The mGlu5 Receptor Protomer-Mediated Dopamine D2 Receptor Trans-Inhibition Is Dependent on the Adenosine A2A Receptor Protomer: Implications for Parkinson’s Disease

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Abstract
The adenosine A2A receptor (A2AR), dopamine D2 receptor (D2R) and metabotropic glutamate receptor type 5 (mGluR5) form A2AR-D2R-mGluR5 heteroreceptor complexes in living cells and in rat striatal neurons. In the current study, we present experimental data supporting the view that the A2AR protomer plays a major role in the inhibitory modulation of the density and the allosteric receptor-receptor interaction within the D2R-mGluR5 heteromeric component of the A2AR-D2R-mGluR5 complex in vitro and in vivo. The A2AR and mGluR5 protomers interact and modulate D2R protomer recognition and signalling upon forming a trimeric complex from these receptors. Expression of A2AR in HEK293T cells co-expressing D2R and mGluR5 resulted in a significant and marked increase in the formation of the D2R-mGluR5 heteromeric component in both bioluminescence resonance energy transfer and proximity ligation assays. A highly significant increase of the high-affinity component of D2R (D2R

Introduction
The first pieces of evidence for antagonistic glutamate receptor with dopamine D2 receptor (D2R) interactions were found in 1983–1984 through the ability of glutamate to reduce the affinity of the high-affinity D2R agonist binding sites in striatal membrane preparations. Subsequently, it was observed that mGluR5 agonists alone or combined with an A2AR agonist (CGS-21680) can reduce the affinity of the high-affinity state of D2R for agonist binding sites in the rat striatum [1]. Co-immunoprecipitation experiments also indicated the existence of A2AR-mGluR5 heteroreceptor complexes in HEK293 cells and rat striatal membrane preparations [2]. The colocation of the receptors in striatal neurons was demonstrated [3, 4] as well as their synergistic interactions as studied with in vivo microdialysis and intracellular signalling in striatal preparations [2, 5, 6].

In 1974, the discovery that the methylxanthines caffeine and theophylline could enhance the contralateral turning behaviour induced by levodopa and dopamine receptor agonists in the hemi-Parkinsonian rat model was one early
finding leading to the hypothesis that antagonistic adenosine-dopamine interactions existed [7, 8]. Today, a considerable amount of molecular and functional experimental data supports the view that \( A_{2A} R \) and \( D_2 R \) form heteroreceptor complexes with antagonistic receptor-receptor interactions on the plasma membrane [9–16].

The existence of \( A_{2A} R-D_2 R-mGluR_5 \) higher-order oligomers was postulated, and it was proposed that the receptor-receptor interactions within this high-order complex are important to modulate the dorsal and ventral striatal-pallidal GABA neurons [2, 3, 8]. Years later, it was proposed that combined treatment with \( A_{2A} R \) and \( mGluR_5 \) agonists targeting \( A_{2A} R-D_2 R-mGluR_5 \) heteroreceptor complexes in the ventral striatal-pallidal GABA pathway can represent a new strategy for the treatment of schizophrenia [17]. Also, the combine treatment with selective \( A_{2A} R \) and \( mGluR_5 \) receptor antagonists represents an alternative therapeutic approach to Parkinson’s disease [18–20].

A combination of bimolecular fluorescence complementation assays and bioluminescence resonance energy transfer assays as well as the sequential resonance energy transfer technique was used to show that \( A_{2A} R-D_2 R-mGluR_5 \) heteroreceptor complexes exist in living cells [21]. In addition, high-resolution immunoelectron microscopy was also used to further demonstrate their existence in striatal glutamate synapses [21]. An integrative role of these receptor complexes in adenosine, dopamine and glutamate transmission was also proposed [8, 22, 23]. Recently, \( A_{2A} R, D_2 R \) and \( mGluR_5 \) receptor-receptor interactions were also found to modulate the activity of the striatal-pallidal GABA neurons based on in vivo dual-probe microdialysis [24].

Herein, new findings that further expand the understanding of \( A_{2A} R-D_2 R-mGluR_5 \) heteroreceptor complexes are presented. Results in cellular models first demonstrated that \( A_{2A} R \) promotes the \( D_2 R \) and \( mGluR_5 \) receptor-receptor interactions, and its participation increases the density of the \( D_2 R-mGluR_5 \) heterocomplexes. Binding and functional experiments indicated that \( A_{2A} R \) and \( mGluR_5 \) upon agonist activation play a significant role in modulating a composition, density and signalling of \( A_{2A} R-D_2 R-mGluR_5 \) heteroreceptor complexes. This was also observed in \( A_{2A} R \) or \( D_2 R \) knockout mice when studying the effects of the \( mGluR_5 \) negative allosteric modulator raseglurant on locomotor activity.

Methods

Plasmid Constructs

The cDNA encoding the rat \( mGluR_5 \) was cloned (without stop codon) in pGFP\(^2\)-N1 vector (PerkinElmer, Waltham, MA, USA) using standard molecular biology techniques.

