Published in final edited form as:

J Neurochem. 2012 November ; 123(3): 373–384. doi:10.1111/j.1471-4159.2012.07956.x.

MOLECULAR DETERMINANTS OF A_{2A}R-D₂R ALLOSTERISM: ROLE OF THE INTRACELLULAR LOOP 3 OF THE D₂R

Víctor Fernández-Dueñas¹, Maricel Gómez-Soler¹, Kenneth A. Jacobson², T. Santhosh Kumar², Kjell Fuxe³, Dasiel O. Borroto-Escuela³, and Francisco Ciruela^{1,*}

¹Unitat de Farmacologia, Departament Patologia i Terapèutica Experimental, Facultat de Medicina, Universitat de Barcelona, L'Hospitalet de Llobregat, 08907 Barcelona, Spain

²Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 20892 Bethesda, USA

³Department of Neuroscience, Karolinska Institutet, 17177 Stockholm, Sweden

Abstract

In the central nervous system (CNS), an antagonistic interaction has been shown between adenosine A_{2A} and dopamine D₂ receptors (A_{2A}Rs and D₂Rs) that may be relevant both in normal and pathological conditions (i.e. Parkinson's disease). Thus, the molecular determinants mediating this receptor-receptor interaction have recently been explored, since the fine tuning of this target (namely the A_{2A}R/D₂R oligomer) could possibly improve the treatment of certain CNS diseases. Here, we used a fluorescence resonance energy transfer (FRET)-based approach to examine the allosteric modulation of the D₂R within the A_{2A}R/D₂R oligomer and the dependence of this receptor-receptor interaction on two regions rich in positive charges on intracellular loop 3 (IL3) of the D₂R. Interestingly, we observed a negative allosteric effect of the D₂R agonist quinpirole on A_{2A}R ligand binding and activation. However, these allosteric effects were abolished upon mutation of specific arginine residues (217–222 and 267–269) on IL3 of the D₂R, thus demonstrating a major role of these positively-charged residues in mediating the observed receptor-receptor interaction. Overall, these results provide structural insights to better understand the functioning of the A_{2A}R/D₂R oligomer in living cells.

Keywords

A_{2A}R; D₂R; FRET; oligomerization; allosterism; fluorescent agonist

Introduction

The existence of oligomeric complexes comprising adenosine and dopamine receptors (i.e. A_{2A}R/D₂R) has been postulated to be relevant for proper striatal function both in normal and pathological conditions (Fuxe *et al.*, 2010). For instance, these A_{2A}R/D₂R oligomers have been implicated in the control of striatal processes such as motor activity control, motor learning or some forms of associative and visual learning (Bornstein and Daw, 2011). Interestingly, the A_{2A}R-D₂R interaction occurs in the somatodendritic area of GABAergic enkephalinergic striatal neurons (Cabello *et al.*, 2009; Ferre *et al.*, 2007a; Ferre *et al.*, 2007b;

*To whom correspondence should be addressed: Francisco Ciruela: Tel: +34 (93) 402 4280/+34 (93) 403 5820, Fax: +34 (93) 402 9082, fciruela@ub.edu.

Conflict of interest

The authors declare no conflict of interest.

Fuxe *et al.*, 1998; Fuxe *et al.*, 2003). At this level, the coexistence of reciprocal antagonistic interactions between these receptors has been further described, namely an intramembrane interaction in which the A_{2A}R mediates inhibition of the D₂R, thus modulating neuronal excitability and neurotransmitter release, and an interaction at the level of adenylate cyclase (AC), in which the D₂R inhibits A_{2A}R-mediated protein phosphorylation and gene expression (Shindou *et al.*, 2002; Svenningsson *et al.*, 2000). Thus, the rationale to study the precise mechanism behind these A_{2A}R-D₂R molecular and functional interactions is well suited since the A_{2A}R/D₂R oligomer has been postulated to be responsible for the neuronal signal integration coming from two different neurotransmitter systems (i.e. dopaminergic and adenosinergic) (Ferre *et al.*, 2007b).

Importantly, in recent years the existence of the A_{2A}R/D₂R heteromer has been revealed in living cells, while in native tissue the results are still not conclusive, by biochemical (i.e. co-immunoprecipitation) and biophysical (i.e. resonance energy transfer-based) approaches (Agnati *et al.*, 2005; Cabello *et al.*, 2009; Canals *et al.*, 2003a; Genedani *et al.*, 2005; Kamiya *et al.*, 2003). In addition, computerized modeling and techniques of pull-down and mass spectrometry have shown the heteromerization between the A_{2A}R and the D₂R to depend on a Coulombic interaction between the third intracellular loop (IL3) of the D₂R and the C-terminal tail of the A_{2A}R (Canals *et al.*, 2003a; Ciruela *et al.*, 2004; Woods and Ferre, 2005; Woods *et al.*, 2005). Thus, two positively charged arginine rich motifs (²¹⁵VLRRRKRV²²³ and ²⁶⁵EVRRRN²⁷¹) located at the N-terminal part of the D₂R-IL3 may interact with two distinct negatively charged motifs from the C-terminal tail of the A_{2A}R. These motifs are the ³⁸⁸HELGVCPEPPGLDDPLAQDGAVGS⁴¹² domain, which contains two adjacent aspartic acid residues, or the ³⁷⁰SAQEpSQGNT³⁷⁸ domain, which contains a phosphorylatable serine residue (S374), and thus their interaction with the D₂R forms electrostatic bonds of covalent-like strength (Canals *et al.*, 2003b; Ciruela *et al.*, 2004; Woods and Ferre, 2005; Woods *et al.*, 2005).

Interestingly, we recently demonstrated that the point mutation of serine 374 to alanine (S374A) reduced A_{2A}R/D₂R heteromerization and blocked the allosteric modulation of the D₂R by the A_{2A}R (Borrito-Escuela *et al.*, 2010), a result that was further confirmed (Navarro *et al.*, 2010). Also, when the S374A point substitution was accompanied by mutation of the two negatively charged aspartates on the A_{2A}R C-terminal tail (D401A/D402A, see above), a synergistic reduction in the physical A_{2A}R/D₂R interaction and the loss of antagonistic allosteric modulation over the A_{2A}R/D₂R interface were observed (Borrito-Escuela *et al.*, 2010). Similarly, it was recently shown the importance of the serine residue on the present A_{2A}R-D₂R interaction (Azdad *et al.*, 2009). Thus, by means of competitive peptides mimicking the serine-containing epitope it was possible to preclude, performing perforated-patch-clamp recordings on brain slices, the ability of the A_{2A}R to counteract the effects of D₂R activation (Azdad *et al.*, 2009). In addition, by using synthetic transmembrane (TM) α-helical peptides of the D₂R, the role of helical interactions within the A_{2A}R/D₂R heteromeric TM interface was also recently explored. Thus, TMs-IV and V of the D₂R may play a critical role in the A_{2A}R/D₂R heteromeric interface since incubation with peptides corresponding to these domains significantly reduced the ability of the A_{2A}R and D₂R to heteromerize. Also, the incubation with TM-IV or TM-V peptides blocked the allosteric modulation normally found in the A_{2A}R/D₂R heteromer (Borrito-Escuela *et al.*, 2010). Overall, the above-mentioned results further corroborated the existence of an electrostatic interaction between the C-terminal tail of the A_{2A}R and IL3 of the D₂R. On the other hand, while the A_{2A}R-mediated allosteric effects on the D₂R have been object of numerous studies, the reciprocal interaction is a less studied aspect of this antagonistic receptor-receptor interaction. Hence, in the present work we aimed to study the D₂R-mediated allosteric modulation of the A_{2A}R functionality within the framework of A_{2A}R/

D₂R oligomers, and to demonstrate that this receptor-receptor allostereism is mostly mediated by two regions rich in positive charges located within IL3 of the D₂R.

