Acta Neuropsychiatrica

cambridge.org/neu

Cannabidiol negatively modulates adenosine A_{2A} receptor functioning in living cells

Nuria Sánchez-Fernández[1,2], Laura Gómez-Acero[1,2], Laura I. Sarasola[1,2], Josep Argerich[1,2], Andy Chevigné[3], Kenneth A. Jacobson[4], Francisco Ciruela[1,2], Víctor Fernández-Dueñas[1,2] and Ester Aso[1,2]

[1]Pharmacology Unit, Department of Pathology and Experimental Therapeutics, School of Medicine and Health Sciences, Institute of Neurosciences, University of Barcelona, L'Hospitalet de Llobregat, Spain; [2]Neuropharmacology and Pain Group, Neuroscience Program, Institut d'Investigació Biomèdica de Bellvitge, IDIBELL, L'Hospitalet de Llobregat, Spain; [3]Immuno-Pharmacology and Interactomics, Department of Infection and Immunity, Luxembourg Institute of Health (LIH), Esch-sur-Alzette, Luxembourg and [4]Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA

Abstract

Objectives: Cannabidiol (CBD) is a phytocannabinoid with great potential in clinical applications. The mechanism(s) of action of CBD require further investigation. Previous studies suggested that adenosine A_{2A} receptors (A_{2A}Rs) could play a role in CBD-induced effects. Here, we evaluated the ability of CBD to modify the function of A_{2A}R. ***Methods:*** We used HEK-293T cells transfected with the cDNA encoding the human A_{2A}R and Gαs protein, both modified to perform bioluminescence-based assays. We first assessed the effect of CBD on A_{2A}R ligand binding using an A_{2A}R NanoLuciferase sensor. Next, we evaluated whether CBD modified A_{2A}R coupling to mini-Gαs proteins using the NanoBiT™ assay. Finally, we further assessed CBD effects on A_{2A}R intrinsic activity by recording agonist-induced cAMP accumulation. ***Results*:** CBD did not bind orthosterically to A_{2A}R but reduced the coupling of A_{2A}R to Gαs protein and the subsequent generation of cAMP. ***Conclusion*:** CBD negatively modulates A_{2A}R functioning.

Significant outcomes

- Cannabidiol does not bind orthosterically to A_{2A}R.
- Cannabidiol reduces the functionality of A_{2A}R

Limitations

- This is an *in vitro* study, and results cannot be directly extrapolated to *in vivo* conditions.
- Putative allosteric binding of CBD to A_{2A}R cannot be confirmed or ruled out with the luminescence-based techniques employed in this study.

Introduction

Cannabidiol (CBD) is a phytocannabinoid isolated from *Cannabis sativa* without psychoactive properties, but with potential benefits against multiple pathological conditions (ElSohly *et al.*, 2017). Several preclinical reports demonstrated protective and anti-inflammatory effects of CBD in a wide spectrum of neurodegenerative diseases, neuroinflammatory processes, stroke, colitis, liver, kidney injury, cardiovascular disease, arthritis, sepsis, diabetes, cancer, and epilepsy models (Pacher *et al.*, 2020). Furthermore, CBD exerted positive effects in experimental models of other neuropsychiatric disorders such as epilepsy, anxiety, schizophrenia, dementia, addiction, and neonatal hypoxic-ischemic encephalopathy (Devinsky *et al.*, 2014). Although its translation to clinical trials is somewhat limited to date, the successful case of Epidiolex®, an oral solution based on a botanical extract containing purified CBD, is notable. Epidiolex® was approved by the US Food and Drug Administration in 2018 for the treatment of Lennox-Gastaut and Dravet syndromes, two rare and debilitating genetic forms of epilepsy in children. Additionally, CBD is currently under clinical evaluation for other conditions, including different forms of pain, obsessive-compulsive disorders, and behavioural problems associated with intellectual disability or autism, among others (ClinicalTrials.gov database).

