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# Adenosine $A_{2A}$-Cannabinoid $CB_1$ Receptor Heteromers in the Hippocampus: Cannabidiol Blunts $\Delta^9$-Tetrahydrocannabinol-Induced Cognitive Impairment

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## Abstract

At present, clinical interest in the plant-derived cannabinoid compound cannabidiol (CBD) is rising exponentially, since it displays multiple therapeutic properties. In addition, CBD can counteract the undesirable effects of the psychoactive cannabinoid $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) that hinder clinical development of cannabis-based therapies. Despite this attention, the mechanisms of CBD action and its interaction with $\Delta^9$-THC are still not completely elucidated. Here, by combining in vivo and complementary molecular techniques, we demonstrate for the first time that CBD blunts the $\Delta^9$-THC-induced cognitive impairment in an adenosine $A_{2A}$ receptor ($A_{2A}$R)-dependent manner. Furthermore, we reveal the existence of $A_{2A}$R and cannabinoid $CB_1$ receptor ($CB_1$R) heteromers at the presynaptic level in CA1 neurons in the hippocampus. Interestingly, our findings support a brain region-dependent $A_{2A}$R-$CB_1$R functional interplay; indeed, CBD was not capable of modifying motor functions presumably regulated by striatal $A_{2A}$R/$CB_1$R complexes, nor anxiety responses related to other brain regions. Overall, these data provide new evidence regarding the mechanisms of action of CBD and the nature of $A_{2A}$R-$CB_1$R interactions in the brain.

**Keywords** Cannabidiol · $\Delta^9$-Tetrahydrocannabinol · Cannabis · Memory · Adenosine 2A receptor · Cannabinoid 1 receptor

## Introduction

In recent years, clinical research has focused increasingly on cannabidiol (CBD), the second most significant plant-derived cannabinoid, after $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC). The reason for this attention lies in the neuroprotective, antipsychotic, anti-inflammatory and anti-epileptic properties exhibited by this compound, both in animal models and human studies [1–4]. In fact, a 1:1 $\Delta^9$-THC/CBD combination (Sativex®/Nabiximols, GW Pharmaceuticals, UK) is currently approved in more than 20 countries for the treatment of spasticity in multiple sclerosis and is under clinical development for other applications. In addition, based on controlled clinical trials testing the safety and efficacy of the drug, a

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botanical extract containing mainly CBD (Epidiolex®, GW Pharmaceuticals, UK) has recently been approved by the US Food and Drug Administration (FDA) for the treatment of seizures associated with two rare and severe forms of epilepsy: Lennox-Gastaut syndrome and Dravet syndrome [5]. CBD has also received orphan designation from the FDA and the European Medicines Agency for the treatment of new-born infants with neonatal hypoxic-ischaemic encephalopathy. Finally, and of equal relevance, CBD has been shown to antagonise some undesirable effects of the psychoactive $\Delta^9$-THC, including intoxication, sedation and tachycardia, while it increases the clinical efficacy of $\Delta^9$-THC as an analgesic, anti-emetic, anti-carcinogenic and neuroprotective agent [6–8].

Despite the multiple and promising clinical applications of CBD, the mechanisms of action of this natural cannabinoid and its interaction with $\Delta^9$-THC are still not completely elucidated. CBD is a promiscuous compound with activity at multiple targets, including TRPV1 channels and PPARγ, adenosine $A_{2A}$, 5-$HT_{1A}$, α3-glycine, α1-adrenal, dopamine $D_2$, $GABA_A$, and μ- and δ-opioid receptors [8]. In contrast to $\Delta^9$-THC, CBD exhibits a very low affinity for the orthosteric sites of $CB_1$ and $CB_2$ receptors ($CB_1$R and $CB_2$R); the main G protein-coupled receptors (GPCRs) belonging to the endogenous cannabinoid system, the affinity being less than three orders of magnitude below that of other selective compounds [8]. However, CBD has been shown in vitro to negatively modulate $CB_1$R activity [9], probably due to CBD's capacity to behave as a non-competitive negative allosteric modulator (NAM) of $CB_1$R [10]. Despite these properties, the functional or pharmacodynamic antagonism of $\Delta^9$-THC by CBD is assumed not only to be mediated by a $CB_1$R mechanism of action but also to be related to CBD's ability to target different receptors or enzymes. Among these, one of the most intriguing is the capacity of CBD to modulate the activity of adenosine receptors (AR), mainly the $A_{2A}$R subtype [8]. Evidence for the participation of $A_{2A}$R in CBD-mediated effects derives from several studies reporting that $A_{2A}$R antagonists block the beneficial effects of CBD in animal models of inflammation [11–15]. It has been proposed that this $A_{2A}$R-dependent activity of CBD is a product of the ability of CBD to bind itself to the equilibrative nucleoside transporter, thereby inhibiting adenosine uptake, resulting in an indirect activation of $A_{2A}$R [16, 17]. It could also depend on the previously demonstrated reciprocal antagonistic functional interaction between $A_{2A}$R and $CB_1$R [18, 19], which may be explained, at least in part, by the existence of $A_{2A}$R-$CB_1$R heteromers [20–22].

Here, we hypothesise that CBD may modulate $\Delta^9$-THC effects via the $A_{2A}$R-$CB_1$R complex. Therefore, the aim of the present study was to investigate the potential participation of the $A_{2A}$R-$CB_1$R interaction in the CBD-mediated reduction of the main negative consequences of $\Delta^9$-THC consumption (i.e. cognitive impairment) that currently hinder clinical development of $\Delta^9$-THC-based therapies.

