



Cannabidiol biases A_{2A}-CB₂ receptor heteromer function by decoupling β-arrestin signaling from complex formation[☆]

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ABSTRACT

Cannabidiol (CBD), a phytocannabinoid with pleiotropic effects, exhibits complex mechanisms of action that remain incompletely understood, particularly its role as an allosteric modulator of G protein-coupled receptor (GPCR) heteromers. Among these, the adenosine A_{2A}-cannabinoid CB₂ receptor heteromer (A_{2A}R-CB₂R) is a functionally relevant complex implicated in diverse physiological processes. This study aimed to characterize the modulatory effects of CBD on A_{2A}R-CB₂R heteromers.

A multi-technique approach was employed using HEK-293T cells co-transfected with A_{2A}R and CB₂R. Real-time NanoBRET assays at 37 °C were used to assess receptor interaction kinetics. Functional consequences were evaluated via β-arrestin II recruitment assays, and complex formation was visualized and quantified using proximity ligation assays (PLA).

CBD acted as a potent allosteric modulator, enhancing the kinetics of agonist-induced A_{2A}R-CB₂R interaction. Notably, this effect was accompanied by functional dissociation: CBD inhibited β-arrestin II recruitment in a probe-dependent manner, with significant modulatory effects at concentrations of 100 nM, and showed a greater inhibition of the selective CB₂R agonist JWH-133-induced signaling than that induced by CGS 21680, a selective A_{2A}R agonist. PLA data confirmed that this functional modulation occurred without altering the extent of heteromer complex formation. These findings reveal that CBD operates as a biased allosteric modulator of the A_{2A}R-CB₂R heteromer. Rather than disrupting heteromer formation, CBD selectively induces a conformational state that uncouples physical receptor interaction from β-arrestin II signaling. This defines a distinct mechanism of action for CBD and highlights A_{2A}R-CB₂R heteromers as promising targets for biased signaling-based therapeutics.

1. Introduction

Phytocannabinoids, the chemically diverse metabolites of *Cannabis*

sativa L., are increasingly recognised as a versatile pharmacological reservoir for human therapeutics [1]. Their clinical relevance is already exemplified by dronabinol (Marinol®, synthetic Δ⁹-

Abbreviations: A_{2A}, Adenosine; A_{2A}R, Receptor; BRET, Bioluminescence Resonance Energy Transfer; BRET₅₀, BRET at half-maximal acceptor concentration; BRET_{MAX}, Maximal BRET signal; BSA, Bovine Serum Albumin; CBD, Cannabidiol; CB₂R, Cannabinoid CB₂ Receptor; C_F, Correction Factor; CI, Confidence Interval; DMEM, Dulbecco's Modified Eagle's Medium; EC₅₀, Half maximal effective concentration; FBS, Fetal Bovine Serum; GPCR, G protein-coupled receptor; IC₅₀, Half maximal inhibitory concentration; mBU, milliBRET units; Nluc, Nanoluciferase; ns, not significant; PBS, Phosphate-Buffered Saline; PEI, Polyethylenimine; PLA, Proximity Ligation Assay; SEM, Standard Error of the Mean; t_{1/2}, Half-time; wt, wild-type; YFP, Yellow Fluorescent Protein.

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tetrahydrocannabinol) for chemotherapy-induced nausea and AIDS-related anorexia, nabiximols (Sativex[®], an oromucosal spray with an ~1:1 THC:CBD ratio) for multiple-sclerosis spasticity, and purified cannabidiol (Epidiolex[®]) for refractory childhood epilepsies [2].

Whereas Δ^9 THC is psychotropic, cannabidiol (CBD) and several other major phytocannabinoids are non-intoxicating, thereby widening the therapeutic window for cannabinoid-based interventions [3]. For decades these benefits were ascribed almost exclusively to the activation of the $G_{i/o}$ -coupled cannabinoid receptors CB₁ and CB₂ [4].

However, mounting evidence reveals a far broader pharmacology: CBD acts as an agonist at the nociceptive TRPV1 ion channel, activates the nuclear receptor PPAR γ , positively modulates the serotonin 5-HT_{1A} $G_{i/o}$ -coupled receptor, modulates the functionality of the adenosine A_{2A} G protein-coupled receptor (GPCR), antagonizes the orphan GPCR GPR55, and directly blocks voltage-gated Na⁺ and L-type Ca²⁺ channels. [5,6]. The ability of a single molecule to influence such a heterogeneous ensemble of proteins complicates the attribution of specific clinical outcomes to individual targets, and suggests that some effects may arise through subtle allosteric modulation rather than straightforward orthosteric binding. Consequently, new integrative approaches, combining poly-pharmacological profiling, high-resolution structural biology, and systems-level signalling analyses, are required to unravel the mechanistic underpinnings of CBD's therapeutic actions. Taken together, these pleiotropic interactions underscore the therapeutic promise of phytocannabinoids and highlight the need for integrative mechanistic studies of readily available molecules.

CBD may interact with the orthosteric centre of the cannabinoid receptors [7], but we have also found that it also acts at an allosteric site of the CB₂ receptor (CB₂R) [8]. The affinity of CBD for the allosteric site in the CB₂R seems to be higher than the affinity for the orthosteric site thus suggesting that its main physiological action is to allosterically modulate CB₂R-mediated endocannabinoid action. Two different laboratories have shown that CBD is also an allosteric modulator of the adenosine A_{2A} receptor [9,10], which is a target of neuroprotection and whose antagonists are proposed for the therapy of Parkinson's disease. In fact, one of them, istradefylline, has been approved in Japan and USA for coadjuvant therapy of the disease [11,12]. A_{2A}R antagonists are also undergoing clinical trials aimed at improving the efficacy of anti-cancer chemotherapy and immunotherapy [13,14,15]. Cannabinoids are also considered for preventing and/or treating some types of cancers [16]. In this context the receptor complex formed by CB₂ and A_{2A} receptors becomes relevant. The two receptors, when expressed in heterologous cells or in native cells establish receptor-receptors interactions whose functionality is not fully deciphered [17,18]. Heteromerization of GPCRs provides versatility and contributes to better understand functional selectivity, i.e. how targeting a given receptor may lead to differential signaling depending on the heteromeric context [19,20].

The aim of this study was to assess the effect of cannabidiol on heteromers formed by adenosine A_{2A} and cannabinoid CB₂ receptors. Conformational changes upon binding of the phytocannabinoid to the receptors have been detected using a resonance energy transfer technique, taking advantage of thermostating at 37 °C. This level of temporal resolution was not achievable at room temperature, underscoring the impact of physiological temperature on receptor behavior. These findings align with recent structural studies of TRPM4, that reveal how physiological temperature can be relevant to study conformational states and ligand interactions that remain undetected at lower temperatures [21]. As a result, it was possible to detect the kinetics of conformational changes in response to agonists of the two receptors in the presence and absence of CBD. CBD accelerated the conformational changes mediated by agonists of the two receptors also affecting the recruitment of β -arrestin II.

In summary, our results show a previously uncharacterized mechanism of action for CBD at the A_{2A}R-CB₂R heteromer. Interestingly, we found that CBD promoted faster agonist-induced conformational changes and simultaneously inhibited β -arrestin II recruitment,

particularly when the heteromer was activated via the CB₂R agonist. This finding of biased signaling is further contextualized by our Proximity Ligation Assays (PLA). While nanoBRET revealed that CBD modulates the kinetics of the agonist-induced interaction, PLA results show that CBD does not significantly alter the total number of detectable A_{2A}R-CB₂R heteromers at a defined endpoint.

2. Methods

2.1. Reagents

CGS 21,680 (A_{2A}R agonist, ref. C141), JWH-133 (CB₂R agonist, ref. SML3627), SCH-58261 (A_{2A}R antagonist, ref. S4568), SR144528 (CB₂R antagonist, ref. SML0800), PolyEthylenImine (PEI, ref. 408727), and Hoechst 33,432 (ref. 14533) were purchased from Merck (St Louis, MO, USA). CBD was purified by PhytoPlant Research S.L.U. from a GOYA variety (CPVO 20180113) following a direct crystallization method that is described elsewhere [22]. The purity was set at > 98 % (data provided by PhytoPlant Research S.L.U., Cordoba, Spain).

Concentrated (10 mM) stock solutions of agonists/antagonists prepared in DMSO (Merck ref. D4540; St Louis, MO, USA) and were stored at -20°; they were thawed and diluted before use.

2.2. Cell culture

HEK-293T cells, batch 70022180, were acquired from the American Type Culture Collection (ATCC). Cells were amplified and frozen in liquid nitrogen in several aliquots.