Short Communication

Cite this article: Sánchez-Fernández N, Gómez-Acero L, Sarasola LI, Argerich J, Chevigné A, Jacobson KA, Ciruela F, Fernández-Dueñas V, and Aso E. (2024) Cannabidiol negatively modulates adenosine A_{2A} receptor functioning in living cells. *Acta Neuropsychiatrica* **36**:320–324.
doi: 10.1017/neu.2023.30

Received: 31 March 2023
Revised: 17 May 2023
Accepted: 17 July 2023
First published online: 22 August 2023

Keywords:
cannabidiol; adenosine 2A receptor; negative allosteric regulation; competitive binding; cyclic AMP; luminescence-based assays

Corresponding authors:
Ester Aso; Email: ester.aso@ub.edu;
Víctor Fernández-Dueñas;
Email: vfernandez@ub.edu

Despite growing interest in its potential clinical applications, the mechanism(s) of action of CBD require further exploration. CBD has a very low affinity for the orthosteric site of CB_1 and CB_2 receptors, the main G protein-coupled receptors (GPCRs) that belong to the endogenous cannabinoid system (McPartland *et al.*, 2015). Alternatively, CBD can act on multiple targets, including TRPV1 channels and PPARγ, adenosine A_{2A}, 5-HT_{1A}, α_3-glycine, α_1-adrenal, dopamine D_2, $GABA_A$, μ- and δ-opioid receptors (McPartland *et al.*, 2015). Additionally, CBD can inhibit the activity of GPR55 (Ryberg *et al.*, 2007), an effect that has been associated with its antiepileptic activity (Sylantyev *et al.*, 2013). In the present study, we probed the putative direct effects of CBD on adenosine A_{2A} receptors (A_{2A}Rs). The relevant role that A_{2A}Rs play in several of the neuropsychiatric disorders in which CBD could offer beneficial effects (i.e. dementia, schizophrenia, epilepsy, depression, anxiety) supports this interest (Domenici *et al.*, 2019). Furthermore, previous preclinical evidence supports the participation of A_{2A}R in CBD-mediated effects. Thus, A_{2A}R antagonists blocked the anti-inflammatory effects of CBD (Liou *et al.*, 2008; Ribeiro *et al.*, 2012; Mecha *et al.*, 2013; Oláh *et al.*, 2014), or the ability of CBD to blunt Δ^9-THC-induced cognitive impairment (Aso *et al.*, 2019). Similarly, the genetic deletion of A_{2A}R reduced the CBD-induced potentiation of the cataleptic and anxiolytic properties of Δ^9-THC (Stollenwerk *et al.*, 2021). This A_{2A}R-dependent activity of CBD was proposed to depend on the ability of CBD to bind to the equilibrative nucleoside transporter (ENT). Thus, inhibition of adenosine uptake would lead to indirect activation of A_{2A}R (Pandolfo *et al.*, 2011). However, a direct effect of CBD on A_{2A}R has not been further investigated. Here we aimed to evaluate the capacity of CBD to bind to the orthosteric site of A_{2A}R and/or to modify its intrinsic activity by using state-of-the-art luminescence-based assays.

Materials and methods

Reagents

The ligands used were CGS21680, ZM241385, and CBD (Tocris Bioscience, Bristol, United Kingdom). MRS7396, a fluorescent selective A_{2A}R orthosteric antagonist derived from SCH442416 and containing a BODIPY630/650 fluorophore, was previously described (Duroux *et al.*, 2017). Other reagents used were Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, St Louis, MO, USA), geneticin (Santa Cruz Biotechnology, Dallas, TX, USA), adenosine deaminase (ADA; Roche Diagnostics GmbH, Mannheim, Germany), zardaverine (Calbiochem, San Diego, CA, USA), and coelenterazine 400a (NanoLight Technologies, Pinetop, AZ, USA).

Plasmid constructs

To perform bioluminescence resonance energy transfer (BRET) experiments and cAMP accumulation assays, we used the A_{2A}R NanoLuciferase (NanoLuc) sensor ($A_{2A}R^{NL}$), previously described (Lanznaster *et al.*, 2019). To perform the NanoBiT™ assay, the cDNA encoding human A_{2A}R was cloned at the BamHI/EcoRV restriction enzyme sites of pIREShyg3-SmBiT (Promega, Madison, WI, USA), as previously described (Sarasola *et al.*, 2022). The construct ($A_{2A}R^{SmBiT}$) was verified by DNA sequencing. The plasmid encoding the mini-Gαs (engineered GTPase domain of Gα subunit; ^{LgBiT}mini-Gαs) linked to LgBiT was previously described (Wan *et al.*, 2018; Meyrath *et al.*, 2021).

