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Adenosine A2A-cannabinoid CB1 receptor heteromers in the hippocampus: cannabidiol blunts delta-9-tetrahydrocannabinol-induced cognitive impairment

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Abstract:	At present, the clinical interest on the plant-derived cannabinoid compound cannabidiol (CBD) is exponentially rising, since it displays multiple therapeutic properties. In addition, CBD can counteract the undesirable effects of the psychoactive cannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC) that dampen the clinical development of cannabis-based therapies. Despite this attention, the CBD mechanism of action and its interaction with Δ^9-THC are still not completely elucidated. Here, by combining in vivo and molecular complementary techniques, we demonstrate for the first time that CBD blunts the Δ^9-THC-induced cognitive impairment in an adenosine A2A receptor (A2AR)-dependent manner. Furthermore, we revealed the existence of A2AR and cannabinoid CB1 receptor (CB1R) heteromers at the pre-synaptic level in CA1 neurons of the hippocampus. Interestingly, our findings supported a brain region-dependent A2AR-CB1R functional interplay, indeed CBD was not able to modify motor functions presumably regulated by striatal A2AR/CB1R complexes, or anxiety responses related to other brain regions. Overall, these data provide new evidences about the mechanisms of action of CBD and the nature of A2AR-CB1R interaction in brain.

Adenosine A_{2A}-cannabinoid CB_1 receptor heteromers in the hippocampus: cannabidiol blunts Δ^9-tetrahydrocannabinol-induced cognitive impairment

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Abstract

At present, the clinical interest on the plant-derived cannabinoid compound cannabidiol (CBD) is exponentially rising, since it displays multiple therapeutic properties. In addition, CBD can counteract the undesirable effects of the psychoactive cannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC) that dampen the clinical development of cannabis-based therapies. Despite this attention, the CBD mechanism of action and its interaction with Δ^9-THC are still not completely elucidated. Here, by combining *in vivo* and molecular complementary techniques, we demonstrate for the first time that CBD blunts the Δ^9-THC-induced cognitive impairment in an adenosine A_{2A} receptor ($A_{2A}R$)-dependent manner. Furthermore, we revealed the existence of $A_{2A}R$ and cannabinoid CB_1 receptor (CB_1R) heteromers at the pre-synaptic level in CA1 neurons of the hippocampus. Interestingly, our findings supported a brain region-dependent $A_{2A}R$-CB_1R functional interplay, indeed CBD was not able to modify motor functions presumably regulated by striatal $A_{2A}R$/CB_1R complexes, or anxiety responses related to other brain regions. Overall, these data provide new evidences about the mechanisms of action of CBD and the nature of $A_{2A}R$-CB_1R interaction in brain.

Keywords: cannabidiol, Δ^9-tetrahydrocannabinol, cannabis, memory, adenosine 2A receptor, cannabinoid 1 receptor.

Introduction

In recent years, clinical research has increasingly focused on cannabidiol (CBD), the second most significant plant-derived cannabinoid after Δ^9-tetrahydrocannabinol (Δ^9-THC). The reason for this attention is based on 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 Δ^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 botanical extract mainly containing CBD (Epidiolex®, GW Pharmaceuticals, UK) has been recently 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 status in treating newborn children with neonatal hypoxic-ischaemic encephalopathy from the FDA and the European Medicines Agency. Finally, although equally relevant, CBD has been demonstrated to antagonize some undesirable effects of the psychoactive Δ^9-THC, including intoxication, sedation and tachycardia, while it increases the clinical efficacy of Δ^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 Δ^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, $GABA_A$, μ- and δ-opioid receptors [8]. In contrast to Δ^9-THC, CBD exhibits a very low affinity for the orthosteric site of CB_1 and CB_2 receptors (CB_1R and CB_2R), the main

G protein-coupled receptors (GPCRs) belonging to the endogenous cannabinoid system, being the affinity at least three orders of magnitude less than that of other selective compounds [8]. However, CBD has been demonstrated *in vitro* to negatively modulate CB_1R activity [9], likely due to the CBD capacity to behave as a non-competitive negative allosteric modulator (NAM) of CB_1R [10]. Despite these CBD properties, the functional or pharmacodynamic antagonism of Δ^9-THC by CBD is assumed to be not only mediated by a CB_1R mechanism of action but to be also related to the CBD ability to targeting different receptors or enzymes. Among them, one of the most intriguing is the capacity of CBD to modulate adenosine receptors (AR) activity, mainly the $A_{2A}R$ subtype [8]. Evidence for the participation of $A_{2A}R$ on 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]. This $A_{2A}R$-dependent activity of CBD has been proposed to occur through the ability of CBD to bind to the equilibrative nucleoside transporter and in consequence to inhibit adenosine uptake, resulting in an indirect activation of $A_{2A}R$ [16, 17]. In addition, it could also depend on the already demonstrated reciprocal antagonistic functional interaction between $A_{2A}R$ and CB_1R [18, 19], which may be explained, at least in part, by the existence of $A_{2A}R$-CB_1R heteromers [20–22].