## Materials and Methods

### Drugs and Reagents

$\Delta^9$-THC was purchased from Sigma-Aldrich Química SL (Madrid, Spain). CBD, the selective $CB_1$R antagonist SR141716A and the selective $A_{2A}$R antagonists SCH442416 and KW-6002 were purchased from Tocris BioScience (Bristol, UK). The cannabinoid compounds $\Delta^9$-THC (1 and 3 mg/kg), CBD (3 mg/kg) and SR141716A (1 mg/kg) were dissolved in 5% ethanol, 5% Tween and 90% saline and injected intraperitoneally (IP). The $A_{2A}$R antagonists SCH442416 (0.1 mg/kg) and KW-6002 (0.1 mg/kg) were dissolved in 1% DMSO for IP administration. In all cases, the administered volume was 10 ml per kg of body weight.

The primary antibodies used were rabbit anti-$CB_1$R (3 μg/ml; Frontier Institute Co. Ltd., Shinko-nishi, Ishikari, Hokkaido, Japan) and goat anti-$A_{2A}$R (3 μg/ml; Frontier Institute Co. Ltd).

### Animals

Twenty-three male mice C57BL/6J (Janvier Labs, France) weighing 31.2 ± 0.8 g at the beginning of the study, and $A_{2A}$R deficient ($A_{2A}R^{-/-}$) [23] were housed 3–4 per cage and maintained under standard animal housing conditions in a 12-h dark-light cycle with unlimited access to food and water. Mice were habituated to their new environment for 1 week after arrival before starting the experimental procedure. Mice were randomly assigned to treatment groups and the experiments were conducted under blind experimental conditions. The University of Barcelona Committee on Animal Use and Care approved the protocol. Animals were housed and tested in compliance with the guidelines provided by the Guide for the Care and Use of Laboratory Animals [24] and following the European Union directives (2010/63/EU). All efforts were made to minimise animal suffering and the number of animals used.

### Behavioural Evaluation

**Two-Object Recognition Test** Object recognition memory was evaluated in a black Plexiglas V-maze with two corridors (30 cm long × 4.5 cm wide × 15 cm high) set at a 120° angle and slightly illuminated. Immediately after the administration of $A_{2A}$R or $CB_1$R selective antagonists or the corresponding vehicle, the mice were placed for 9 min in the V-maze where two identical objects were situated at the ends of the arms; the

amount of time the mice spent exploring each object was recorded. $\Delta^9$-THC and CBD were injected immediately after this training session. Twenty-four hours later, the animals were, again, placed in the V-maze where one of the two familiar objects had been replaced by a novel object. The amount of time the animals spent exploring these two objects was recorded. The object recognition index (RI) was calculated as the difference between the amount of time spent exploring the novel ($T_N$) and familiar ($T_F$) objects, divided by the total time spent exploring the two objects [$RI = (T_N\text{-}T_F)/(T_N + T_F)$]. Animals exhibiting memory impairments showed a lower RI.

**Locomotor Activity and Anxiety Levels** $A_{2A}R$ or $CB_1R$ selective antagonists or the corresponding vehicle were administered 10 min before $\Delta^9$-THC and CBD administration. Fifteen minutes after the natural cannabinoid treatment, spontaneous locomotor activity and anxiety levels were evaluated in an open field black Plexiglas arena (30 cm long × 30 cm wide) slightly illuminated, into which mice were placed individually and video-recorded for 30 min. The distance travelled (locomotor activity) and time spent in the 20 $cm^2$ central zone (anxiety levels) were analysed by SpotTracker 2D software (ImageJ, NIH, US) and a customised Matlab application for calculations and plotting (The MathWorks, Inc., Natick, MA, US).

A timeline of the behavioural evaluation is included in Fig. 1.

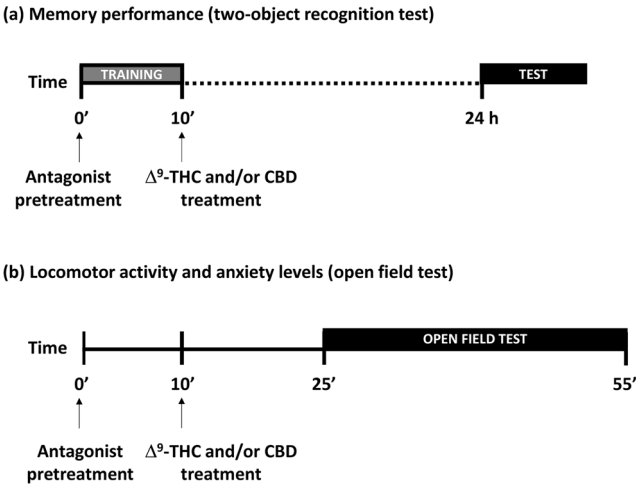


**Fig. 1** Timeline for behavioural evaluation. **a** Memory performance was evaluated by the two-object recognition test. Immediately following administration of $A_{2A}R$ or $CB_1R$, selective antagonists or the corresponding vehicle (0 min), the mice were placed for 9 min in the V-maze for the training session. $\Delta^9$-THC and CBD were injected immediately after this training (10 min). Twenty-four hours later, the animals were placed in the V-maze again, for the test session. **b** Locomotor activity and anxiety levels were evaluated in the open field test. $A_{2A}R$ or $CB_1R$ selective antagonists or the corresponding vehicle were administered 10 min before the injection of $\Delta^9$-THC and CBD. Fifteen minutes after the natural cannabinoid treatment, spontaneous locomotor activity and anxiety levels were evaluated in an open field for 30 min

## Fixed Brain Tissue Preparation

Mice were anaesthetised and perfused intracardially with 100–200 ml of ice-cold 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; 8.07 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$, 137 mM NaCl, 0.27 mM KCl, pH 7.2). Brains were post-fixed in the same solution of PFA at 4 °C for 12 h. Coronal sections (25 μm) were processed using a vibratome (Leica Lasertechnik GmbH, Heidelberg, Germany). Slices were collected in Walter's antifreeze solution (30% glycerol, 30% ethylene glycol in PBS, pH 7.2) and kept at − 20 °C until processing.