Materials and methods

Plasmid Constructs

We created an A_{2A}R sensor (A_{2A}R^{CFP}) to perform FRET experiments with the fluorescent agonist MRS5424, a nucleoside labelled strategically with Alexa Fluor 532. To this end, a pcDNA3.1 vector (Invitrogen, Carlsbad, CA, U.S.A.) containing (from 5' to 3') a signal peptide that targets the receptor to the cell membrane, the fluorescent protein CFP and the parathyroid hormone receptor type 1 (PTH1R) was used as a template (kindly provided by Dr. J. P. Vilardaga, University of Pittsburgh, Pittsburgh, Pennsylvania, USA). Thus, the cDNA encoding the A_{2A}R (Cabello *et al.*, 2009) was amplified by a polymerase chain reaction using the primers FA2ABam (5'-CGCGGATCCATGCCCATCATGGGCTCC-3') and RA2AXho (5'-CGCCTCGAGTCAGGACACCCTGTCTCC-3'), and subcloned into the BamHI/XhoI sites replacing the PTH1R in the above-mentioned template vector.

The mutations at the intracellular loop 3 (IL3) of the D₂R were introduced into the cDNA encoding the wild type D₂R cloned in pEYFP-N1 (i.e. D₂R^{YFP}) (Clontech, Heidelberg, Germany) (Cabello *et al.*, 2009). Thus, two regions rich in positive charges (amino acids 217–222 and 267–269) within the IL3 were sequentially mutated to alanine by using the QuickChangeTM site-directed mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands) and following the manufacturer's instructions. Accordingly, the 217–222 region was first mutated from RRRRKR to AAAAKA, and thereafter the obtained 217–222 mutant D₂R cDNA was used as a template for the second set of mutations (267R–269R to 267A–269A). The mutagenic primers were designed using Stratagene's web-based program: F217–222A (5'-ATCAAGATCTACATTGTCTCGCCGAGCCGCCAAGGCAGTCAACACCAAACGC A GCAGC-3') and R217–222A (5'-GCTGCTGCGTTTGGTGTGACTGCCTTGGCGGCTGCGGCGAGGACAATGTAGAT C TT-3') for the first set of mutations, and F267–269A (5'-TGGGAGTTTCCCAGTGAAGCGGGCGGCAGTGGTGCAGGAGGCTGC-3') and R267–269A (5'-GGCAGCCTCTGCACCACTGCCGCCGCGTTCCTGTTGAACTCCC-3') for the second. The resulting mutated D₂R construct, D₂R_{mut}^{YFP}, was verified by DNA sequencing with BIGDYE[®] terminator V3.1 cycle sequencing kit (Applied Biosystems Hispania S.A., Alcobendas, Spain).

Finally, in order to visualize the presence of the D₂R during our real-time FRET experiments we tagged D₂R constructs at their N-terminus with the O⁶-alkylguanine-DNA alkyltransferase (AGT), which is a 24-kDa protein that acts as a suicide enzyme to covalently transfer modifications from DNA bases onto it. Interestingly, once the AGT is fused to the protein of interest it is possible to stain it by means of fluorescent AGT substrates, which if non-permeable allow the identification of cell surface proteins over periods of hours due to the fact that the complex formed is extremely stable (Ciruela *et al.*, 2010). To this end, a pcDNA3.1 vector containing (from 5' to 3') the signal peptide of the metabotropic glutamate 5 receptor, the AGT protein and adenosine A₁ receptor was used as a template (kindly provided by Dr. J. P. Pin, Université de Montpellier and Institut de Génomique Fonctionnelle, Montpellier, France). Thus, the cDNA encoding the D₂R and the D₂R_{mut} were amplified by polymerase chain reaction using the primers FD2SNAP (5'-GCCGCTCGAGGATCCACTGAATCTGTCCTGG-3') and RD2SNAP (5'-GCCGAAGCTTTCAGCAGTGGAGGATCTTCAGGAAGGCC-3'), and subcloned into the XhoI/HindIII sites replacing the A₁ receptor in the above-mentioned template vector.

Synthesis of the A_{2A}R fluorescent agonist

APEC^{Alexa532} (MRS5424) is a functional agonist at the adenosine A_{2A}R, in which the fluorescent dye Alexa Fluor 532 is covalently attached to a functionalized A_{2A}R agonist 2-[[2-[4-[2-(2-aminoethyl)-aminocarbonyl]ethyl]phenyl]ethylamino]-5'-N-ethyl-carboxamidoadenosine (APEC). Briefly, similar to the method previously described for a related APEC conjugate (Brand *et al.*, 2008), MRS5424 was synthesized as follows: Alexa Fluor 532 carboxylic acid, *N*-succinimidyl ester (1.0 mg, 1.38 μmol) was dissolved in anhydrous DMF (200 μL). Freshly prepared sodium tetraborate labeling buffer (0.1 M, 1 mL, pH 8.5) and APEC (1.12 mg, 2.07 μmol), which was dissolved in anhydrous DMF (200 μL), were added. The reaction mixture was protected from light and after stirring for 18 h, the mixture was diluted with H₂O (600 μL) and purification was performed by HPLC with a Luna 5μ RP-C18(2) semipreparative column (250 X 10.0 mm; Phenomenex, Torrance, CA, USA) under the following conditions: flow rate of 2 mL/min; 10 mM triethylammonium acetate (TEAA)-CH₃CN from 100:0 (v/v) to 70:30 (v/v) in 30 min. The homogeneous product was isolated in the triethylammonium salt form with an HPLC retention time of 13.5 min. Analytical purity of this conjugate was checked using a Hewlett-Packard 1100 HPLC equipped with a Zorbax SB-Aq 5μm analytical column (50 × 4.6 mm; Agilent Technologies Inc, Palo Alto, CA, USA). Mobile phase: linear gradient solvent system: 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)-CH₃CN from 80:20 to 40:60 in 13 min; the flow rate was 0.5 mL/min (retention time 9.08 min). Peaks were detected by UV absorption with a diode array detector at 254, 275, and 280 nm, and the yield of MRS5424 was 0.67 mg (31%). ESI-HRMS *m/z* 1150.4142 [M + H]⁺, C₅₅H₆₃N₁₁O₁₃S₂H⁺: Calcd. 1150.4127).

Cell culture, transfection and membrane preparation

Human embryonic kidney (HEK)-293 cells were grown 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 penicillin and 5% (v/v) fetal bovine serum at 37°C and in an atmosphere of 5% CO₂. HEK-293 cells growing in 25 cm² flasks or six-well plates containing 18 mm coverslips were used for western blot analysis and fluorescence imaging, respectively, were transiently transfected with the cDNA encoding the specified proteins using TransFectinTM Lipid Reagent (Bio-Rad Laboratories, Hercules, CA, USA). Membrane suspensions from transfected HEK-293 cells were obtained as described previously (Burgueno *et al.*, 2003; Burgueno *et al.*, 2004).

cAMP determinations

The functionality of both the D₂R^{YFP} and D₂R_{mut}^{YFP} and their allosteric modulation on activity of the A_{2A}R were assessed by means of a dual luciferase reporter assay (Promega, Stockholm, Sweden). In brief, upon co-transfection of a plasmid containing the cAMP response element (CRE) fused to firefly luciferase it is possible to indirectly detect variations of cAMP, since the expression of the reporter leads to increased levels of luminescence, which are directly proportional to newly generated cAMP. Thus, cells were co-transfected with plasmids corresponding to the following constructs as follows: 1 μg firefly luciferase- encoding experimental plasmid (pGL4-CRE-luc2p; Promega), 1 μg of D₂R^{YFP} or D₂R_{mut}^{YFP} expression vectors, 1 μg of A_{2A}R^{CFP} expression vector (only in the allosteric studies), and 50 ng *Renilla* luciferase-encoding internal control plasmid (phRG-B; Promega). Approximately 36 h post-transfection, after the cells were treated for 4 h with forskolin (Sigma-Aldrich, St. Louis, MO, USA), quinpirole (Sigma-Aldrich), CGS21680 (Tocris Bioscience, Ellisville, MI, USA) and/or ZM 241385 (Tocris Bioscience), they were harvested with passive lysis buffer (Promega) and the luciferase activity of cell extracts was determined in a FLUOStar Optima plate-reader (BMG Labtech) using a 30-nm bandwidth excitation filter at 535 nm. Firefly luciferase was measured as firefly luciferase

luminescence over a 15 s reaction period. The luciferase values were normalized against *Renilla* luciferase luminescence values.