Here, we hypothesize that CBD may modulate Δ^9-THC effects via the $A_{2A}R$-CB_1R complex. Therefore, the aim of the present study consisted of investigating the potential participation of the $A_{2A}R$-CB_1R interaction on the CBD-mediated reduction of the main negative consequences of Δ^9-THC consumption (i.e. cognitive impairment) that dampen clinical development of Δ^9-THC-based therapies.

Materials and methods

Drugs and reagents

Δ^9-THC was purchased from Sigma-Aldrich Química SL (Madrid, Spain). CBD, the selective CB_1R antagonist SR141716A and the selective $A_{2A}R$ antagonists SCH442416 and KW-6002 were purchased from Tocris BioScience (Bristol, UK). The cannabinoid compounds Δ^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 intra-peritoneally (i.p.). The $A_{2A}R$ antagonists SCH442416 (0.1 mg/kg) and KW-6002 (0.1 mg/kg) were dissolved in 1% DMSO for i.p. administration. In all the cases, the administration volume was 10 mL/kg body weight.

The primary antibodies used were rabbit anti-CB_1R (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

Male C57BL/6J (Janvier Labs, France) weighting 31.2 ± 0.8 g, at the beginning of the study, and $A_{2A}R$ deficient ($A_{2A}R^{-/-}$) mice [23] were housed 3-4 per cage and maintained under standard animal housing conditions in a 12-h dark-light cycle with free 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 minimize animal suffering and the number of animals used.

Behavioral evaluation

Two-object recognition test: Object-recognition memory was evaluated in a black Plexiglas V-maze with two corridors (30cm long x 4.5cm wide, and 15 cm high walls) set at a 120° angle and slightly illuminated. Immediately after the administration of $A_{2A}R$ or CB_1R selective antagonists or the corresponding vehicle, mice were placed for 9 min in the V-maze where two identical objects were situated at the end of the arms; the time that mice spent exploring each object was recorded. Δ^9-THC and CBD were injected immediately after this training session. Twenty-four hours after, animals were placed again in the V-maze where one of the two familiar objects was replaced by a novel object. The time that the animals spent exploring the two objects was recorded. Object recognition index (RI) was calculated as the difference between the time spent exploring the novel (T_N) and the familiar object (T_F) divided by the total time spent exploring the two objects [$RI=(T_N-T_F)/(T_N+T_F)$]. 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 minutes before the Δ^9-THC and CBD administration. 15 minutes after the natural cannabinoids treatment, spontaneous locomotor activity and anxiety levels were evaluated in an open-field black Plexiglas arena (30 cm long x 30 cm wide) slightly illuminated, where mice were individually placed and videotaped for 30 min. Distance traveled (locomotor activity) and time spent in central zone (20 cm long x 20 cm wide, anxiety levels) were analyzed by the

SpotTracker 2D software (ImageJ, NIH, US) and a customized Matlab application for calculations and plotting (The MathWorks, Inc., Natick, MA, US).

A timeline for behavioral evaluation is included in Figure 1.

Fixed brain tissue preparation

Mice were anesthetized and perfused intracardially with 100-200 ml 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 during 12 h. Coronal sections (25 μm) were processed using a vibratome (Leica Lasertechnik GmbH, Heidelberg, Germany). Slices were collected in Walter's Antifreezing 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 previously described [25]. Briefly, ultrathin sections 80-nm thick 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 28ºC 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 from the CA1 region in hippocampus were then photographed from the selected ultrathin sections at a final magnification of 50,000X.

Proximity ligation assay

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

Statistical analysis

In behavioural experiments, memory performance and locomotor activity were analyzed with three-way ANOVA with antagonist, Δ^9-THC and CBD treatments as between factors, followed by two-way ANOVA for antagonist pre-treatment with Δ^9-THC and CBD treatments as between factors, and Dunnett's *post hoc* when required. Memory performance with a sub-effective dose of Δ^9-THC was analysed with two-way ANOVA with SCH442416 and Δ^9-THC treatments as between factors, followed by Dunnet's *post hoc* test. In the case of the effect of CBD on memory performance after the combination of both $A_{2A}R$ and CB_1R antagonists, data were analyzed with three-way ANOVA with

SR141716A, SCH442416 and CBD treatments as between factors, followed by Dunnett's *post hoc*. P-LISA quantifications were analysed by 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 Δ^9-THC-induced cognitive impairment, but not locomotor or anxiogenic, effects preferentially through pre-synaptic $A_{2A}R$-CB_1R

