The mGlu$_5$ Receptor Protomer-Mediated Dopamine D$_2$ Receptor Trans-Inhibition Is Dependent on the Adenosine A$_{2A}$ Receptor Protomer: Implications for Parkinson's Disease

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Abstract

The adenosine A$_{2A}$ receptor (A$_{2A}$R), dopamine D$_2$ receptor (D$_2$R) and metabotropic glutamate receptor type 5 (mGluR$_5$) form A$_{2A}$R-D$_2$R-mGluR$_5$ heteroreceptor complexes in living cells and in rat striatal neurons. In the current study, we present experimental data supporting the view that the A$_{2A}$R protomer plays a major role in the inhibitory modulation of the density and the allosteric receptor-receptor interaction within the D$_2$R-mGluR$_5$ heteromeric component of the A$_{2A}$R-D$_2$R-mGluR$_5$ complex in vitro and in vivo. The A$_{2A}$R and mGluR$_5$ protomers interact and modulate D$_2$R protomer recognition and signalling upon forming a trimeric complex from these receptors. Expression of A$_{2A}$R in HEK293T cells co-expressing D$_2$R and mGluR$_5$ resulted in a significant and marked increase in the formation of the D$_2$R-mGluR$_5$ heteromeric component in both bioluminescence resonance energy transfer and proximity ligation assays. A highly significant increase of the the high-affinity component of D$_2$R (D2R$_{Ki High}$) values was found upon cotreatment with the mGluR$_5$ and A$_{2A}$R agonists in the cells expressing A$_{2A}$R, D$_2$R and mGluR$_5$ with a significant effect observed also with the mGluR$_5$ agonist alone compared to cells expressing only D$_2$R and mGluR$_5$. In cells co-expressing A$_{2A}$R, D$_2$R and mGluR$_5$, stimulation of the cells with an mGluR$_5$ agonist like or D$_2$R antagonist fully counteracted the D$_2$R agonist-induced inhibition of the cAMP levels which was not true in cells only expressing mGluR$_5$ and D$_2$R. In agreement, the mGluR$_5$-negative allosteric modulator raseglurant significantly reduced the haloperidol-induced catalepsy in mice, and in A$_{2A}$R knockout mice, the haloperidol action had almost disappeared, supporting a functional role for mGluR$_5$ and A$_{2A}$R in enhancing D$_2$R blockade resulting in catalepsy. The results represent a relevant example of integrative activity within higher-order heteroreceptor complexes.

Keywords Adenosine A$_{2A}$ receptor · Dopamine D$_2$ receptor · Metabotropic glutamate receptor 5 · Heteroreceptor complex · Receptor-receptor interaction · Allosteric modulation

Introduction

The first pieces of evidence for antagonistic glutamate receptor with dopamine D$_2$ receptor (D$_2$R) interactions were found in 1983–1984 through the ability of glutamate to reduce the affinity of the high-affinity D$_2$R agonist binding sites in striatal membrane preparations. Subsequently, it was observed that mGluR$_5$ agonists alone or combined with an A$_{2A}$R agonist (CGS-21680) can reduce the affinity of the high-affinity state of D$_2$R for agonist binding sites in the rat striatum [1]. Co-immunoprecipitation experiments also indicated the existence of A$_{2A}$R-mGluR$_5$ 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

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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_2R form heteroreceptor complexes with antagonistic receptor-receptor interactions on the plasma membrane [9–16].

The existence of $A_{2A}R$-D_2R-$mGluR_5$ 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_2R-$mGluR_5$ 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} 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_2R-$mGluR_5$ 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_2R 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_2R-$mGluR_5$ heteroreceptor complexes are presented. Results in cellular models first demonstrated that $A_{2A}R$ promotes the D_2R and $mGluR_5$ receptor-receptor interactions, and its participation increases the density of the D_2R-$mGluR_5$ heterocomplexes. Binding and functional experiments indicated that $A_{2A}R$ and $mGluR_5$ upon agonist activation play a significant role in modulating the composition, density and signalling of $A_{2A}R$-D_2R-$mGluR_5$ heteroreceptor complexes. This was also observed in $A_{2A}R$ or D_2R 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.

The D_2R^{Rluc} construct used has been described previously in Borroto-Escuela et al. 2010 [25].

Drugs and Chemicals

The $A_{2A}R$ agonist 4-[2-[[6-Amino-9-(N-ethyl-β-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_2R antagonist 4-[4-(4-Chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone 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 μM) 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 (1×10^6 cells/well), 96-well plates (1×10^4 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 $D_2R^{-/-}$ 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

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]. Mice were housed in groups of five in standard cages with access to food and water ad libitum while maintained under a 12-h dark/light cycle (starting at 7:30 AM), 22 °C temperature and 66% humidity (standard conditions). All animal experimentation was carried out in a period comprehended between 9:00 AM and 6:00 PM by a researcher blind to drug treatments.