Cell culture and transfection

Human embryonic kidney (HEK)-293T cells were grown in DMEM supplemented with 1 mM sodium pyruvate (Biowest, Nuaillé, France), 2 mM L-glutamine (Biowest), 100 U/mL streptomycin (Biowest), 100 mg/mL penicillin (Biowest), and 5% (*v*/*v*) foetal bovine serum (Invitrogen Corporation, Camarillo, CA, USA) at 37°C and in an atmosphere of 5% CO_2. Cells were transiently transfected with the indicated cDNA construct using polyethylenimine (PEI, 1 mg/mL, Sigma Aldrich), as previously described (Longo *et al.*, 2013). Finally, HEK-293T cells stably expressing $A_{2A}R^{NL}$ were grown in the presence of geneticin (1 mg/mL).

NanoBRET experiments

The NanoBRET assay was performed as previously described (Lanznaster *et al.*, 2019). Briefly, HEK-293T cells expressing the $A_{2A}R^{NL}$ construct were resuspended in Hank's balanced salt solution (HBSS; Thermo Fisher, Waltham, MA, USA) containing ADA (0.5 U/mL) and plated on white 96-well plates coated with poly-ornithine (Corning, Corning, NY, USA) at a density of 20,000 cells/well. After 24 h, cells were challenged with the fluorescent A_{2A}R antagonist (MRS7396) in the absence/presence of ZM241385 or CBD and incubated for 1 h at 37°C. Subsequently, coelenterazine 400a was added at a final concentration of 1 μM, and the readings were performed after 15 min using a CLARIOStar microplate reader (BMG Labtech, Durham, NC, USA). Donor and acceptor emission were measured at 490 ± 10 nm and 650 ± 40 nm, respectively. The raw NanoBRET ratio was calculated by dividing the 650 nm emission by the 490 nm emission and the values fitted by nonlinear regression using GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). The results were expressed as a percentage of the maximum signal obtained (mBU; miliBRET units).

NanoBiT assay

The NanoBiT™ assay (Promega) was performed as previously described (Sarasola *et al.*, 2022). Briefly, transient transfected HEK-293T cells with $A_{2A}R^{SmBiT}$ and ^{LgBiT}mini-Gαs were resuspended in HBSS containing ADA (0.5 U/mL) and transferred (90 μl) into white 96-well plates (Corning). Subsequently, coelenterazine 400a was added (1 μM) to each well. After 15-minute incubation, basal luminescence was determined using a CLARIOstar plate reader (BMG Labtech). Immediately after the initial measurement (basal), the ligands were added, and the luminescent signal was measured every 5 min for 30 min. The luminescence signal (RLU) was normalised as follows: $(RLU_{sample} - RLU_{basal)} / RLU_{basal}$.

cAMP assay

cAMP accumulation was measured using the LANCE® Ultra cAMP Kit (PerkinElmer, Waltham, MA, USA) as previously described (Lanznaster *et al.*, 2019). Briefly, HEK-293T cells stably expressing the $A_{2A}R^{NL}$ construct were first incubated for 1 h at 37°C with stimulation buffer (BSA 0.1%, ADA 0.5 units/mL, zardaverine 2 μM; in serum-free DMEM) and later with CGS21680 (100 nM) and increasing concentrations of ZM241385 or CBD for 30 min at 37°C. Subsequently, cells were transferred (1000 cells/well) into white 384-well plates (Corning), in which reagents were added following the manufacturer's instructions. After 1 h at room temperature, time-resolved fluorescence resonance energy transfer (TR-FRET) was determined by measuring light emission at

https://doi.org/10.1017/neu.2023.30 Published online by Cambridge University Press