Immunoprecipitation, gel electrophoresis and immunoblotting

For immunoprecipitation, membrane suspensions of transiently transfected HEK-293 cells were solubilized in ice-cold radio immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS and 1 mM EDTA) for 30 min on ice. The solubilized preparation was then centrifuged for 30 min at 13200 rpm at 4°C. The supernatant (1 mg/mL) was processed for immunoprecipitation, each step of which was conducted with constant rotation at 0–4 °C. The supernatant was incubated overnight with 1 µg of mouse anti-A_{2A}R monoclonal antibody (clone 7F6-G5-A2; Millipore, Temecula, CA, USA). Then, a suspension of protein A crosslinked to agarose beads (40 µL, Sigma-Aldrich) was added, and the mixture was incubated overnight. The beads were washed twice with ice-cold RIPA buffer, twice with ice cold RIPA buffer diluted 1:10 in Tris-buffered saline (TBS; 50 mM Tris HCl pH 7.4, 100 mM NaCl) and once with TBS and aspirated to dryness with a 28-gauge needle. Subsequently, 30 µL of SDS-PAGE sample buffer (8 M urea, 2% SDS, 100 mM dithiothreitol, 375 mM Tris, pH 6.8) was added to each sample. Immune complexes were dissociated by heating to 37° C for 2 h and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gel electrophoresis was performed using 10 % polyacrylamide gels. Proteins were transferred to PVDF membranes using a semi-dry transfer system and immunoblotted with either rabbit anti-A_{2A}R polyclonal antibody (Ciruela *et al.*, 2004) or rabbit anti-D₂R polyclonal antibody (Millipore), and then horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1/60000; Thermo Fisher Scientific, Inc., Rockford, IL, USA). The immunoreactive bands were developed using a chemiluminescent detection kit (Pierce) (Ciruela and McIlhinney, 1997).

BRET assay

For BRET experiments, HEK-293 cells, transiently transfected with constant (1 µg) amount of plasmid encoding A_{2A}R^{Rluc} and increasing amounts (0.5–6 µg) of plasmids encoding for D₂R^{YFP} or D₂R^{mutYFP}, were rapidly washed twice in PBS, detached, and resuspended in the same buffer. Cell suspensions (20 µg protein) were distributed in duplicate into 96-well microplate black plates with a transparent bottom (Corning 3651) (Corning, Stockholm, Sweden) for fluorescence measurement or white plates with a white bottom (Corning 3600) for BRET determination. For BRET¹ measurement, *h*-coelenterazine substrate (Life Technologies Corp., Grand Island, NY, USA) was added at a final concentration of 5 µM, and readings were performed 1 min after using the POLARstar Optima plate-reader (BMG Labtech, Durham, NC, USA) that allows the sequential integration of the signals detected with two filter settings [485 nm (440–500 nm) and 530 nm (510–560 nm)]. The BRET ratio was defined as previously described (Canals *et al.*, 2003b; Ciruela *et al.*, 2004; Woods and Ferre, 2005; Woods *et al.*, 2005).

SNAP labelling

Eventually, we identified cells to perform FRET experiments and also quantified the expression of the D₂R constructs by means of the SNAP-tag technology (see above). Briefly, cells transfected with the D₂R^{AGT} and D₂R^{mutAGT} constructs were seeded into 18 mm diameter glass coverslips or in 96-wells plates and then stained with 5 µM or 100 µM, respectively, of the SNAP-Surface 647 ligand (New England BioLabs, Ipswich, MA, USA) in supplemented DMEM during 30 min at 37°C, 5% CO₂. Finally, cells were washed three times with phenol red-free Hank's balanced salt solution containing 1 g/l glucose (HBSS: 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 4.2 mM NaHCO₃, 1.3 mM CaCl₂, 0.5 mM MgCl₂, 0.6 mM MgSO₄, 5.6 mM glucose, pH 7.4) and imaged at 688

nm upon excitation at 647 nm with a Cy5 filter set (Zeiss, Oberkochen, Germany) or fluorescence measured at 680 nm upon excitation at 640 nm in a POLARstar Optima plate-reader (BMG Labtech).

Microscopic FRET measurements

Binding of MRS5424 in cells expressing the A_{2A}R^{CFP} or PTHR1^{CFP} construct was determined by real-time single-cell FRET experiments. In brief, transiently transfected cells seeded into 18 mm diameter glass coverslips were mounted in an Attofluor holder and placed on an inverted Axio Observer microscope (Zeiss, Oberkochen, Germany) equipped with a 63X oil immersion objective and a dual-emission photometry system (TILL Photonics, Gräfelfing, Germany). Then, cells were continuously superfused with the fluorescent ligand dissolved in HBSS, applied with the aid of a focal drug application system (OCTAFLOWTM, ALA Scientific Instruments, Westbury, NY, USA). A Polychrome V (Till Photonics) was used as the light source and signals detected by avalanche photodiodes were digitized using a Digidata 1440A analog/digital converter (Molecular Devices, Sunnyvale, CA, USA). pCLAMP (Molecular Devices) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA) softwares were used for data collection and analysis. FRET was measured upon excitation at 430 ± 10 nm (beam splitter dichroic long-pass (DCLP) 460 nm) and an illumination time set to 10 ms at 10 Hz. Then, the emission light intensities were determined at 535 ± 15 nm (F₅₃₅) and 480 ± 20 nm (F₄₈₀) with a beam splitter DCLP of 505 nm. No corrections for spillover between channels or direct Alexa532 excitation were made. The increase in FRET ratio (F₅₃₅/F₄₈₀) was fitted to the equation: $r(t) = A \times (1 - e^{-t/\tau})$, where τ is the time constant (s) and A is the magnitude of the FRET signal. When necessary for calculating τ , agonist-independent changes in FRET due to photobleaching were subtracted. Cell images were taken with a Zeiss AxioCamMR3 and processed with the Axiovision 4.8 software (Zeiss).

Statistics

The number of samples (n) in each experimental condition is indicated in the figure legends. When two experimental conditions were compared, statistical analysis was performed using an unpaired *t* test. Otherwise, statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post-hoc test. Statistical significance was set as $P < 0.05$.