## Immunoelectron Microscopy

Double-labelling, post-embedding, immunogold detection of $A_{2A}R$ and $CB_1R$ was performed, as described elsewhere [25]. Briefly, ultrathin sections (80 nm) from Lowicryl-embedded blocks of hippocampus were picked up on coated nickel grids and incubated on drops of a blocking solution consisting of 2% human serum albumin (HSA) in 0.05 M TBS and 0.03% Triton X-100 (TBST). The grids were incubated with a mixture of anti-$A_{2A}R$ and anti-$CB_1R$ polyclonal antibodies (10 μg/ml in TBST with 2% HSA) at 28 °C, overnight. The grids were incubated on drops of rabbit anti-goat IgG or goat anti-rabbit IgG, conjugated to 10 nm and 20 nm colloidal gold particles, respectively (BBI Solutions, Cardiff, UK) in 2% HSA and 0.5% polyethylene glycol in TBST. The grids were then washed in TBS and counterstained for electron microscopy with saturated aqueous uranyl acetate followed by lead citrate. Ultrastructural analyses were performed in a Jeol-1010 electron microscope. Randomly selected areas of the CA1 region of the hippocampus from among the ultrathin sections were then photographed at a final magnification of 50,000 times.

## Proximity Ligation Assay

Proximity ligation in situ assay (P-LISA), using the Duolink detection kit (Olink Bioscience, Uppsala, Sweden), was performed, as described elsewhere [25]. Fluorescence images were acquired using a Leica TCS 4D confocal scanning laser microscope (Leica Lasertechnik GmbH), taking a 60× N.A. = 1.42 oil objective from the selected brain area (i.e. CA1 region in the hippocampus). High-resolution images were acquired as a Z-stack with a 0.2 μm Z-interval with a total thickness of 5 μm. Nonspecific nuclear signal was eliminated from P-LISA images by subtracting DAPI labelling. The particle analyser function of ImageJ (NIH) was used to count particles larger than 0.3 $\mu m^2$ for P-LISA signal and larger than 100 $\mu m^2$ to distinguish neuronal from glia nuclei, as described elsewhere [26].

### Statistical Analysis

In behavioural experiments, memory performance and locomotor activity were analysed using three-way ANOVA with antagonist, $\Delta^9$-THC and CBD treatments as between factors, followed by two-way ANOVA for antagonist pre-treatment with $\Delta^9$-THC and CBD treatments as between factors, and Dunnett's post hoc test when required. Memory performance with a sub-effective dose of $\Delta^9$-THC was analysed using two-way ANOVA with SCH442416 and $\Delta^9$-THC treatments as between factors, followed by Dunnett's post hoc test. To determine the effect of CBD on memory performance after the combination of both $A_{2A}R$ and $CB_1R$ antagonists, data were analysed using three-way ANOVA with SR141716A, SCH442416 and CBD treatments as between factors, followed by Dunnett's post hoc test. P-LISA quantifications were analysed using Student's *t* test. In all the experiments, the significance level was set at $p < 0.05$ and the number of animals used was $n = 5–8$ per group, as indicated in figure legends.

## Results

### CBD Blunts $\Delta^9$-THC-Induced Cognitive Impairment, but Not Locomotor or Anxiogenic Effects, Preferentially through Presynaptic $A_{2A}R$-$CB_1R$

The involvement of the adenosinergic system in cannabinoid-mediated effects in the central nervous system (CNS) has been widely studied [18]. Here, we aimed to examine the participation of $A_{2A}R$ on the modulatory effects of CBD in $\Delta^9$-THC-mediated memory impairment. To this end, we first assessed the effects of $\Delta^9$-THC in the two-object recognition test, which evaluates mainly hippocampus- and perirhinal cortex-dependent declarative memory performance [27]. Previous findings indicated that the hippocampus plays a crucial role in the $CB_1R$ agonist-induced memory impairment in the two-object recognition test, evaluated in both an open field [28] and a V-maze [29–31]. As expected, $\Delta^9$-THC (3 mg/kg) induced a significant ($P < 0.01$; Table S1) reduction in the recognition index when administered immediately after training, which was reversed by pre-treatment with the selective $CB_1R$ antagonist SR141716A (1 mg/kg) ($P < 0.05$; Table S1) (Fig. 2a). Importantly, co-administration of CBD (3 mg/kg) completely abolished $\Delta^9$-THC-induced memory impairment (Fig. 2a). Next, we assessed the $A_{2A}R$ blockade of the CBD modulation of $\Delta^9$-THC effects in memory, using preferential pre- and postsynaptic $A_{2A}R$ antagonists (i.e. SCH-442416 and KW-6002, respectively) [32]. Interestingly, while CBD was still able to preclude $\Delta^9$-THC-induced memory impairment in the presence of KW-6002 (0.1 mg/kg) ($P < 0.05$; Table S1), pre-treatment with SCH442416 (0.1 mg/kg) significantly ($P < 0.01$; Table S1) reduced CBD modulation of $\Delta^9$-THC-induced memory impairment (Fig. 2a). These results pointed to a potential involvement of presynaptic $A_{2A}R$ in the CBD-mediated modulation of $\Delta^9$-THC effects. Further evidence of the participation of presynaptic $A_{2A}R$ in the cognitive impairment mediated by $\Delta^9$-THC was obtained by the evaluation of a sub-effective dose of $\Delta^9$-THC (1 mg/kg) [30] in combination with SCH442416. Accordingly, pre-treatment with this preferential presynaptic $A_{2A}R$ antagonist (Fig. 2b), but not with the preferential postsynaptic $A_{2A}R$ antagonist KW-6002 (Fig. 2c), resulted in a cognitive impairment induced by the otherwise sub-effective $\Delta^9$-THC dose ($P < 0.01$). These results again suggest a potential functional interplay between $CB_1R$ and $A_{2A}R$ at the presynaptic level, controlling the $\Delta^9$-THC effects.