Drugs and Chemicals

The \( A_{2A} R \) agonist 4-[2-[[6-Amino-9-(N-ethyl-\( \beta \)-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride (CGS-21680), the selective \( A_{2A} R \) antagonist 4-[2-[7-Amino-2-(2-furyl)]1,2,4 triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl]phenol (ZM-241385), the \( mGluR_5 \) agonist (RS)-2-Chloro-5-hydroxyphenylglycine sodium salt (CHPG), the \( mGluR_5 \) antagonist 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) and the \( D_2 R \) antagonist 4-[4-(4-Chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butane hydrochloride (haloperidol) were purchased from Tocris Bioscience (UK), and the \( mGluR_5 \) negative allosteric modulator 2-[3-Fluorophenyl]ethynyl]-4,6-dimethyl-3-pyridinamine hydrochloride (raseglurant) was purchased from Hello Bio (Republic of Ireland). The concentrations of CGS-21680 (100 nM) and ZM-241385 (1 \( \mu \)M) were chosen in agreement with our previous studies [26, 27]. The concentrations of CHPG (500 nM) and MPEP (300 nM) have been selected on the basis of previous studies suggesting that, in this concentration range, the compounds selectively act as agonist or antagonist of \( mGluR_5 \), respectively [18, 24, 28, 29]. Finally, the dose of haloperidol (1 mg/kg) and raseglurant (1 mg/kg) used in mouse behavioural experiments was previously described [30, 31]. Also, isobutyl-1-methylxanthine (IBMX) and 4-(3-butoxy-4-methoxybenzyl) imidazolidone (Ro 20-1724) were purchased from Tocris Bioscience (Bristol, UK).

Cell Culture and Transfection

Human embryonic kidney 293T (HEK293T cells (American Type Culture Collection, Manassas, VA, USA) cells were grown in Dulbecco’s Modified Eagle’s Medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin/streptomycin and 10% (v/v) foetal bovine serum at 37 °C in an atmosphere of 5% CO\(_2\). Cells were plated in 6-well plates (1 \( \times \) 10\(^6\)cells/well), 96-well plates (1 \( \times \) 10\(^6\)cells/well) or in 75 cm\(^2\) flasks and cultured overnight prior to transfection or experimental procedures. Cells were transiently transfected using linear polyethyleneimines (Polysciences Inc., Warrington, PA, USA) according to the manufacturer’s instructions.

Animals

\( A_{2A} R^{-/-} \) and \( D_2 R^{-/-} \) mice generated on a CD-1 genetic background [30, 32] and the corresponding wild-type littermates weighing 20–25 g were used. The animal protocol (no. 7085) was approved by the University of Barcelona.
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optimal neurotransmission is likely to have an improved
function when the endogenous agonist is present. This is of particular interest for CNS targets where spatial and temporal aspects of endogenous receptor signal-
tors (NAM) and positive allosteric modulators (PAM)) have
mGluR5 allosteric modulators (negative allosteric modula-
tors) instead of a full antagonist was used to establish the oligomeric order of receptor com-
exes, as well as the proportion of receptors engaged in
dimers or oligomers (BRETmax). In the current work, bio-
luminescence resonance energy transfer (BRET2) saturation
assays were carried out using plasmids encoding for
mGluR5-negative allosteric modulator) instead of a full antagonist was based on the theoretical advantages that allosteric modu-
ators offer compared with their competitive counterparts.

The rationale for the use of raseglurant (a mGluR5-neg-
atives) are of particular interest for CNS targets where optimal neurotransmission is likely to have an improved
therapeutic outcome as opposed to sustained receptor block-
ade or activation.

Haloperidol-Induced Catalepsy

Mice (n = 10) were randomly assigned to treatment groups, and behavioural testing was performed blind to treatment. The dopamine D2 receptor (D2R) antagonist, haloperidol (1 mg/kg, s.c.), was administered to induce catalepsy. Thirty minutes after the haloperidol administration, mice experienced a full cataleptic response. At this time point, for each mouse, the state of catalepsy was tested by gently placing their front limbs over an 8-cm-high horizontal bar. The intensity of catalepsy was assessed by measuring the time the mice remain in this position being completely immobile for a maximum of 120 s. Only mice that remained cataleptic for the entire 120 s were used for subsequent drug testing. After 30 min of the baseline measurement vehicle (0.5% methylcellulose and 2% DMSO), PBF509 was administered orally via gavage (3, 10 or 30 mg/kg, p.o.), and the cata-
lepsy was then determined at 15, 30 and 60 min PBF509 administration. For each time point, the number of responding mice and the total cataleptic time for each animal were determined.

Membrane Preparation

HEK293T cells or mouse striata were homogenized in ice-
cold 10 mM Tris HCl, pH 7.4, 1 mM EDTA and 300 mM
KCl buffer containing a protease inhibitor cocktail (Roche,
Penzberg, Germany) using a Polytron for three periods of 10
s each. The homogenate was centrifuged for 10 min at 1000
× g. The resulting supernatant was centrifuged for 30 min at
12,000 × g. The membranes were dispersed in 50 mM Tris
HCl (pH 7.4) and 10 mM MgCl2, washed and resuspended in the same medium. Protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

Bioluminescence Resonance Energy Transfer
Saturation Assay

BRET2 saturation curves have been particularly used with the aim to establish the oligomeric order of receptor complex,
es, as well as the proportion of receptors engaged in
dimers or oligomers (BRETmax). In the current work, bio-
luminescence resonance energy transfer (BRET2) saturation
assays were carried out using plasmids encoding for D2R
and mGluR5 according to previously published methods
[9, 26, 35, 36]. The netBRET2 ratio was defined as the BRET
to co-expressed Rluc and GFP2 constructs normalized against the BRET ratio for the Rluc expression construct alone: netBRET2 ratio = [GFP2 emission at 515 ± 30 nm]/
(Rluc emission 410 ± 80 nm)-cf. The correction factor, cf, corresponds to (emission at 515 ± 30 nm)/(emission at 410 ± 80 nm) found with the receptor-Rluc construct expressed alone in the same experiment. The maximal value of BRET (netBRET50max) corresponds to the situation when all available donor molecules are paired up with acceptor molecules [8]. Also, saturation assay was used to compare the relative affinity of receptors for each other and their probability to form a complex, the so-called BRET50, which represents the acceptor/donor ratio giving 50% of the maximal signal. The ratio is calculated from fluorescence and bioluminescence values expressed as arbitrary units. BRET50 values should not be regarded as a common or classical value to expressed affinities as Molar units. Pairs with low BRET50 value thought to form oligomers or an increased tendency to dimerize, while high BRET50 values indicate weak interaction or the absence of interaction between the investigated receptors. The specificity of D2 R-Rluc-mGluR5, GFP2 interactions was assessed by comparison with co-expression of A1R-GFP2 and D2 R-Rluc.