Results

Role of the D₂R IL3 Arg-rich domains on A_{2A}R/D₂R oligomerization

It is well established that a Coulombic interaction between the C-terminal tail of the A_{2A}R and IL3 of the D₂R is involved in the A_{2A}R/D₂R oligomerization (Ciruela *et al.*, 2004). Interestingly, while the role of the A_{2A}R C-terminal tail in this phenomenon has been largely studied (Borrito-Escuela *et al.*, 2010; Borrito-Escuela *et al.*, 2010) the impact of Arg-rich domains of the D₂R IL3 on the oligomeric function has been less explored. Thus, we aimed to investigate the role of two regions rich in positive charges within the D₂R IL3 on the negative allosteric receptor-receptor interaction associated with the A_{2A}R/D₂R oligomer. To this end, and by means of site-directed mutagenesis, we generated a mutated D₂R construct (D₂R_{mut}^{YFP}) where the arginine (Arg) residues in IL3 of the D₂R were substituted by alanine (Ala) (Fig. 1a). First, we examined whether the Arg to Ala substitutions affected the functionality of the receptor, since it had to be challenged in further experiments with the selective agonist quinpirole, and a loss of efficacy could lead to misinterpretation of the results. Therefore, by means of a luciferase reporter assay, we determined changes in CRE transcription, which is increased after the activation of the cAMP response element-binding (CREB). Specifically, we examined the ability of both

D₂R^{YFP} and D₂R_{mut}^{YFP} to antagonize the forskolin-mediated activation of AC through coupling to G_{αo/i}, as previously described (Obadiah *et al.*, 1999). Interestingly, both D₂R constructs equally reduced the forskolin-mediated cAMP accumulation after quinpirole (25 nM) challenge (Fig. 1b), suggesting that the Arg to Ala substitutions did not affect the receptor functionality.

Next, we evaluated the ability of the D₂R_{mut}^{YFP} to oligomerize with the A_{2A}R. To this end, we examined the physical interaction with the A_{2A}R by means of a classical biochemical approach (i.e. co-immunoprecipitation experiments) and by a biophysical assay (i.e. BRET experiments). Thus, from extracts of HEK-293 cells transiently co-transfected with A_{2A}R^{CFP} plus D₂R^{YFP} or D₂R_{mut}^{YFP}, the mouse anti-A_{2A}R antibody co-immunoprecipitated a protein of molecular weight ~75 kDa corresponding to the D₂R^{YFP} (either the wild type or the mutant) (Fig. 2a, IP: anti-A_{2A}R, upper panel, lanes 4 and 5). Importantly, this protein band did not appear in singly transfected (A_{2A}R^{CFP}, D₂R^{YFP} or D₂R_{mut}^{YFP}) cells immunoprecipitated with the same antibody (Fig. 2a, IP: anti-A_{2A}R, upper panel, lanes 1, 2 and 3). Finally, we confirmed the presence of the A_{2A}R by means of a rabbit polyclonal antibody (Fig. 2a, IP: anti-A_{2A}R, lower panel, lanes 1, 4 and 5). Overall, these results suggested that by mutating the Arg residues of IL3 of the D₂R, its interaction with the A_{2A}R was not qualitatively altered.

Also, we analyzed the degree of the A_{2A}R-D₂R interaction by means of BRET experiments. Thus, a BRET saturation curve was constructed in HEK-293 cells co-transfected with a constant amount of an A_{2A}R^{Rluc} construct while increasing the concentrations of plasmids containing D₂R^{YFP} or D₂R_{mut}^{YFP}. Interestingly, a positive BRET signal was obtained for the transfer of energy between A_{2A}R^{Rluc} and both the D₂R^{YFP} and the D₂R_{mut}^{YFP}. The BRET signal, as reflected in the BRET ratio, increased as a hyperbolic function of the concentration of the D₂R^{YFP} fusion constructs (Fig. 2b). However, the pairing of A_{2A}R^{Rluc} and D₂R_{mut}^{YFP} led to a substantial reduction in the maximal BRET signal (BRET_{max}) when compared to the A_{2A}R^{Rluc} and D₂R^{YFP} pair (45±2 mBU and 81±2 mBU, respectively) (Fig. 2b). Importantly, when cells singly expressing the A_{2A}R^{Rluc} and D₂R^{YFP}-expressing cells were mixed, no BRET signal was observed (Fig. 2c). Overall, the results obtained suggested that while the Arg to Ala substitutions within the D₂R IL3 did not abolish A_{2A}R/D₂R oligomerization, the proximity between the two receptors was significantly reduced. Alternatively, it could also be possible that the mutations would lead to a lower proportion of oligomers, thus the partial BRET signal detected would be the sum of a smaller number of direct receptor-receptor interactions.

D₂R-mediated allosteric modulation of A_{2A}R agonist binding

Antagonistic A_{2A}R-mediated allosteric modulation of D₂R function has been largely studied; however, the reverse condition, i.e. D₂R-mediated allosteric modulation of the A_{2A}R, is still elusive. Consequently, we decided to investigate both the possible allosteric modulation exerted by the D₂R on A_{2A}R function when these receptors are forming part of an oligomer, and also, the contribution of Arg-rich domains of IL3 of the D₂R to this receptor-receptor allosterism. To this end, we implemented a real-time FRET-based A_{2A}R agonist binding procedure in cells co-expressing both A_{2A}Rs and D₂Rs. Accordingly, we recorded the FRET engaged between a novel fluorescent A_{2A}R agonist synthesized in this study (MRS5424) and the A_{2A}R^{CFP} construct (Fig. 3b). First, we checked that the agonist-receptor interaction was observed at the cell surface of the transfected cells, and that the binding was completely blocked in the presence of an A_{2A}R-specific antagonist, ZM241,385 (Fig. 3a). And thereafter, a specific FRET signal between MRS5424 and the A_{2A}R^{CFP} was measured in a real-time mode (Fig. 3c). Thus, under these experimental conditions we analyzed changes in the FRET signal exerted by a quinpirole challenge on cells expressing A_{2A}R^{CFP} plus either D₂R or D₂R_{mut}. It is important to mention here that to ensure the

presence of both A_{2A}R and D₂R within the same assayed cell, we engineered a D₂R^{AGT} construct (Fig. 4a), which allowed the subsequent visualization of the receptor at the cell surface (Fig. 4b). Indeed, in Fig. 4b it is possible to visualize that the A_{2A}R^{CFP} and the D₂R-AGT fusion constructs (D₂R^{AGT} and D₂R_{mut}^{AGT}) labelled with the SNAP substrate are co-distributed at the plasma membrane of co-transfected cells, thus providing the ideal conditions to assess our real-time FRET-based A_{2A}R agonist binding experiments.

First, we recorded FRET in cells singly transfected with the A_{2A}R^{CFP}. Thus, upon excitation at 430 nm (CFP excitation) the signals recorded from single HEK-293 cells expressing A_{2A}R^{CFP} challenged with the fluorescent agonist were analyzed at emissions of 480 nm (Fig. 3b, lower panel, CFP) and 535 nm (Fig. 3b, lower panel, Alexa532), and the corresponding normalized ratio F₅₃₅/F₄₈₀ plotted (Fig. 3b, upper panel). After addition of MRS5424 (2 μM), a symmetrical decrease in CFP emission and an increase in Alexa532 emission indicated that the change was indeed due to an increase in FRET between A_{2A}R^{CFP} and MRS5424 as a consequence of the ligand binding (i.e. increase in the ratio F₅₃₅/F₄₈₀) (Fig. 3b). Interestingly, control experiments with cells expressing PTHR^{CFP} showed no FRET when MRS5424 was superfused on these cells (data not shown). On the other hand, the selection of the MRS5424 concentration (2 μM) was based on previous results (Fernández-Dueñas, unpublished data), indicating that this concentration elicited the best-defined response to be subsequently modulated with the D₂R agonist.