The involvement of the adenosinergic system in cannabinoid-mediated effects in the central nervous system (CNS) have been largely studied [18]. Here, we aimed to examine the participation of $A_{2A}R$ on the modulatory effects of CBD in Δ^9-THC-mediated memory impairment. To this end, we first assessed the effects of Δ^9-THC in the two-object recognition test, which evaluates mainly hippocampal- and perirhinal cortex-dependent declarative memory performance [27]. Previous findings indicate that the hippocampus plays a crucial role in the CB_1R agonists-induced memory impairment in the two-object recognition test evaluated both in an open field [28] or in a V-maze [29–31]. As expected, Δ^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 with a 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 Δ^9-THC-induced memory impairment (Fig. 2a). Next, we assessed the effect $A_{2A}R$ blockade in the CBD modulation of Δ^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 Δ^9-THC-induced memory impairment in the presence of KW-6002 (0.1 mg/kg) ($P < 0.05$, Table S1*)*, pretreatment with SCH442416 (0.1 mg/kg) significantly ($P < 0.01$, Table S1*)* reduced CBD modulation of Δ^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 Δ^9-THC effects. Further evidence of the participation of presynaptic $A_{2A}R$ on the cognitive impairment mediated by Δ^9-THC was obtained by the evaluation of a sub-effective dose of Δ^9-THC

(1 mg/kg) [30] in combination with SCH442416. Accordingly, pretreatment 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 Δ^9-THC dose ($P < 0.01$). These results suggest again a potential functional interplay between CB_1R and $A_{2A}R$ at the presynaptic level, which would be controlling Δ^9-THC effects.

Interestingly, we unexpectedly observed that CBD produced a 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 for 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. Noteworthy, the marked decrease in the recognition index produced by CBD in mice pretreated with SR14176A was prevented ($P < 0.001$) when animals also received SCH442416, indicating that the CBD-mediated cognitive impairment upon CB_1R blockade 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, all these phenomena could be related to receptor-receptor (*i.e.* $A_{2A}R$-CB_1R) allosteric interactions, as it has been shown for many GPCR heteromers (for review see [33]); however, further work will be needed to substantiate this view.

Moreover, we investigated whether CBD might modulate Δ^9-THC-mediated hypolocomotion and anxiety, which are also CB_1R-dependent effects but regulated by other brain structures different from hippocampus, mainly striatum and amygdala respectively. As expected, Δ^9-THC (3 mg/kg) significantly reduced the total distance traveled by 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 either by CBD (3 mg/kg) or SCH442416 (0.1 mg/kg) and KW-6002 (0.1 mg/kg) (Fig. 3a and 3c). On the other hand, a significant *per se* effect of SR14176A (hypolocomotion) and KW-6002 (hyperlocomotion) ($P < 0.05$ and $P < 0.01$, respectively, Table S1*)* was also observed (Fig. 3a and 3c), which is consistent with previous data [34, 35]. Similarly, Δ^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 modified by CBD or SCH442416 (Fig. 3b). SR14176A blocked the Δ^9-THC effect although 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 about an interaction between pre-synaptic $A_{2A}R$ and CBD. In contrast to control mice, pretreatment with KW-6002 prevented Δ^9-THC-treated mice to exhibit a significant reduction in the time spent in central area, despite a tendency (Fig. 3b). However, we cannot discard in this case a bias in the anxiety evaluation in the open field due to the KW-6002 effect in locomotor activity (Fig. 3a).

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

$A_{2A}R$ and CB_1R heteromerize in presynaptic terminals at the CA1 region in the mouse hippocampus

Our observations in the two-object recognition test clearly demonstrated that the CBD capacity to block Δ^9-THC-mediated memory impairment was $A_{2A}R$-dependent. Since these Δ^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 such brain

area, which might represent a novel physical substrate for such CBD-mediated modulation of Δ^9-THC effects. We focused in the CA1 region of the hippocampus since it was demonstrated 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]. First, 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 described technique providing enough sensitivity to evaluate receptor's close proximity within a named GPCR heteromer in native conditions [26]. Thus, by using proper antibody combinations, the $A_{2A}R/CB_1R$ heteromer expression in mouse hippocampus (i.e. CA1) was addressed by the P-LISA assay. Indeed, red dots reflecting a positive P-LISA signal was observed in the CA1 region of the hippocampus from wild-type mice (Fig. 5a), thus allowing the visualization 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 assay. Indeed, when the P-LISA signal was quantified, wild-type animals showed 24 ± 3 dots/field while $A_{2A}R^{-/-}$ mice displayed only 13 ± 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). Interestingly, we included a pre-synaptic marker, namely the vGlut1, to disclose where the P-LISA signal occurred. A close analysis of the P-LISA signal together with the vGlut1 staining demonstrated a significant

high degree of co-localization (70 % ± 3%, $P < 0.001$) (Fig. 5c and d). Overall, our results demonstrated that $A_{2A}R/CB_1R$ heteromers are highly enriched in the pre-synaptic terminals at the CA1 area of the hippocampus (Fig. 5c and d).