**In Situ PLA in Cultured Cells**

In situ proximity ligation assay (PLA) in cultured cells was performed using the Duolink in situ PLA detection kit (Sigma-Aldrich, St. Louis, MO, USA), following the protocol described previously [11, 37, 38] using mouse monoclonal anti-D2 R (2 μg/ml, MABN53; Millipore, Billerica, MA, USA) and rabbit polyclonal anti-mGluR5 (2 μg/ml, Ab25675; Millipore) primary antibodies. PLA control experiments employed only one primary antibody. The PLA signal was visualized and quantified by using a TCS-SL confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) and the Duolink ImageTool software. High magnifications of the microphotographs were taken and visualized using multiple z-scan projections.

The background signal was estimated from both PLA control experiments and from PLA experiments performed on non-transfected HEK293T cells (HEK293T cell line expresses endogenously small amount of D2 R, A2A R and mGluR5). In general, the positive PLA values obtained in these experiments were residuals. The assay cut-off value was set to two standard deviations over the background signal. Therefore, samples with values below this cut-off were negative for the interaction of interest, while samples with values higher than the threshold were positive.

**Immunohistofluorescence and In Situ PLA in Mouse Brain**

Mice were anaesthetized and intracardially perfused with 50–200 ml of ice-cold 4% formaldehyde solution (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS; 1.47 mM KH2PO4, 8.07 mM Na2HPO4, 137 mM NaCl, 0.27 mM KCl with pH 7.2). The brains were post-fixed overnight in the same 4% formaldehyde solution at 4 °C. The vibratome (Leica Lasertechnik GmbH, Heidelberg, Germany) was used to make coronal section (50 μm). Slices were collected and kept in Walter’s antifreezing solution (30% glycerol, 30% ethylene glycol in PBS with pH 7.2) at −20 °C until further processing [39].

For immunohistofluorescence (IHF), experiments coronal brain slices were washed three times with PBS for 10 min at 22 °C, then permeabilized with 0.3% Triton X-100 in PBS (2 h at 22 °C) and rinsed (3×) with washing solution (PBS containing 0.05% Triton X-100, 10 min at 22 °C). Blocking of the slices was performed with washing solution containing 10% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 h at 22 °C. To avoid unspecific binding, the slices were incubated with secondary anti-mouse IgG (no. 715-005-150; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in washing solution (2 h at 22 °C). Then, the slices were incubated with mouse anti-mGluR5 monoclonal (20 μg/ml, MABN540; Millipore) and rabbit anti-D2 R polyclonal (1 μg/ml, D2 R-Rb-Af960; Frontier Institute Co. Ltd, Shinkonishi, Ishikari, Hokkaido, Japan) in washing solution with 5% NDS overnight at 4 °C. Subsequently, the slices were washed twice with a washing solution containing 1% NDS (10 min at 22 °C). Next, the slices were incubated with anti-Cy2 donkey anti-rabbit (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and anti-Cy3 donkey anti-mouse (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in washing solution with 1% NDS for 2 h at 22 °C. Finally, slices were washed two times with washing solution containing 1% NDS (10 min at 22 °C), two times with PBS (10 min at 22 °C) and then mounted with Duolink® in situ mounting medium with DAPI (Sigma-Aldrich). The Leica TCS 4D confocal scanning laser microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) was used to capture the fluorescence striatal images.

For in situ PLA in mouse brain, the Duolink in situ PLA detection kit (Sigma-Aldrich) was used as previously described [37, 39, 40]. Thus, the experimental procedure until the secondary antibody incubation step was the same as the IHF (see above). Subsequently, the following steps were performed according to the manufacturer’s protocol. Images were acquired and analysed as previously described [39]. The background signal was estimated from PLA control experiments, and the assay cut-off value was performed as described above.

**Radioligand Competition Binding Experiments**

For the binding experiments, membrane preparations (60 μg protein/ml) were obtained from HEK293T cells expressing
was determined by liquid scintillation spectrometry. forskolin were defined as basal and control, respectively. non-treated cells and non-transfected cells treated only with the POLARstar Optima plate reader (BMG Lab Technologies). Readings of luminescence intensity were performed using to the manufacturer’s specifications (Promega, Sweden). 

