alexa Fluor-555, Life Technologies, Carlsbad, CA, USA) in NGS-T-PB. After incubation, sections were rinsed and mounted onto glass slides in Mowiol mounting media. The stained sections of the brain were analysed at 10× objective using a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany) equipped with a digital camera Leica DFC 300FX (Leica Microsystems). The images were processed using the ImageJ analysis software. c-Fos-positive neurons in each brain area were quantified using the automatic 'particle counting' option with a fixed configuration that only detected pixels that matched a certain range of size and circularity. Additionally, a fixed threshold interval was set in order to distinguish the c-Fos positive neurons from the background. Six representative brain sections of each mouse were quantified along the rostro-caudal trajectory of the brain nuclei, and the average number of c-Fos-positive neurons was calculated for each mouse. The data are expressed as the mean number of c-Fos-positive cells mm^{-2} in seven to nine mice per group.

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology(Curtis *et al.*, 2015). Values are expressed as mean ± SEM. Statistical significance was determined by two-way ANOVA

for the analysis of behavioural experiments and immunofluorescence studies, using genotype/antagonist pretreatment and treatment as between-subject factors of variation, followed by Newman–Keuls *post hoc* when ANOVA revealed significant effect ($P < 0.05$). CB$_1$ receptor expression detected by immunoblotting was compared between genotypes by Student's unpaired *t*-test (two-tailed). All data were analysed using Statistica (StatSoft, Tulsa, OK, USA) software. *P*-values <0.05 were considered to be statistically significant.

Drugs

THC (THC-Pharm-GmbH, Frankfurt, Germany) was dissolved in 5% tween-80 and physiological saline solution and administered by the i.p. route in a volume of 10 mL·kg^{-1} body weight. The OX$_1$ receptor antagonist SB-334867 (5 mg·kg^{-1}) (Tocris, Bristol, UK) was dissolved in 1% (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich, Saint Louis, MO, USA) and 10% DMSO in distilled water. The OX$_2$ receptor antagonist TCS-OX-229 (10 mg·kg^{-1}) (Tocris) was dissolved in physiological saline. Both antagonists were administered by the i.p. route in a volume of 5 mL·kg^{-1} 30 min before THC injection. Doses of orexin receptor antagonists were based on previous studies (Plaza-Zabala *et al.*, 2010, 2012b).

Results

Orexins modulate THC-induced hypothermia and supraspinal antinociception through OX$_2$ receptor signalling

The possible orexinergic modulation of the acute effects induced by THC was studied in PPO-deficient mice, OX$_1$ KO mice and in C57BL6/J mice pretreated with the OX$_1$ antagonist SB-334867 (5 mg·kg^{-1}) or the OX$_2$ antagonist TCS-OX-229 (10 mg·kg^{-1}). Locomotion, temperature and nociception were evaluated in sequence after acute THC injection at 5 and 10 mg·kg^{-1}. THC treatment decreased similarly spontaneous locomotor activity in PPO KO and WT mice (Figure 1A). PPO KO animals presented lower basal activity levels in comparison with WT mice ($P < 0.05$), as previously reported (Plaza-Zabala *et al.*, 2010). The decrease in locomotion induced by THC was not modified in OX$_1$ KO and control animals (Figure 1C). In agreement with the genetic approach, pretreatment with the antagonists SB-334867 and TCS-OX-229 did not change THC-induced hypolocomotor activity (Figure 1B and 1D).

Interestingly, THC-induced hypothermia was modified in PPO KO in comparison with WT mice (Figure 1E). *Post hoc* comparisons showed a significant reduction of the hypothermic effects of THC in these mutant animals ($P < 0.05$) (Figure 1E). THC-induced hypothermia remained unaffected by SB-334867 pretreatment (Figure 1F) and in OX$_1$ KO mice (Figure 1G). In contrast, TCS-OX-229 decreased the effect of THC in body temperature. *Post hoc* analysis showed that hypothermia was significantly reduced in TCS-OX-229-pretreated mice at the highest dose of THC ($P < 0.05$) (Figure 1H), suggesting that orexins modulate the hypothermic effects of THC through the activation of OX$_2$ receptors.

