

# Uncovering Caffeine's Adenosine A<sub>2A</sub> Receptor Inverse Agonism in Experimental Parkinsonism

Víctor Fernández-Dueñas,<sup>†</sup> Maricel Gómez-Soler,<sup>†</sup> Marc López-Cano,<sup>†</sup> Jaume J. Taura,<sup>†</sup> Catherine Ledent,<sup>‡</sup> Masahiko Watanabe,<sup>§</sup> Kenneth A. Jacobson,<sup>||</sup> Jean-Pierre Villardaga,<sup>\*,⊥</sup> and Francisco Ciruela<sup>\*,†</sup>

<sup>†</sup>Unitat de Farmacologia, Departament Patologia i Terapèutica Experimental, Facultat de Medicina, IDIBELL-Universitat de Barcelona, L'Hospitalet de Llobregat, 08907 Barcelona, Spain

<sup>‡</sup>IRIBHM, Université Libre de Bruxelles, Brussels, Belgium

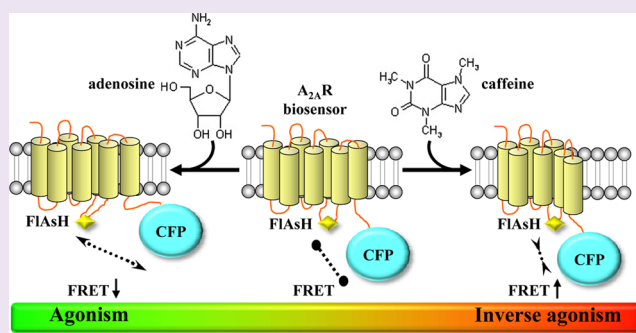
<sup>§</sup>Department of Anatomy, Hokkaido University School of Medicine, Sapporo, Japan

<sup>||</sup>Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, United States

<sup>⊥</sup>Laboratory for G Protein-Coupled Receptor Biology, Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, United States

## Supporting Information

**ABSTRACT:** Caffeine, the most consumed psychoactive substance worldwide, may have beneficial effects on Parkinson's disease (PD) therapy. The mechanism by which caffeine contributes to its antiparkinsonian effects by acting as either an adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R) neutral antagonist or an inverse agonist is unresolved. Here we show that caffeine is an A<sub>2A</sub>R inverse agonist in cell-based functional studies and in experimental parkinsonism. Thus, we observed that caffeine triggers a distinct mode, opposite to A<sub>2A</sub>R agonist, of the receptor's activation switch leading to suppression of its spontaneous activity. These inverse agonist-related effects were also determined in the striatum of a mouse model of PD, correlating well with increased caffeine-mediated motor effects. Overall, caffeine A<sub>2A</sub>R inverse agonism may be behind some of the well-known physiological effects of this substance both in health and disease. This information might have a critical mechanistic impact for PD pharmacotherapeutic design.



Caffeine is considered a putative drug for the treatment of Parkinson's disease (PD).<sup>1,2</sup> Its antiparkinsonian effect is thought to be mediated by blocking the well-known adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R)-mediated tonic inhibition of dopamine D<sub>2</sub> receptor (D<sub>2</sub>R) signaling in the striatopallidal pathway.<sup>1</sup> Caffeine is proposed to act as a weak ( $\mu$ M) A<sub>2A</sub>R antagonist;<sup>3</sup> however, recent structural studies suggest that caffeine may behave as an inverse agonist.<sup>4</sup> Indeed, the comparison of crystal structures of an engineered A<sub>2A</sub>R in complex with either 4-[2-[7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-a][1,3,5]triazin-5-yl-amino]ethyl]phenol (ZM241385), a well-known potent inverse agonist, or caffeine revealed the same cardinal structural features, suggesting that caffeine can stabilize the receptor in an inverse agonistic conformation.<sup>4</sup> Nonetheless, a parallel study predicting A<sub>2A</sub>R-based ligand binding to isolated receptor conformations from the crystal structures predicted caffeine to be a neutral antagonist.<sup>5</sup> The intrinsic efficacy of caffeine, antagonist vs inverse agonist, on the A<sub>2A</sub>R is thus under intense debate as its determination would have critical consequences not only for understanding the mechanism underlying the

antiparkinsonian action of this drug but also for designing new PD pharmacotherapies. This is particularly relevant given that constitutive A<sub>2A</sub>R signaling is thought to increase in certain pathological conditions including PD.<sup>6,7</sup> Therefore, inverse agonists<sup>8</sup> able to block A<sub>2A</sub>R's constitutive activity would be promising therapeutic drugs. Here, we found that caffeine acted as an A<sub>2A</sub>R inverse agonist in cells and in a tissue model for PD.