**cAMP Functional Assay**

Intracellular cAMP levels were determined using a cAMP-Glo™ assay detection kit (Promega, Madison, WI, USA). HEK293T cells expressing either D2R and mGluR5 or A2A R, D2R and mGluR5, or A2A R, D2R and mGluR5 were plated at a density of 10,000 cells/well in 96-well microtiter plates coated with poly-L-lysine (Sigma-Aldrich) and incubated overnight. Culture medium was then removed; cells were washed with 1 × PBS before the induction buffer (red phenol/serum-free DMEM containing 500 μM IBMX and 100 μM Ro 20-1724) was added. The cells were incubated for 1 h prior to drug incubation. To examine the G1 protein-mediated inhibition of adenylyl cyclase, the levels of cAMP were first raised with 5 μM forskolin for 10 min. Drug dilutions were prepared in the induction buffer, and the temperature- and carbon dioxide-equilibrated drug dilutions (37 °C cell culture incubator for 30 min) were added as indicated, and cells were then incubated at 37 °C for 30 min. The assay was performed accordingly to the manufacturer’s specifications (Promega, Sweden). Readings of luminescence intensity were performed using the POLARstar Optima plate reader (BMG Lab Technologies, Offenburg, Germany). cAMP levels in non-transfected, non-treated cells and non-transfected cells treated only with forskolin were defined as basal and control, respectively.

**Gel Electrophoresis and Immunoblotting**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE) was performed using 7% polyacrylamide gels. Proteins were transferred to Hybond-LPF polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Chicago, IL, USA) using the Trans-Blot Turbo™ transfer system (Bio-Rad, Hercules, CA, USA) at 200 mA/membrane for 30 min. PVDF membranes were blocked with 5% (wt/vol) dry non-fat milk in phosphate-buffered saline (PBS; 8.07 mM Na2HPO4, 1.47 mM KH2PO4, 137 mM NaCl, 0.27 mM KCl, pH 7.2) containing 0.05% Tween-20 (PBS-T) during 1 h at 20 °C before being immunoblotted with the indicated antibody in blocking solution overnight at 4 °C. PVDF membranes were washed with PBS-T three times (5 min each) before incubation with either a HRP-conjugated rabbit anti-mouse IgG (1/10,000) or HRP-conjugated goat anti-rabbit IgG (1/30,000) in blocking solution at 20 °C during 2 h. After washing the PVDF membranes with PBS-T three times (5 min each), the immunoreactive bands were developed using a chemiluminescent detection kit (Thermo Fisher Scientific) and detected with an Amersham Imager 600 (GE Healthcare Europe, Barcelona, Spain).

**Statistical Analysis**

The number of independent experiments (n) in each group is indicated in figure legends. Data are represented as mean ± standard error of mean (SEM). Outliers were assessed by the ROUT method [43]; thus, subjects were excluded assuming a Q-value of 1% in GraphPad Prism 9 (San Diego, CA, USA). Data normality was assessed by the Shapiro-Wilk normality test (p < 0.05). When two groups were evaluated, unpaired Student’s t-test or Mann-Whitney U-test was used. Comparisons among more than two experimental groups were performed by one-, two- or three-way factor analysis of variance (ANOVA) followed by either Dunnett’s, Šídák’s or Tukey post hoc test using GraphPad Prism 9, as indicated in the figure legends. A p-value ≤ 0.05 was considered significant.

**Results**

**BRET.2 Experiments: Transient Co-expression of A2AR with D2R and mGluR5 Had a Significant Impact on D2R-mGluR5 Heteroreceptor Complex Formation**

HEK293T cells were transiently transfected with constant amounts of D2R<sub>Rluc</sub> and increasing amounts of plasmids encoding for mGluR5<sub>GFP2</sub> with/without transient co-expression of A2AR. The transient co-expression of A2AR with D2R<sub>Rluc</sub> and mGluR5<sub>GFP2</sub> had a significant impact on D2R<sub>Rluc</sub>-mGluR5<sub>GFP2</sub> heteroreceptor complex formation (Fig. 1A). Transient co-expression of A2AR promoted a significant increase of netBRET<sup>2</sup>max ratio value (0.084 ± 0.003 AU) compared to that found in cells without transient co-expression of A2AR (0.043 ± 0.002 AU) (Fig. 1B). When the A2AR was coexpressed with D2R<sub>Rluc</sub> and mGluR5<sub>GFP2</sub>, these receptors hence showed an increased ability to heteromerize.

Also, saturation assay was used to compare the relative affinity of receptors for each other and their probability to form a complex, the so-called RET50, which represents the acceptor/donor ratio giving 50% of the maximal signal. The netBRET<sup>2</sup>50 ratio value for D2R<sub>Rluc</sub>-mGluR5<sub>GFP2</sub> heteromerization was significantly reduced by transient co-expression of A2AR from (1.58 ± 0.09 AU) to (0.94 ± 0.11 AU) (Fig. 1C) indicating increased affinity of the two
receptor protomers for each other. Pairs with low BRET$_{50}$ value thought to form oligomers or an increased tendency to dimerize, while high BRET$_{50}$ values indicate weak interaction or the absence of interaction between the investigated receptors.

**Proximity Ligation Assay Experiments: Transient Co-expression of A$_{2A}$R Promoted the Formation D$_2$R-mGluR$_5$ Heteroreceptor Complexes in HEK Cells**

The role of A$_{2A}$R in the dynamics of the D$_2$R-mGluR$_5$ heteromers was also evaluated by in situ proximity ligation assays (PLA) in transiently co-transfected HEK293T cells. The PLA results were in line with the results from the BRET$_2$ assays. The in situ PLA demonstrated the existence of D$_2$R-mGluR$_5$ heteroreceptor complexes in cells to a low degree without transient co-expression of A$_{2A}$R (Fig. 2A). Furthermore, the transient co-expression of A$_{2A}$R highly significantly promoted the formation of D$_2$R-mGluR$_5$ heteroreceptor complexes as shown by the marked increase in the number of PLA-positive D$_2$R-mGluR$_5$ complexes, while this was significantly reduced in HEK293T cells without co-expressing A$_{2A}$R (Fig. 2B and D). Few and weak PLA clusters were detected in the PLA-negative controls (lack of D$_2$R antibodies) representing background labelling (Fig. 2C).