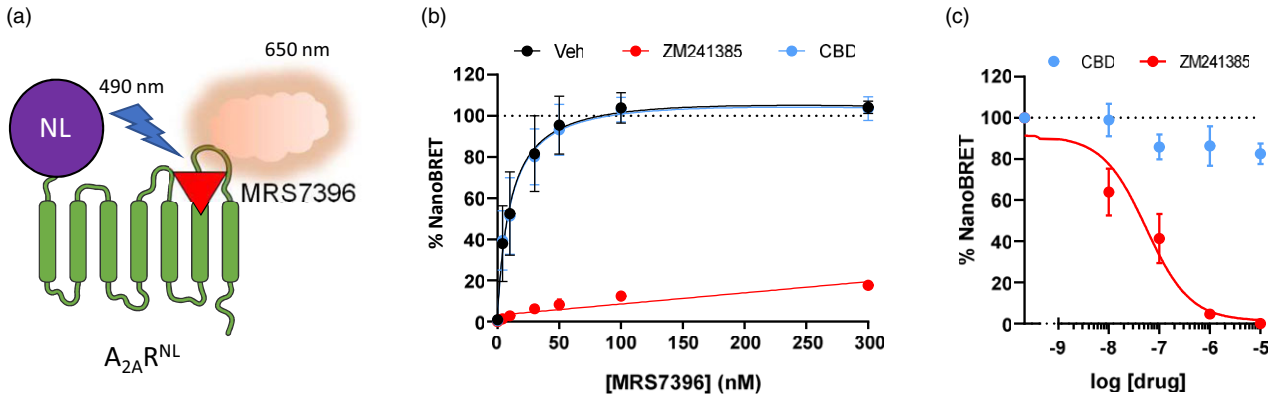

Figure 1. Determination of CBD effects on $A_{2A}R$ ligand binding affinity**. (a)** Schematic representation of the NanoBRET assay. A nanoluciferase is linked to the N-terminal part of the $A_{2A}R$ ($A_{2A}R^{NL}$). When the nanoluciferase substrate coelenterazine is added, $A_{2A}R^{NL}$ (donor) emits light at 490–10 nm. Light excites the fluorescent selective $A_{2A}R$ ligand, MRS7396 (acceptor), which subsequently emits fluorescence at 650-80 nm. **(b)** NanoBRET saturation binding curves obtained by challenging $A_{2A}R^{NL}$ expressing HEK-293T cells with increasing concentrations of MRS7396 in the absence/presence of CBD (1 μM) or ZM241385 (1 μM). **(c)** NanoBRET signals obtained by challenging $A_{2A}R^{NL}$ expressing HEK-293T cells with a fixed concentration of MRS7396 (30 nM, normalised to 100%) in the presence of increasing concentrations of CBD or ZM241385. The represented data are mean ± SEM of three independent experiments each performed in triplicate.

620 nm and 665 nm using a CLARIOstar plate reader (BMG Labtech).

Statistics

Data are represented as mean ± standard error of mean (SEM) with statistical significance set at $P < 0.05$. The number of samples (n) in each experimental condition is indicated in the legend of the corresponding figure. Outliers were assessed using the ROUT method (Motulsky & Brown, 2006); thus, no sample was excluded assuming a Q value of 1% in GraphPad Prism 9. Comparisons between experimental groups were performed using one-way factor analysis of variance (ANOVA) followed by Dunnett's multiple comparisons *post hoc* test using GraphPad Prism 9 as indicated.