Next, we analysed the D₂R-mediated allosteric modulation of A_{2A}R agonist binding in cells co-expressing the A_{2A}R^{CFP} and D₂R^{AGT} constructs. Interestingly, the proteins were shown to co-localize at the cell surface (Fig. 4b), and it could also be assessed that the plasma membrane expression of the D₂R^{AGT} constructs were unaffected due to the mutations (Fig. 4c). Thus, upon identification of a doubly-transfected cell (as in Fig. 4b), we proceeded to analyze the binding of MRS5424 at the A_{2A}R^{CFP} in the absence or the presence of the D₂R agonist quinpirole (100 μM). Importantly, MRS5424 binding was unaffected by quinpirole in cells transfected only with the A_{2A}R^{CFP} (data not shown). Thus, upon D₂R^{AGT} co-expression and quinpirole challenge, a significantly lower FRET signal was observed, indicating that D₂R activation diminished the binding of the A_{2A}R agonist (Fig. 5a, left panel). Noteworthy, in this Figure the inverse of the decline of CFP fluorescence (1/F_{CFP}) was used as readout of the ligand binding event, because the diminution of CFP fluorescence was exclusively due to FRET and also to avoid any contamination of the FRET determinations. Furthermore, we analyzed the kinetic data by fitting the CFP decline to a monoexponential decay curve, and an increase was observed in the association rate constant upon addition of quinpirole (0.134 ± 0.025 s⁻¹ with quinpirole vs 0.028 ± 0.005 s⁻¹ with buffer). This indicated that the association of the fluorescent ligand was significantly affected by the conformational change of the D₂R upon quinpirole activation. Conversely, in cells co-expressing the A_{2A}R^{CFP} and D₂R_{mut}^{AGT} the binding of the fluorescent A_{2A}R ligand was unaffected by quinpirole superfusion (Fig. 5a, right panel). In addition, when adjusting the CFP decline to a monoexponential decay curve, the kinetic data revealed that the association rate constant was not as affected by the D₂R_{mut}^{AGT} (0.046 ± 0.007 s⁻¹) as with the wild-type D₂R^{AGT}. Therefore, Arg mutations diminished the ability of the D₂R to exert a complete allosteric modulation of binding at the A_{2A}R. Overall, these results demonstrated that the IL3 Arg-rich domains of D₂R played a major role in the allosteric effects of the D₂R within the A_{2A}R/D₂R oligomer, since the negative allosteric modulation exerted by the D₂R (74.3±3.1%) disappeared (94.4±4.3%; p<0.05) following mutagenesis (Fig. 5b).

Functional consequences of the D₂R allosteric modulation

In view of the previous results, one would expect that D₂R-mediated allosteric modulation of the A_{2A}R would lead to functional consequences on A_{2A}R signaling. Therefore, we evaluated the effects of the D₂R^{AGT} or the D₂R_{mut}^{AGT} challenge on A_{2A}R-mediated AC

stimulation by measuring cAMP accumulation in co-transfected cells. It is important to mention here that in cells singly transfected with A_{2A}R^{CFP} the selective A_{2A}R agonist CGS21680 (50 nM) led to an increase of cAMP accumulation, and while the A_{2A}R antagonist ZM241385 (1 μM) totally blocked this effect, the D₂R agonist quinpirole (25 nM) did not produce any effect on A_{2A}R-mediated cAMP accumulation (data not shown). Interestingly, in doubly A_{2A}R^{CFP}-D₂R^{AGT} transfected cells quinpirole partially and significantly (~60%; $p < 0.01$) diminished the effects of CGS21680 on cAMP accumulation (Fig. 6a, red bars, CGS+Quin). On the other hand, when cells expressing A_{2A}R^{CFP} and D₂R^{mut}^{AGT} were challenged with quinpirole, a significant (~80%; $p < 0.01$) reduction of A_{2A}R agonist-mediated cAMP accumulation was observed (Fig. 6a, red bars, CGS+Quin). Curiously, when inhibition of D₂R^{AGT}- and D₂R^{mut}^{AGT}-mediated cAMP accumulation was compared, it was shown to be significantly ($p < 0.05$) different (Fig. 6a). Apparently, these contradictory results might be readily explained if we consider that the establishment of an A_{2A}R/D₂R functional oligomer (i.e. displaying receptor-receptor allostery) would allow direct A_{2A}R-D₂R cross-talk, providing a balanced outcome of G_{αs}- and G_{αi}-mediated AC modulation (Fig. 6b). On the contrary, when no direct functional A_{2A}R-D₂R cross-talk would exist, a shift to more G_{αi}- coupled D₂Rs would happen, thus leading to an increased AC inhibition outcome (Fig. 6b).

Overall, the results of this study show that the Arg-rich domains of the D₂R IL3 played a key functional role in the A_{2A}R/D₂R oligomer. Thus, when mutating these residues it could be observed that although i) there were no changes on membrane density of D₂R and ii) no effect on D₂R signalling, iii) it was affected but not abolished A_{2A}R-D₂R heteromerization, and iv) a selective abolition of the allosteric interactions between A_{2A}R and D₂R occurred.

Discussion

The molecular interactions between the A_{2A}R and the D₂R when engaged in an oligomeric complex have been postulated to play a major role in the development and also the treatment of neurodegenerative disorders, such as Parkinson's disease (for review see (Fuxe *et al.*, 2010)). As a result, the elucidation of the mechanisms mediating such receptor-receptor interactions has become a main goal during recent years. Consequently, as commented earlier, it was previously shown that a Coulombic interaction established between the IL3 of the D₂R and the C-terminal tail of the A_{2A}R plays a pivotal role in the phenomenon of A_{2A}R/D₂R oligomerization (Canals *et al.*, 2003b; Ciruela *et al.*, 2004; Woods and Ferre, 2005; Woods *et al.*, 2005). Thus, in the present work, we aimed to examine the allosteric modulation that the D₂R exerts on the A_{2A}R within the A_{2A}R/D₂R oligomer. In addition, we also aimed to demonstrate that this receptor-receptor interaction is mostly mediated by the two highly positively-charged regions located at the IL3 of the D₂R, not only at the level of the binding of A_{2A}R agonists but also with respect to the functionality of the latter receptor.

Hence, we performed site-directed mutagenesis in order to generate a D₂R mutant in which the positively charged Arg residues were replaced by uncharged alanine residues. Interestingly, the mutation of the Arg residues did not affect the functionality of the receptor, since it was able to inhibit forskolin-induced cAMP accumulation at the same level as the wild-type D₂R. Accordingly, it could be assumed that the results obtained when examining the allosteric effects of the D₂R agonist were not due to an aberrant signalling pathway as a result of the mutations. Interestingly, by means of co-immunoprecipitation experiments we found that the Arg-rich domains of IL3 of the D₂R seemed nonessential for A_{2A}R and D₂R oligomerization since D₂R^{mut} still co-immunoprecipitated with the A_{2A}R. However, the Arg→Ala substitutions within the D₂R IL3 probably reduced the molecular proximity between these two receptors as indicated in our BRET assays. These results are consistent with those previously obtained concerning the role of negatively charged residues

on the A_{2A}R C-terminal tail, which in fact may interact electrostatically with IL3 of the D₂R in A_{2A}R/D₂R oligomerization (Borrito-Escuela *et al.*, 2010). Although these Coulombic interactions disappeared, the complex was still formed, since the protein-protein interaction also depends on other receptor regions, for instance the TMs-IV and V of the D₂R (Borrito-Escuela *et al.*, 2010). Needless to say, it could also be possible for the receptor-receptor interaction to be wholly affected and that we were observing a lower proportion of oligomers formed. However, when assessing the expression levels of the D₂R^{AGT} constructs we did not find differences in the presence of the wild-type and the mutant receptor at the cell surface. Nevertheless, it could be concluded that the mutations affected in some manner the present receptor-receptor interaction, thus the possible effects on the presumably antagonistic allosteric interaction were subsequently examined.