Cells from each aliquot were used until passage 18. HEK-293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland, UK) supplemented with 2 mM L-glutamine, 100 μ g/mL sodium pyruvate, 100 U/mL penicillin/streptomycin, MEM non-essential amino acids solution (1/100) and 5 % (v/v) heat-inactivated fetal bovine serum (FBS) (all supplements were from Invitrogen, Paisley, Scotland, UK) and maintained at 37° in a humid atmosphere of 5 % CO₂.

2.3. Cell transfection

HEK-293T cells were transiently transfected with the corresponding cDNA(s) by the PEI (PolyEthylenImine) method. Briefly, cDNA diluted in 150 mM NaCl (PanReac AppliChem ref. 131659; Barcelona, Spain) was mixed (10 min) with PEI (5.5 mM in nitrogen residues) and prepared in 150 mM NaCl for 10 min. The cDNA-PEI complexes were placed in contact with HEK-293T cells and were incubated for 4 h in a serum-free medium. Then, the medium was replaced by a fresh supplemented culture medium, and cells were maintained at 37° in a 5 % CO₂ humid atmosphere. 48 h after transfection, cells were washed, detached, and resuspended in the assay buffer.

2.4. Expression vectors

pcDNA3.1-based plasmids encoding for A_{2A}R-NanoLuc, A_{2A}R-YFP, CB₂R wt, CB₂R-YFP and D₄R-YFP fusion proteins were used. A_{2A}R-Nanoluc plasmid was generated by VectorBuilder company and express fusion protein in C-terminal end. Plasmids encoding YFP fusion protein were generated by subcloning the coding region of each receptor to be in-frame with pEYFP-N1 vector (PerkinElmer, Waltham, MA, US) to provide plasmids that express the CB₂ and D₄ receptors with YFP proteins fused on the C-terminal end.

2.5. Immunocytochemistry

Cells were fixed in 4 % paraformaldehyde (ref. 100496; Merck) for 15 min and washed twice with PBS containing 20 mM glycine (ref. A1067; PanReac) before permeabilization with PBS-glycine containing

0.2 % Triton X-100 (ref. 142314.1611, PanReac) for 10 min incubation. Then, samples were blocked with PBS-gly with 1 % bovine serum albumin (BSA) (ref. A2153, Merck) for 1 h. Subsequently, cells were incubated with a mouse monoclonal anti-Nanoluc primary antibody (1/100, N7000, Promega Biotech Ibérica, Madrid, Spain) for 1 h and then were washed and incubated with a secondary Cy3-conjugated anti-mouse IgG antibody (1/200, ref. 115–165-003, Jackson ImmunoResearch, West Grove, PA, USA) (red) for 2 h. CB₂R-YFP was detected based on its intrinsic fluorescence using appropriate excitation settings. In all cases, nuclei were stained with Hoechst 33,432 (1/100 from 1 mg/mL stock; Sigma-Aldrich) for 10 min. Samples were washed several times with 1 % PBS-glycine-BSA solution and preserved at 4° in the dark using EpreDia™ Immu-mount® (ref. 10622689, ThermoFisher) until imaging. Confocal images were acquired on a Zeiss LSM 880 microscope (Zeiss, Jena, Germany) using a 63x oil-immersion objective.

2.6. Bioluminescence resonance energy transfer (nanoBRET) assays

Nanoluc (Nluc) offers significantly higher luminescence intensity than traditional *Renilla luciferase*, which takes advantage of the bright and engineered Nanoluc luciferase to improve signal-to-noise ratios and sensitivity over conventional Rluc-based assays. HEK-293T cells were transiently cotransfected with a constant amount of cDNA encoding for A_{2A}R-Nluc (30 ng) and with increasing amounts of cDNA corresponding to either CB₂R-YFP (0.5–2.5 µg), or with D₄R-YFP (0.25–1 µg), as a negative control for nanoBRET assay.

To assess cell amount, protein concentration was determined using a Bradford assay kit (Bio-Rad, Munich, Germany) using BSA dilutions as standards, and the total of cells was adjusted to 20 µg of protein using a Bradford assay kit (ref. 5000205, Bio-Rad, Munich, Germany). To quantify protein-YFP expression, fluorescence was read in a Glomax® Discover plate reader (ref. GM3000, Promega Biotech Ibérica, Madrid, Spain) equipped with a head-on photon counting photomultiplier tube (PMT) detector, using an excitation at 475 nm with a wavelength-matched LED.

For nanoBRET measurements, the equivalent to 20 µg protein cell suspension was distributed in 96-well microplates (white plates, ref. 11457009) Porvair, Leatherhead, UK) and furimazine (5 µM) was added (ref. T15359, Tebubio; Barcelona, Spain). 30 s after furimazine addition, the readings were collected using a Glomax® Discover plate reader, which allows the integration of the signals detected in the short-wavelength filter at 460 nm and the long-wavelength filter at 530 nm.

To quantify receptor-Rluc expression, luminescence readings were collected 10 min after adding furimazine (5 µM) using a Glomax® Discover plate reader. MilliBRET units (mBU) are defined as:

$$mBU = \frac{\lambda_{530nm} \text{ (long wavelength emission)}}{\lambda_{460nm} \text{ (short wavelength emission)}} - C_F \times 1000; \text{ where correction factor } (C_F), \text{ corresponds to } [(\text{long-wavelength emission})/(\text{short-wavelength emission})] \text{ for cells only expressing the Nanoluc-containing donor.}$$

For kinetics measurements, cells were cotransfected with a constant amount of cDNA encoding for A_{2A}R-Nluc (30 ng) and CB₂R-YFP (1.5 µg). 48 h later, during the assay, cells were maintained at 37° in Glomax® Discover plate reader to maintain physiological conditions and ensure optimal cell viability and receptor function. Cells were treated with CBD or vehicle for 30 min. Then, furimazine (5 µM) was added and subsequently cells were treated with the CGS 21680, JWH-133 or vehicle. After that, nanoBRET readings were collected every minute for a total of 70 min. Kinetics fits represent the ΔBRET, i.e., the changes in BRET ratios relative to non-treated BRET between A_{2A}R and CB₂R receptors.

BRET_{MAX} is defined as the maximal achievable value of nanoBRET, and this circumstance occurs when all available donor receptors are paired up with acceptor receptors. Accordingly, the half-time (mins) is defined as the time at which 50 % of the BRET_{MAX} is achieved.

2.7. β-Arrestin II recruitment assays

β-Arrestin II recruitment was analysed by determining the bioluminescence energy transfer signal between β-arrestin II-Nanoluc (donor) and the YFP-labeled receptor (acceptor). HEK-293T cells were transiently transfected as described in *Cell transfection* methods section with 20 ng of cDNA coding for β-arrestin II-Nluc, 1.5 µg of cDNA coding for A_{2A}R-YFP and 1.5 µg of cDNA coding for CB₂R wt. 48 h later, for nanoBRET measurements, the equivalent to 20 µg protein cell suspension was distributed in 96-well microplates (white plates, ref. 11457009) Porvair, Leatherhead, UK).

During the assay, cells were maintained at 37° in Glomax® Discover plate reader to maintain physiological conditions and ensure optimal cell viability and receptor function. Cells were treated for 15 min with vehicle (basal), with the A_{2A}R or CB₂R antagonist (100 nM SCH 58261 or 100 nM SR 144528, respectively) or with CBD (100 nM or 1 µM). Then, furimazine (5 µM) was added and, subsequently, cells were treated with vehicle (basal) or with the A_{2A}R and/or CB₂R agonist (100 nM CGS 21680, 100 nM JWH-133). NanoBRET readings were collected 15 min after adding furimazine.

2.8. Proximity ligation assay (PLA)

The interaction and alterations in the formation of heteromeric complexes between A_{2A} and CB₂ receptors in HEK-293T cells were detected using the PLA detection Kit (ref. DUO92008, Sigma-Aldrich; St Louis, MO, USA) following the instructions of the supplier. HEK-293T cells were grown on glass coverslips and transfected using *Cell transfection* method. After 48 h, cells were maintained at 37° and treated for 30 min with CBD (100 nM) or vehicle. After that, cells were treated for 1 h with CGS 21680 (100 nM) or JWH-133 (100 nM) and, immediately after, fixed in 4 % paraformaldehyde (ref. 100496, Merck; St Louis, MO, USA) for 15 min.