The specificity of the PLA-positive D$_2$R-mGluR$_5$ complexes, shown as red blobs in the mouse dorsal striatum (Fig. 3A), was demonstrated using D$_2$R$^{-/-}$ mice (Fig. 3C).

![Fig. 1](image1.png) Effect of A$_{2A}$R expression in D$_2$R-mGluR$_5$ heteromer formation assessed by BRET$_2$ assay in HEK293T cells. Cells were transiently transfected with plasmids encoding the D$_2$R tagged with Rluc (i.e. D$_2$R$^{Rluc}$) and mGluR$_5$ with GFP2 (i.e. mGluR$_5^{GFP2}$) in the absence (blue squares) or presence (red circles) of A$_{2A}$R expression. The A$_{1R}^{GFP2}$-D$_2$R$^{Rluc}$ pair was used as a control (grey triangles). BRET$_2$ saturation curves (A) were constructed by co-transfecting a constant amount of the plasmid for D$_2$R$^{Rluc}$ and increasing amounts of the mGluR$_5^{GFP2}$ plasmid or A$_{2A}$R$^{GFP2}$ plasmid. Curves are based on mean values of four independent experiments performed in quadruplicates. The netBRET$_2$$_{max}$ (B) and netBRET$_2$$_{50}$ (C) values from the BRET$_2$ saturation curves shown in A are represented. BRET ratio is calculated from fluorescence and bioluminescence values expressed as arbitrary units. Results are expressed as mean±SEM (n=4, each determination performed in quadruplicates). ****p<0.0001 and **p<0.01, Student’s t-test

![Fig. 2](image2.png) In situ PLA assessment of D$_2$R-mGluR$_5$ heteromer formation in the absence (A) or presence (B) of A$_{2A}$R (see “Methods”). The in situ PLA-positive D$_2$R-mGluR$_5$ heteroreceptor complexes were shown as red blobs (arrows) and nuclei in blue (DAPI staining). A negative in situ PLA control (C) was included by incubating the cells in the absence of the primary anti-D$_2$R antibody. D Quantification of D$_2$R-mGluR$_5$ complexes. The number of PLA blobs (red clusters) per positive cell (n=4×50 cells) was assessed as described in Methods. Results were expressed as mean±SEM (n=4 independent experiments). ****p<0.0001 and **p<0.01, Student’s t-test
In the sections from the mouse striatum, the appearance of the red PLA-positive D2R-GluR5 complexes, shown as mean number of red blobs/Nucleus, was markedly and highly significantly reduced (Fig. 3D). Furthermore, the loss of the red D2R-mGluR5 blobs to the same high degree in the A2AR−/− mice (Fig. 3B, D) likely reflects the requirement of D2R-mGluR5 heterocomplexes to be part of an A2AR-D2R-mGluR5 to be expressed in the mouse striatum, probably by dorsal striatal-pallidal GABAergic neurons. In this way, it forms D2R-mGluR5 complexes that are close enough to be visualized by PLA.

**[3H]-Raclopride/Quinpirole Competition Experiments: the A2AR and mGluR5 Protomers Interact and Modulate D2R Protomer Recognition**

In HEK293T cells expressing D2R and mGluR5, the mGluR5 agonist CHPG (500 nM) reduced the affinity of the high-affinity state (K_i,High) of the D2R for the agonist quinpirole with no effects on its low-affinity state (K_i,Low). Co-treatment with A2AR agonist CGS-21680 (100 nM) did not significantly alter the D2R K_i,High and K_i,Low values obtained when the cells were treated only with CHPG (500 nM) (Fig. 4A and Table 1). In HEK293T cells expressing A2AR, D2R and mGluR5, mGluR5 agonist stimulation also reduced the affinity of the high-affinity state (K_i,High) of the D2R for the agonist quinpirole with no statistically significant effects on its low-affinity state (K_i,Low) (Fig. 4A and Table 2). However, the transient co-expression of A2AR by itself (without agonist stimulation) potentiates mGluR5 agonist effects on the high-affinity D2R agonist binding sites (Fig. 4B, Tables 1 and 2). Finally, the co-stimulation of A2AR and mGluR5 synergistically increased in the K_i,High values of the D2R protomer upon co-expression of the A2AR (Table 2). Nevertheless, in cells expressing A2AR, D2R and mGluR5, further analysis should be performed to test the effect of combination treatment of A2AR (ZM-241385) and mGluR5 (CHPG) to figure out if the expression of A2AR, without agonist stimulation and its corresponding constitutive activity, is responsible for increased in the K_i,High values of the D2R protomer upon co-expression of the A2AR.

In both HEK293T cells expressing D2R and mGluR5 or A2AR, D2R and mGluR5, the incubation with A2AR antagonist ZM-241385 (1 μM) and mGluR5 antagonist MPEP (300 μM) alone or in combination resulted in an almost complete blockade of the mGluR5 increase of the D2R K_i,High values and A2AR agonist-induced increase of mGluR5 agonist effects on the high-affinity D2R agonist binding sites (Tables 1 and 2).