A limited number of strategies can be applied to measure inverse agonism of ligands acting at G protein-coupled receptors (GPCRs). The most conventional among them consists of determining alterations in basal cell signaling upon receptor overexpression. Accordingly, we evaluated the effects of caffeine on basal levels of both 3',5'-cyclic adenosine monophosphate (cAMP) and phosphorylation of ERK in cells transiently expressing the A<sub>2A</sub>R. We found that 2-[p-(2-carboxyethyl)phenyl-ethylamino]-5'-N-ethylcarboxamidoad-

Received: April 23, 2014

Accepted: September 30, 2014

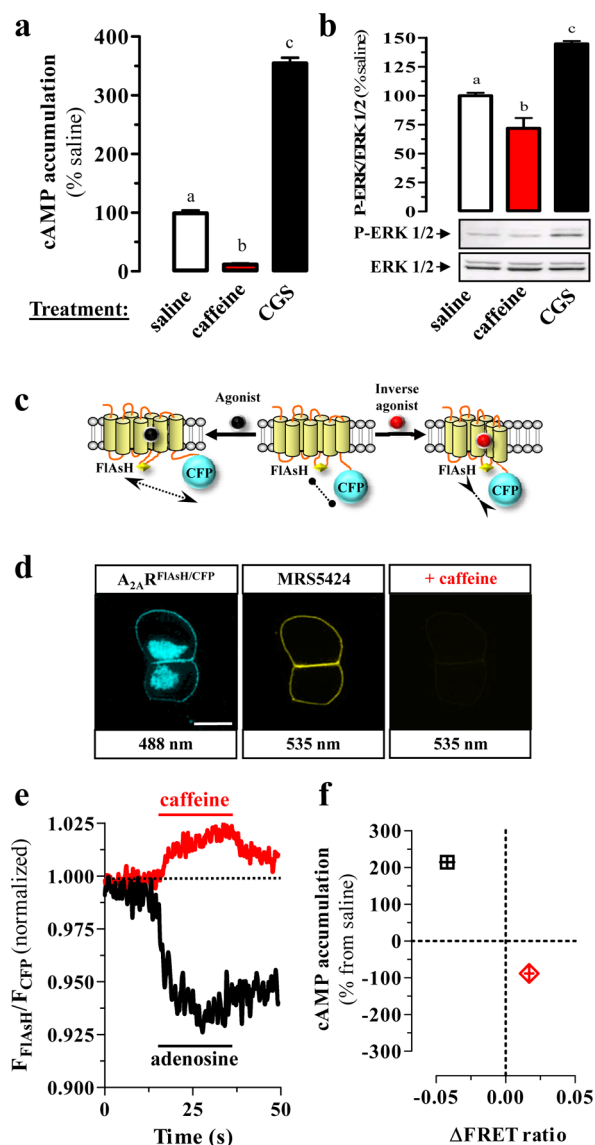
Published: September 30, 2014

nosine (CGS21680), a well-established selective  $A_{2A}R$  agonist, induced cAMP accumulation by  $\sim 3.5$ -fold over the basal, whereas caffeine reduced the basal level of cAMP by  $\sim 10$ -fold (Figure 1a). Interestingly, a dose-dependent effect was found for caffeine, which was comparable, although with lower potency and efficacy, to that obtained for the well-described inverse agonist ZM241385 (Figure S1, Supporting Information). In addition, an inhibitory effect of caffeine was also observed in basal levels of  $A_{2A}R$ -mediated ERK phosphorylation (Figure 1b). These signaling data supported the view that caffeine acts as an inverse agonist by reducing the constitutive activity of the  $A_{2A}R$ .

To further confirm the intrinsic efficacy of caffeine, we used an approach that is independent of downstream biochemical responses by recording its action directly at the level of the receptor. To this end, we used an intramolecular biosensor based on Förster resonance energy transfer (FRET) for the  $A_{2A}R$  ( $A_{2A}R^{F_{\text{FlAsH}}/CFP}$ )<sup>9</sup> as depicted in Figure 1c. This type of GPCR biosensor permits the measurement of changes in receptor conformation upon ligand binding in live cells and the rigorous measurement of the intrinsic efficacy of an agonist (full, partial, or inverse) directly at the level of the receptor and independently from variation in receptor number and/or cell conditions.<sup>9–11</sup> We first ascertained that caffeine bound to and displaced a full agonist from the  $A_{2A}R$  biosensor by using MRS5424,<sup>12</sup> a fluorescent  $A_{2A}R$  agonist (Figure 1d, left panel). HEK293 cells expressing the receptor biosensor perfused with MRS5424 alone were selectively labeled at the plasma membrane (Figure 1d, middle panel). Perfusion of caffeine in addition to MRS5424 blocked most of the cell fluorescence (Figure 1d, right panel), indicating that MRS5424 and caffeine competed for the same binding site on the receptor.