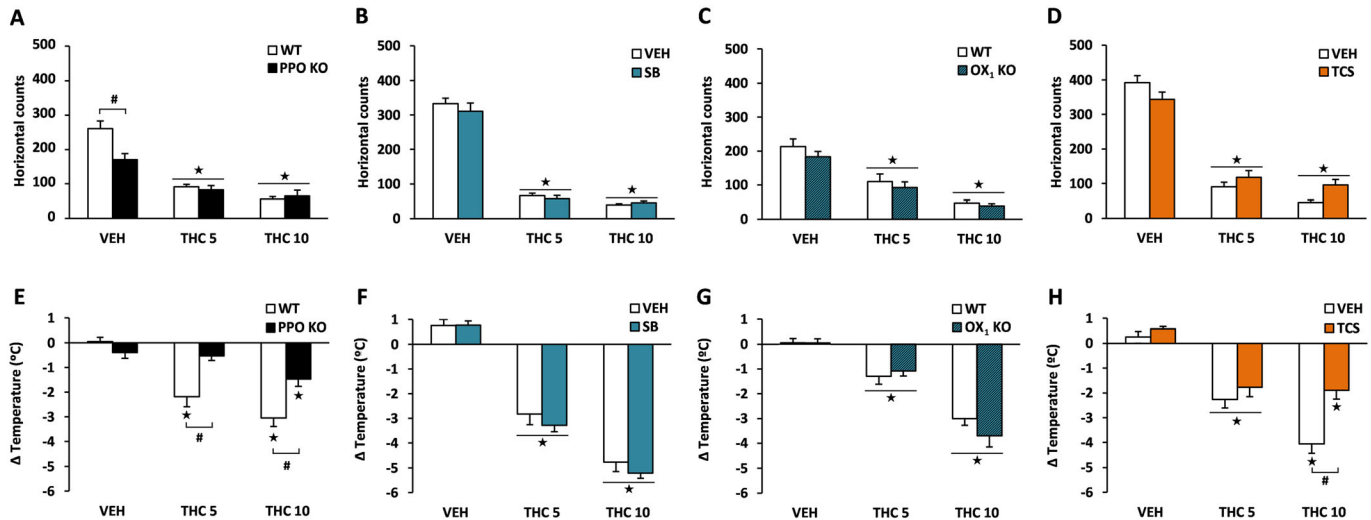In the hot plate test, PPO KO and WT mice showed different antinociceptive responses after THC administration.

Figure 1

Orexinergic modulation of the hypolocomotor and hypothermic effects induced by THC. Locomotor activity (A–D) and body temperature (E–H) were measured after acute administration of THC (5 and 10 mg·kg^{-1}) in PPO KO mice (A, E), OX$_1$ KO mice (C, G) and in mice pretreated with the OX$_1$ antagonist SB-334867 (5 mg·kg^{-1}) (B, F) or the OX$_2$ antagonist TCS-OX-229 (10 mg·kg^{-1}) (D, H). Data are expressed as mean ± SEM (n = 10–11 in A, E; 8–9 in C, G; 13–14 in B, F; and 11–12 in D, H). ★$P < 0.05$ when comparing with control group; #$P < 0.05$ comparison between genotypes or with VEH-pretreated mice (Newman–Keuls test). VEH, vehicle; SB, SB-334867; TCS, TCS-OX-229.

Thus, THC at 5 and 10 mg·kg^{-1} increased the jumping latency in WT mice ($P < 0.05$), whereas this effect was abolished in PPO KO animals ($P < 0.05$) (Figure 2A). THC-induced antinociception remained unaltered by SB-334867 pretreatment (Figure 2B) and in mice lacking the OX$_1$ receptor (Figure 2C). However, TCS-OX-229 administration altered the antinociceptive effects of THC. *Post hoc* comparisons revealed that THC administration increased the jumping latency in vehicle-pretreated mice ($P < 0.05$), and this effect was reduced by TCS-OX-229 pretreatment in mice receiving THC at 5 and 10 mg·kg^{-1} ($P < 0.05$) (Figure 2D). Latency times for the jumping response are shown in the Supporting Information Fig. S1. The spontaneous latency of this response in the hot plate test was lower in PPO KO than in WT mice, as shown in the Supporting Information Fig. S1a. This result suggests a higher sensitivity of PPO-deficient mice to painful stimuli under these experimental conditions. In the tail immersion test, THC-induced antinociception remained unaffected in PPO KO mice (Figure 2E) and in mice lacking the OX$_1$ receptor (Figure 2G). In agreement, the pretreatment with the antagonists SB-334867 (Figure 2F) or TCS-OX-229 (Figure 2H) did not modify the antinociceptive effects of THC. Latency times for the tail-flick response are shown in the Supporting Information Fig. S1. These results suggest that THC-induced supraspinal antinociception is regulated by orexins through OX$_2$ receptor signalling.