Then, cells were washed with PBS containing glycine (20 mM) to quench the aldehyde groups and permeabilized with the same buffer containing Triton X-100 0.05 % (ref. 142314.1611, PanReac; Barcelona, Spain) for 15 min and successively washed with PBS. Then, samples were incubated (1 h) at 37° with a blocking solution (ref. DUO82007, Sigma Aldrich; St Louis, MO, USA) in a pre-heated humidity chamber. After overnight incubation with the antibody diluent medium having a mixture of equal amounts of rabbit anti-A_{2A}R (AB1559, Merck; St Louis, MO, USA) (1/100) and mouse anti-CB₂R (sc-293188, Santa Cruz; Dallas, Texas) antibodies (1/100), cells were incubated with Duolink *in situ* Probe anti-Mouse PLUS (ref. DUO82001, Sigma-Aldrich; St Louis, MO, USA) and anti-Rabbit MINUS (ref. DUO82005, Sigma-Aldrich; St Louis, MO, USA) for 1 h at 37 °C. After that, ligation and amplification were conducted as indicated by the supplier. In all cases, nuclei were stained with Hoechst 33,432 (1/100 from 1 mg/mL stock; Sigma-Aldrich; St Louis, MO, USA) for 10 min. Samples were preserved at 4° in the dark using EpreDia™ Immu-mount® (ref. 10622689, ThermoFisher; Waltham, MA, USA) until imaging. Confocal images were acquired on a (Zeiss, Jena, Germany) using a 63x oil-immersion objective equipped with an apochromatic 63 × oil immersion objective (N.A. 1.4) and 405 nm (for nuclei detection) and 561 nm (for red dots detection) laser lines. For each image, a stack of the two channels and five Z-plane with a step size of 1 µm were acquired.

For the quantification of A_{2A}R-CB₂R heteromers in the obtained PLA confocal images, a custom-designed image analysis pipeline for Cell-profiler was used. The pipeline included the following processing modules: “ColorToGray” to split each RGB image into the blue (nuclei) and red (PLA dots) channels; “GaussianFilter” applied to the blue channel to smooth and correct the shape of the nuclei, facilitating their subsequent detection; and “IdentifyPrimaryObjects”, used to detect all nuclei in the image by setting the expected minimum and maximum diameters and applying Otsu’s thresholding method. Next, “IdentifySecondaryObjects” segmented the image by defining an approximate

region surrounding each nucleus. In the red channel, the “EnhanceOrSuppressFeatures” module (with the enhance speckles option) improved the detection of PLA dots. “IdentifyPrimaryObjects” was then used again to apply an appropriate threshold for detecting these red dots. To associate detected PLA dots with individual nuclei, the “RelateObjects” module was used. Quality control images for each analysed field were generated using the “OverlayOutlines” and “SaveImages” modules. Finally, all quantitative data were exported to a.csv file using the “ExportToSpreadsheet” module.

2.9. Data handling and statistical analysis

GraphPad Prism 10.4.1 software (San Diego, CA, USA) was used for data analysis. Prior to performing parametric tests (ANOVA, *t*-test), data were assessed for normal distribution using the Shapiro-Wilk test. Homogeneity of variances was confirmed using Brown-Forsythe test. All data sets met these assumptions. One-way ANOVA followed by *post hoc* Dunnett’s test was used when multiple comparison analysis. Statistical comparisons of the number of PLA dots per cell across experimental

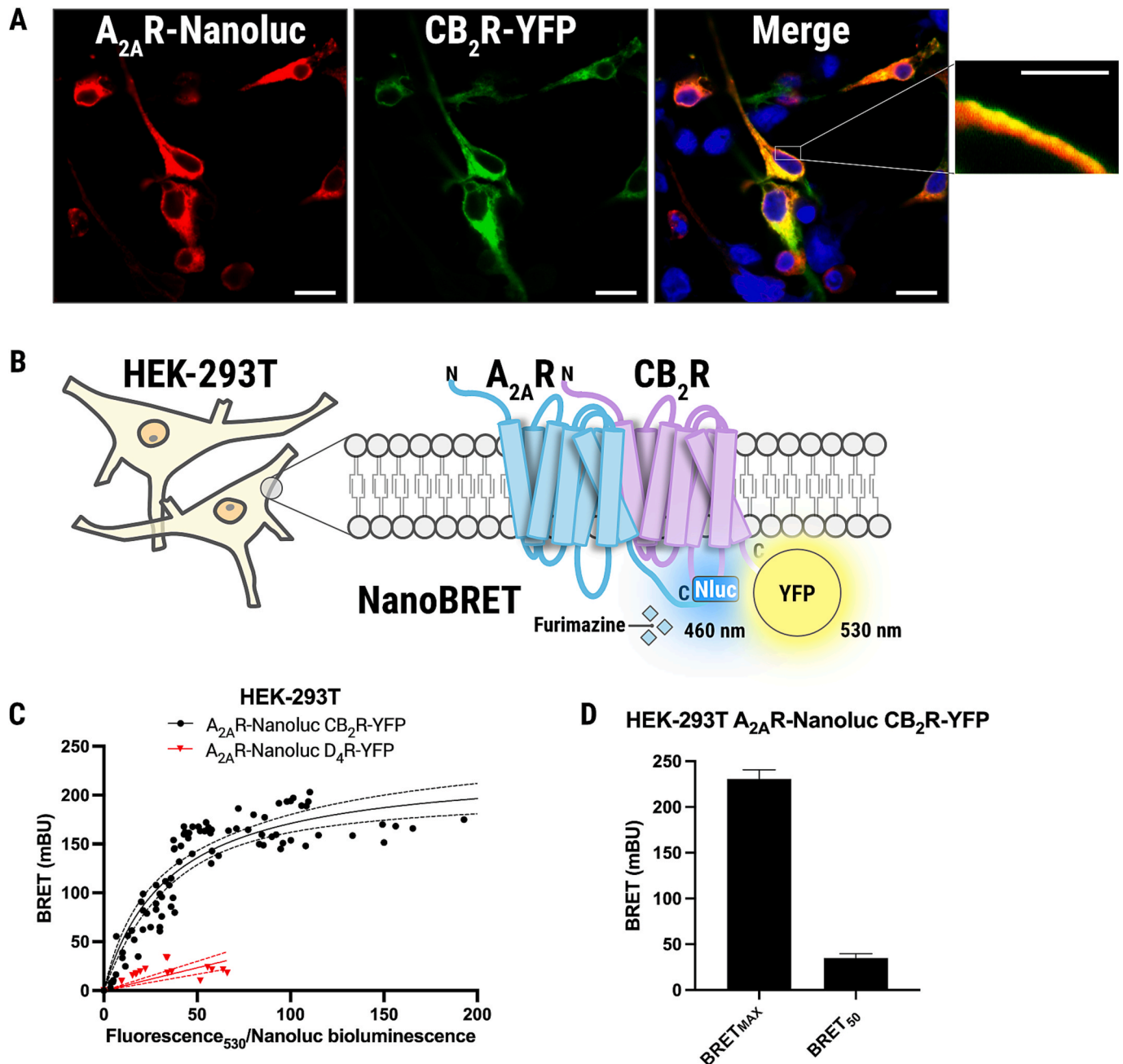


Fig. 1. Specific interaction between A_{2A} and CB₂ receptors in HEK-293T cells. **A)** Immunocytochemistry was performed in HEK-293T cells coexpressing A_{2A}R-Nluc and CB₂R-YFP. Representative confocal microscopy image showing co-localization (yellow) of A_{2A}R-Nluc (detected by an anti-Nluc mouse monoclonal antibody and a secondary Cy3-conjugated anti-mouse IgG, red) and CB₂R-YFP (green) in co-transfected HEK-293T cells. Nuclei were stained with Hoechst (blue). Scale bar = 10 μ m. **B)** Schematic representation of the NanoBRET assay principle to detect interaction between A_{2A}R-Nluc and CB₂R-YFP. **C)** NanoBRET assays were performed in HEK-293T cells transfected with a constant amount of cDNA for A_{2A}R-Nluc (30 ng) and increasing amounts of cDNA for CB₂R-YFP (0.5 to 2.5 μ g) (black circles, hyperbolic fit) or, for the negative control, with increasing amounts of cDNA for D₄R-YFP (0.25 to 1 μ g) (red triangles, linear fit). BRET signal is expressed as millibRET units (mBU) and it is plotted as a function of the acceptor/donor ratio (YFP Fluorescence/Nluc Bioluminescence). Dashed lines indicate the 99 % confidence interval (CI) of the fit. **D)** BRET_{MAX} and BRET₅₀ parameters (mean $\pm$ SEM) derived from the hyperbolic fit for A_{2A}R-Nluc/CB₂R-YFP nanoBRET shown in C). Values shown in each graph were obtained from five independent experiments performed in triplicates.

conditions were performed using unpaired *t*-test two tailed. Statistical analysis was undertaken only when each group size was at least $n = 3$, n being the number of independent experiments (technical replicates were not treated as independent experiments). Differences were considered significant when $p \leq 0.05$.