Next, we performed FRET studies in live cells expressing the  $A_{2A}R^{F_{\text{FlAsH}}/CFP}$  biosensor to compare the action of adenosine and caffeine directly at the level of the receptor. Thus, we evaluated changes on FRET signals from a cyan fluorescent protein (CFP) sequence introduced in the C-terminus of the  $A_{2A}R$  to FlAsH, a selectively reactive fluorescein in the third intracellular loop, upon ligand challenging. We observed that adenosine caused a fast decrease in FRET, whereas caffeine caused FRET to increase with slower kinetics (Figure 1e). Both these opposite changes and the distinct kinetics of change in intramolecular FRET (see also Figure S2, Supporting Information) are evidence of the capacity of the  $A_{2A}R$  to adopt distinct conformations in response to adenosine or caffeine, which correlates with the distinct functional efficacies of these two ligands (Figure 1f). Of note, the magnitude of caffeine-induced FRET changes was lower than for adenosine-mediated changes. This fact may be attributed to close energy transfer efficiencies in inverse agonism and basal conformational states. Thus, we performed additional photobleaching experiments and observed that caffeine mediated a slight but significant increase of FRET efficiency compared to control conditions (Figure S3, Supporting Information). These results are in agreement with similar studies done with the  $\alpha_{2A}$ -adrenergic receptor.<sup>10</sup> Overall, an inverse agonism ascribed to caffeine could be postulated, an observation consistent with that obtained not only for  $\alpha_{2A}$ - but also  $\beta_1$ -adrenergic receptors, in which inverse agonists were also found to trigger a very distinct off mode of the receptor's activation switch.<sup>10,13</sup>

The inhibitory effect of inverse agonists on constitutive GPCR signaling is usually straightforwardly assessed in heterologous cell systems, but not in native tissues.<sup>14</sup> To test



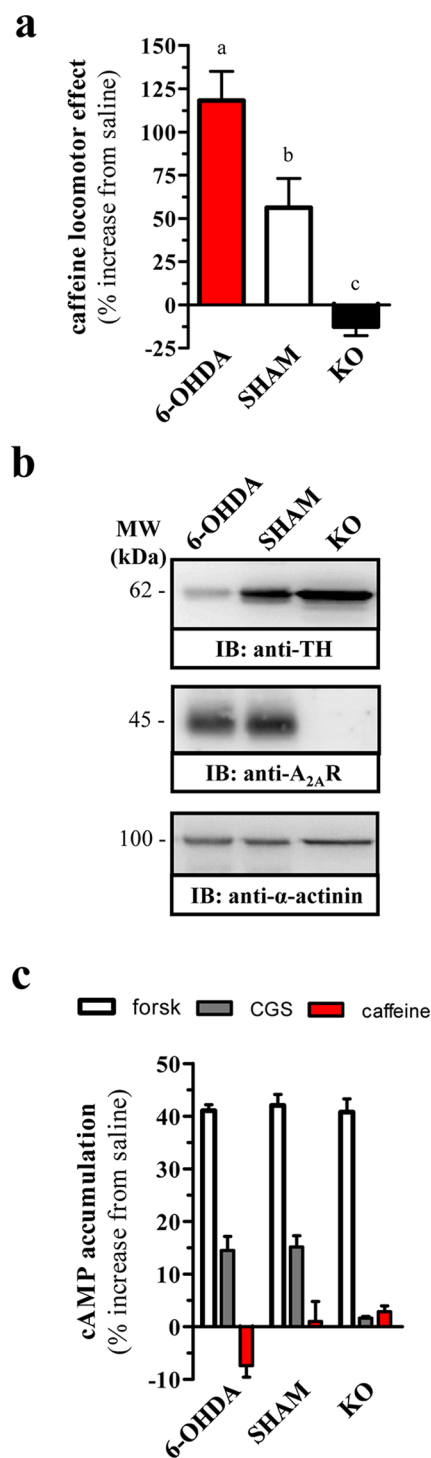
**Figure 1.** Intrinsic efficacy of caffeine. (a) cAMP measurement in HEK293 cells expressing  $A_{2A}R$  and incubated with saline, 200 nM CGS21680, and 100  $\mu\text{M}$  caffeine. Saline-stimulated cAMP was set as 100%, and bars represent the mean  $\pm$  SEM of four independent experiments. Letters (a, b, and c) designate a significant difference between treatments ( $P < 0.05$ ). (b) Extracellular signal regulated kinase 1/2 (ERK1/2) activation in HEK293 cells expressing  $A_{2A}R$  and incubated with saline, 200 nM CGS21680 and 100  $\mu\text{M}$  caffeine. Saline-stimulated cAMP was set as 100%, and bars represent the mean  $\pm$  SEM of four independent experiments. Letters (a, b, and c) designate a significant difference between treatments ( $P < 0.05$ ). (c) Design of the predicted FRET-based  $A_{2A}R$  sensor conformations in response to a full agonist (left) or to an inverse agonist (right). (d) Specific MRS5424 binding to the  $A_{2A}R^{F_{\text{FlAsH}}/CFP}$  in the absence (middle) or presence (right) of caffeine. Scale bar: 10  $\mu\text{m}$ . Images are representative of four independent experiments. (e) FRET changes of the  $A_{2A}R^{F_{\text{FlAsH}}/CFP}$  in response to a full or an inverse agonist. Shown are the changes of the calculated FRET ratio ( $F_{\text{FlAsH}}/F_{\text{CFP}}$ ), induced by rapid superfusion of adenosine (100  $\mu\text{M}$ ) or caffeine (300  $\mu\text{M}$ ). Traces are representative of at least five separate experiments. (f) Plot of the effects on cAMP accumulation of adenosine and caffeine versus the changes that they induced in the normalized FRET ratio ( $F_{\text{FlAsH}}/F_{\text{CFP}}$ ). The  $\Delta$ FRET ratio (A-1) induced by each single  $A_{2A}R$  ligand (adenosine (100  $\mu\text{M}$ ) or caffeine (300  $\mu\text{M}$ );  $n = 5$ ) is represented vs the effects on cAMP accumulation (setting saline-stimulated effects to 0%) achieved for the same ligand (adenosine (100  $\mu\text{M}$ ) or caffeine (300  $\mu\text{M}$ );  $n = 4$ ).