THC-induced anxiolytic-like effects, but not anxiogenic- or amnesic-like effects, are modulated by orexins through OX$_2$ receptor signalling

Low doses of cannabinoids induce anxiolytic-like effects, whereas high doses produce anxiogenic-like responses (Berrendero and Maldonado, 2002; Valjent *et al.*, 2002). The involvement of orexins in the anxiolytic-like and anxiogenic-like effects of THC was studied by using the elevated plus maze. After exposure to an anxiogenic dose of THC (5-mg·kg^{-1}), both PPO KO and WT mice spent similarly less time in the open arms than vehicle-treated animals (Figure 3A). A similar result was found in OX$_1$ KO animals (Figure 3C). Moreover, neither OX$_1$ nor OX$_2$ receptor blockade altered the reduction of time spent in the open arms induced by THC at 5 mg·kg^{-1} (Figure 3B and 3D). On the contrary, injection of an anxiolytic dose of THC (0.3 mg·kg^{-1}) increased the percentage of time spent in the open arms in WT animals ($P < 0.05$) but not in PPO-deficient mice ($P < 0.05$) (Figure 3E). In addition, the anxiolytic-like effects of THC at 0.3 mg·kg^{-1} were maintained in SB-334867-pretreated animals (Figure 3F) and in OX$_1$ KO mice (Figure 3G) but not in TCSOX229-pretreated mice (Figure 3H). No significant differences in the total number of entries were observed between groups in any of the experiments performed (Supporting Information Fig. S2). These data point to an orexinergic modulation of THC-induced anxiolytic-like effects, but not anxiogenic-like effects, through OX$_2$ receptor signalling.

We evaluated the amnesic-like effects of THC in the novel object recognition task, as previously reported (Puighermanal *et al.*, 2009). THC (10 mg·kg^{-1}) after the training session reduced similarly the DI in PPO KO and WT mice (Figure 4A). A similar response was observed in OX$_1$ KO animals (Figure 4C). THC-induced amnesic-like effects also remained unaffected by SB-334867 and TCS-OX-229 administration (Figure 4B and 4D), confirming that under these experimental conditions, the orexin system is not involved in this THC pharmacological response. No significant differences in the total time of exploration were observed between groups (Supporting Information Fig. S3).
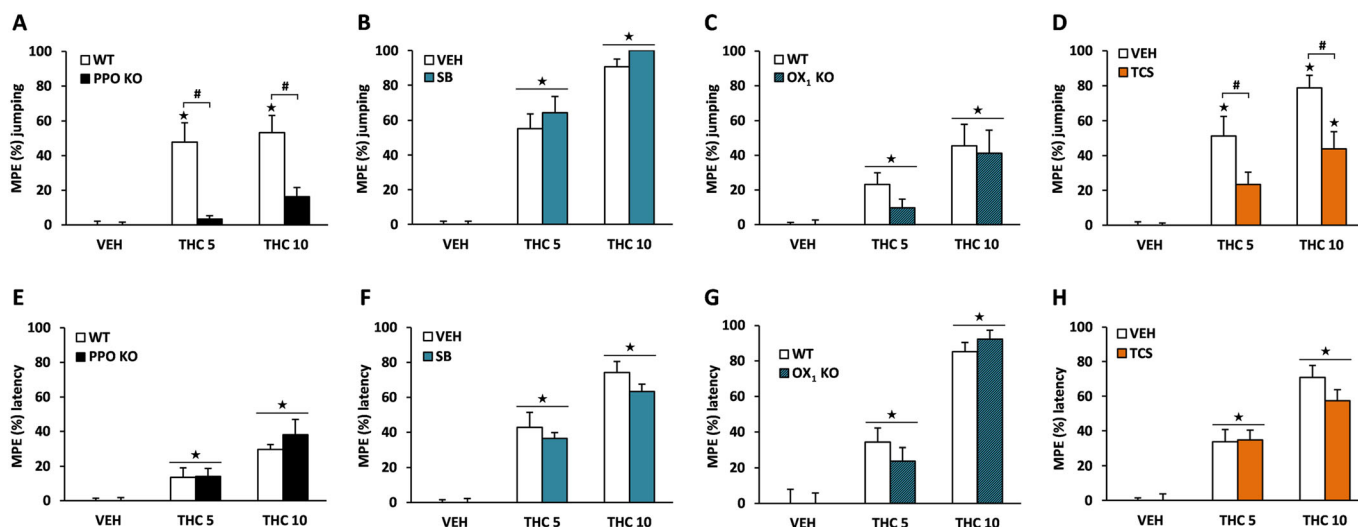
Figure 2