Results

To assess the impact of CBD on $A_{2A}R$ functionality, we initially evaluated whether CBD modified the binding of MRS7396, a fluorescent $A_{2A}R$ antagonist. To this end, we took advantage of a previously reported NanoBRET-based $A_{2A}R$ binding assay (Fig. 1a) (Lanznaster *et al.*, 2019). HEK-293T cells permanently expressing the $A_{2A}R^{NL}$ construct were challenged with increasing concentrations of MRS7396, which upon binding to the receptor can act as a compatible acceptor in a BRET process (Fig. 1a). As expected, a saturable hyperbolic curve was obtained for the total binding of MRS7396, which was blocked upon incubation with a saturating concentration (1 μM) of the unlabelled $A_{2A}R$ antagonist ZM241385 (nonspecific binding; Fig. 1b). The analysis of the specific binding of MRS7396 revealed a dissociation constant (K_D) of 8.5 ± 3.2 nM and a maximum binding capacity (B_{max}) of 98.9 ± 8.4 %. Next, upon the same experimental conditions, we examined the ability of CBD to attenuate MRS7396 binding. Differently from ZM241385, CBD (1 μM) was unable to modify the specific binding of MRS7396 to the $A_{2A}R$ (Fig. 1b). Accordingly, no significant differences in affinity (K_D) and receptor capacity (B_{max}) were found in the presence of CBD (K_D = 8.2 ± 2.7 nM; B_{max} = 96.9 ± 7.2 %; $P = 0.992$, $F_{(2, 34)} = 0.0079$). In addition, we also assessed whether CBD could modulate orthosteric binding of $A_{2A}R$ by performing a competition binding assay with a fixed concentration of MRS7396 (30 nM). Again, while ZM241385 blocked $A_{2A}R$ binding, CBD did not significantly modify the NanoBRET signal (Fig. 1c; $P = 0.269$, $F_{(4, 10)} = 1.518$). Collectively, these results indicate that CBD does not bind orthosterically to $A_{2A}R$.

Subsequently, we aimed to determine whether CBD affected $A_{2A}R$ signalling. To this end, we first evaluated the interaction of $A_{2A}R$ with mini-Gαs protein using the NanoBiT™ complementation assay (Sarasola *et al.*, 2022). Accordingly, cells were transiently transfected with the $A_{2A}R^{SmBiT}$ and ^{LgBiT}mini-Gαs constructs, which once expressed allow the reconstitution of the split nanoluciferase and real-time recordings of receptor-effector coupling induced by agonists (Fig. 2a). Cells were challenged with the selective $A_{2A}R$ agonist CGS21680 (100 nM), which induced a rapid increase in the luminescent signal, reaching a peak at 15 min that remained stable for 30 min. This effect was absent in cells challenged with CBD alone (Fig. 2b). Notably, this agonist-dependent $A_{2A}R$ interaction with mini-Gαs protein was completely blocked when co-incubating cells with ZM241385 (50 nM, Fig. 2b). Next, we assessed CGS21680-induced $A_{2A}R$ coupling to Gαs protein in the presence of increasing concentrations of CBD. Interestingly, CBD dose-dependently blocked $A_{2A}R$ coupling to Gαs protein, both decreasing the maximum peak and the density of the effect (Fig. 2b). Of note, differently from ZM241385, CBD only led to a partial blockade of CGS21680-mediated effects.

Finally, to further characterise the effects of CBD on the intrinsic activity of $A_{2A}R$, we evaluated agonist-induced cAMP accumulation in cells permanently expressing the $A_{2A}R^{NL}$ construct and challenged with CGS21680 in the presence/absence of CBD. Interestingly, although CBD itself did not modify cAMP levels, it was able to dose-dependently block CGS21680-induced cAMP levels (Fig. 2c). Again, differently from ZM241385, a full-antagonist, CBD partially blocked $A_{2A}R$ agonist increase of cAMP levels. Overall, these results are compatible with the notion that CBD acts as a weak $A_{2A}R$ negative allosteric modulator.

Discussion

CBD is a promising drug for several pathologies (ElSohly *et al.*, 2017). Accordingly, unravelling its precise mechanism of action is relevant in the progress towards its clinical development. Here, we

https://doi.org/10.1017/neu.2023.30 Published online by Cambridge University Press