our hypothesis that caffeine acts as an  $A_{2A}R$  inverse agonist under physiological conditions, we generated 6-hydroxydopamine (6-OHDA)-lesioned mice as an animal model for PD,<sup>15</sup> a disease related to  $A_{2A}R$  increased functionality.<sup>6,7</sup> We examined the effect of caffeine on locomotor activity in both control (sham) and 6-OHDA-lesioned mice. The administration of caffeine (10 mg/kg) produced hyper-motility in both 6-OHDA-lesioned and sham animals. This increased mobility was selectively mediated via the  $A_{2A}R$  since caffeine did not affect locomotion in  $A_{2A}R$  deficient ( $A_{2A}R$ -KO) mice (Figure 2a). The caffeine-induced motor effect was markedly intensified in the 6-OHDA-lesioned mice (Figure 2a). These data confirmed the  $A_{2A}R$ -dependent caffeine locomotor effect as previously reported<sup>16,17</sup> and further showed that the 6-OHDA lesion resulted in enhanced  $A_{2A}R$  activity that would be responsible of caffeine-mediated locomotion effects.

We next evaluated the expression of  $A_{2A}R$  and quantified the extent of the 6-OHDA lesion by the loss of tyrosine hydroxylase (TH) (Figure 2b). We observed a significant reduction ( $P < 0.05$ ) of TH expression upon 6-OHDA lesion, confirming dopamine denervation. Conversely, we did not find a significant difference ( $P > 0.05$ ) in levels of  $A_{2A}R$  expression between sham and 6-OHDA-lesioned mice. Various studies have shown either no alteration or an increase of striatal  $A_{2A}R$  expression under dopaminergic denervation,<sup>18,19</sup> indicating that the increase in  $A_{2A}R$  basal function described in PD<sup>6,7</sup> would be mostly explained by mechanisms other than receptor over-expression alone.

The last set of experiments was designed to further confirm our hypothesis. Thus, cAMP accumulation was determined in striatal synaptosomes from sham and 6-OHDA-lesioned mice in response to forskolin, CGS21680, or caffeine. Two positive controls validated the reliability of the approach used: (1) forskolin, a direct activator of adenylyl cyclases, induced cAMP production in all conditions; and (2) the selective  $A_{2A}R$  agonist CGS21680 did not induce cAMP generation in  $A_{2A}R$ -KO animals (Figure 2c). Caffeine did not exert any effect on sham or  $A_{2A}R$ -KO mice, but decreased cAMP levels ( $P < 0.05$ ) in 6-OHDA-lesioned mice (Figure 2c). Interestingly, the prototypic inverse agonist ZM241385 (100 nM) also decreased cAMP basal levels with similar efficacy (~10%) only in 6-OHDA-lesioned mice. Hence, in normal physiological conditions where basal cAMP accumulation is probably not exclusively dependent on  $A_{2A}R$  activity, an effect of caffeine could not be detected. However, we observed a clear inverse agonistic action of caffeine under pathological conditions where constitutive  $A_{2A}R$  activity would be increased. It could be then postulated that  $A_{2A}R$  inverse agonists would be more efficient than neutral antagonists in the management of PD. Indeed, several putative  $A_{2A}R$  blockers have recently been in clinical trials, and one of the most promising is preladenant,<sup>20,21</sup> which has been precisely characterized as an inverse agonist.<sup>5</sup> However, we did not observe significant differences in the efficacy of caffeine and a more potent drug, i.e., ZM241385, in the tissue model for PD. However, it would seem likely that depending on the pathological status the choice of a low- or high-potency drug would permit the fine modulation not only of  $A_{2A}R$  activity but also drug-mediated adverse effects.