Orexinergic modulation of the antinociceptive effects induced by THC. The supraspinal component of THC-induced antinociception was evaluated in the hot plate test (A–D) and the spinal component in the tail immersion test (E–H). The nociceptive thresholds evaluated were the jumping response and the latency to tail flick, respectively, after the acute administration of THC (5 and 10 mg·kg^{-1}) in PPO KO mice (A, E), OX$_1$ KO mice (C, G) and in mice pretreated with the OX$_1$ antagonist SB-334867 (5 mg·kg^{-1}) (B, F) or the OX$_2$ antagonist TCS-OX-229 (10 mg·kg^{-1}) (D, H). Data are expressed as mean ± SEM of the maximum possible effect (MPE %), calculated as MPE (%) = (individual latency − mean control latency)/(cut-off time − mean control latency) × 100 ($n = 10–11$ in A, E; 8–9 in C, G; 13–14 in B, F; and 11–12 in D, H). ★$P < 0.05$ when comparing with control group; #$P < 0.05$ comparison between genotypes or with VEH-pretreated mice (Newman–Keuls test). VEH, vehicle; SB, SB-334867; TCS, TCS-OX-229.
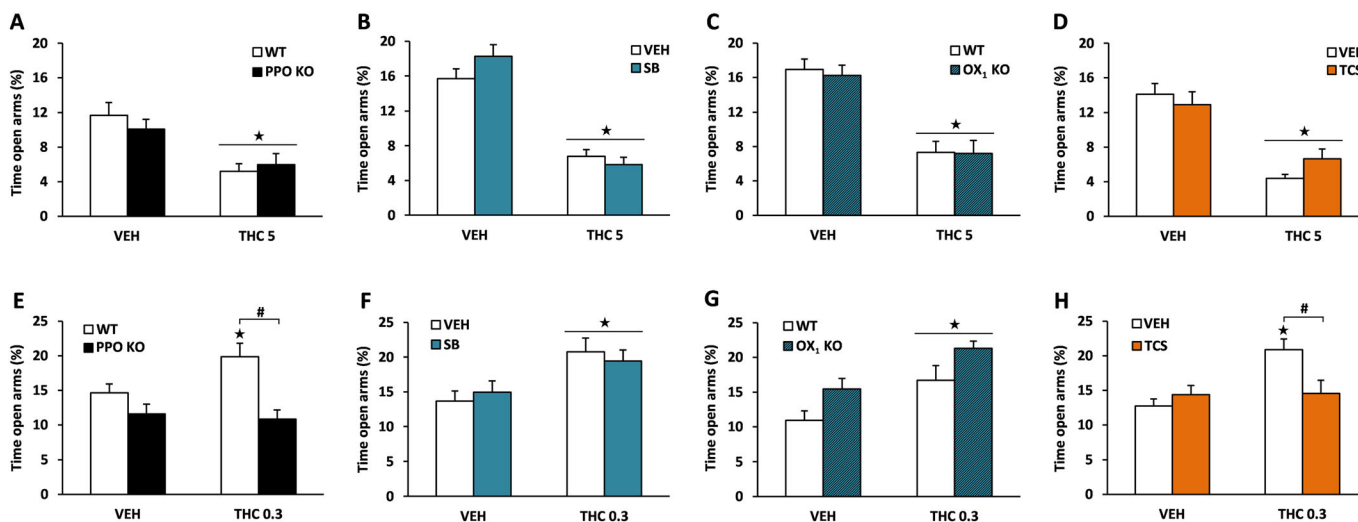

Figure 3

Orexinergic modulation of the anxiogenic-like or anxiolytic-like effects induced by THC. Anxiogenic-like effects (A–D) were evaluated 5 h after the acute injection of THC (5 mg·kg^{-1}), whereas anxiolytic-like effects (E–H) were measured 30 min after THC administration (0.3 mg·kg^{-1}). The time spent in the open and the closed arms was recorded for 5 min in PPO KO mice (A, E), OX$_1$ KO mice (C, G) and in mice pretreated with the OX$_1$ antagonist SB-334867 (5 mg·kg^{-1}) (B, F) or the OX$_2$ antagonist TCS-OX-229 (10 mg·kg^{-1}) (D, H). Data are expressed as mean ± SEM of the percentage of time spent in the open arms ($n = 7–8$ in A, C, G; 9–12 in B, D; 14–15 in E; 10–12 in F; and 16–18 in H). ★$P < 0.05$ when comparing with control group; #$P < 0.05$ comparison between genotypes or with VEH-pretreated mice (Newman–Keuls test). VEH, vehicle; SB, SB-334867; TCS, TCS-OX-229.
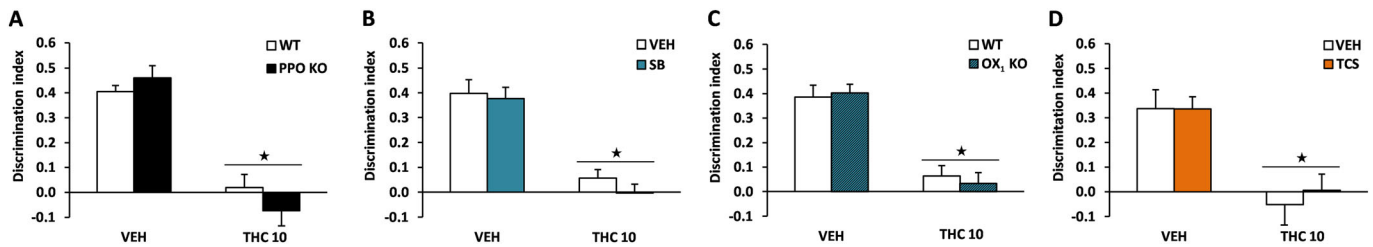
Figure 4