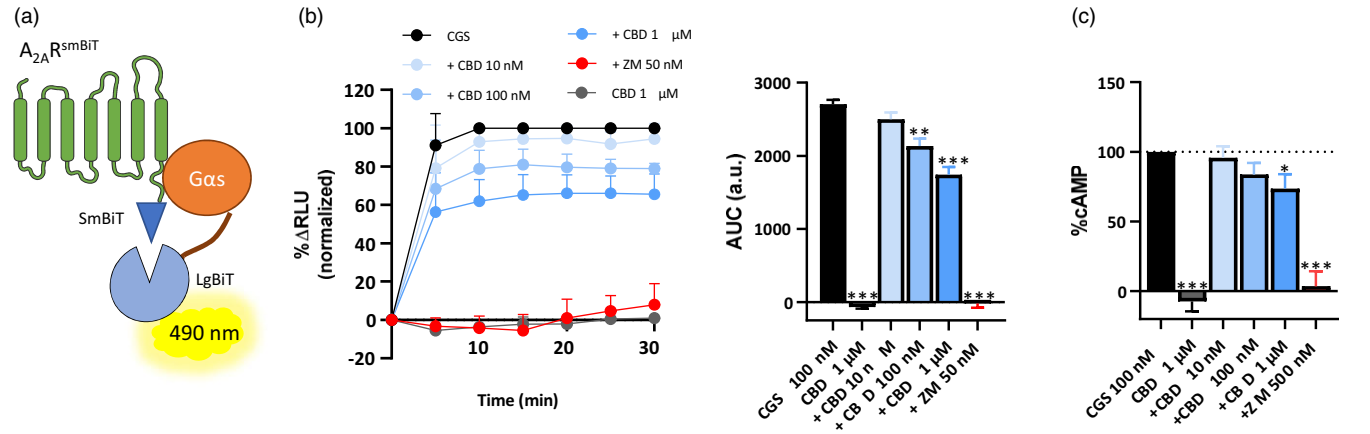

Figure 2. Assessment of CBD effects on $A_{2A}R$ intrinsic activity**. (a)** Schematic representation of the NanoBIT™-based assay. The two fragments of nanoluciferase, small (SmBIT) and large (LgBIT), are fused to $A_{2A}R$ and mini-Gαs protein, respectively. Then, upon agonist binding, $A_{2A}R$ intrinsic activity is assessed by receptor recruitment of Gαs, which induces an increase on luminescence due to nanoluciferase reconstitution. **(b)** Representative time-course of $A_{2A}R$ agonist-mediated Gαs recruitment. The selective $A_{2A}R$ agonist CGS21680 was challenged to $A_{2A}R^{SmBiT}$ and ^{LgBiT}mini-Gαs expressing HEK-293T cells in the absence/presence of increasing concentrations of CBD or ZM241385. The luminescent signal obtained after reconstitution of the nanoluciferase was assessed by calculating the area under the curve for each condition. Data are shown as mean ± SEM of three independent experiments with five replicates. $^*P < 0.05$, $^{***}P < 0.001$, one-way ANOVA with Dunnett's *post hoc* test. **(c)** cAMP accumulation was assessed on HEK-293T cells permanently expressing the $A_{2A}R^{NL}$. Cells were challenged with the selective $A_{2A}R$ agonist CGS21680 (100 nM, normalised to 100% of effect) in the absence/presence of increasing concentrations of CBD. Data are expressed as mean ± SEM of four independent experiments performed in triplicates. $^*P < 0.05$, one-way ANOVA with Dunnett's *post hoc* test.

reveal that CBD does not affect the binding of an $A_{2A}R$ orthosteric ligand, but it is capable of negatively modulating agonist-induced interaction with the Gαs protein at sub-micromolar concentrations (≥100 nM), thus reducing receptor signalling (i.e. cAMP generation). Therefore, we disclose a new non-competitive interaction of CBD with $A_{2A}R$.

The effect of CBD on $A_{2A}R$ could operate through a new allosteric site at the receptor. However, further experiments (i.e. using labelled CBD) would be needed to confirm this hypothesis. On the other hand, we cannot rule out other mechanisms of action for CBD different from classical allosteric drugs. In this sense, previous evidence indicates that other lipids, including the endogenous cannabinoid anandamide at micromolar concentrations, might act as allosteric modulators of other GPCRs through a membrane-perturbing effect that is sensitive to receptor conformation (Lanzafame *et al.*, 2004; Van der Westhuizen *et al.*, 2015). Further studies are needed to assess this putative CBD-mediated membrane effect on $A_{2A}R$-Gαs protein coupling. Similarly, CBD could indirectly modify $A_{2A}R$ functioning by interacting with equilibrative nucleoside transporter 1 (ENT1), as was previously demonstrated in striatal synaptosomes (Pandolfo *et al.*, 2011). However, this hypothetical CBD effect on ENT seems not to play a relevant role *in vivo*, since a recent study demonstrated that CBD lacks the ability to substantially raise endogenous adenosine levels by using the hypothermia mouse model (Xiao *et al.*, 2023). These discrepancies between *in vitro* and *in vivo* studies could be also explained by the fact that $A_{2A}R$ can form heteromers with other GPCRs, including CB_1R (Carriba *et al.*, 2007; Ferré *et al.*, 2010; Aso *et al.*, 2019), in physiological conditions different from that obtained in heterologous expression systems. The assembly of $A_{2A}R$-containing heteromers leads to changes in the agonist recognition, signalling, and trafficking, which might result in different $A_{2A}R$ activity in the presence of CBD.