In conclusion, the present study characterized caffeine as an  $A_{2A}R$  inverse agonist both in heterologous and endogenous systems. Behavioral experiments using a PD model supported the hypothesis that  $A_{2A}R$  inverse agonists are promising drugs



**Figure 2.** Evaluation of the effects of caffeine in a mouse model of Parkinson's disease. (a) Locomotor activity assessed in the open-field paradigm. Caffeine-evoked locomotor activity is expressed as percentage increase compared to baseline activity (which is set to 0% after saline injection). 6-OHDA ( $n = 6$ ); SHAM ( $n = 6$ ); KO ( $n = 8$ ); vertical bars indicate mean  $\pm$  SEM, and letters (a, b, and c) designate a significant difference between groups ( $P < 0.05$ ). (b) Western blot analysis of striatal synaptosomes showing that TH expression is reduced upon 6-OHDA lesion, while  $A_{2A}R$  is not altered. Load control used for quantitating was  $\alpha$ -actinin. A representative blot of four samples is shown. (c) cAMP measurement of striatal synaptosomes and incubated with 10  $\mu$ M forskolin, 200 nM CGS21680, and 100  $\mu$ M caffeine. Basal cAMP levels were set as 0%, and bars represent the mean  $\pm$  SEM of four independent experiments.

for the pharmacotherapy of neurological diseases that are linked to A<sub>2A</sub>R constitutive activity.

## METHODS

**Reagents.** The primary antibodies used were goat anti-A<sub>2A</sub>R polyclonal antibody (Frontier Institute Co. Ltd., Shinko-nishi, Hokkaido, Japan) and rabbit anti-TH polyclonal antibody (Millipore, Temecula, CA, USA). The secondary antibodies were horseradish peroxidase (HRP)-conjugated rabbit antigoat and goat antirabbit IgG (Pierce Biotechnology, Rockford, IL, USA). Ligands used were MRS5424, previously characterized and containing an AlexaFluor532 fluorophore,<sup>12</sup> and forskolin from Sigma-Aldrich (St. Louis, MO, USA); and CGS21680, ZM241385, and caffeine from Tocris Bioscience (Ellisville, MO, USA). Adenosine deaminase was purchased from Roche Diagnostics GmbH (Mannheim, Germany) and zardaverine from Calbiochem (San Diego, CA, USA).

**Plasmids and Transfection.** The cDNA encoding the human A<sub>2A</sub>R and the previously characterized A<sub>2A</sub>R<sup>FLAsH/CFP</sup> FRET biosensor<sup>9</sup> were used. Human embryonic kidney (HEK) 293 cells were grown at 37 °C, 5% CO<sub>2</sub>, in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL streptomycin, 100 mg mL<sup>-1</sup> penicillin, and 5% (v/v) fetal bovine serum. Cells were seeded into six-well plates at 300,000 cells/well and transiently transfected using Transfectin (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions.

**Animals.** CD-1 mice (Charles River Laboratories, L'Arbresle, France) and A<sub>2A</sub>R-KO mice<sup>22</sup> weighing 20–25 g were used. They were housed in standard cages with ad-libitum access to food and water and maintained under controlled standard conditions (12 h dark/light cycle starting at 7:30 AM, 22 °C temperature, and 66% humidity). The University of Barcelona Committee on Animal Use and Care approved the protocol, and animals were housed and tested in compliance with the guidelines described in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996) and following the European Community, law 86/609/CCE.

**Surgery.** Experimental parkinsonism was induced by a bilateral 6-OHDA injection at the dorsal striatum, as previously described.<sup>15</sup> In brief, mice anesthetized with a ketamine/xylazine combination (75 mg/kg/10 mg/kg, intraperitoneally; Sigma-Aldrich) were immobilized in an adapted digital stereotaxic device (Stoelting Co., Wood Dale, IL, USA). Left and right striatums were injected with 6-OHDA (8 μg/4 μL in 0.05% ascorbic acid; Sigma-Aldrich) by means of a Hamilton syringe (model 701, Reno, NV, USA). Desipramine hydrochloride (10 mg/kg; Sigma-Aldrich) was injected 30 min before lesion to minimize the uptake of 6-OHDA by noradrenergic neurons. Stereotaxic coordinates were respected to bregma: AP = +1.0 mm, ML = ± 1.7 mm, and DV = -2.9 mm. The 6-OHDA solution was injected manually at a rate of 1 μL/min, and after the injection, the needle was left in place for 5 min before slowly retracting it to prevent reflux. Mice were then quickly warmed and returned to their cages. Also, a number of mice were injected with saline to discriminate the possible effects of surgery.

**Open Field Test.** In order to determine the effects of caffeine on locomotor activity, mice were individually placed in an open-field Plexiglas arena (40 cm diameter) illuminated at 5 Lux light intensity. Activity was videotaped for 5 min and distance moved analyzed by means of the OpenField.vi software, kindly provided by Dr. Ricardo Borges (Universidad de La Laguna, Tenerife, Spain). After testing each animal, the apparatus was thoroughly cleaned with cotton pads wetted with 40% ethanol.