3. Results

3.1. Adenosine A_{2A} and cannabinoid CB_2 receptors form heteromeric complexes

To determine whether A_{2A} and CB_2 receptors physically interact under our experimental conditions, immunocytochemical and bioluminescence resonance energy transfer (BRET) assays were performed in co-transfected HEK-293T cells. The degree of colocalization in HEK-293T cells expressing the two receptors was marked (shown as yellow) and suggestive of a receptor-receptor heteromeric interaction (Fig. 1A).

To confirm this A_{2A} R- CB_2 R interaction, nanoBRET assays were performed using cells expressing A_{2A} R C-terminally fused to nanoluc (Nluc) as the donor and CB_2 R C-terminally fused to yellow fluorescent protein (YFP) as the acceptor. This produced a saturable hyperbolic curve, indicative of a direct physical interaction (Fig. 1C). In contrast, the negative control using cells expressing A_{2A} R-Nluc as a donor and dopamine D_4 receptor fused to YFP as an acceptor, produced a linear signal between BRET units and the increase of the acceptor/donor ratio. Analysis of the saturation curve yielded a $BRET_{MAX}$ of 230.8 ± 9.9 mBU and a $BRET_{50}$ of 34.9 ± 4.8 mBU; the latter suggests a high apparent affinity between the two receptors within the complex (Fig. 1D). Collectively, these results confirm that A_{2A} R and CB_2 R form constitutive heteromers, validating the HEK-293T cell expression system to study this complex.

3.2. Cannabidiol allosterically modulates the agonist-induced interaction of the A_{2A} R- CB_2 R heteromer

Although agonist binding to a GPCR induces conformational rearrangements, detecting these changes has been technically challenging. Even advanced techniques that measure donor-acceptor distances, such as FRET and BRET, have often failed to yield relevant results. In this study, we successfully detected conformational changes in cells expressing A_{2A} R-Nluc (donor) and CB_2 R-YFP (acceptor) upon agonist stimulation, using real-time NanoBRET measurements at 37 °C for all kinetics assays. The constant temperature was maintained with the Glomax® Discover plate reader, ensuring reliable detection of conformational rearrangements. Specifically, agonist induced conformational rearrangements within the heteromer alter the distance and/or orientation between the A_{2A} R-Nluc and the CB_2 R-YFP, resulting in measurable variations in resonance energy transfer over time. The donor/acceptor ratio was chosen to be in the intermediate zone (according to the saturation curve in Fig. 1), i.e. neither using very low (first part of the curve in Fig. 1C) nor very high donor-to-acceptor (saturation part of the curve in Fig. 1C) ratios to operate within the optimal dynamic range of the assay. Treatment with vehicle did not produce any noticeable change in the BRET signal over time. In contrast, time-course measurements of nanoBRET following treatment with the selective A_{2A} R agonist, CGS 21680, revealed a concentration-dependent effect that reached a plateau at approximately 60 min. Notably, at the highest concentration tested (10 μ M), the BRET signal displayed a sustained increase that persisted throughout the entire recording period (70 min) (Fig. 2A). When cells were treated with the selective CB_2 R agonist, JWH-133, nanoBRET showed a time-dependent increase. Interestingly, the curves obtained with 10 nM, 100 nM, and 1 μ M concentrations were similar, indicating no further effect at higher concentrations within that

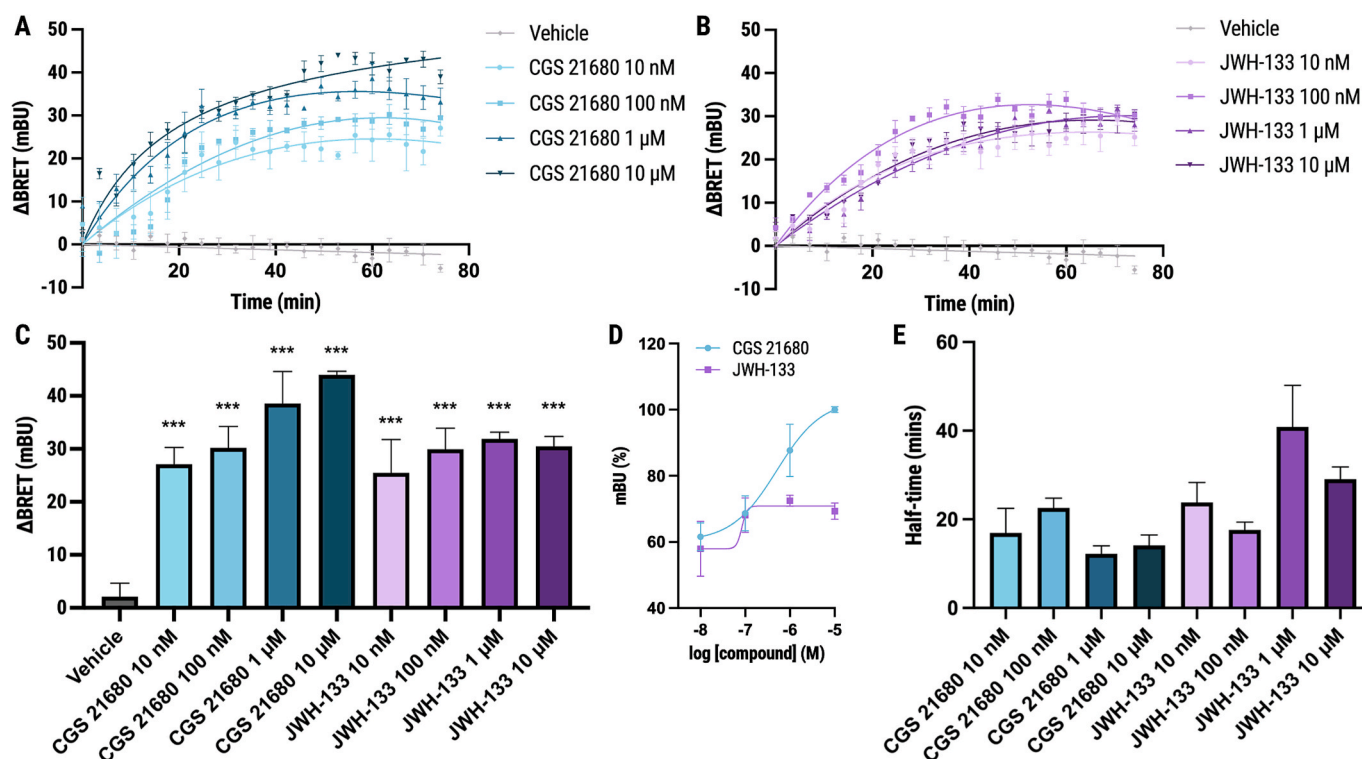


Fig. 2. Modulation of the A_{2A} R- CB_2 R heteromers formation by selective agonists. NanoBRET assays were performed in HEK-293T cells co-expressing A_{2A} R-Nluc and CB_2 R-YFP. Kinetics of the Δ BRET signal (mBU increase with respect to non-treated cells) over time (min) following stimulation with increasing concentrations (10 nM, 100 nM, 1 μ M and 10 μ M) of A_{2A} R (CGS 21680, A) or CB_2 R (JWH-133, B) selective agonist. Curves represent fits to a one-site model. C) Magnitude of $\Delta BRET_{MAX}$ (mBU) at the endpoint. Bars represent mean $\pm$ SEM. *** $p < 0.001$ versus vehicle (One-way ANOVA, Dunnett's test). D) Concentration-response curves for both CGS21680 and JWH-133 with mBU normalized to CGS21680 10 μ M maximum effect E) Half-time ($t_{1/2}$, min) to reach half-maximal signal, derived from kinetic fits. Bars represent mean $\pm$ SEM. Values are the mean $\pm$ SEM. of five independent experiments performed in triplicates.

range. At a high concentration (1 μ M and 10 μ M), the BRET signal increased more rapidly but ultimately reached the same plateau at the same time ($\sim$ 70 min) as the other concentrations (Fig. 2B). The bar graph showing the maximal increase in nanoBRET revealed a concentration-dependent increase with CGS 21680, while the response to JWH-133 remained similar across all concentrations tested (Fig. 2C). To better characterize these distinct pharmacological profiles, Δ BRET_{MAX} values were plotted as concentration–response curves (Fig. 2D), normalizing the maximal effect of 100 % at 10 μ M CGS 21680. This analysis confirmed that CGS 21680 induced a graded response without reaching saturation. In contrast, the effect of JWH-133 saturated at 100 nM, yet this maximal response was still less pronounced than the effect induced by higher concentrations (1 μ M and 10 μ M) of CGS 21680. The calculated half-time values were consistently around 20 min across all tested concentrations of both CGS 21680 and JWH-133. At the highest concentrations of JWH-133, there was a trend toward increased half-time values; however, this difference did not reach statistical significance (Fig. 2E).