Interestingly, we observed that CBD produced an unexpected significant ($P < 0.05$; Table S1) cognitive impairment in the presence of the $CB_1R$ antagonist SR14176A (Fig. 2a), thus indicating a non-$CB_1R$-dependent activity of CBD. In order to further investigate this unpredicted CBD property and its potential relationship with the adenosinergic system, we combined both $A_{2A}R$ and $CB_1R$ antagonists (i.e. SCH442416 and SR14176A, respectively) with CBD administration. Notably, the marked decrease in the recognition index produced by CBD in mice pre-treated with SR14176A was prevented ($P < 0.001$) when animals also received SCH442416, indicating that the effect of a $CB_1R$ blockade upon CBD-mediated cognitive impairment was $A_{2A}R$-dependent (Fig. 2c). In addition, we observed that when CBD- and SR14176A-treated animals were also challenged with THC (Fig. 2a), the CBD/SR14176A-mediated cognitive impairment was abolished. Overall, these phenomena could be related to receptor-receptor (i.e. $A_{2A}R$-$CB_1R$) allosteric interactions, as has been shown for many GPCR heteromers (for review see [33]); however, further work will be needed to substantiate this interpretation.

Moreover, we investigated whether CBD might modulate $\Delta^9$-THC-mediated hypolocomotion and anxiety, which are also $CB_1R$-dependent effects but regulated by brain structures other than the hippocampus; mainly the striatum and amygdala, respectively. As expected, $\Delta^9$-THC (3 mg/kg) significantly reduced the total distance travelled by the mice in the open field, an effect that was abolished by SR14176A (1 mg/kg) pre-treatment ($P < 0.001$; Table S1), but not modified by either CBD (3 mg/kg) or SCH442416 (0.1 mg/kg) and KW-6002 (0.1 mg/kg) (Fig. 3a, c). On the other hand, significant per se effects of SR14176A (hypolocomotion) and KW-6002 (hyperlocomotion) ($P < 0.05$ and $P < 0.01$, respectively; Table S1) were observed (Fig. 3a, c), which is consistent with previously recorded data [34, 35]. Similarly, $\Delta^9$-THC (3 mg/kg) significantly reduced the time spent by mice in the central area of the open field, revealing the expected anxiogenic effect ($P < 0.001$; Table S1), which was not