Although we evaluated the effects of CBD in cultured cells expressing $A_{2A}R$, these results could be relevant for many disorders in which $A_{2A}R$ activity increases. For example, in certain inflammatory processes and cardiovascular diseases, but also in pathological conditions that affect the central nervous system, such as Alzheimer's disease, Parkinson's disease, attention deficit hyperactivity disorder, fragile X syndrome, depression, or anxiety (Domenici *et al.*, 2019). $A_{2A}Rs$, which are widely expressed both in neurons and glia, are mainly found in the dorsal and ventral striatum and other nuclei of the basal ganglia, where they play a key role in the control of voluntary movements, as well as in motivational, emotional and cognitive processes (Sebastião and Ribeiro, 2009). In this way, $A_{2A}Rs$ are involved in regulating the release of neurotransmitters and contribute to the homeostatic control of synaptic transmission and brain function (Sebastião and Ribeiro, 2009). In general, our results are consistent with the positive effects reported for CBD in various brain disorders that can be associated with an exacerbated $A_{2A}R$ function, where CBD would tone down $A_{2A}R$ hyperactivity.

Overall, the present study provides evidence on the ability of CBD to negatively modulate $A_{2A}R$ signalling. The CBD-mediated negative modulation of $A_{2A}R$ function is restricted to the receptor-effector coupling and does not interfere with the binding of the orthosteric ligand. Accordingly, we provide a new and genuine pharmacological way to modulate the adenosinergic system in pathological conditions in which $A_{2A}R$ function is increased.

Acknowledgements. We thank Centres de Recerca de Catalunya Programme/Generalitat de Catalunya for IDIBELL institutional support and Maria de Maeztu MDM-2017-0729 to Institut de Neurociencies, Universitat de Barcelona. We are also grateful to the CannaLatan network members (CYTED-Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo) for the fruitful discussion about the results.

Author contribution. EA, VFD, and FC conceived and designed the study and wrote the manuscript. NSF performed all the experiments and contributed to the manuscript preparation. FC and AC designed the cDNA constructs used in this study. LGA, LIS, and JA cloned and validated cDNA constructs. KAJ provided the fluorescent selective $A_{2A}R$ antagonist.

Financial support. This study was supported by Ministerio de Ciencia, Innovación y Universidades–Agencia Estatal de Investigación-FEDER funds/European Regional Development Fund – 'a way to build Europe' grant

RTI2018-097773-A-I00 to EA and PID2020-118511RB-I00 to FC. Founded by MCIN/AEI /10.13039/501100011033 'ESF Investing in your future' grant PRE2018–084480 to JA, grant PRE2019-088153 to NSF and grant FPU19/03142 to LGA. Also supported by 'Acció instrumental de formació de científics i tecnòlegs' (SLT017/20/000114) of the Departament de Salut de la Generalitat de Catalunya to LIS. The study was also supported by the Luxembourg Institute of Health (LIH), Luxembourg National Research Fund (INTER/FNRS grants 20/15084569 to AC) and the National Institute of Diabetes and Digestive and Kidney Diseases NIDDK Intramural Research Program (ZIADK031117 to KAJ).

Competing interests. None of the authors declare any conflict of interest.

Ethical standard. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on experimentation with cells and DNA constructs.

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https://doi.org/10.1017/neu.2023.30 Published online by Cambridge University Press