After establishing the conditions for reliable measurement of agonist-induced conformational changes, we next assessed the potential of CBD to modulate the effects of 100 nM CGS 21680 (a selective A_{2A}R agonist) and 100 nM JWH-133 (a selective CB₂R agonist). CBD was tested at the same concentration as the agonists (100 nM), as well as at a higher concentration (1 μ M). Given that the reported K_i of CBD for the orthosteric site of human CB₂R is approximately 4 μ M, and that its interaction at nanomolar concentrations is consistent with allosteric

rather than orthosteric binding [8], direct competition at the orthosteric site is unlikely at 100 nM. Moreover, CBD does not bind to the orthosteric site of A_{2A}R [9,10]. Therefore, at 100 nM, CBD is not expected to occupy orthosteric sites on either receptor, supporting an allosteric mechanism of action.

BRET results obtained in cells co-expressing A_{2A}R-Nluc and CB₂R-YFP were consistent and revealing. The experimental timeline is illustrated in Fig. 3A. Briefly, cells were preincubated for 30 min with either vehicle or CBD before recording data upon addition of the Nluc substrate (furimazine) and either CGS 21680 or JWH-133. Compared to the time-course response of CGS 21680 in vehicle-pretreated cells (dotted blue curve in Fig. 3B), the response in the presence of CBD was significantly faster, although it reached a similar plateau (solid green curve in Fig. 3B). Data quantification revealed that CBD did not alter the maximum BRET signal achieved at approximately 60 min (Fig. 3C). Strikingly, however, it reduced the half-time (t_{1/2}) significantly to approximately 30 % ($p < 0.001$ for both 100 nM and 1 μ M CBD) in comparison with cells only treated with CGS 21680 alone (Fig. 3D).

Similarly, we investigated the effect of CBD on the interaction induced by the selective CB₂R agonist, JWH-133. Pre-incubation with CBD also resulted in a significantly faster kinetic response compared to cells treated with JWH-133 alone (Fig. 3E, compare solid green vs. dashed purple curves). However, in this case, CBD not only significantly accelerated the interaction, reducing the half-time (t_{1/2}) to approximately 30 % compared to JWH-133 alone ($p < 0.01$ for both 100 nM and 1 μ M CBD) but also it inhibited the maximal BRET signal (Δ BRET_{MAX}),

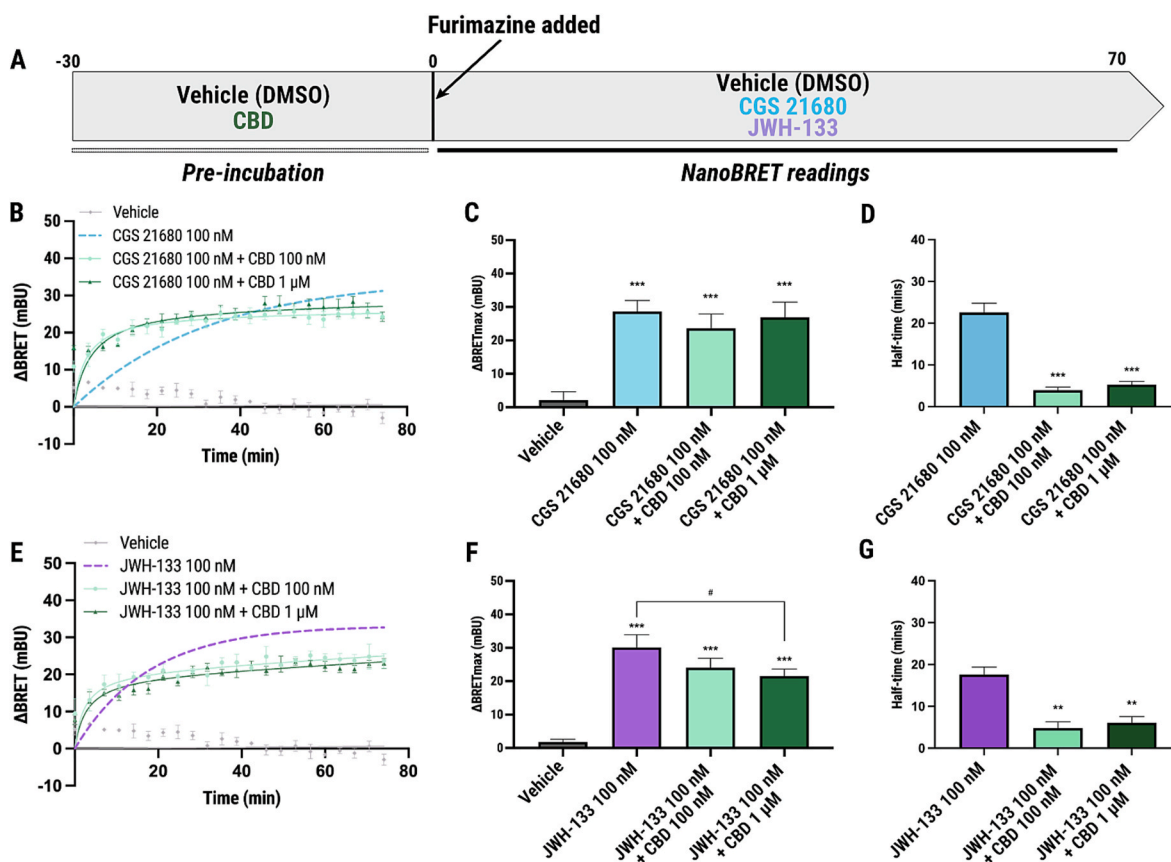


Fig. 3. Modulation of the A_{2A}R–CB₂R heteromers formation by cannabidiol (CBD). NanoBRET assays were performed in HEK-293T cells co-expressing A_{2A}R–Nluc and CB₂R–YFP. **A**) Schematic workflow and experimental timeline of nanoBRET assays. The diagram illustrates the sequence of assay steps and their relative timing. Cells were pre-treated for 30 min with vehicle or indicated concentrations of CBD before adding 5 μ M furimazine and stimulation with 100 nM CGS 21680 or 100 nM JWH-133. Then, nanoBRET readings were acquired for 70 min every minute. Kinetics of Δ BRET (mBU increase with respect to non-treated cells) vs time (min) show the modulation of CBD (green lines; **B**, **E**) when compared to the response of the agonist alone (100 nM CGS 21680 or 100 nM JWH-133, dashed reference line). **C**, **F**) Quantification of Δ BRET_{MAX} (milliBU) at the endpoint. *** $p < 0.001$ vs Vehicle; # $p < 0.05$ vs 100 nM JWH-133 (One-way ANOVA, Dunnett's test). **D**, **G**) Half-time (t_{1/2}, min) derived from kinetic fits. ** $p < 0.01$, *** $p < 0.001$ vs agonist alone (One-way ANOVA, Dunnett's test). Values are representing the mean $\pm$ SEM. of five independent experiments performed in triplicates.

causing a significant reduction of 20 % with 100 nM CBD and 30 % with 1 μ M CBD (Fig. 3F). This dual effect was further reflected in the kinetic parameters, where CBD significantly decreased the half-time of the response (Fig. 3G), consistent with an accelerated, yet less pronounced, BRET values.

3.3. Cannabidiol induces biased signaling at the $A_{2A}R$ -CB $_2$ R heteromer by inhibiting β -arrestin recruitment

Since CBD modulated the BRET signal upon activation of $A_{2A}R$ or CB $_2$ R, we next examined whether it also influenced ligand-induced β -arrestin II recruitment to $A_{2A}R$.

First, we optimized the conditions for the BRET-based assay in cells co-expressing β -arrestin II-Nluc, $A_{2A}R$ -YFP and CB $_2$ R, to monitor β -arrestin II recruitment to the adenosine receptor (Fig. 4A). The selected setup is schematized in Fig. 4B. Upon addition of CGS 21680, a significant increase in the nanoBRET signal was observed at 15 min (Fig. 4C). Interestingly, activation of CB $_2$ R, the partner receptor in the heteromer, using JWH-133 also resulted in a significant recruitment of β -arrestin II to the adenosine receptor. However, simultaneous activation with both CGS 21680 (100 nM) and JWH-133 (100 nM) did not

produce any additive or synergistic effect (Fig. 4C).