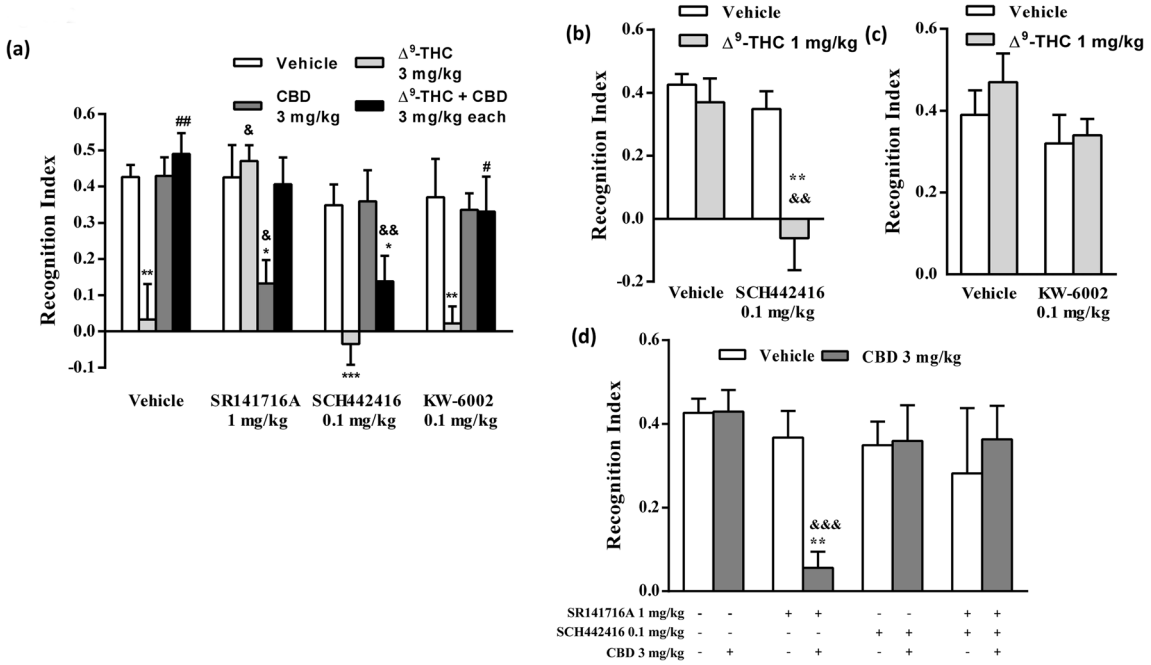


**Fig. 2** CBD modulatory effects on $\Delta^9$-THC-mediated memory impairment are $A_{2A}R$ dependent. **a** The mice were treated with $\Delta^9$-THC (3 mg/kg), CBD (3 mg/kg) or a $\Delta^9$-THC/CBD combination (3 mg/kg of each compound) in the absence or presence of SR141716A (1 mg/kg), SCH442416 (0.1 mg/kg) or KW-6002 (0.1 mg/kg). $\Delta^9$-THC induces memory impairment in mice, which was prevented by the pre-treatment with SR141716A. CBD co-treatment completely blocked the $\Delta^9$-THC-induced memory impairment in a presynaptic $A_{2A}R$-dependent manner. CBD administration significantly reduced memory performance in mice pre-treated with SR141716A. **b** A sub-effective dose of $\Delta^9$-THC (1 mg/kg) resulted in a cognitive impairment effect when the presynaptic $A_{2A}$ receptors were blocked by SCH442416, but not when they were blocked by KW-6002. **c** The memory impairment produced by CBD in mice pre-treated with SR14176A was prevented by the co-administration of SCH442416. Data are expressed as mean values ± SEM ($n = 5$–8 animals per group). Three-way ANOVA (antagonist pre-treatment, $\Delta^9$-THC and CBD treatments as between factors), two-way ANOVA (antagonist pre-treatment, $\Delta^9$-THC or CBD treatments as between factors), and Dunnett's post hoc test were used for statistical analysis (see "Materials and methods" section and Table S1 for details). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle-treated mice. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to $\Delta^9$-THC-treated mice. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ compared to non-receiving antagonist group of mice

modified by CBD or SCH442416 (Fig. 3b). SR14176A blocked the $\Delta^9$-THC effect although it induced per se an increase in the anxiety levels of mice ($P < 0.01$). Interestingly, SCH442416 pre-treatment potentiated an anxiogenic effect of CBD ($P < 0.05$; Fig. 3b), providing further evidence of an interaction between presynaptic $A_{2A}R$ and CBD. In contrast to the control mice, pre-treatment with KW-6002 prevented $\Delta^9$-THC-treated mice from exhibiting a significant reduction in the time spent in the central area, despite a tendency (Fig. 3b). However, we cannot discount a bias in the anxiety evaluation in the open field due to the effect of KW-6002 on locomotor activity (Fig. 3a).

Overall, these results revealed a role for $CB_1R$-$A_{2A}R$ functional interplay in the CBD-mediated modulation of $\Delta^9$-THC effects, mainly in the hippocampus, but not in the striatum or amygdala.

## $A_{2A}R$ and $CB_1R$ Heteromerise in Presynaptic Terminals at the CA1 Region in the Mouse Hippocampus

Our observations from the two-object recognition test clearly demonstrated that CBD capacity to block $\Delta^9$-THC-mediated memory impairment was $A_{2A}R$-dependent. Since these $\Delta^9$-THC effects are known to occur via $CB_1R$ activation in the hippocampus, we aimed to demonstrate the existence of a putative $A_{2A}R$-$CB_1R$ interaction in this brain area, which might represent a novel physical substrate for such CBD-mediated modulation of $\Delta^9$-THC effects. We focused on the CA1 region of the hippocampus since it has been shown to play a relevant role in the memory processing related to the object recognition test [36] and specifically in the $CB_1R$-dependent memory impairment [28]. Firstly, we detected hippocampal $A_{2A}R$ and $CB_1R$ at the subcellular level using double-labelling immunogold electron microscopy. Interestingly, immunoparticles for $A_{2A}R$ and $CB_1R$ showed a high degree of co-distribution in axon terminals projecting to dendritic spines (Fig. 4), thus pointing to the possibility that these two receptors might be forming heteromers under native conditions.

Subsequently, to confirm the existence of $A_{2A}R/CB_1R$ heteromers in the hippocampus (i.e. CA1), we implemented the P-LISA approach, a well-respected technique providing enough sensitivity to evaluate a receptor's