Discussion

By combining *in vivo* and molecular complementary techniques, here we provide compelling evidence about an $A_{2A}R$-CB_1R interaction occurring in the hippocampus. Of note, the $A_{2A}R/CB_1R$ oligomer would likely result to be relevant for CBD capacity to mitigate the cognitive impairment induced by the psychoactive cannabis derivative Δ^9-THC in a declarative and spatial memory task. Although other brain areas contribute to the two-object recognition test performance, including perirhinal cortex and striatum [27, 37], previous findings reveal a crucial role for hippocampus in the Δ^9-THC-induced memory impairment [30, 31] suggesting this brain areas as the main target for this CBD modulation of $A_{2A}R$-CB_1R interaction. Interestingly, the pre-treatment with the preferentially pre- and post-synaptic $A_{2A}R$ antagonists SCH442416 and KW-6002, respectively [32], demonstrated for the first time that this CBD effect was mostly dependent on the activity of pre-synaptic $A_{2A}R$ receptors. Indeed, further evidence about the involvement of pre-synaptic $A_{2A}R$ receptors in the regulation of CB_1R activity on memory tasks derived from the fact that a sub-effective dose of Δ^9-THC resulted in memory impairment in those animals previously pre-treated with SCH442416, but not with KW-6002. These results are in line with the already 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 signaling and $A_{2A}R$ blockade might facilitate CB_1R activity. Nevertheless, a recent report demonstrated that a synthetic CB_1R agonist-mediated memory disruption was prevented by adenosine $A_{2A}Rs$ blockade [38], which suggested that the activity of $A_{2A}Rs$ might also facilitate the CB_1R signaling under certain conditions.

Interestingly, our *in vivo* results also provide evidence supporting a functional cross-talk between both receptors. Thus, the pre-treatment with the selective CB_1R

antagonist SR141716A resulted in a facilitation of a CBD-induced memory impairment in mice, similar to that observed upon $A_{2A}R$ stimulation [39], which was dependent on pre-synaptic $A_{2A}R$ as demonstrated by blocking such effect upon SCH442416 co-administration. Additionally, we cannot disclose the possibility that $A_{2A}R$ might potentiate the CBD-mediated negative allosteric modulation of CB_1R activity [40], which could imply a CB_1R conformational rearrangement less favorable to Δ^9-THC binding and activation. Based on the predicted allosteric interactions existent in the context of GPCRs oligomerization (for review see [41]), this more inactive state of the CB_1R receptor would be precisely the expected when $A_{2A}R$ constitutive activity would not be 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 pre-synaptic hippocampal $A_{2A}R$-CB_1R heteromers *in vivo*. Importantly, $A_{2A}R$-CB_1R heteromers have been previously described in 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 was no previous evidence about 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 pre-synaptic $A_{2A}R$-CB_1R heteromers in hippocampal CA1 neurons, precisely where Δ^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 also 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 preclude the Δ^9-THC-induced locomotor activity depression, it prevented Δ^9-THC-mediated memory impairment. Thus, although $A_{2A}R$

activity has been demonstrated to modulate 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 what we observed on memory formation, which appears to be mainly a pre-synaptic 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 occurs at the pre-synaptic level in the CA1 region of the hippocampus, recent findings demonstrated that the expression of the $A_{2A}R/CB_1R$ heteromers in pre-synaptic corticostriatal projections of the dorsal striatum is almost negligible, but abundantly present in the somatodendritic compartment and terminals of post-synaptic GABAergic medium spiny neurons [21]. Thus, the additional partners differentially interacting with $A_{2A}R$, either presynaptically (*e.g.*, A_1R) [43] or postsynaptically (*e.g.*, D_2R and $mGluR_5$) [44] could be crucial to determine CBD functional selectivity associated to $A_{2A}R/CB_1R$ heteromers expression and its different physiological effects on memory or motor functions. Additional evidence about the functional selectivity of CBD depending on brain region derives from the present results on the anxiety levels evaluated in the open field. Anxiety is a complex behavior integrating neurocognitive and sensory processing, in which amygdala plays a central role [45]. Thus, our results demonstrate that CBD do not modify the CB_1R-dependent anxiogenic effects induced by Δ^9-THC, suggesting a differential activity of CBD in the amygdala and other anxiety-related brain structures apart from hippocampus. Interestingly, we demonstrate for the first time that the blockade of presynaptic $A_{2A}R$ induced an increase in the anxiety levels of CBD-treated mice, which provide further evidence about the contribution of $A_{2A}R$ to the CBD effects and might contribute to

clarify the molecular substrate underlying the role of CBD in the treatment of anxiety-related disorders [46].

In conclusion, our results demonstrate that CBD blunts the Δ^9-THC-induced memory impairment in an $A_{2A}R$-dependent manner and that these receptors form heteromers with the CB_1R at the pre-synaptic level in CA1 neurons of the hippocampus. Altogether, our data provide new evidence about CBD mechanisms of action, which might be relevant to understand the multiple beneficial effects described for this natural compound. In addition, these results may lead to consider how to gain a better benefit/risk profile when clinically using cannabis derivatives, especially by avoiding undesired cognitive side effects associated to Δ^9-THC consumption.