Given the unexpected result observed with JWH-133, we investigated whether β -arrestin II recruitment could be blocked by $A_{2A}R$ and/or CB $_2$ R antagonists. As expected, the effect of CGS 21680 was completely abolished by preincubation with the selective $A_{2A}R$ antagonist SCH 58261. Remarkably, the CB $_2$ R antagonist SR 144528 also fully inhibited β -arrestin II recruitment to $A_{2A}R$ (Fig. 4D). Likewise, the effect of the CB $_2$ R agonist JWH-133 was entirely suppressed by both SCH 58261 and SR 144528 (Fig. 4E). This bidirectional cross-antagonism provides strong evidence that the $A_{2A}R$ -CB $_2$ R heteromer operates as an integrated signaling unit, in which the activation and signaling efficacy of one receptor is strictly dependent on the conformational state of its partner.

After establishing that activation of either receptor specifically leads to β -arrestin II recruitment, we examined the modulatory effects of CBD under both conditions—namely, activation of CB $_2$ R with 100 nM JWH-133 and activation of $A_{2A}R$ with 100 nM CGS 21680. CBD significantly reduced β -arrestin II recruitment at 15 min in a concentration-dependent manner. The inhibitory effect displayed relatively low potency when co-administered with CGS 21680, yielding an IC_{50} of 176.5 ± 11 nM. In contrast, co-treatment with JWH-133 resulted in a markedly lower IC_{50} of 44.5 ± 14.3 nM, indicating greater sensitivity of the CB $_2$ R-

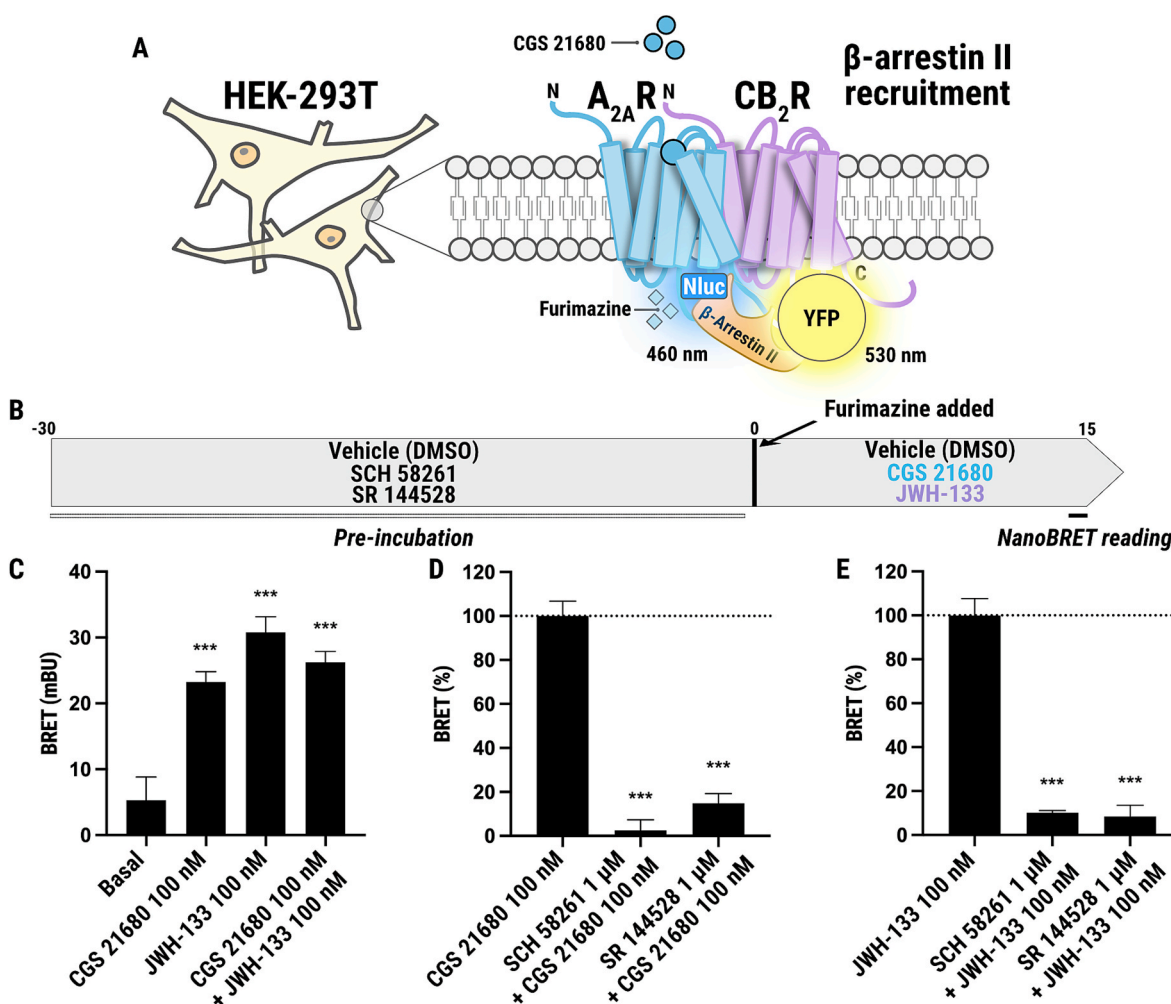


Fig. 4. $A_{2A}R$ -CB $_2$ R heteromer β -arrestin II recruitment. β -Arrestin II recruitment in HEK-293T co-expressing $A_{2A}R$ -YFP, CB $_2$ R, and β -arrestin II-Nluc using nanoBRET. **A)** Assay schematic. **B)** Schematic workflow and experimental timeline of β -arrestin II recruitment assays. The diagram illustrates the sequence of assay steps and their relative timing. **C)** BRET induction (mBU) at the endpoint (15 min) by CGS 21680 (100 nM), JWH-133 (100 nM), or both, compared to Basal (vehicle). *** p < 0.001 vs Basal (One-way ANOVA, Dunnett's test). **D)** BRET (%) relative to 100 nM CGS-21680 alone (100 %) in the presence of preincubated SCH 58261 (1 μ M) or SR 144528 (1 μ M) for 10 min. *** p < 0.001 vs 100 nM CGS-21680 (One-way ANOVA, Dunnett's test). **E)** BRET (%) relative to 100 nM JWH-133 (100 %) in the presence of SCH 58261 (1 μ M) or SR 144528 (1 μ M) is shown. *** p < 0.001 vs 100 nM JWH-133 (One-way ANOVA, Dunnett's test). Note the cross-antagonism in D and E. Bars are representing the mean $\pm$ SEM. of five independent experiments performed in triplicates.

mediated response to CBD modulation (Fig. 5A, C). The inhibitory effect of 100 nM CBD in combination with 100 nM agonists was statistically significant, and more pronounced in the case of JWH-133 (Fig. 5B, D). Specifically, β -arrestin II recruitment to the $A_{2A}R$ -CB $_2R$ heteromer induced by 100 nM JWH-133 was reduced by approximately 60 % in the presence of CBD (Fig. 5D). These results indicate that CBD acts as a significantly more potent modulator of the CB $_2R$ -initiated β -arrestin II signaling pathway than of the $A_{2A}R$ -initiated pathway within the heteromeric complex.

3.4. Cannabidiol modulates $A_{2A}R$ -CB $_2R$ heteromers function without altering the overall agonist-induced complexes

The results described above demonstrate that CBD interacts with the $A_{2A}R$ -CB $_2R$ heteromer, initially interpreted as an effect mediated by CBD-induced conformational changes. However, we considered the possibility that these observations could also stem from alternative mechanisms—such as receptor rearrangements leading to heteromer disruption (reflected as decreased BRET signals), or conversely, the formation of additional heteromeric complexes (reflected as increased BRET signals).

To distinguish between these scenarios and assess whether the observed effects were elicited by agonist treatment alone or by CBD co-administration, we employed the *in situ* proximity ligation assay (PLA). This powerful imaging technique enables the visualization and quantification of protein–protein interactions within intact cells or tissues, and allows detection of changes in receptor heteromerization either under pathological conditions or following pharmacological treatments *in vitro*.

A schematic representation of the technique used to detect $A_{2A}R$ -CB $_2R$ heteromers, visualized as red fluorescent puncta in confocal images, is shown in Fig. 6A. The negative control, in which one of the primary antibodies was omitted, produced no detectable signal (data not shown). As additional proof of specificity, no signal was observed in non-transfected cells (Fig. 6B). In contrast, when both primary antibodies against $A_{2A}R$ and CB $_2R$ were applied to co-transfected HEK-293T cells, a robust PLA signal was detected, indicating the presence of $A_{2A}R$ -CB $_2R$ heteromers (Fig. 6B).