Interestingly, regarding the allosteric modulation of the D₂R on A_{2A}R binding, the generation of the mutant variant of the D₂R permitted to demonstrate the relevance of the positively-charged residues located at the IL3 in such receptor-receptor interaction. Thus, upon co-transfecting both the D₂R_{mut}^{AGT} and the A_{2A}R^{CFP}, quinpirole treatment did not affect FRET between the fluorescent A_{2A}R agonist and A_{2A}R^{CFP}, as compared with the wild-type D₂R. Previous studies have revealed that an intramembrane interaction occurs between the A_{2A}R and the D₂R; thus, it was shown that the A_{2A}R inhibits the D₂R-mediated neuronal excitability and neurotransmitter release and that the D₂R negatively modulates A_{2A}R ligand binding to inhibit A_{2A}R-mediated protein phosphorylation and gene expression (Canals *et al.*, 2003b; Ciruela *et al.*, 2004; Woods and Ferre, 2005; Woods *et al.*, 2005). However, to our knowledge this is the first time the negative allosteric modulation of the D₂R on A_{2A}R agonist binding has been visualized in a real-time mode. Thus, we were able to determine that D₂R activation partially inhibited and also slowed the binding association of the fluorescent A_{2A}R agonist. Furthermore, the results obtained clearly indicated that this important interaction was abolished by mutating the IL3 of the D₂R. In fact, we also evaluated the allosteric modulation of the D₂R on A_{2A}R agonist-mediated cAMP accumulation, and it was shown that the D₂R agonist quinpirole, by activating the D₂R_{mut}, was able to completely counteract A_{2A}R agonist-mediated activation. Interestingly, it has been previously shown that the antagonistic allosteric A_{2A}R/D₂R interaction favors a rapid onset of β -arrestin-2 D₂R signalling through G_{ai/o} uncoupling (Canals *et al.*, 2003b; Ciruela *et al.*, 2004; Trincavelli *et al.*, 2012; Woods and Ferre, 2005; Woods *et al.*, 2005). Thus, when the receptor-receptor allosteric interaction disappears, as in the absence of Arg-rich domains of the D₂R IL3, the D₂R would be able to mostly signal through G_{ai/o} protein to inhibit cAMP accumulation. Consequently, it could be considered that in the absence of A_{2A}R-D₂R allosteric modulation a major outcome of G_{ai/o}-signalling depending cascades occurs (Fig. 6b). Interestingly, this condition might be exacerbated by the fact that the levels of G_{ai/o} protein may be higher than the G_{as} (Borrito-Escuela *et al.*, 2011), thus contributing to the enhanced inhibition of AC activity. In this regard, it is currently thought that upon agonist stimulation and the subsequent receptor conformational change, this information is transmitted into the IL3 (Li *et al.*, 1996). Thus, it would seem likely that changing some residues (Arg→Ala) of this domain would disrupt its normal function. Consequently, the lack of an allosteric modulation of the D₂R_{mut} could also be explained, not only because a reduced direct receptor-receptor interaction within the A_{2A}R/D₂R oligomer, but also by means of other mechanisms located at the intracellular signal transmission pathways (Zurn *et al.*, 2009). However, our results point to a scenario in which the mutation of the Arg residues at the IL3 reduced the physical interaction between the D₂R and the A_{2A}R, and therefore partially precluded the D₂R-mediated allosteric modulation of the A_{2A}R.

In conclusion, the findings of this study provide further evidence of the existence of antagonistic A_{2A}R-D₂R interactions. In particular, the Arg residues (217–222 and 267–269)

on IL3 of the D₂R have been identified to play a major key role in such receptor-receptor allosteric interaction. Thus, it would be feasible to target this region in order to allosterically modulate the functionality of the A_{2A}R/D₂R oligomer.

Acknowledgments

This work was supported by grants SAF2011–24779 and Consolider-Ingenio CSD2008–00005 from Ministerio de Ciencia e Innovación and ICREA Academia-2010 from the Catalan Institution for Research and Advanced Studies to FC. Also, VF-D, MG-S and FC belong to the “Neuropharmacology and Pain” accredited research group (Generalitat de Catalunya, 2009 SGR 232). Support to KAJ and TSK from the NIDDK Intramural Research Program of the National Institutes of Health, Bethesda, MD, USA is acknowledged. We also thank Esther Castaño, Eva Julià, and Benjamín Torrejón, from the Scientific and Technical Services (SCT)-Bellvitge Campus of the University of Barcelona for the technical assistance.

References

- Agnati LF, Fuxe K, Torvinen M, Genedani S, Franco R, Watson S, Nussdorfer GG, Leo G, Guidolin D. New methods to evaluate colocalization of fluorophores in immunocytochemical preparations as exemplified by a study on A_{2A} and D₂ receptors in Chinese hamster ovary cells. *J Histochem Cytochem.* 2005; 53:941–953. [PubMed: 16055748]
- Azad K, Gall D, Woods AS, Ledent C, Ferre S, Schiffmann SN. Dopamine D₂ and adenosine A_{2A} receptors regulate NMDA-mediated excitation in accumbens neurons through A_{2A}-D₂ receptor heteromerization. *Neuropsychopharmacology.* 2009; 34:972–986. [PubMed: 18800071]
- Bornstein AM, Daw ND. Multiplicity of control in the basal ganglia: computational roles of striatal subregions. *Curr Opin Neurobiol.* 2011; 21:374–380. [PubMed: 21429734]
- Borroto-Escuela DO, Marcellino D, Narvaez M, Flajolet M, Heintz N, Agnati L, Ciruela F, Fuxe K. A serine point mutation in the adenosine A_{2A}R C-terminal tail reduces receptor heteromerization and allosteric modulation of the dopamine D₂R. *Biochem Biophys Res Commun.* 2010; 394:222–227. [PubMed: 20197060]
- Borroto-Escuela DO, Romero-Fernandez W, Tarakanov AO, Ciruela F, Agnati LF, Fuxe K. On the existence of a possible A_{2A}-D₂-beta-Arrestin2 complex: A_{2A} agonist modulation of D₂ agonist-induced beta-arrestin2 recruitment. *J Mol Biol.* 2011; 406:687–699. [PubMed: 21256133]
- Borroto-Escuela DO, Romero-Fernandez W, Tarakanov AO, et al. Characterization of the A_{2A}R-D₂R interface: focus on the role of the C-terminal tail and the transmembrane helices. *Biochem Biophys Res Commun.* 2010; 402:801–807. [PubMed: 21040702]
- Brand F, Klutz AM, Jacobson KA, Fredholm BB, Schulte G. Adenosine A(2A) receptor dynamics studied with the novel fluorescent agonist Alexa488-APEC. *Eur J Pharmacol.* 2008; 590:36–42. [PubMed: 18603240]
- Burgueno J, Canela EI, Mallol J, Lluís C, Franco R, Ciruela F. Mutual regulation between metabotropic glutamate type 1alpha receptor and caveolin proteins: from traffick to constitutive activity. *Exp Cell Res.* 2004; 300:23–34. [PubMed: 15383311]
- Burgueno J, Enrich C, Canela EI, Mallol J, Lluís C, Franco R, Ciruela F. Metabotropic glutamate type 1alpha receptor localizes in low-density caveolin-rich plasma membrane fractions. *J Neurochem.* 2003; 86:785–791. [PubMed: 12887677]
- Cabello N, Gandia J, Bertarelli DC, Watanabe M, Lluís C, Franco R, Ferre S, Lujan R, Ciruela F. Metabotropic glutamate type 5, dopamine D(2) and adenosine A(2a) receptors form higher-order oligomers in living cells. *J Neurochem.* 2009; 109:1497–1507. [PubMed: 19344374]
- Canals M, Marcellino D, Fanelli F, et al. Adenosine A_{2A}-dopamine D₂ receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J Biol Chem.* 2003a; 278:46741–46749. [PubMed: 12933819]
- Canals M, Marcellino D, Fanelli F, et al. Adenosine A_{2A}-dopamine D₂ receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J Biol Chem.* 2003b; 278:46741–46749. [PubMed: 12933819]
- Ciruela F, Burgueno J, Casado V, et al. Combining mass spectrometry and pull-down techniques for the study of receptor heteromerization. Direct epitope-epitope electrostatic interactions between