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Figure Legends

Figure 1. Timeline for behavioral evaluation. (a) Memory performance was evaluated in the two-object recognition test. Immediately after the administration of $A_{2A}R$ or CB_1R selective antagonists or the corresponding vehicle (minute 0), mice were placed for 9 min in the V-maze for the training session. Δ^9-THC and CBD were injected immediately after this training (minute 10). Twenty-four hours after, animals were placed again in the V-maze 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 minutes before the Δ^9-THC and CBD administration. 15 minutes after the natural cannabinoids treatment, spontaneous locomotor activity and anxiety levels were evaluated in an open-field for 30 minutes.

Figure 2. CBD modulatory effects on Δ^9-THC-mediated memory impairment are $A_{2A}R$-dependent. (a) Mice were treated with Δ^9-THC (3 mg/kg), CBD (3 mg/kg) or a Δ^9-THC/CBD (3 mg/kg each compound) combination in the absence or presence of SR141716A (1 mg/kg), SCH442416 (0.1 mg/kg) or KW-6002 (0.1 mg/kg). Δ^9-THC induced memory impairment in mice, which were prevented by the pre-treatment with SR141716A. CBD co-treatment completely blunted the Δ^9-THC-induced memory impairment in a pre-synaptic $A_{2A}R$-dependent manner. CBD administration significantly reduced memory performance in mice pre-treated with SR141716A. **(b)** A sub-effective dose of Δ^9-THC (1 mg/kg) resulted in a cognitive impairment effect when the pre-synaptic $A_{2A}R$ were blocked by SCH442416, but not by KW-6002 **(c). (d)** The memory impairment produced by CBD in mice pre-treated with SR14176A was prevented by the co-administration of SCH442416. Data are expressed as the mean values ± s.e.m. (n = 5-8 animals per group). Three-way ANOVA (antagonist pre-treatment, Δ^9-THC and CBD

treatments as between factors), two-way ANOVA (antagonist pre-treatment, Δ^9-THC or CBD treatments as between factors), and Dunnett's *post hoc* were used for statistical analysis (See Material and methods section and Table S1 per details). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle-treated mice. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to Δ^9-THC-treated mice. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ compared to non-receiving antagonist group of mice.

Figure 3. CBD do not affect Δ^9-THC-mediated locomotor activity depression or anxiety levels increase. (a) Δ^9-THC (3 mg/kg) significantly reduced the distance traveled by mice in the open-field, an effect that was abolished by SR14176A (1 mg/kg) pre-treatment, but not modified either by CBD (3 mg/kg) nor SCH442416 (0.1 mg/kg) and KW-6002 (0.1 mg/kg). SR14176A reduced the locomotor activity and KW-6002 increased the total distance traveled in control mice. **(b)** Δ^9-THC (3 mg/kg) reduced the time spent in central zone in the open field by mice. This Δ^9-THC anxiogenic effect was reduced by SR14176A (1 mg/kg) pre-treatment, but not modified either by CBD (3 mg/kg) nor SCH442416 (0.1 mg/kg). Intriguingly, KW-6002 (0.1 mg/kg) pretreatment reduced the anxiogenic effect of Δ^9-THC. SR14176A increased the anxiety levels of control mice**.** SCH442416 pretreatment potentiated the anxiogenic effects of CBD. Data are expressed as the mean values ± s.e.m (n = 7-8 animals per group). Three-way ANOVA (antagonist pre-treatment, Δ^9-THC and CBD treatments as between factors), two-way ANOVA (antagonist pre-treatment, Δ^9-THC or CBD treatments as between factors), and Dunnett's *post hoc* were used for statistical analysis (See Material and methods section and Table S1 per details). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle-treated mice. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ compared to non-receiving antagonist group of mice. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ 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.

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

Figure 5. $A_{2A}R$-CB_1R heterodimers are present in presynaptic terminals at 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 of P-LISA signal density between $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice. Values correspond to the mean ± s.e.m. (dots/nuclei) of at least 6 animals for condition. $^{**}P < 0.01$, 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 hippocampal CA1 region of $A_{2A}R^{+/+}$ mice. Scale bar: 100 μm. **(d)** The high percentage of co-localization 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.