Orexinergic modulation of the amnesic-like effects induced by THC. Amnesic-like effects (A–D) were evaluated in the novel object recognition task. THC (10 mg·kg^{-1}) was administered 20 min after the training session in PPO KO mice (A), OX$_1$ KO mice (C) and in mice pretreated with the OX$_1$ antagonist SB-334867 (5 mg·kg^{-1}) (B) or the OX$_2$ antagonist TCS-OX-229 (10 mg·kg^{-1}) (D) immediately after the training trial. Data are expressed as mean ± SEM of the discrimination index (n = 7–9 for all experiments). ★$P < 0.05$ when comparing with control group. VEH, vehicle; SB, SB-334867; TCS, TCS-OX-229.

Prepro-orexin knockout and wild-type mice show similar CB$_1$ receptor protein levels

We next evaluated whether the behavioural modifications observed in PPO-deficient mice could be a consequence of altered basal levels of CB$_1$ receptors in KO animals. Western blot analysis revealed no differences in CB$_1$ receptor expression levels in the striatum, hippocampus, amygdala and hypothalamus of PPO-deficient mice (Figure 5). These results suggest that the reduction in THC-induced hypothermic, antinociceptive and anxiolytic-like effects observed in KO mice is not due to a lower expression of CB$_1$ receptors in these mutant animals.

THC-induced c-Fos expression is reduced in the central amygdala, lateral septum and the preoptic area of prepro-orexin knockout mice

Acute administration of THC induces c-Fos expression, a marker of neuronal activity, in several brain regions (Valjent *et al.*, 2002; Allen *et al.*, 2003). We investigated the possible modulation of this neuronal activation induced by THC (10 mg·kg^{-1}) in WT and PPO-deficient mice in different brain structures. Immunofluorescence analysis showed an increase in c-Fos expression in the shell and core of the nucleus accumbens, dorsal striatum, lateral septum area, paraventricular thalamic nucleus, medial preoptic area, paraventricular hypothalamic nucleus, central amygdala and periaqueductal grey (Table 1; Figure 6) in WT and KO mice. Interestingly, a reduction in c-Fos expression was observed in the central amygdala of PPO-deficient mice ($P < 0.05$) in comparison with WT animals (Table 1). Moreover, a similar decrease in c-Fos expression was found in the preoptic area and lateral septum of KO mice, although no significant genotype × treatment interaction was revealed in these two brain areas (Table 1). These results suggest that the central amygdala, lateral septum and medial preoptic area could be involved in the altered responses induced by THC in PPO-deficient mice.