We next evaluated whether CBD could modulate agonist-induced $A_{2A}R$ -CB $_2R$ heteromer formation. Pre-treatment with 100 nM CBD did not result in a statistically significant change in the number of heteromers induced by either CGS 21680 (Fig. 6C) or JWH-133 (Fig. 6D). These findings suggest that the modulatory effect of CBD does not

involve a substantial alteration in the overall number of heteromeric complexes detectable at the 1-hour time point.

4. Discussion

In this study, we expand on the concept of cannabidiol (CBD) as a pleiotropic agent by demonstrating, for the first time, that it directly modulates the dynamics of $A_{2A}R$ -CB $_2R$ heteromer interactions, affecting downstream signaling without altering the overall prevalence of heteromeric complexes. Using real-time NanoBRET at physiological temperature, we showed that pre-treatment with CBD (100 nM) significantly accelerates the conformational rearrangements elicited by the $A_{2A}R$ agonist CGS 21680, reducing the half-time to reach BRET_{max} by an order of magnitude, while leaving the overall amplitude of the response unchanged at endpoint. A similar result was observed when the CB $_2$ agonist JWH-133 was applied, indicating that the modulatory activity spans both protomers within the heteromeric complex. In contrast to its effect with CGS 21680, co-treatment with JWH-133 revealed that CBD 1 μ M not only accelerated the kinetics but also partially inhibited the maximal BRET signal. Importantly, proximity ligation assays revealed that CBD, at the conditions of the assay, did not significantly alter the fraction of cells exhibiting $A_{2A}R$ -CB $_2R$ heteromers under agonist-stimulated conditions (Fig. 6C, D).

A notable observation from the agonist modulation experiments was the concentration-dependent effect of CGS 21680, which, in contrast to JWH-133, did not reach a plateau even at the highest concentration tested (10 μ M) (Fig. 2D). While the possibility of a complex binding mechanism, such as a second low-affinity site in $A_{2A}R$, cannot be fully discarded, the most likely explanation for this non-saturating response resides in our experimental design, which combines a high-efficacy agonist with the high density of the CB $_2R$ -YFP acceptor relative to the limiting $A_{2A}R$ -Nluc donor. Nevertheless, our data suggest that the concentrations needed for full conformational engagement of $A_{2A}R$ within a heteromeric complex might be higher than classically assumed, a point of consideration for future *ex vivo* studies.

These findings fit with previous reports showing that CBD acts as a negative allosteric modulator at individual GPCRs. Raich *et al.* and Sánchez-Fernández *et al.* independently showed that CBD does not compete orthosterically at the A_{2A} receptor but attenuates G $_s$ coupling and downstream cAMP/ERK signaling [9,10]. Likewise, Martínez-Pinilla *et al.* identified an allosteric binding site on CB $_2R$, through which CBD can dampen receptor activation [8]. Our data extend these observations into the context of receptor heteromers, where allosteric

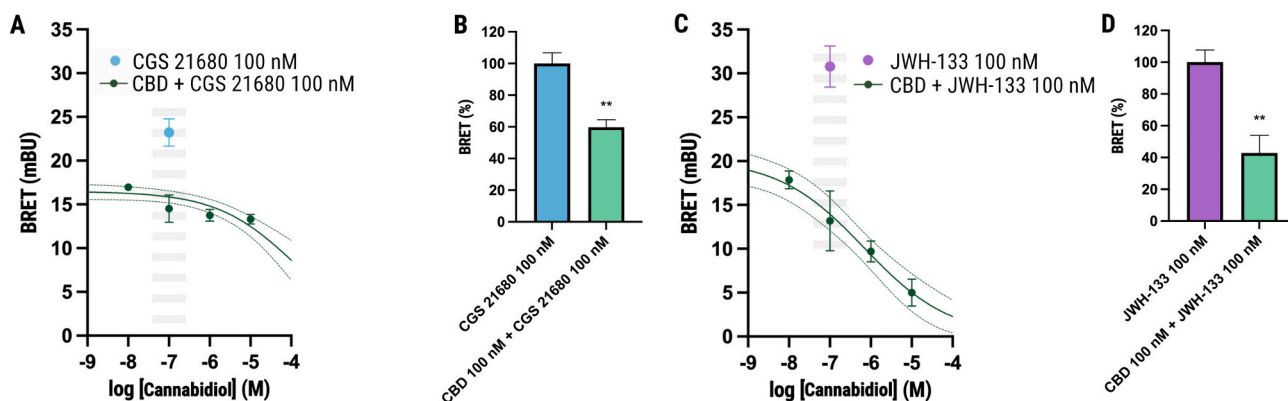


Fig. 5. Modulation by CBD of β -arrestin II recruitment. β -Arrestin II recruitment was determined by NanoBRET (as in Fig. 4). Cells were pretreated for 30 min with increasing concentrations of CBD (10 nM, 100 nM, 1 μ M or 10 μ M) and subsequently treated with 100 nM CGS 21680 (A) or 100 nM JWH-133 agonist (C). BRET (mBU) is plotted vs Log [Cannabidiol] (M). Control conditions (CGS 21680 or JWH-133 alone) are shown for reference. Curves represent non-linear fits (log (compound) vs response) and dashed lines indicate the confidence interval (90 % CI). Bars are shown relative to the response induced by CGS 21680 alone (blue, B) or JWH-133 alone (violet, D). BRET (%) induced by CGS 21680 (100 nM) or JWH-133 (100 nM) in the presence of CBD (100 nM) is shown in green (B, D). ***p < 0.01 vs CGS 21680 100 nM or vs JWH-133 100 nM, respectively (One-way ANOVA, Dunnett's test). Values are representing the mean $\pm$ SEM. of three independent experiments performed in triplicates.