**Membrane and Synaptosomal Preparations.** Membrane suspensions from transfected HEK-293 cells were obtained as described previously.<sup>12</sup> To prepare mouse striatal membranes, the procedure was the following: mouse striata were dissected and rapidly homogenized in ice-cold 10 mM Tris HCl (pH 7.4), 1 mM EDTA, and 300 mM KCl buffer with Polytron for three periods of 10 s each. The homogenate was centrifuged for 10 min at 1000g. The resulting supernatant was centrifuged for 30 min at 12 000g. The membranes

were dispersed in 50 mM Tris HCl (pH 7.4) and 10 mM MgCl<sub>2</sub>, washed, and resuspended in the same medium. However, striatal-purified synaptosomes were obtained by discontinuous Percoll gradients.<sup>23</sup> In brief, striatum was homogenized in HEPES 10 mM medium containing 0.32 M sucrose (pH 7.4) and then centrifuged for 2 min at 2000g and 4 °C. The supernatant was spun at 9500g for 12 min and the pellet gently resuspended in 2 mL of the 0.32 M sucrose solution. This synaptosomal suspension was placed onto a Percoll discontinuous gradient containing: 0.32 M sucrose, 1 mM EDTA, 0.25 mM DL-dithiothreitol, and 3, 10, or 23% Percoll (GE Healthcare, Piscataway, NJ, USA), pH 7.4. After centrifugation at 25 000g for 10 min at 4 °C, the synaptosomes were recovered from the 10 and 23% Percoll bands, and they were diluted in a final volume of 30 mL of HEPES buffer medium (HBM): 140 mM NaCl, 5 mM KCl, 5 mM NaHCO<sub>3</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 10 mM glucose, and 10 mM HEPES (pH 7.4). Following further centrifugation at 22 000g for 10 min, the synaptosome pellet was resuspended in 1 mL of HBM. Finally, the synaptosomal suspension was spun at 3000g for 10 min and pellets containing the synaptosomes stored on ice.

**Gel Electrophoresis and Immunoblotting.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) was performed using 10% polyacrylamide gels. Proteins were transferred to PVDF membranes using a semidry transfer system and immunoblotted with the indicated antibody and then HRP-conjugated rabbit antigoat (1:30000) or goat antirabbit IgG (1:30000). The immunoreactive bands were developed using a chemiluminescent detection kit (Pierce).

**cAMP and ERK Phosphorylation Assays in Cells.** Transiently transfected HEK293 cells were grown overnight in serum-free DMEM containing adenosine deaminase (0.5 U/mL), preincubated with the phosphodiesterase inhibitor zardaverine (50 μM) for 10 min, and then stimulated with the distinct ligands for 15 min at 37 °C. Then, we performed either the [<sup>3</sup>H]-cAMP assay protocol (GE Healthcare, Piscataway, NJ, USA) as described in the manufacturer's manual or ERK phosphorylation determination. Radioactivity was determined in scintillation vials using a Packard 1600 TRI-CARB scintillation counter (PerkinElmer, Waltham, MA, USA). However, ERK phosphorylation was detected by immunoblotting using a mouse antiphospho-ERK1/2 antibody and rabbit anti-ERK1/2 antibody (Sigma-Aldrich).

**cAMP Assay in Tissues.** As previously described,<sup>23</sup> mouse striatal purified synaptosomes were first incubated for 2 h at 37 °C, in the presence of BSA (16 μM) and adenosine deaminase (2 units/mg of protein). Zardaverine (50 μM) was included for 30 min during the incubation. Subsequently, the distinct ligands were added for 30 min prior to lysis. Synaptosomes were collected by centrifugation at 13 000g for 1 min at 4 °C, resuspended in the lysis buffer, and transferred to a 384-well assay plate. The homogeneous time-resolved fluorescence assay was performed following manufacturer's instructions (Cisbio Bioassays, Bagnol sur-Cèze, France). A RUBYstar plate-reader (BMG Labtech, Durham, NC, USA) was used to detect the TR-FRET signal.

**Microscopic FRET Measurements.** Single-cell real-time FRET measurements were performed as previously described.<sup>10,12</sup> Briefly, HEK293 cells expressing the A<sub>2A</sub>R<sup>FLAsH/CFP</sup> were seeded on poly-D-lysine-coated coverslips and allowed to grow overnight in the presence of adenosine deaminase (0.5 U/mL). A<sub>2A</sub>R<sup>FLAsH/CFP</sup> transfected cells were then FLAsH-labeled as described before,<sup>9</sup> mounted in an Attofluor holder (Life Technologies, Carlsbad, CA, USA), and placed on a Zeiss inverted microscope (Axio Observer D1; Zeiss, Oberkochen, Germany) equipped with a 63× oil immersion objective and a dual emission photometry system (TILL Photonics, Gräfelfing, Germany). The sample was illuminated with a polychrome V monochromator (Till Photonics). Excitation light was set at 436 ± 10 nm (DCLP of 460 nm), and the excitation time was 10 ms at 10 Hz. Emission lights were recorded at 535 ± 15 nm (F<sub>535</sub>) and 480 ± 20 nm (F<sub>480</sub>) (DCLP of 505 nm). The FRET ratio (F<sub>535</sub>/F<sub>480</sub>) was corrected by the corresponding spillover of CFP emission into the 535 nm channel and by the cross-talk due to direct FLAsH excitation at 436 nm. Eventually, FRET efficiency between donor (CFP) and acceptor

(FLAsH) fluorophores was determined by the donor recovery after acceptor photobleaching according to the equation:

$$\text{FRET efficiency} = 1 - (\text{CFP}_{\text{pre}}/\text{CFP}_{\text{post}})$$

where  $\text{CFP}_{\text{pre}}$  and  $\text{CFP}_{\text{post}}$  are the CFP emissions ( $F_{480}$ ) before and after photobleaching FLAsH by 5–10 min of illumination at 500 nm.