Discussion

This study reveals that orexins modulate THC-induced hypothermia, supraspinal antinociception and anxiolytic-like effects through OX$_2$ receptor signalling. In contrast, these neuropeptides are not involved in the hypolocomotion,
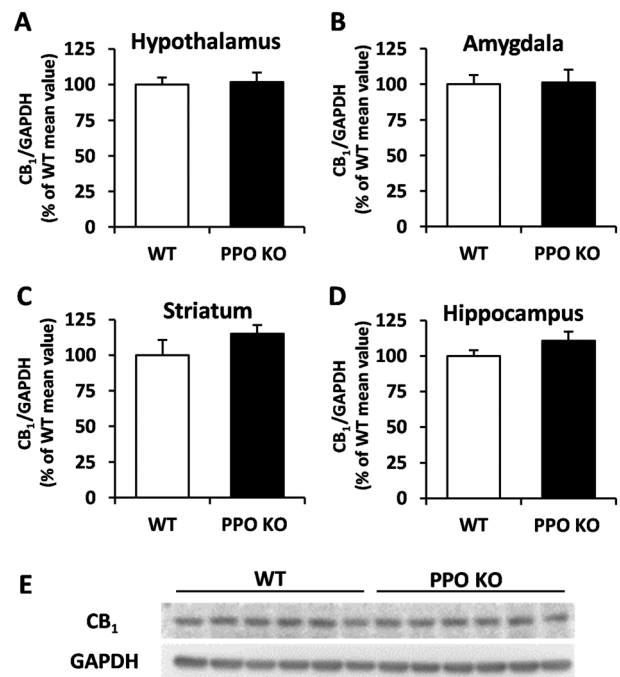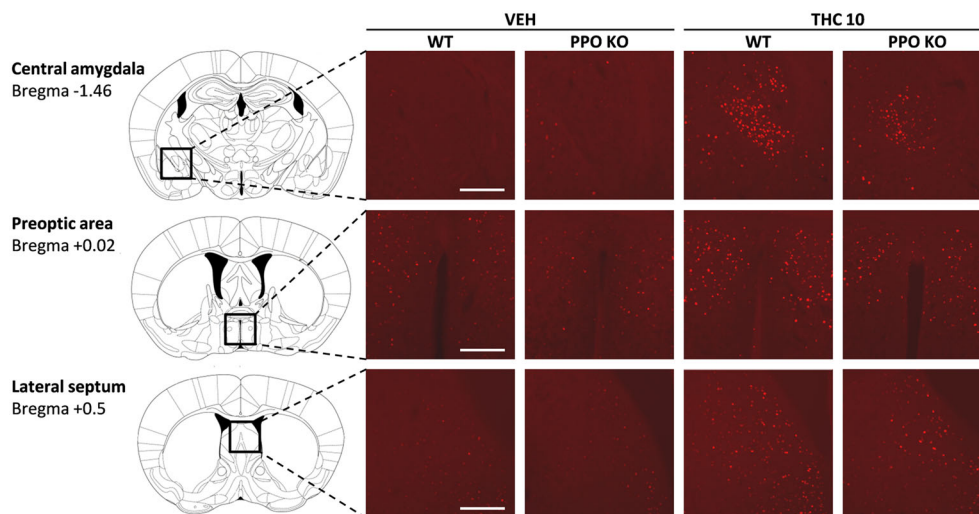

Figure 5

CB$_1$ receptor levels in WT and PPO KO mice. Immunoblot analysis was used to determine total CB$_1$ receptor levels in the hypothalamus (A), amygdala (B), striatum (C) and hippocampus (D) of PPO KO and WT mice under basal conditions. Data are expressed as mean ± SEM of the densitometric value for CB$_1$ receptors related to GAPDH, as a percentage of the control (WT) group (n = 6 mice per group). Displayed are representative blots showing CB$_1$ receptor expression in the hypothalamus of six WT and six KO mice, as well as GAPDH levels as a loading control for each corresponding sample (E).

spinal antinociception, amnesic- and anxiogenic-like effects produced by this cannabinoid. Immunoblot analyses show similar levels of CB$_1$ receptors in WT and PPO KO mice ruling out a possible bias due to different expressions of these receptors in mutant animals. Furthermore, THC administration induced c-Fos immunoreactivity in WT and PPO KO mice in several brain areas, and this effect was significantly reduced

Table 1

Number of c-Fos + cells mm^{-2} in WT and PPO KO animals after THC administration (10 mg·kg^{-1})

	WT-VEH	PPO KO-VEH	WT-THC	PPO KO-THC	ANOVA
Nucleus accumbens (shell)	54 ± 10	52 ± 9	163 ± 12	131 ± 19	*t*
Nucleus accumbens (core)	84 ± 14	57 ± 14	150 ± 22	149 ± 14	*t*
Dorsal striatum	20 ± 4	10 ± 3	80 ± 12	86 ± 16	*t*
Lateral septum	149 ± 29	88 ± 21	**325 ± 34**	**242 ± 27**	***t***, *g****
Preoptic area	165 ± 17	143 ± 22	**298 ± 17**	**219 ± 26**	***t***, *g****
Paraventricular nucleus of the hypothalamus	248 ± 39	336 ± 81	877 ± 84	726 ± 146	*t*
Paraventricular nucleus of the thalamus	259 ± 59	243 ± 47	822 ± 52	722 ± 63	*t*
Central amygdala	39 ± 5	40 ± 5	**825 ± 79**	**503 ± 109**	***t***, *g**, *t* × *g*****
Periaqueductal grey	176 ± 27	116 ± 11	229 ± 30	216 ± 25	*t*

Results are expressed as mean ± SEM. Displayed are significant effects (*t* = treatment, *g* = genotype) and interactions (*t* × *g* = treatment × genotype) revealed by two-way ANOVA. *$P < 0.05$, **$P < 0.01$.

Figure 6

Representative images obtained by fluorescence microscopy showing c-Fos immunoreactivity in the central amygdala, the medial preoptic area and the lateral septum of PPO KO and WT mice receiving acute injections of vehicle or THC (10 mg·kg^{-1}) (*n* = 7–9 mice per group). The scale bar represents 200 μm.

in the central amygdala, medial preoptic area and lateral septum in mutant mice.