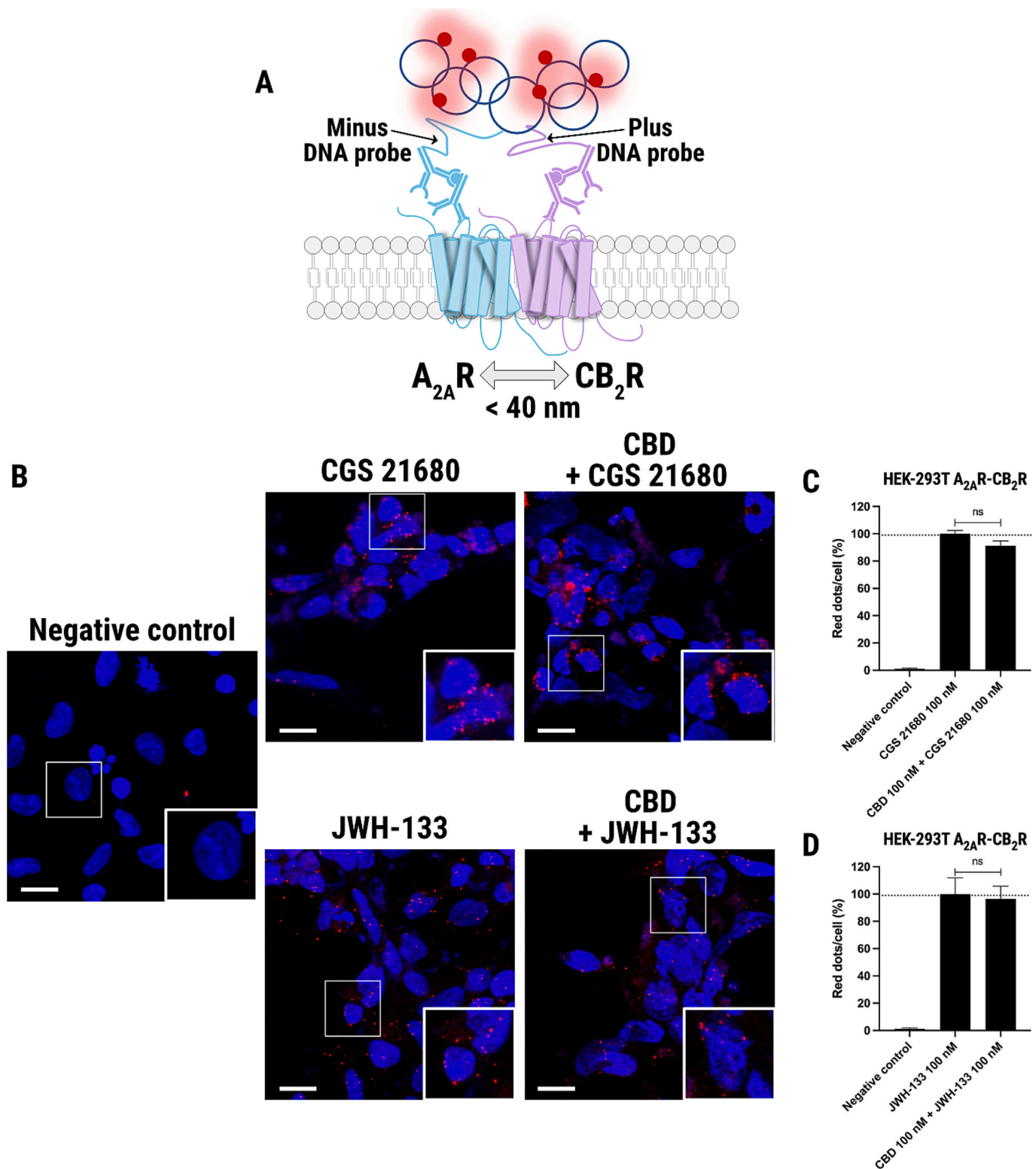


Fig. 6. Proximity Ligation Assays reveal agonist-induced $A_{2A}R$ - CB_2R heteromerization and its modulation by CBD in HEK-293T cells. Proximity Ligation Assays (PLA) were performed in HEK-293T cells co-expressing A_{2A} and CB_2 receptors. (A) Schematic representation of the PLA assay, detecting $A_{2A}R$ - CB_2R interaction (<40 nm). Cells were pre-treated (30 min) with vehicle (DMSO) or CBD (100 nM), before stimulation (1 h) with vehicle (DMSO), CGS 21680 (100 nM), or JWH-133 (100 nM). (B) Representative confocal microscopy images for each condition. Red dots indicate PLA signals ($A_{2A}R$ - CB_2R heteromers); nuclei were stained with Hoechst (blue). Insets show zoomed cells. PLA assay conditions: Negative control (non-transfected cells), CGS 21680, CBD + CGS 21680, JWH-133, CBD + JWH-133. Scale bar = 10 μ m. (C-D) Quantification of PLA signals. Data are expressed as red dots/cell (%) (Mean $\pm$ SEM), normalized to CGS 21680 (C) or JWH-133 (D) conditions (set to 100 %, dashed line). ns vs CGS 21680 (C) or JWH-133 (D) (Unpaired *t*-test two tailed). Each bar represents quantification from at least 200 cells per condition, analyzed across *n* = 3 independent experiments performed in duplicates.

modulation of one protomer may propagate conformational constraints across the dimer interface, consistent with the structural model of A_{2A}R-CB₂R heteromer allosterism described by [18], primarily involving TM6 in the agonist-bound state. Therefore, our findings that CBD modulates the agonist-bound complex are consistent with an action on this pre-formed, likely TM6-mediated, heteromeric assembly, where it induces subtle conformational changes that lead to biased signaling.

Additionally, a recent comprehensive review has highlighted the interactions of this phytocannabinoid with a diverse array of targets, including cannabinoid and non-cannabinoid GPCRs, peroxisome proliferator activated –PPARγ– nuclear receptors, transient receptor potential ion channels, membrane transporters, and metabolic enzymes [23]. Taken together these discoveries underscore a role as a multi-functional molecule with context-dependent therapeutic potential. Our demonstration of cross-protomer allosteric modulation in A_{2A}R-CB₂R heteromers introduces a crucial kinetic perspective to this poly-pharmacology. Using temperature-controlled measurements at 37 °C, we uncover rapid, intermediate receptor states and show that CBD selectively accelerates conformational transitions without affecting efficacy. These results emphasize the necessity of physiological assay conditions for resolving the intricate, heteromer-selective mechanisms of CBD action. Therefore, we suggest the routine use of temperature-controlled instrumentation, not only to capture early binding kinetics but also to monitor downstream events like cAMP production, kinase phosphorylation, and β-arrestin II recruitment, each of which may exhibit qualitatively and quantitatively more physiological behaviors at 37 °C.

Mechanistically, the CBD-induced acceleration of heteromer rearrangements could reflect stabilization of intermediate conformers that favor faster transition to the active state, even as the final active ensemble remains quantitatively similar. Such a “kinetic bias” could selectively influence temporal aspects of signaling, quickly altering responses to endogenous agonists. This kinetic tuning might underlie the reported capacity of CBD to exert context-dependent functional outcomes, e.g., anti-inflammatory versus neuroprotective effects, by reshaping the signaling “tempo” rather than its magnitude. Moreover, our observation that CBD diminishes β-arrestin II recruitment to the A_{2A}R protomer upon stimulation of either receptor suggests a broader impact on receptor desensitization and internalization pathways, which could translate into prolonged receptor availability at the cell surface. These findings suggest that CBD’s kinetic and allosteric modulation of A_{2A}R-CB₂R heteromers could be exploited in conditions where dynamic receptor crosstalk drives disease processes—such as neurodegeneration, chronic inflammation, and cancer. Indeed CBD has attracted attention across a range of indications [24,25,26,27], and is approved as Epidiolex® for pediatric epilepsy [28,29,30].

From a clinical perspective, targeting A_{2A}R-CB₂R heteromers with CBD may offer distinct advantages. A_{2A}R antagonists, such as istradefylline, are already approved for Parkinson’s disease and are being evaluated to, among other, boost cancer immunotherapy, while CB₂ receptor modulators are in development for neuroinflammation and pain. The ability of CBD to modulate both receptors within a single heteromeric framework may offer unique therapeutic advantages, coordinated fine-tuning of multiple signaling axes with a single molecule, reducing the need for combination therapies and potentially lowering side-effect burden. Conversely, the pleiotropic profile of CBD underscores the importance of understanding heteromer composition in target tissues, as receptor co-expression patterns will dictate the net pharmacological outcome.

While HEK-293T cells enable precise mechanistic dissection under controlled conditions, receptor overexpression may limit physiological relevance. Thus, the CBD-biased modulation of the A_{2A}R-CB₂R complex demonstrated here awaits validation in primary cells, such as microglia, where this heteromer is relevant. Even so, our results provide key mechanistic evidence supporting future *in vivo* exploration of A_{2A}R-CB₂R as a therapeutic target.

Our findings have significant biological relevance for neuro-inflammation, a context where A_{2A}R and CB₂R are co-expressed and upregulated in microglia [17]. In this setting, sustained A_{2A}R activation is often associated with pro-inflammatory responses [31], while CB₂R signaling is considered neuroprotective [32]. In this state, occurs the tonic inhibition of the CB₂R by A_{2A}R, that become functionally dominant [18]. We propose a novel mechanism for CBD’s intervention based on this principle: rather than directly potentiating CB₂R, targeting A_{2A}R with CBD could reduce the A_{2A}R-CB₂R pro-inflammatory drive and shift its net output toward a less harmful state. This biased modulation fine-tunes the dominant partner instead of merely activating the suppressed one, offering a more subtle approach to reduce neuroinflammation.

Finally, our work highlights that receptor heteromers provide an additional layer of complexity in polypharmacology. While most structure-activity studies focus on individual targets [33], the existence of heteromeric interfaces opens new allosteric sites and signaling modalities. Future drug discovery efforts may benefit from screening for modulators that selectively impact heteromer kinetics or protomer crosstalk, as exemplified by ability of the phytochemistry to bias conformational transitions in the A_{2A}R-CB₂R heteromers. In this light, CBD also emerges as a blueprint for designing polypharmacological probes that exploit heteromer-specific allostery to achieve precise spatiotemporal control over signaling networks.

5. Informed consent statement

Not applicable.

Author contributions

RRS and RF contributed to the conceptualization of the study and the experimental design. RF was also responsible for funding acquisition, validation, and writing the original draft. RRS conducted the investigation, supervised the work, and contributed to writing the original draft. GN participated in preliminary discussions and provided general advisement. MP and CGF provided resources, contributed to the experimental design, and participated in validation. CFV and VSM provided key resources and shared their expertise regarding the handling of specific reagents. All authors reviewed and approved the submitted version of the manuscript.

CRedit authorship contribution statement

Rafael Rivas-Santisteban: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Carlos Ferreira-Vera:** Resources, Methodology. **Verónica Sánchez de Medina:** Resources, Methodology. **Gemma Navarro:** Validation. **Mercè Pallàs:** Validation, Resources, Conceptualization. **Christian Grinán-Ferré:** Validation, Resources, Conceptualization. **Rafael Franco:** Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: RRS, GN, MP, CGF and RF declare no conflicts of interest. CFV and VSM declare that they work for PhytoPlant Research SL, a research company that does not directly sell any product (web page: <https://www.phytoplantresearch.com/>).

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Not applicable.

Data availability

Data can be obtained from the corresponding author, RRS, upon reasonable request.

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