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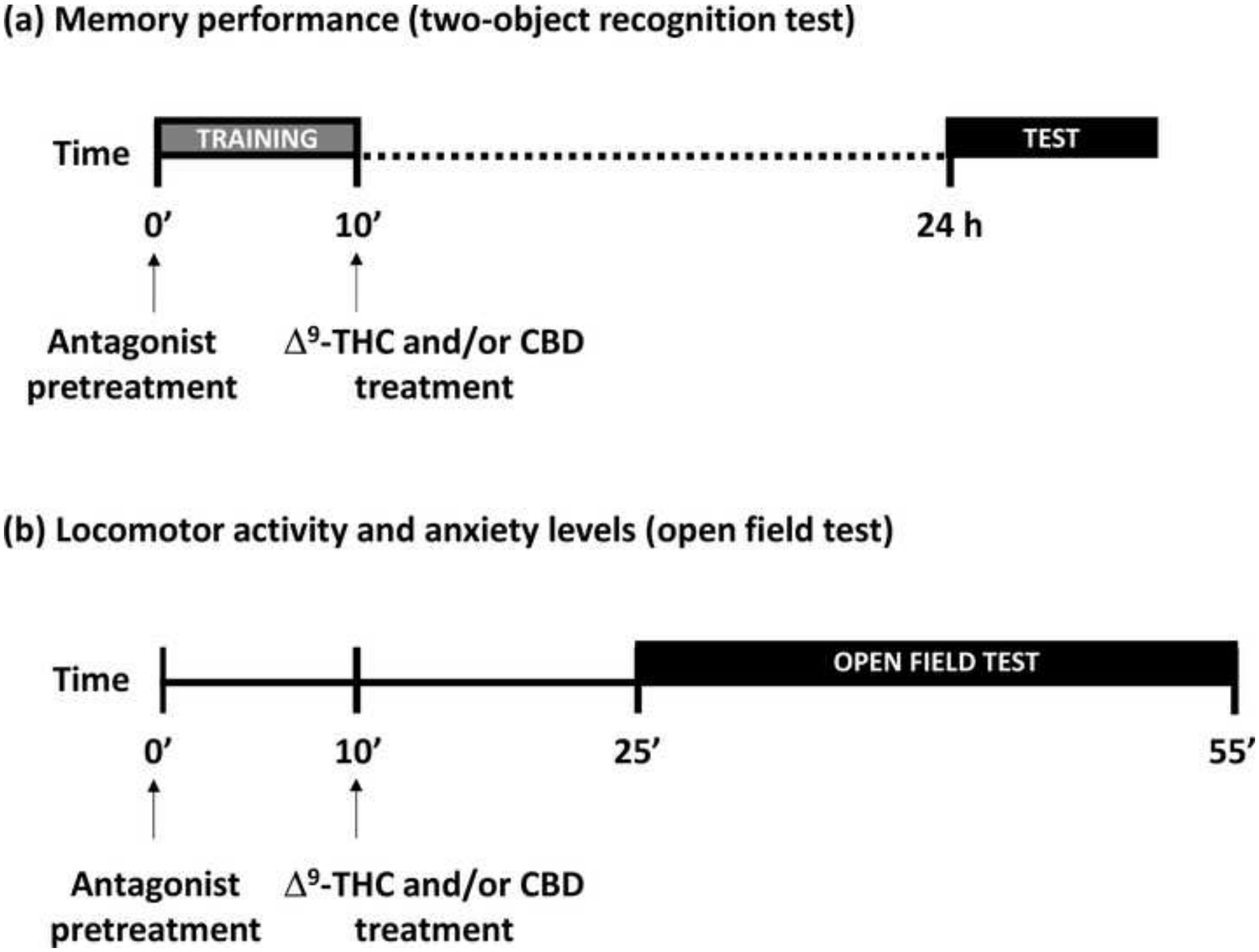