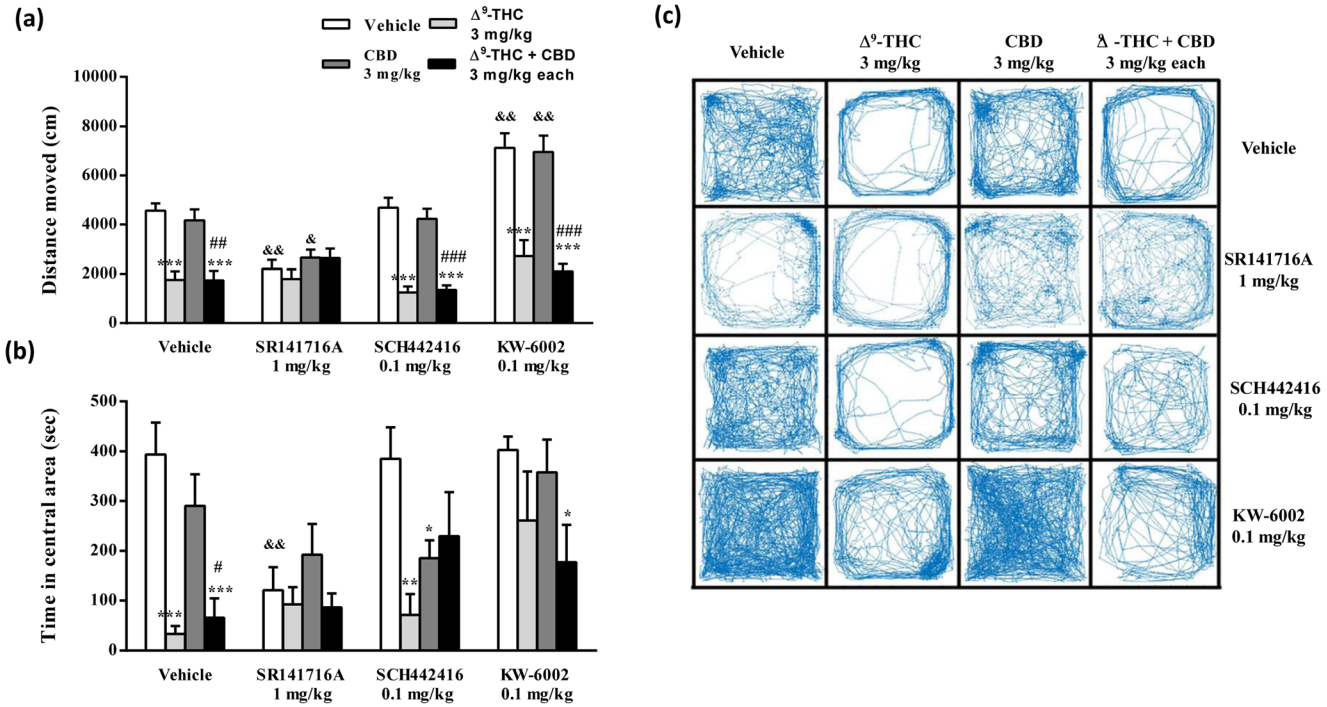


**Fig. 3** CBD does not affect $\Delta^9$-THC-mediated locomotor activity depression or anxiety levels increase. **a** $\Delta^9$-THC (3 mg/kg) significantly reduced the distance travelled by mice in the open field, an effect that was abolished by SR14176A (1 mg/kg) pre-treatment, but not modified by either CBD (3 mg/kg) or SCH442416 (0.1 mg/kg) and KW-6002 (0.1 mg/kg). SR14176A reduced the locomotor activity and KW-6002 increased the total distance travelled, in control mice. **b** $\Delta^9$-THC (3 mg/kg) reduced the time spent in the central zone in the open field. This $\Delta^9$-THC anxiogenic effect was reduced by SR14176A (1 mg/kg) pre-treatment, but not modified by either CBD (3 mg/kg) or SCH442416 (0.1 mg/kg). Intriguingly, KW-6002 (0.1 mg/kg) pre-treatment reduced the anxiogenic effect of $\Delta^9$-THC. SR14176A increased the anxiety levels of control mice**.** SCH442416 pre-treatment potentiated the anxiogenic effects of CBD. Data are expressed as the mean values ± SEM ($n = 7$–8 animals per group). Three-way ANOVA (antagonist pre-treatment, $\Delta^9$-THC and CBD treatments as between factors), two-way ANOVA (antagonist pre-treatment, $\Delta^9$-THC or CBD treatments as between factors) and Dunnett's post hoc test were used for statistical analysis (see "Material and methods" section and Table S1 for details). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle-treated mice. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ compared to the non-receiving antagonist group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to CBD-treated mice. **c** Representative plots showing the animals' tracking during the 30-min evaluation of locomotor activity in the open field arena

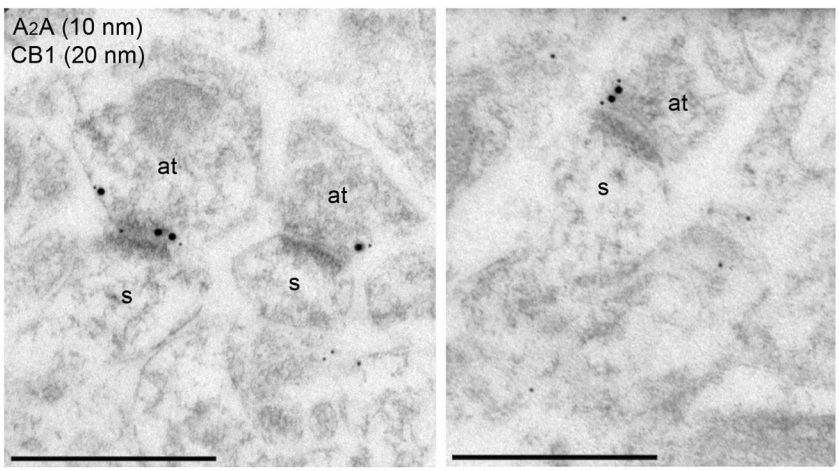


**Fig. 4** $A_{2A}R$-$CB_1R$ co-clustering in presynaptic terminals in the CA1 area of the hippocampus. Electron micrographs showing immunoreactivity for $A_{2A}R$ and $CB_1R$ in the hippocampus (CA1 region) as revealed using a double-labelling, post-embedding immunogold technique. Immunoparticles specifically recognising $A_{2A}R$ (10 nm size) and $CB_1R$ (20 nm size) were detected along the extrasynaptic and perisynaptic plasma membranes of the same presynaptic axon terminals, establishing synaptic contact with dendritic shafts (s). Scale bar: 0.2 μm

proximity within a named GPCR heteromer in native conditions [26]. Thus, using proper antibody combinations, the $A_{2A}R/CB_1R$ heteromer expression in mouse hippocampus (i.e. CA1) was addressed using P-LISA. Indeed, red dots reflecting a positive P-LISA signal were observed in the CA1 region of hippocampus from wild-type mice (Fig. 5a), thus allowing for visualisation of the $A_{2A}R/CB_1R$ receptor-receptor interaction. Interestingly, in hippocampal slices from $A_{2A}R^{-/-}$ mice, the P-LISA signal was negligible (Fig. 5a), thus reinforcing the specificity of our P-LISA approach. Indeed, when the P-LISA signal was quantified, wild-type animals showed $24 \pm 3$ dots/field while $A_{2A}R^{-/-}$ mice displayed only $13 \pm 2$ dots/field, under the same experimental conditions (Fig. 5b). Thus, a marked and significant ($P < 0.01$) reduction in the P-LISA signal was observed in $A_{2A}R^{-/-}$ striatal slices (Fig. 5b). In addition, we included a presynaptic marker, namely the vGlut1, to disclose where the P-LISA signal occurred. A close analysis of the P-LISA signal together with vGlut1 staining revealed a significantly high degree of colocalisation ($70\% \pm 3\%$, $P < 0.001$) (Fig. 5c, d). Overall, our results demonstrated that $A_{2A}R/CB_1R$ heteromers are highly enriched in the presynaptic terminals in the CA1 area of the hippocampus (Fig. 5c, d).