The hypolocomotion induced by THC was not modified in mice lacking the PPO gene. However, a lower basal locomotor activity was observed in PPO KO mice in comparison with WT animals. This effect is consistent with previous studies (España *et al.*, 2007; Anaclet *et al.*, 2009; Plaza-Zabala *et al.*, 2010) and suggests that orexins contribute to the maintenance of wakefulness by enhancing locomotion (Anaclet *et al.*, 2009). Indeed, the i.c.v. administration of orexin-A has been shown to increase arousal and locomotor activity in rats (Hagan *et al.*, 1999; Mang *et al.*, 2012). In contrast to the effects observed in locomotion, THC-induced hypothermia was abolished in PPO KO mice. This effect seems to be mediated by OX$_2$ receptor activation because the OX$_2$ antagonist TCS-OX2-29, but not the OX$_1$ antagonist SB-334867, reduced this cannabinoid response. The hypothermic effects of THC were also maintained in OX$_1$ KO mice. Orexins are involved in thermoregulation (Messina *et al.*, 2014), although hypothermic (Jászberényi *et al.*, 2002) and hyperthermic (Monda *et al.*, 2004, 2007) effects have been described following orexin-A administration. Interestingly, the enhancement in c-Fos expression induced by THC in the medial preoptic area was reduced in PPO KO mice, indicating that this hypothalamic region could be important to explain this cannabinoid/orexin interaction. Indeed, the preoptic area is an important thermoregulatory centre (McKinley *et al.*, 2015) containing a dense projection of orexin-positive nerve terminals (Peyron *et al.*, 1998) and high density of OX$_2$ receptor mRNA (Marcus *et al.*, 2001). Therefore, our

results suggest that cannabinoid exposure could induce the release of orexins in the medial preoptic area. The subsequent activation of OX_2 receptors by orexins in this hypothalamic region would be important for the modulation of the hypothermic effects of THC.

The antinociceptive responses induced by THC in the tail immersion test were not modified in PPO KO and OX_1 KO mice or after OX_1 or OX_2 antagonist pretreatment. In contrast, THC-induced antinociception in the hot plate test was reduced in PPO KO animals as well as in TCS-OX-229-pretreated mice. These results suggest that orexins, through OX_2 receptor signaling, are involved in the supraspinal but not in the spinal antinociceptive effects of THC. Interestingly, the endocannabinoid 2-arachidonoylglycerol also participates in the supraspinal antinociception induced by orexin-A (Ho *et al.*, 2011). Several studies support an important role for orexins in the control of nociception (Chiou *et al.*, 2010). Thus, orexins induced antinociceptive effects in different assays for thermal, mechanical and chemical stimuli (Mobarakeh *et al.*, 2005). OX_1 receptor activation has been classically implicated in the antinociceptive effects of orexins (Jeong and Holden, 2009; Ho *et al.*, 2011; Heidari-Oranjaghi *et al.*, 2012), although a role for OX_2 receptors has also been recently suggested (Azhdari-Zarmehri *et al.*, 2013). The central amygdala could participate in the regulation by orexins of THC-induced antinociception because a reduction in c-Fos expression was observed in PPO KO mice following THC administration. This brain area receives ascending nociceptive signals, has efferent projections to areas that are involved in pain modulation and is crucial in controlling pain threshold and its emotional component (Palazzo *et al.*, 2011). Although the expression of OX_2 receptors in the central amygdala is low, a recent study has related an increased orexin transmission at OX_2 receptors in this brain region with compulsive-like heroin self-administration behaviour (Schmeichel *et al.*, 2015). Other brain areas could also participate in the modulation of this nociceptive interaction, although no changes in c-Fos expression between WT and PPO KO mice were observed in important regions for pain regulation, such as the periaqueductal grey matter or the nucleus accumbens.

The anxiogenic-like effects of THC were not modified in mice lacking the PPO gene, the OX_1 receptor or after pharmacological blockade of OX_1 or OX_2 receptors. However, orexin peptides and particularly OX_1 receptors have been shown to be involved in anxiety and panic states (Pich and Melotto, 2014), as well as in the anxiogenic-like effects of other drugs of abuse, such as nicotine (Plaza-Zabala *et al.*, 2010). In contrast, THC-induced anxiolytic-like effects were abolished in PPO KO animals and in mice pretreated with the OX_2 antagonist TCS-OX-229. So far, few studies have evaluated the possible role of OX_2 receptors in anxiety. Recently, an interesting study suggests that orexinergic activity in the basolateral amygdala may be responsible for bidirectional modulation of anxious behaviour (Arendt *et al.*, 2014). Knocking down the OX_2, but not the OX_1 receptor, in this brain area increased anxiety as measured by reduced social preference and reduced time spent in the centre of an open field (Arendt *et al.*, 2014). These data indicate that OX_2 receptor activation in the basolateral amygdala could have the potential to alleviate anxiety. In agreement, our results