- adenosine A2A and dopamine D2 receptors. *Anal Chem.* 2004; 76:5354–5363. [PubMed: 15362892]
- Ciruela F, McIlhinney RA. Differential internalisation of mGluR1 splice variants in response to agonist and phorbol esters in permanently transfected BHK cells. *FEBS Lett.* 1997; 418:83–86. [PubMed: 9414100]
- Ciruela F, Vilardaga JP, Fernandez-Duenas V. Lighting up multiprotein complexes: lessons from GPCR oligomerization. *Trends Biotechnol.* 2010; 28:407–415. [PubMed: 20542584]
- Ferre S, Agnati LF, Ciruela F, Lluís C, Woods AS, Fuxe K, Franco R. Neurotransmitter receptor heteromers and their integrative role in ‘local modules’: the striatal spine module. *Brain Res Rev.* 2007a; 55:55–67. [PubMed: 17408563]
- Ferre S, Ciruela F, Quiroz C, et al. Adenosine receptor heteromers and their integrative role in striatal function. *Scientific World Journal.* 2007b; 7:74–85. [PubMed: 17982579]
- Fuxe K, Agnati LF, Jacobsen K, et al. Receptor heteromerization in adenosine A2A receptor signaling: relevance for striatal function and Parkinson’s disease. *Neurology.* 2003; 61:S19–23. [PubMed: 14663004]
- Fuxe K, Ferre S, Zoli M, Agnati LF. Integrated events in central dopamine transmission as analyzed at multiple levels. Evidence for intramembrane adenosine A2A/dopamine D2 and adenosine A1/dopamine D1 receptor interactions in the basal ganglia. *Brain Res Brain Res Rev.* 1998; 26:258–273. [PubMed: 9651540]
- Fuxe K, Marcellino D, Borroto-Escuela DO, Guescini M, Fernandez-Duenas V, Tanganelli S, Rivera A, Ciruela F, Agnati LF. Adenosine-dopamine interactions in the pathophysiology and treatment of CNS disorders. *CNS Neurosci Ther.* 2010; 16:e18–42. [PubMed: 20345970]
- Genedani S, Guidolin D, Leo G, Filaferro M, Torvinen M, Woods AS, Fuxe K, Ferre S, Agnati LF. Computer-assisted image analysis of caveolin-1 involvement in the internalization process of adenosine A2A-dopamine D2 receptor heterodimers. *J Mol Neurosci.* 2005; 26:177–184. [PubMed: 16012191]
- Kamiya T, Saitoh O, Yoshioka K, Nakata H. Oligomerization of adenosine A2A and dopamine D2 receptors in living cells. *Biochem Biophys Res Commun.* 2003; 306:544–549. [PubMed: 12804599]
- Kull B, Ferre S, Arslan G, Svenningsson P, Fuxe K, Owman C, Fredholm BB. Reciprocal interactions between adenosine A2A and dopamine D2 receptors in Chinese hamster ovary cells co-transfected with the two receptors. *Biochem Pharmacol.* 1999; 58:1035–1045. [PubMed: 10509756]
- Li Y, Mende U, Lewis C, Neer EJ. Maintenance of cellular levels of G-proteins: different efficiencies of α_s and α_o synthesis in GH3 cells. *Biochem J.* 1996; 318 (Pt 3):1071–1077. [PubMed: 8836158]
- Navarro G, Ferre S, Cordomi A, et al. Interactions between intracellular domains as key determinants of the quaternary structure and function of receptor heteromers. *J Biol Chem.* 2010; 285:27346–27359. [PubMed: 20562103]
- Obadiah J, Avidor-Reiss T, Fishburn CS, Carmon S, Bayewitch M, Vogel Z, Fuchs S, Levavi-Sivan B. Adenylyl cyclase interaction with the D2 dopamine receptor family; differential coupling to G_i , G_z , and G_s . *Cell Mol Neurobiol.* 1999; 19:653–664. [PubMed: 10384262]
- Shindou T, Nonaka H, Richardson PJ, Mori A, Kase H, Ichimura M. Presynaptic adenosine A2A receptors enhance GABAergic synaptic transmission via a cyclic AMP dependent mechanism in the rat globus pallidus. *Br J Pharmacol.* 2002; 136:296–302. [PubMed: 12010779]
- Svenningsson P, Lindskog M, Ledent C, Parmentier M, Greengard P, Fredholm BB, Fisone G. Regulation of the phosphorylation of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa in vivo by dopamine D1, dopamine D2, and adenosine A2A receptors. *Proc Natl Acad Sci U S A.* 2000; 97:1856–1860. [PubMed: 10677546]
- Trincavelli ML, Daniele S, Orlandini E, et al. A new D(2) dopamine receptor agonist allosterically modulates A(2A) adenosine receptor signalling by interacting with the A(2A)/D(2) receptor heteromer. *Cell Signal.* 2012; 24:951–960. [PubMed: 22230688]
- Woods AS, Ciruela F, Fuxe K, Agnati LF, Lluís C, Franco R, Ferre S. Role of electrostatic interaction in receptor-receptor heteromerization. *J Mol Neurosci.* 2005; 26:125–132. [PubMed: 16012186]

- Woods AS, Ferre S. Amazing stability of the arginine-phosphate electrostatic interaction. *J Proteome Res.* 2005; 4:1397–1402. [PubMed: 16083292]
- Zurn A, Zabel U, Vilardaga JP, Schindelin H, Lohse MJ, Hoffmann C. Fluorescence resonance energy transfer analysis of alpha 2a-adrenergic receptor activation reveals distinct agonist-specific conformational changes. *Mol Pharmacol.* 2009; 75:534–541. [PubMed: 19106230]

Fig. 1. Characterization of the D₂R construct

(a) Schematic representation of the D₂R_{mut} construct. The mutations performed at the IL3 are indicated. The D₂R structure was suited using the PDB 1JGJ of rhodopsin and prepared using PyMOL (PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA, USA). (b) Determination of cAMP accumulation by means of the luciferase reporter assay system. HEK-293 cells transiently transfected with pGL4-CRE-luc2p/phRG-B plus D₂R^{YFP} (red bars) or D₂R_{mut}^{YFP} (blue bars) were incubated either with forskolin (1 μ M) plus HBSS buffer (solid bars) or quinpirole (25 nM) (empty bars). Light emission was normalized assigning the 100% of effect to that obtained when incubating cells with forskolin. Data are expressed as the mean $\pm$ SEM of three independent experiments. (*) indicate statistically significant differences ($P < 0.05$; Student t test) when comparing HBSS vs quinpirole treatment in both D₂R^{YFP} and the D₂R_{mut}^{YFP} transfected cells.