## Discussion

By combining in vivo and complementary molecular techniques, we provide compelling evidence of an $A_{2A}R$-$CB_1R$ interaction occurring in the hippocampus. Of note, the $A_{2A}R/CB_1R$ oligomer would seem likely to be relevant to the capacity of CBD to mitigate cognitive impairment induced by the psychoactive cannabis derivative $\Delta^9$-THC, in a declarative and spatial memory task. Although other brain areas contribute to performance in the two-object recognition test, including the perirhinal cortex and striatum [27, 37], previous findings reveal the crucial role of the hippocampus in $\Delta^9$-THC-induced memory impairment [30, 31] suggesting this brain area as the main target for CBD modulation of $A_{2A}R$-$CB_1R$ interaction. Interestingly, pre-treatment with the preferentially pre- and postsynaptic $A_{2A}R$ antagonists SCH442416 and KW-6002, respectively [32], demonstrated for the first time that this CBD effect was mostly dependent on the activity of presynaptic $A_{2A}R$ receptors. Indeed, further evidence of the involvement of presynaptic $A_{2A}R$ receptors in the regulation of $CB_1R$ activity on memory tasks derived from the observation that a sub-effective dose of $\Delta^9$-THC resulted in memory impairment in those animals previously pre-treated with SCH442416, but not with KW-6002. These results are in line

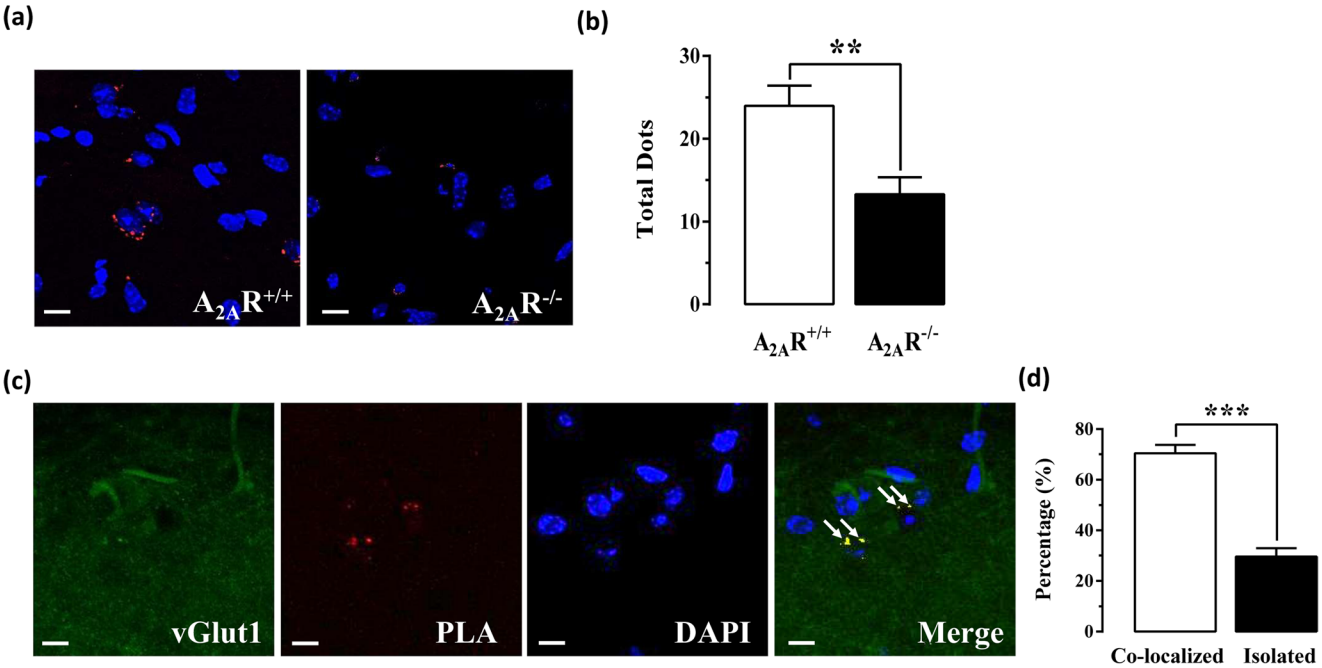


**Fig. 5** $A_{2A}R$-$CB_1R$ heterodimers are present in presynaptic terminals in the CA1 area of the hippocampus. **a** Photomicrographs of dual recognition of $A_{2A}R$ and $CB_1R$ with the proximity ligation in situ assay (P-LISA) in the CA1 area of the hippocampus of wild-type ($A_{2A}R^{+/+}$) and $A_{2A}R$ knockout ($A_{2A}R^{-/-}$) mice. Scale bar: 100 μm. **b** Quantification of P-LISA signals for $A_{2A}R$ and $CB_1R$ proximity confirmed the significant difference in P-LISA signal density between $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice. Values correspond to the mean ± SEM (dots/nuclei) among at least 6 animals. $^{**}P < 0.01$, Student's *t* test. **c** Representative photomicrographs showing specific presynaptic marker vGlut1 immunostaining, the $A_{2A}R/CB_1R$ heteromer detected by P-LISA, and nuclei staining (DAPI) in the hippocampal CA1 region of $A_{2A}R^{+/+}$ mice. Scale bar: 100 μm. **d** The high percentage of colocalisation of the $A_{2A}R$ and $CB_1R$ heterodimers' P-LISA signal with the vGlut1 immunostaining demonstrates that $A_{2A}R$ and $CB_1R$ heterodimers preferentially occur at the presynaptic level in CA1. $^{***}P < 0.001$, Student's *t* test

with the known opposing functional interaction between $A_{2A}R$ and $CB_1R$ [18]. Thus, activation of $A_{2A}R$ may result in an inhibition of $CB_1R$ signalling and an $A_{2A}R$ blockade might facilitate $CB_1R$ activity. Nevertheless, a recent report demonstrated that a synthetic $CB_1R$ agonist-mediated memory disruption was prevented by an adenosine $A_{2A}R$ blockade [38], which suggested that the activity of $A_{2A}Rs$ might also facilitate $CB_1R$ signalling under certain conditions.