**cAMP Functional Experiments: the A2AR and mGluR5 Protomers Interact and Modulate D2R Protomer Signalling**

In cells expressing D2R and mGluR5 forming D2R-mGluR5 heterocomplexes (Fig. 2), the D2R agonist activation with quinpirole (100 nM) induced a G_i protein-mediated inhibition of adenylyl cyclase that first was raised with 5 μM forskolin (Fig. 5A). This effect was highly significantly blocked by the D2R antagonist raclopride (1 μM). In these cells, the mGluR5 agonist CHPG stimulation significantly counteracted the D2R agonist-induced reduction of cAMP accumulation (Fig. 5A). The significant effect of CHPG (500 nM) was significantly reduced by the mGluR5 antagonist MPEP (300 μM). The co-treatment with the A2AR agonist did not enhance the counteraction of the inhibitory D2R signalling by CHPG (Fig. 5A).

Likewise, quinpirole significantly reduced the cAMP level in cells expressing A2AR, D2R and mGluR5 (Fig. 5B). The mGluR5 agonist CHPG had an improved ability to counteract the adenylyl cyclase inhibition produced by the D2R agonist in these cells, yielding cAMP levels similar to those obtained after blocking D2R signalling with raclopride (Fig. 5B). Upon A2AR and mGluR5 agonist co-activation, a larger counteraction of the D2R agonist action was found compared to that obtained with such a co-treatment performed in cells expressing only D2R.
induced decrease of cAMP accumulation (Fig. 5B). Such effects followed by the Tukey post hoc test. Data are means ± SEM; n=4, each determination performed in triplicate. Statistical analysis was performed by one-way ANOVA followed by the Tukey post hoc test. §§§(p<0.01); significant increased compared to vehicle. §§(p<0.001); significant increased compared to cells incubated with CHPG. †††(p<0.001); significant reduced compared to cells incubated with CHPG and CGS-21680

Table 1 Values for quinpirole binding site affinities to the D2-likeR by [3H]-raclopride/quinpirole competition assays in HEK293T cells transiently expressing D2R and mGluR5 incubated with agonist(s) or antagonist(s) as indicated

|                 | K_i,High (nM) | K_i,Low (nM) |
|-----------------|---------------|--------------|
| + Vehicle       | 1.1 ± 0.4     | 155 ± 73     |
| + CHPG          | 83 ± 7***     | 166 ± 63     |
| + CHPG + CGS-21680 | 99 ± 9*** | 283 ± 179     |
| + CHPG + MPEP   | 5.6 ± 2***    | 40 ± 18      |
| + CHPG + MPEP + CGS-21680 | 16 ± 1*** | 63 ± 19       |
| + CGS-21680     | 4.3 ± 2.2     | 127 ± 62     |
| + CGS-21680 + ZM-241385 | 5.7 ± 0.9 | 21 ± 62       |

K_i,High, D2R high-affinity value and K_i,Low, D2R low-affinity value. Data are means ± SEM; n=4, each determination performed at least intriplicate. Statistical analysis was performed by one-way ANOVA followed by the Tukey post hoc test. ***(p<0.001); significant increased compared to vehicle. §§§(p<0.01); significant increased compared to cells incubated with CHPG. †††(p<0.001); significant reduced compared to cells incubated with CHPG and CGS-21680

Table 2 Values for quinpirole binding site affinities to the D2-likeR by [3H]-raclopride/quinpirole competition assays in HEK293T cells transiently expressing A2AR, D2R and mGluR5 incubated with agonist(s) or antagonist(s) as indicated

|                 | K_i,High (nM) | K_i,Low (nM) |
|-----------------|---------------|--------------|
| + Vehicle       | 1.6 ± 1       | 45 ± 18      |
| + CHPG          | 411 ± 46***   | 106 ± 41     |
| + CHPG + CGS-21680 | 1781 ± 72*** | 49 ± 14      |
| + CHPG + MPEP   | 3.4 ± 1.7***  | 19 ± 6       |
| + CHPG + MPEP + CGS-21680 + ZM-241385 | 43 ± 10††† | 158 ± 65     |
| + CGS-21680     | 743 ± 53***   | 115 ± 23     |
| + CGS-21680 + ZM-241385 | 13 ± 4  | 52 ± 12      |

K_i,High, D2R high-affinity value and K_i,Low, D2R low-affinity value. Data are means ± SEM; n=4, each determination performed at least in triplicate. Statistical analysis was performed by one-way ANOVA followed by the Tukey post hoc test. ***(p<0.001); significant increased compared to vehicle. §§§(p<0.01); significant increased compared to cells incubated with CHPG. †††(p<0.001); significant reduced compared to cells incubated with CHPG and CGS-21680

A 2A R agonist produces similar increases in cAMP levels as found after the A2AR agonist GGS in HEK293T cells co-expressing D2R, A2AR and mGluR5 (Fig. 5C). Therefore, we should consider also that mGluR5 might simply activate Gs, inducing cAMP accumulation, independently of D2-Gi-induced inhibition of adenylate cyclase (Fig. 5 A−C).
Catalepsy is a nervous condition characterized by loss of muscle control and fixity of posture. It is considered a symptom of certain nervous disorders such as Parkinson’s diseases and epilepsy [44]. It is also a characteristic symptom of cocaine withdrawal, as well as one of the features of catatonia. The catalepsy is mainly produced by haloperidol-induced blockade of D2R complexes in the dorsal striatal-pallidal GABA neurons within the dorsal striatum [44–46]. These GABA neurons mediate motor inhibition, counteracted by D2R agonist-induced activation of the D2R homo- and heterocomplexes like the D2R-A2AR or the D2R-mGluR5 heterocomplexes [24, 47–50]. The D2R activation of the dorsal striatal-pallidal GABA neurons is also essential for maintenance of normal locomotor activity.