Fig. 2. Role of the D_2R IL3 Arg-rich domains on $A_{2A}R/D_2R$ oligomerization

(a) Co-immunoprecipitation experiments. Membrane suspensions of HEK-293 cells transiently expressing $A_{2A}R^{CFP}$ (lane 1), D_2R^{YFP} (lane 2), $D_2R_{mut}^{YFP}$ (lane 3), $A_{2A}R^{CFP}$ plus D_2R^{YFP} (lane 4), $A_{2A}R^{CFP}$ plus $D_2R_{mut}^{YFP}$ (lane 5) were solubilized and processed for immunoprecipitation using mouse anti- $A_{2A}R$ monoclonal antibody (IP). Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted using rabbit anti- D_2R polyclonal antibody and rabbit affinity-purified anti- $A_{2A}R$ polyclonal antibody (IB). The presented blots are representative of three different experiments with similar qualitative results. (b) BRET experiments. BRET saturation curves for the $A_{2A}R/D_2R$ oligomers ($A_{2A}R^{Rluc}/D_2R^{YFP}$: filled red circles and $A_{2A}R^{Rluc}/D_2R_{mut}^{YFP}$: filled blue circles) at increasing expression

levels of the YFP-tagged vectors. Plotted on the X-axis is the fluorescence value obtained from the YFP, normalized with the luminescence value of $A_{2A}R^{Rluc}$ 10 min after *h*-coelenterazine incubation. Results are expressed as mean $\pm$ SEM (n=8 in triplicate). (c) Comparison of the $BRET_{max}$ obtained in panel b. Values represent percentages of maximal saturable BRET responses ($BRET_{max}$). Mean $\pm$ S.E.M.; (n=8 in triplicate). ***: $A_{2A}R^{Rluc}/D_2R_{mut}^{YFP}$ group is significantly different compared to the $A_{2A}R^{Rluc}/D_2R^{YFP}$ group ($p<0.001$); +++: control group ($A_{2A}R^{Rluc}+D_2R_{mut}^{YFP}$) is significantly different compared to the $A_{2A}R^{Rluc}/D_2R_{mut}^{YFP}$ and $A_{2A}R^{Rluc}/D_2R^{YFP}$ groups ($p<0.001$).

Fig. 3. Determination of $A_{2A}R$ agonist binding by real-time FRET in single living cells
 (a) Specific MRS5424 binding to the $A_{2A}R^{CFP}$. HEK-293 cells transiently transfected with $A_{2A}R^{CFP}$ were superfused with MRS5424 (2 μ M) during 5 min, washed and observed on an inverted microscope without (upper image) or with (down image) ZM241,385 (1 μ M), a specific $A_{2A}R$ antagonist. Scale bar: 10 μ m. (b) Schematic representation of the FRET experiment between the fluorescent $A_{2A}R$ agonist MRS5424 (APEC^{Alexa532}) and the $A_{2A}R^{CFP}$ construct. The $A_{2A}R$ and CFP structures were prepared using PyMOL and the PDBs 3EML and 1EMA, respectively. (c) Time-resolved changes in CFP and Alexa532 fluorescence emission signals in single cells transfected with $A_{2A}R^{CFP}$. The emission intensities of CFP (F_{480} , blue trace), Alexa 532 (F_{535} , yellow trace) and the ratio F_{535}/F_{480} (black trace) in response to MRS5424 were recorded simultaneously from single HEK-293 cells expressing the $A_{2A}R^{CFP}$. Shown are the changes induced by rapid superfusion with 2 μ M MRS5424. The increase of the ratio F_{535}/F_{480} was fitted by a simple monoexponential

curve giving a time constant (τ) in this experiment of 12.0 ± 0.3 s. Traces are representative of eight separate experiments.

Fig. 4. Determination of $A_{2A}R$ agonist binding by real-time FRET in single living cells
 (a) Schematic representation of the $A_{2A}R^{CFP}$ and D_2R^{AGT} constructs. The schematic AGT (O6-alkylguanine-DNA alkyltransferase) diagram was prepared using PyMOL and PDB 1EH6. (b) Cell surface localisation of the $A_{2A}R^{CFP}$ and D_2R^{AGT} constructs in living cells. HEK-293 cells were transiently transfected with $A_{2A}R^{CFP}$ and either D_2R^{AGT} or $D_2R_{mut}^{AGT}$, stained with the SNAP substrate and visualized at 480 nm (CFP) and 688 nm (SNAP) upon excitation at 430 nm and 647 nm, respectively, using an inverted microscope. Superposition of images (merge) revealed $A_{2A}R^{CFP}$ and D_2R^{AGT} co-distribution at the cell surface. Scale bar: 10 μ m. (c) Cell surface quantification of the $A_{2A}R^{CFP}$ and D_2R^{AGT} constructs in living cells. HEK-293 cells were transiently transfected with $A_{2A}R^{CFP}$ and

either D_2R^{AGT} or $D_2R_{mut}^{AGT}$, stained with the SNAP substrate and plated in wells, where fluorescence at 680 nm was measured upon excitation at 640 nm using a POLARstar Optima plate-reader. Results are expressed as mean $\pm$ SEM (n=3 in triplicate), after the subtraction of the fluorescence measured in mock cells (AU=arbitrary units).

Fig 5. Real-time FRET determinations of the D_2R -mediated modulation of $A_{2A}R$ agonist binding

(a) Time-resolved changes in FRET signals (see Fig. 3) in cells transfected with $A_{2A}R^{CFP}$ plus D_2R^{AGT} or $A_{2A}R^{CFP}$ plus D_2R^{mutAGT} in the absence (black trace) or presence (red and blue traces) of quinpirole ($100 \mu M$) were recorded. The FRET increase ($1/F_{480}$) was fitted by a simple monoexponential curve and the magnitude of the FRET signal (A, see Materials and methods) calculated for each experimental condition. Traces are representative of five separate experiments with similar qualitative and quantitative results. (b) The variation of the A value for each experimental condition was plotted. Data represents the average $\pm$ S.E.M. values of five independent experiments. Asterisk indicates data significantly different from the control condition (i.e. in the absence of quinpirole): $*p < 0.05$ by ANOVA with Student-Newman-Keuls multiple comparison post-hoc test.

Fig. 6. Functional consequences of the D₂R allosteric modulation

(a) Allosteric modulation of the A_{2A}R^{CFP} activity. HEK-293 cells transiently transfected with pGL4-CRE-luc2p/phRG-B and A_{2A}R^{CFP} plus D₂R^{AGT} (red bars) or D₂R^{mut}^{AGT} (blue bars) were incubated with forskolin (1 μ M), CGS21680 (50 nM), CGS + ZM241385 (50 nM + 1 μ M, respectively), or CGS21680 + quinpirole (50 nM + 25 nM, respectively) and the cAMP accumulation determined by means of the luciferase reporter assay system (see Materials and methods). Light emission was normalized assigning the 100% of effect to that obtained when incubating cells with forskolin. Data are expressed as the mean $\pm$ SEM of three independent experiments. (*) indicates statistically significant differences ($P < 0.05$; Student t test) when comparing cells transfected with the D₂R^{AGT} or the D₂R^{mut}^{AGT}. (b) Schematic representation of the oligomeric antagonistic A_{2A}R-D₂R interaction. In the left part, the native A_{2A}R/D₂R oligomer is shown, in which a balance between G_{αs} and G_{αi/o} coupling would exist, and the bidirectional negative allosteric receptor-receptor interaction may outline the final AC signalling efficacy. Indeed, at the G-protein level the expected antagonistic interaction can be observed since the A_{2A}R protomer via G_{αs} activates AC, while the D₂R protomer would partially lose its G_{αi/o} coupled AC inhibition. In the right part, the mutated A_{2A}R/D₂R oligomer is shown. This oligomer lacks the negative allosteric receptor-receptor interaction and non A_{2A}R-D₂R trans-inhibition phenomena are established. Thus, in the absence of the powerful antagonistic A_{2A}R-D₂R interaction at the level of D₂R recognition and G_{αi/o} coupling, an enhanced AC inhibition is observed.