Interestingly, our in vivo results also provide evidence supporting a functional cross-talk between both receptors. Thus, pre-treatment with the selective $CB_1R$ antagonist SR141716A resulted in facilitation of a CBD-induced memory impairment in mice, similar to that observed upon $A_{2A}R$ stimulation [39], which was dependent on presynaptic $A_{2A}R$, as demonstrated by the blocking of the same effect upon SCH442416 co-administration. Additionally, we cannot discount the possibility that $A_{2A}R$ might potentiate CBD-mediated negative allosteric modulation of $CB_1R$ activity [40], which could imply a $CB_1R$ conformational rearrangement less favourable to $\Delta^9$-THC binding and activation. Based on the predicted allosteric interactions existing in the context of GPCRs oligomerisation (for review see [41]), this more inactive state of the $CB_1R$ receptor would be precisely as expected if $A_{2A}R$ constitutive activity were not blocked.

The functional hippocampal $A_{2A}R$-$CB_1R$ interaction described here was further extended with compelling data (i.e., immunoelectron microscopy and P-LISA) supporting the existence of presynaptic hippocampal $A_{2A}R$-$CB_1R$ heteromers in vivo. Importantly, $A_{2A}R$-$CB_1R$ heteromers have previously been observed in the dorsal striatum, where they play a relevant role in the modulation of corticostriatal pathways regulating motor activity, cognitive functions and emotional control [18–22], but there is no previous evidence of the presence of similar $A_{2A}R$-$CB_1R$ heteromers in the hippocampus of mice. Our immunoelectron microscopy and P-LISA experiments unequivocally demonstrate the existence of presynaptic $A_{2A}R$-$CB_1R$ heteromers in hippocampal CA1 neurons, precisely where $\Delta^9$-THC exerts its effects leading to memory impairment [30]. Thus, we uncovered the putative physical substrate (i.e., $A_{2A}R/CB_1R$ heteromer) for the functional interplay of adenosinergic and endocannabinoid systems controlling memory formation. In addition, our results indicate that CBD may differentially manipulate $A_{2A}R/CB_1R$ heteromer function in a brain region- and/or subsynaptic-dependent manner. Indeed, while CBD was unable to counter the $\Delta^9$-THC-induced locomotor activity depression, it prevented $\Delta^9$-THC-mediated memory impairment. Thus, although $A_{2A}R$ activity has been demonstrated as modulating the $CB_1R$-mediated regulation of striatum-associated motor responses [20–22], CBD was unable to modulate the striatal $A_{2A}R/CB_1R$ heteromer, in contrast to our observation of memory formation, which appears mainly to be a presynaptic hippocampal $A_{2A}R/CB_1R$ heteromer-related task. Accordingly, it could be concluded that CBD might display functional selectivity [42] depending on its brain region and/or subsynaptic distribution. Indeed, whereas we demonstrate that $A_{2A}R/CB_1R$ heteromers occur at the presynaptic level in the CA1 region of the hippocampus, recent findings demonstrate that the expression of $A_{2A}R/CB_1R$ heteromers in presynaptic corticostriatal projections of the dorsal striatum is almost negligible, but is abundantly present in the somatodendritic compartment and terminals of postsynaptic GABAergic medium spiny neurons [21]. Thus, the additional partners interacting differentially with $A_{2A}R$, either presynaptically (e.g. $A_1R$) [43] or postsynaptically (e.g. $D_2R$ and $mGluR_5$) [44], could be crucial in determining the CBD functional selectivity associated with $A_{2A}R/CB_1R$ heteromer expression and its different physiological effects on memory or motor functions. Additional evidence of the functional selectivity of CBD being dependent on brain region derives from our results on the anxiety levels exhibited in the open field. Anxiety is a complex behaviour integrating neurocognitive and sensory processing, in which the amygdala plays a central role [45]. Thus, our results demonstrate that CBD does not modify the $CB_1R$-dependent anxiogenic effects induced by $\Delta^9$-THC, suggesting a differential activity of CBD in the amygdala and anxiety-related brain structures other than the hippocampus. Interestingly, we demonstrated for the first time that a blockade of presynaptic $A_{2A}R$ induced an increase in the anxiety levels of CBD-treated mice, which provides further evidence of the contribution of $A_{2A}R$ to CBD effects and might contribute towards clarifying the molecular substrate underlying the role of CBD in the treatment of anxiety-related disorders [46].

In conclusion, our results demonstrate that CBD blunts $\Delta^9$-THC-induced memory impairment in an $A_{2A}R$-dependent manner and that these receptors form heteromers with the $CB_1R$ at the presynaptic level in CA1 neurons in the hippocampus. Altogether, our data provide new knowledge about the mechanisms of CBD action, which might be relevant for understanding the multiple beneficial effects ascribed to this natural compound. In addition, these results may assist in the building of better benefit/risk profiles for the clinical use of cannabis derivatives, specifically through avoiding the undesired cognitive side effects associated with $\Delta^9$-THC consumption.

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## Compliance with Ethical Standards

The University of Barcelona Committee on Animal Use and Care approved the protocol. Animals were housed and tested in compliance with the guidelines provided by the Guide for the Care and Use of Laboratory Animals [24] and following the European Union directives (2010/63/EU). All efforts were made to minimise animal suffering and the number of animals used.

**Conflict of Interest** The authors declare that they have no conflict of interest.

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