The catalepsy induced by the D2R antagonist haloperidol was evaluated in 10-min time intervals from 60 to 90 min after the injection of haloperidol (Fig. 6). In wild-type mice, the mGluR5-negative allosteric modulator raseglurant produced in this time period a significant reduction of the catalepsy time which was in the order of 25% (Fig. 6). In contrast, such a reduction of catalepsy was not observed by raseglurant treatment of A2AR−/− mice. Furthermore, in vehicle-treated A2AR−/− animals, the injection of haloperidol (Fig. 6). In wild-type mice, the catalepsy induced by the D2R antagonist haloperidol was evaluated in 10-min time intervals from 60 to 90 min after the injection of haloperidol (Fig. 6). In wild-type mice, the mGluR5-negative allosteric modulator raseglurant produced in this time period a significant reduction of the catalepsy time which was in the order of 25% (Fig. 6). In contrast, such a reduction of catalepsy was not observed by raseglurant treatment of A2AR−/− mice. Furthermore, in vehicle-treated A2AR−/− animals,
D2 R is a hub receptor [51] which interacts not only with dorsal striatum [4, 16, 40]. The results indicate that the tor complexes in subcortical limbic areas as well as the many other GPCRs including dopamine isoreceptors D2 R-dependent behaviour in mice. Raseglurant reverses haloperidol (see Methods). Wild-type (circles), was measured as the time spent with both front paws resting on the idol-induced catalepsy. Haloperidol-induced cataleptic behaviour could be related to the development of an increased affin-

Discussion

The field of dopamine D2Rs changed markedly with the discovery of many types of D2R homo- and heteroreceptor complexes in subcortical limbic areas as well as the dorsal striatum [4, 16, 40]. The results indicate that the D2R is a hub receptor [51] which interacts not only with many other GPCRs including dopamine isoreceptors but also with ion-channel receptors, receptor tyrosine kinases, scaffolding proteins and dopamine transporters [24, 52, 53]. Disturbances in several of these D2R heteroreceptor complexes may contribute to the development of brain disorders through changes in the balance of diverse D2R homo- and heteroreceptor complexes mediating the dopamine signal, especially to the ventral striato-pallidal GABA pathway [37, 52, 54]. Of high relevance was the discovery of A2A R-D2R and A2A R-mGluR5 heteroreceptor complexes in native tissue [4, 16, 40, 55, 56]. Furthermore, the existence of the D2R-mGluR5 heterodimers in the biomembranes of living cells was demonstrated by bimolecular fluorescence complementation experiments in cellular models [21]. Although when tested by FRET microscopy in tsA 201 cells, D2R did not associate with mGluR5 [57]. Nevertheless, by combination of bimolecular fluorescence complementation and bioluminescence resonance energy transfer techniques, as well as the sequential resonance energy transfer technique, the occurrence of an A2A R-D2R-mGluR5 heteroreceptor complexes was observed in living cells. Furthermore, by co-immunoprecipitation, experiments validated the existence of an association of mGluR5, D2R and A2A R in rat striatum homogenates [21].

Herein, we present new findings that further expand the understanding of A2A R-D2R-mGluR5 heteroreceptor complexes. Also, strong evidences which support that the expression of the A2A R is necessary to facilitate the association of D2R and mGluR5 in a complex.

Our new findings are that transient co-expression of A2A R in HEK293T cells together with D2 R Rluc and mGluR5 GFP resulted in a significant and marked increase in the formation of the D2R-mGluR5 heterodimer, a component of the A2A R-D2R-mGluR5 hetero complex, based on the increase in the BRET2 max values. Such an increase could be related to the development of an increased affinity of the two D2R and mGluR5 protomers for each other due to allosteric changes related to the formation of the A2A R-D2R-mGluR5 complex. In line with this hypothesis, the BRET2 values were significantly reduced for the D2R-mGluR5 heteromeric component of this trimeric heteroreceptor complex.

These results are also supported by the demonstration with PLA that an increased density of PLA-positive D2R-mGluR5 clusters was observed when A2A R expression had been added to the cells compared to cells only expressing D2R and mGluR5. In agreement, in the mouse dorsal striatum, the D2R-mGluR5 complexes were significantly reduced in the A2A R−/− mice. Thus, it becomes clear that the expression of the A2A R in the mouse dorsal striatum is necessary to facilitate that the D2R and mGluR5 form a complex. It underlines that the multiple receptor protomers in the high-order heteroreceptor complexes are dependent
on each other to improve or facilitate the formation of such complexes in the dorsal striatum.

The different results obtained on haloperidol-induced catalepsy in wild-type mice vs A2A R−/− mice are of substantial interest since they can indicate a functional role of the A2A R-D2R-mGluR5 heteroreceptor complexes in the dorsal striatum as previously discussed [8, 17]. There was a marked reduction in the haloperidol-induced catalepsy in the A2A R−/− mice compared to wild-type mice. Thus, in the absence of the A2A R, the D2R antagonist haloperidol appears to have a substantially reduced potency to block the D2R which can be caused by the loss of the antagonistic A2A R-D2R interaction [9, 58]. According to the current findings in cell lines, the D2R-mGluR5 heterocomplexes should be also formed to a much lower degree in the absence of A2A R in view of their dependency of A2A R according to the PLA experiments performed. The counteraction of the D2R-mediated inhibitory actions on cAMP signalling by CHPG, a mGluR5 agonist, was in our cell line also more effective in cells co-expressing besides D2R and mGluR5, also A2A R.