Ligands were superfused using a pressure-driven solenoid valve perfusion system (Octaflo; ALA Scientific Instruments, Westbury, NY, USA). The fluorescence signals were detected by avalanche photodiodes, digitized using an analog/digital converter (Digidata 1440; Molecular Devices, Sunnyvale, CA, USA), and recorded using pCLAMP (Molecular Devices). GraphPad Prism (GraphPad Software, La Jolla, CA, USA) software was used for data analysis. The change in the FRET ratio was fitted to the equation

$$r(t) = A(1 - e^{-t/\tau})$$

where  $\tau$  is the time constant (s) and  $A$  is the magnitude of the signal. For each measurement, changes in the fluorescence emission produced by photobleaching were subtracted.

**Statistics.** The number of samples ( $n$ ) in each experimental condition is indicated in figure legends. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparison posthoc test. Statistical significance is indicated for each experiment.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Additional supplemental figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## ■ AUTHOR INFORMATION

### Corresponding Authors

\*(F.C.) E-mail: [fciruela@ub.edu](mailto:fciruela@ub.edu).

\*(J.P.V.) E-mail: [jpv@pitt.edu](mailto:jpv@pitt.edu).

### Author Contributions

F.C., V.F.-D., and J.P.V. designed research; V.F.-D., M.G.-S., M.L.-C., J.J.T., C.L., and M.W. performed research; V.F.-D. and F.C. analyzed data; and V.F.-D., F.C., K.A.J., and J.P.V. wrote the paper.

### Notes

The authors declare the following competing financial interest(s): J.P.V. holds a patent on the technology of measuring GPCR activation/deactivation by FRET (EP 1581811B1; US8084575).

## ■ ACKNOWLEDGMENTS

This work was supported by grants SAF2011-24779, Consolider-Ingenio CSD2008-00005, and PCIN-2013-019-C03-03 from Ministerio de Economía y Competitividad and ICREA Academia-2010 from the Catalan Institution for Research and Advanced Studies (to FC), by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) of the National Institutes of Health (NIH) under Award number R01 DK087688 (to J.P.V.), and by NIDDK Intramural Research Program (to K.A.J.). V.F.-D., M.G.-S. and F.C. belong to the "Neuropharmacology and Pain" accredited research group (Generalitat de Catalunya, 2014 SGR 1251). We thank E. Castaño and B. Torrejón from the Scientific and Technical Services (SCT) group at the Bellvitge Campus of the University of Barcelona for their technical assistance.