Figure 2

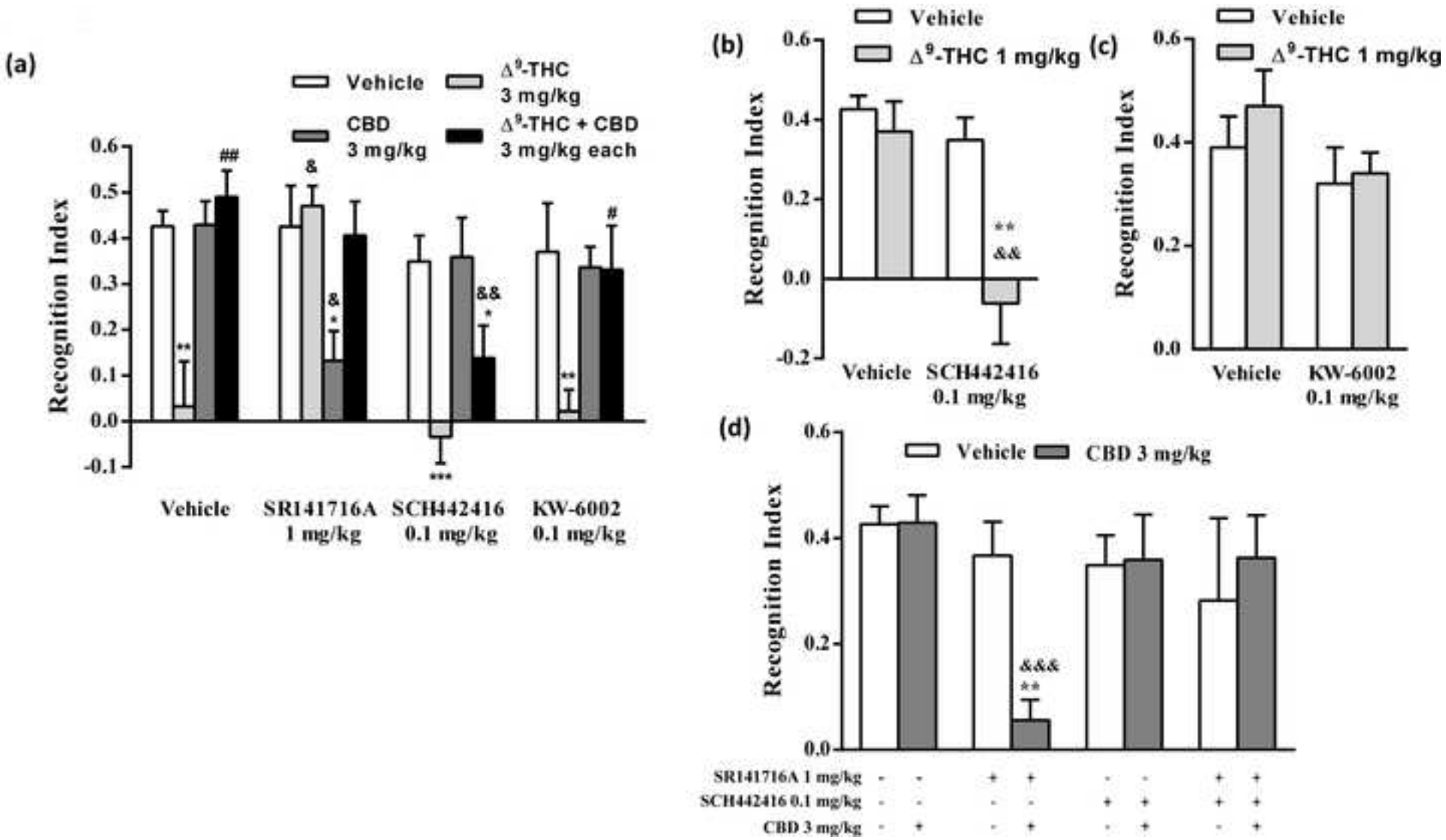

Figure 3