It seems likely that the formation of the A2A R-D2R-mGluR5 complex enhances the affinity of the D2R and mGluR5 protomers for each other in this complex. It is of high interest that the biochemical binding experiments reveal that the mGluR5 CHPG agonist-induced increase in D2R K_i,High values becomes significantly higher in the A2A R-D2R-mGluR5 complex compared to the D2R-mGluR5 complex despite the absence of A2A R agonist exposure. Thus, although agonist activation of the A2A R seems necessary to exert negative allosteric modulation of the D2R protomer agonist binding via heteroreceptor complexes, an increased constitutive activity of the A2A R protomer could explain the above results.

As expected, the combined incubation with CHPG and CGS-21680 led to an even stronger increase in the D2R K_i,High values of the A2A R-D2R-mGluR5 complex, demonstrating the impact of the A2A R protomer on the D2R-mGluR5 allosteric interactions, which can involve both constitutive and A2A R agonist-induced inhibition of D2R agonist binding. Our findings represent one of the first examples of integrative activity within a higher-order heteroreceptor complex and show how one receptor (A2A R) can substantially modulate the structure and recognition of a participating receptor heterodimer (D2R-mGluR5) in such a trimeric receptor complex.

The pharmacological analysis of the A2A R-D2R-mGluR5 complex and its impact on cAMP levels indicated that the A2A R can modulate the effects of the D2R-mGluR5 interactions on cAMP signalling. It was found that when the A2A R-D2R-mGluR5 complex was likely to be formed through the expression also of the A2A R, the mGluR5 agonist had an increased ability to counteract the D2 agonist-induced G_{i/o}-mediated inhibition of the cAMP levels in comparison with the counteraction observed in the absence of A2A R expression. The same was also true for the combined treatment with the mGluR5 agonist CHPG and the A2A R agonist CGS-21680 when the A2A R was coexpressed. A stronger counteraction of the D2R-induced inhibition of the cAMP levels was observed when A2A R expression was present.

Taken together, our work on cell lines gives strong indications that, in the A2A R-D2R-mGluR5 complex, the A2A R protomer enhances the formation of the D2R-mGluR5 component of the complex with enhanced inhibition of D2R agonist binding recognition and its G_{i/o}-mediated cAMP signalling. The inhibitory effects by A2A R and mGluR5 on D2R recognition and signalling reveal a significant molecular integration in A2A R-D2R-mGluR5 complexes, likely formed also in the dorsal striatum. The A2A R and mGluR5 antagonists targeting the A2A R-D2R-mGluR5 complexes in dorsal striatum may reduce the haloperidol-induced catalepsy by removal of the A2A R and mGluR5 protomer-mediated allosteric inhibition of the D2R protomer. Understanding of the trimeric complexes formed by these GPCRs could provide novel strategies for development of drugs against neuropsychiatric and neurodegenerative diseases by targeting their antagonistic receptor-receptor interactions.

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**Author Contribution** We confirm and declare that all authors meet the criteria for authorship according to the ICMJE, including approval of the final manuscript, and they take public responsibility for the work and have full confidence in the accuracy and integrity of the work of other group authors. They have substantially contributed to the conception or design of the work. Also, they have participated in the acquisition, analysis and interpretation of data for the current version. They have also helped revising it critically for important intellectual content and final approval of the version to be published. In addition, they have contributed to this last version of the manuscript in writing assistance, technical editing and language editing. Conceptualization, Francisco Ciruela Alférez, Kjell Fuxe and Dasiel O. Borroto-Escuela; formal analysis, Wilber Romero-Fernandez, Jaume J. Taura, René A. J Crans, Marc Lopez-Cano, Ramon Fores-Pons, Manuel Narváez, Jens Carlsson, Francisco Ciruela Alférez, Kjell Fuxe and Dasiel O. Borroto-Escuela; funding acquisition, Francisco Ciruela Alférez, Kjell Fuxe and Dasiel O. Borroto-Escuela; investigation, Wilber Romero-Fernandez, Jaume J. Taura, René A. J Crans, Marc Lopez-Cano, Ramon Fores-Pons, Manuel Narváez, Jens Carlsson, Francisco Ciruela Alférez, Kjell Fuxe and Dasiel O. Borroto-Escuela; methodology, Wilber Romero-Fernandez, Jaume J. Taura, René A. J Crans, Marc Lopez-Cano, Ramon Fores-Pons, Manuel Narváez, Jens Carlsson, Francisco Ciruela Alférez, Kjell Fuxe and Dasiel O. Borroto-Escuela; project administration, Francisco Ciruela Alférez, Kjell Fuxe and Dasiel O. Borroto-Escuela; supervision, Francisco Ciruela Alférez, Kjell Fuxe and Dasiel O. Borroto-Escuela; visualization, Wilber Romero-Fernandez, Jaume J. Taura, René A. J Crans, Marc Lopez-Cano, Ramon Fores-Pons, Manuel
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**Data Availability** The datasets generated during and/or analysed during the current study are available (upon request) in the Fuxe Lab repository at the Department of Neuroscience, Karolinska Institutet (contact email: Kjell.Fuxe@ki.se).

**Declarations**

**Ethics Approval** This study was performed in line with the principles of the Declaration of Helsinki. The animal protocol (no. 7085) was approved by the University of Barcelona Committee on Animal Use and Care. Animals were housed and tested in compliance with the guidelines provided by the Guide for the Care and Use of Laboratory Animals [33] and following the European Union directives (2010/63/EU), the ARRIVE guidelines [34].

**Consent to Participate** Not applicable. The current research work does not involve human subjects.

**Consent for Publication** Not applicable. This manuscript does not contain individual personal’s data in any form.

**Conflict of Interest** The authors declare no competing interests.

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