## ■ REFERENCES

- (1) Schwarzschild, M. A., Agnati, L., Fuxe, K., Chen, J. F., and Morelli, M. (2006) Targeting adenosine A2A receptors in Parkinson's disease. *Trends Neurosci.* 29, 647–654.
- (2) Prediger, R. D. S. (2010) Effects of caffeine in Parkinson's disease: from neuroprotection to the management of motor and non-motor symptoms. *J. Alzheimers Dis.* 20 (Suppl 1), S205–20.
- (3) Xu, K., Bastia, E., and Schwarzschild, M. (2005) Therapeutic potential of adenosine A(2A) receptor antagonists in Parkinson's disease. *Pharmacol. Ther.* 105, 267–310.
- (4) Doré, A. S., Robertson, N., Errey, J. C., Ng, I., Hollenstein, K., Tehan, B., Hurrell, E., Bennett, K., Congreve, M., Magnani, F., Tate, C. G., Weir, M., and Marshall, F. H. (2011) Structure of the adenosine A(2A) receptor in complex with ZM241385 and the xanthines XAC and caffeine. *Structure* 19, 1283–93.
- (5) Bennett, K. A., Tehan, B., Lebon, G., Tate, C. G., Weir, M., Marshall, F. H., and Langmead, C. J. (2013) Pharmacology and structure of isolated conformations of the adenosine A<sub>2A</sub> receptor define ligand efficacy. *Mol. Pharmacol.* 83, 949–58.
- (6) Varani, K., Vincenzi, F., Tosi, A., Gessi, S., Casetta, I., Granieri, G., Fazio, P., Leung, E., MacLennan, S., Granieri, E., and Borea, P. A. (2010) A2A adenosine receptor overexpression and functionality, as well as TNF-alpha levels, correlate with motor symptoms in Parkinson's disease. *FASEB J.* 24, 587–98.
- (7) Ramlackhansingh, A. F., Bose, S. K., Ahmed, I., Turkheimer, F. E., Pavese, N., and Brooks, D. J. (2011) Adenosine 2A receptor availability in dyskinetic and nondyskinetic patients with Parkinson disease. *Neurology* 76, 1811–1816.
- (8) Bond, R. A., and IJzerman, A. P. (2006) Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery. *Trends Pharmacol. Sci.* 27, 92–6.
- (9) Hoffmann, C., Gaietta, G., Bunemann, M., Adams, S. R., Oberdorff-Maass, S., Behr, B., Vilardaga, J. P., Tsien, R. Y., Ellisman, M. H., and Lohse, M. J. (2005) A FLAsH-based FRET approach to determine G protein-coupled receptor activation in living cells. *Nat. Methods* 2, 171–176.
- (10) Vilardaga, J. P., Steinmeyer, R., Harms, G. S., and Lohse, M. J. (2005) Molecular basis of inverse agonism in a G protein-coupled receptor. *Nat. Chem. Biol.* 1, 25–28.
- (11) Vilardaga, J. P. (2011) Studying ligand efficacy at G protein-coupled receptors using FRET. *Methods Mol. Biol.* 756, 133–148.
- (12) Fernandez-Duenas, V., Gomez-Soler, M., Jacobson, K. A., Kumar, S. T., Fuxe, K., Borroto-Escuela, D. O., and Ciruela, F. (2012) Molecular determinants of A(2A) R-D(2) R allosterism: role of the intracellular loop 3 of the D(2) R. *J. Neurochem.* 123, 373–384.
- (13) Rochais, F., Vilardaga, J.-P., Nikolaev, V. O., Bünemann, M., Lohse, M. J., and Engelhardt, S. (2007) Real-time optical recording of beta1-adrenergic receptor activation reveals supersensitivity of the Arg389 variant to carvedilol. *J. Clin. Invest.* 117, 229–235.
- (14) De Ligat, R. A., Kourounakis, A. P., and IJzerman, A. P. (2000) Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery. *Br. J. Pharmacol.* 130, 1–12.
- (15) Branchi, I., D'Andrea, I., Armida, M., Carnevale, D., Ajmone-Cat, M. A., Pèzzola, A., Potenza, R. L., Morgese, M. G., Cassano, T., Minghetti, L., Popoli, P., and Alleva, E. (2010) Striatal 6-OHDA lesion in mice: Investigating early neurochemical changes underlying Parkinson's disease. *Behav. Brain Res.* 208, 137–43.
- (16) El Yacoubi, M., Ledent, C., Ménard, J. F., Parmentier, M., Costentin, J., and Vaugeois, J. M. (2000) The stimulant effects of caffeine on locomotor behaviour in mice are mediated through its blockade of adenosine A(2A) receptors. *Br. J. Pharmacol.* 129, 1465–73.
- (17) Halldner, L., Adén, U., Dahlberg, V., Johansson, B., Ledent, C., and Fredholm, B. B. (2004) The adenosine A1 receptor contributes to the stimulatory, but not the inhibitory effect of caffeine on locomotion: a study in mice lacking adenosine A1 and/or A2A receptors. *Neuropharmacology* 46, 1008–17.

(18) Kaelin-Lang, A., Liniger, P., Probst, A., Lauterburg, T., and Burgunder, J. M. (2000) Adenosine A2A receptor gene expression in the normal striatum and after 6-OH-dopamine lesion. *J. Neural Transm.* 107, 851–859.

(19) Pinna, A., Corsi, C., Carta, A. R., Valentini, V., Pedata, F., and Morelli, M. (2002) Modification of adenosine extracellular levels and adenosine A(2A) receptor mRNA by dopamine denervation. *Eur. J. Pharmacol.* 446, 75–82.

(20) Vallano, A., Fernandez-Duenas, V., Pedros, C., Arnau, J. M., and Ciruela, F. (2011) An update on adenosine A2A receptors as drug target in Parkinson's disease. *CNS Neurol. Disord. Drug Targets* 10, 659–669.

(21) Pinna, A. (2009) Novel investigational adenosine A2A receptor antagonists for Parkinson's disease. *Expert Opin. Investig. Drugs* 18, 1619–1631.

(22) Ledent, C., Vaugeois, J. M., Schiffmann, S. N., Pedrazzini, T., El Yacoubi, M., Vanderhaeghen, J. J., Costentin, J., Heath, J. K., Vassart, G., and Parmentier, M. (1997) Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* 388, 674–678.

(23) Martín, R., Durroux, T., Ciruela, F., Torres, M., Pin, J.-P., and Sánchez-Prieto, J. (2010) The metabotropic glutamate receptor mGlu7 activates phospholipase C, translocates munc-13-1 protein, and potentiates glutamate release at cerebrocortical nerve terminals. *J. Biol. Chem.* 285, 17907–17.