show that the OX_2 receptor mediates the anxiolytic-like effects of THC, which could have important therapeutic implications. The role played by orexins in the basolateral amygdala was not evaluated because THC did not induce c-Fos expression in this brain region, as previously reported (Valjent *et al.*, 2002). Future experiments will be necessary to investigate whether these receptors also participate in the anxiolytic-like effects of endocannabinoids (Busquets-Garcia *et al.*, 2011) and the brain areas responsible for this interaction.

The amnesic-like effects induced by THC in the object recognition test were not mediated by orexins. These hypothalamic neuropeptides facilitate memory processing because they are critically involved in hippocampus-dependent social recognition memory (Yang *et al.*, 2013), emotional memories (Sears *et al.*, 2013; Soya *et al.*, 2013; Flores *et al.*, 2014b) and improvement of cognitive performance in sleep-deprived nonhuman primates (Deadwyler *et al.*, 2007). The detrimental effects of THC on learning and memory could be aggravated in mice lacking the PPO gene, although we did not observe this effect probably due to a ceiling effect in the object recognition test.

In conclusion, this study reveals a novel role for orexins and OX_2 receptors in specific acute pharmacological effects of THC, providing interesting data of the interaction between orexins and cannabinoids. The activation of OX_1 and OX_2 receptors modulates the effects induced by cannabinoids in a different way. Therefore, the blockade of OX_1 receptors could abolish the reinforcing properties of cannabinoids (Flores *et al.*, 2014a) without affecting other pharmacological responses of these compounds.

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Author contributions

A.F. conducted the behavioural, biochemical and immunohistochemical experiments and wrote the manuscript. M.J-H. conducted the behavioural and immunohistochemical experiments. R.M. funded the project and wrote the manuscript. F.B. conceptualized, supervised and funded the project, participated in experimental design and wrote the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of pre-clinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Nociceptive thresholds evaluated in the hot plate test (A, B, C, D) and the tail immersion test (E, F, G, H), expressed in seconds. The jumping response and the latency to tail-flick, respectively, were determined after the acute administration of THC (5 and 10 mg kg-1) in PPO KO mice (A, E), in OX1 KO mice (C, G), and in mice pre-treated with the OX1 antagonist SB-334867 (5 mg kg-1) (B, F) or the OX2 antagonist TCS-OX-229 (10 mg kg-1) (D, H). Data are expressed as mean ± SEM (*n* = 9-14 mice for each group). PPO, prepro-orexin; VEH, vehicle; SB, SB-334867; TCS, TCS-OX-229. ★$P < 0.01$ when comparing with control group; #$P < 0.05$ comparison between genotypes or with VEH pre-treated mice (Newman–Keuls test)

Figure S2 No significant differences in exploration parameters were observed between groups during the evaluation of anxiety-like behaviour in the elevated plus maze. Anxiogenic-like effects (A, B, C, D) were evaluated 5 hours after the acute injection of THC at 5 mg kg^{-1}, whereas anxiolytic-like effects (E, F, G, H) were measured 30 minutes after the administration of THC at 0.3 mg-kg-1. The total number of entries was recorded for 5 minutes in PPO KO mice (A, E), OX1 KO mice (C,G), and in mice pre-treated with the OX1 antagonist SB-334867 (5 mg kg^{-1}) (B, F) or the OX2 antagonist TCS-OX2-29 (10 mg kg^{-1}) (D, H). Data are expressed as mean ± s.e.m. (*n* = 7-12 mice for each group in anxiogenic-like approach, *n* = 8-18 in anxiolytic-like approach). PPO, prepro-orexin; VEH, vehicle; SB, SB-334867; TCS, TCS-OX2-29.

Figure S3 No significant differences in the total exploration time was observed between groups during the evaluation of amnesic-like behaviour. Amnesic-like effects (A, B, C, D) were evaluated in the novel object recognition task. THC (10 mg kg^{-1}) was administered 20 minutes after the training session to PPO KO mice (A), OX1 KO mice (C), and to mice receiving the OX1 antagonist SB-334867 (5 mg kg^{-1}) (B) or the OX2 antagonist TCS-OX2-29 (10 mg kg^{-1}) (D) immediately after the training trial. Data are expressed as mean ± s.e.m. (*n* = 7-9 mice for each group). PPO, prepro-orexin; VEH, vehicle; SB, SB-334867; TCS, TCS-OX2-29.