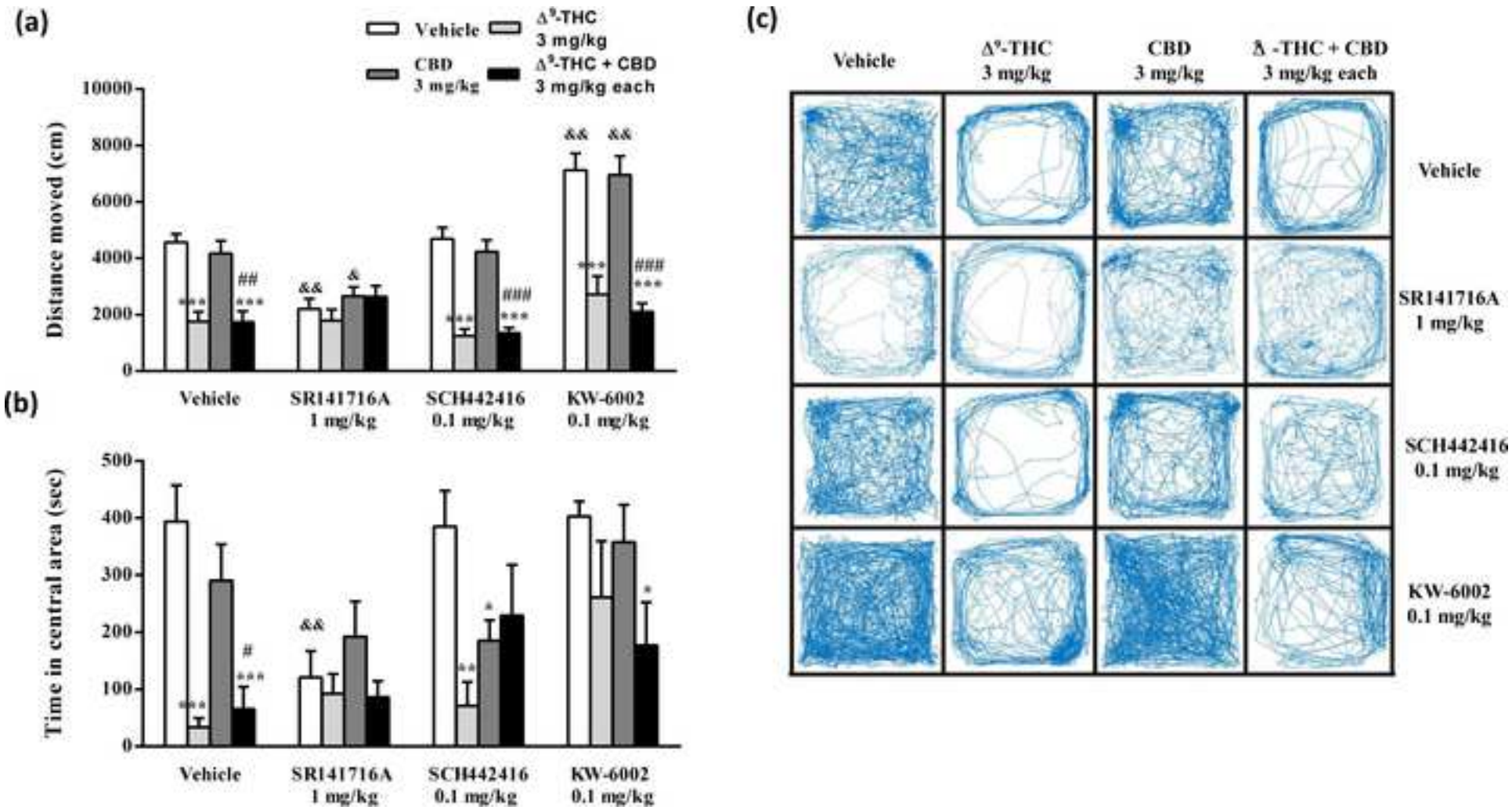

Figure 4

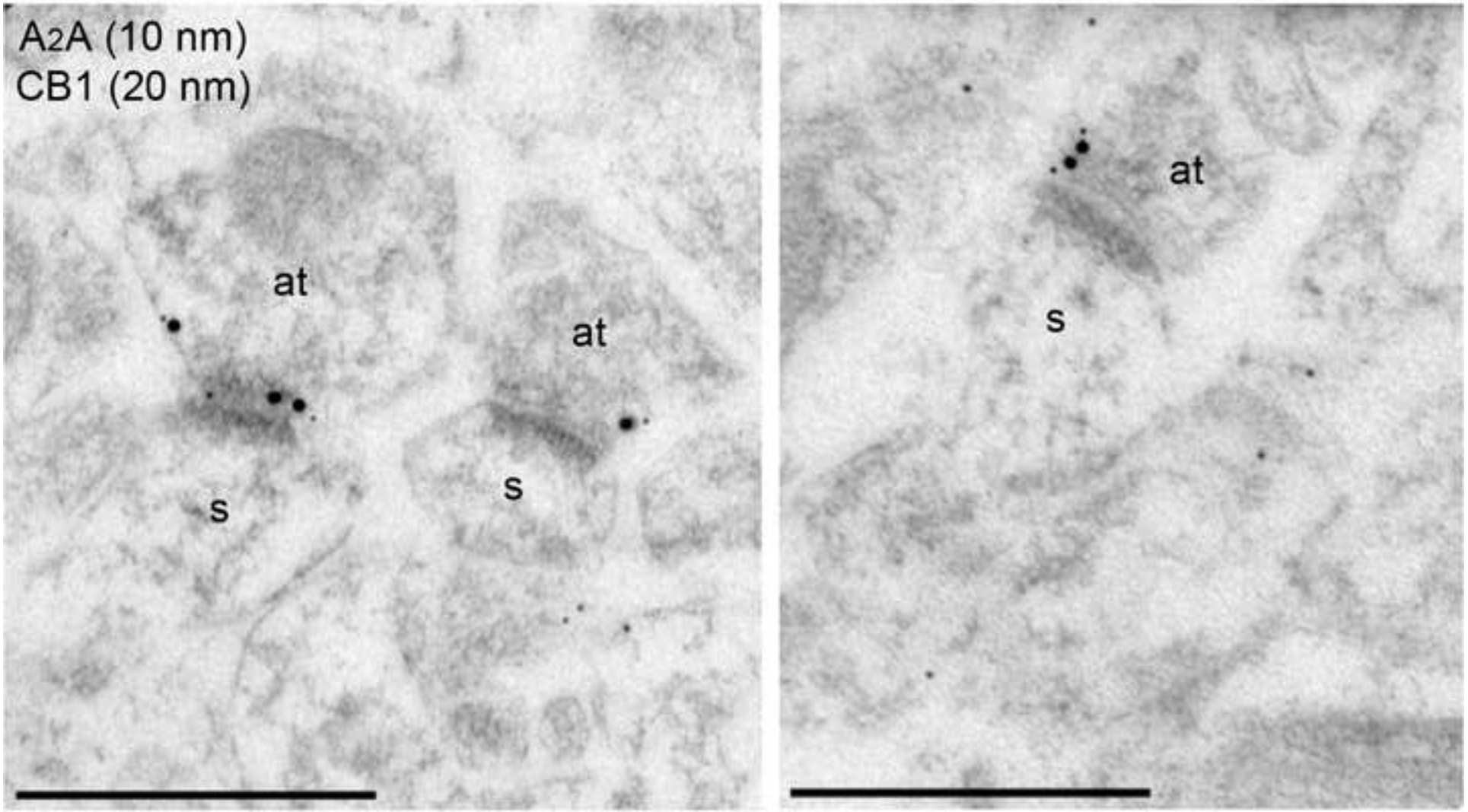

Figure 5