Locomotor Activity Tests

Mice spontaneous or drug-induced locomotor activity was assessed by the open field test. In brief, animals were administered intraperitoneal (i.p.) with raseglurant (1 mg/kg) or vehicle-saline with 5% DMSO and 5% Tween 20 30 min before the testing session. Non-habituated mice were placed in the centre of an activity field arena (30×30 cm, surrounded by four 50-cm-high black-painted walls) equipped with a camera above to record activity and connected to the light source. The total distance travelled was analysed using SPOT tracker function from ImageJ (NIH, Bethesda, MD, USA), as previously described [30].

Catalepsy Test

Mouse catalepsy was induced by the administration (i.p.) of haloperidol (1 mg/kg) [30]. After 1 h, haloperidol-induced catalepsy was measured as the duration in seconds of an abnormal upright posture in which the forepaws of the mouse were placed on a horizontal wooden bar (0.6 cm of diameter) that was located 4.5 cm above the floor. Subsequently, mice were administered (i.p.) with either vehicle (i.e. saline with 5% DMSO and 5% Tween) or raseglurant (1 mg/kg). After 20 min, a second haloperidol-induced catalepsy measurement was performed.

The rationale for the use of raseglurant (a mGluR5-negative allosteric modulator) instead of a full antagonist was based on the theoretical advantages that allosteric modulators offer compared with their competitive counterparts. mGluR5 allosteric modulators (negative allosteric modulators (NAM) and positive allosteric modulators (PAM)) have the potential for greater subtype selectivity when compared to orthosteric ligands. Also, mGluR5 NAM and PAM do not possess intrinsic activity and are assumed to be quiescent in the absence of an endogenous agonist and only modulate receptor function when the endogenous agonist is present. In this manner, NAM and PAM have the potential to retain spatial and temporal aspects of endogenous receptor signalling. This is of particular interest for CNS targets where optimal neurotransmission is likely to have an improved therapeutic outcome as opposed to sustained receptor blockade or activation.

Haloperidol-Induced Catalepsy

Mice ($n = 10$) were randomly assigned to treatment groups, and behavioural testing was performed blind to treatment. The dopamine D_2 receptor (D_2R) 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 catalepsy 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 $1000 \times g$. The resulting supernatant was centrifuged for 30 min at $12{,}000 \times g$. The membranes were dispersed in 50 mM Tris HCl (pH 7.4) and 10 mM $MgCl_2$, 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

$BRET^2$ saturation curves have been particularly used with the aim to establish the oligomeric order of receptor complexes, as well as the proportion of receptors engaged in dimers or oligomers (BRETmax). In the current work, bioluminescence resonance energy transfer ($BRET^2$) saturation assays were carried out using plasmids encoding for D_2R^{Rluc} and $mGluR_5^{GFP2}$ according to previously published methods [9, 26, 35, 36]. The netBRET^2 ratio was defined as the BRET ratio for co-expressed Rluc and GFP^2 constructs normalized against the BRET ratio for the Rluc expression construct alone: netBRET^2 ratio = [(GFP^2 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 (netBRET^{2}max) 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 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. The specificity of D_2R^{Rluc}-mGluR$_5^{GFP2}$ interactions was assessed by comparison with co-expression of A_1R^{GFP2} and D_2R^{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-D$_2$R (2 µg/ml, MABN53; Millipore, Billerica, MA, USA) and rabbit polyclonal anti-mGluR$_5$ (2 µg/ml, AB5675; 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 microphotograph 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 D$_2$R, A$_{2A}$R and mGluR$_5$). 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 KH$_2$PO$_4$, 8.07 mM Na$_2$HPO$_4$, 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-mGluR$_5$ monoclonal (20 µg/ml, MABN540; Millipore) and rabbit anti-D$_2$R polyclonal (1 µg/ml, D$_2$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

either D_2R and $mGluR_5$ or $A_{2A}R$, D_2R and $mGluR_5$, and [^{3}H]-raclopride (Novandi Chemistry AB, Södertälje, Sweden) competition assays with minor modifications were performed according to previously published methods [26, 27, 41]. [^{3}H]-raclopride (75 Ci/mmol), a D_2-like receptor antagonist competing [42] with quinpirole for binding to D_2-like receptors in HEK293T membrane preparations, was used to determine the D_2R high-affinity ($K_{i, High}$) and D_2R low-affinity ($K_{i, Low}$) values. (+)-Butaclamol, a selective D2R antagonist (100 µM, Sigma-Aldrich), was used to determine the non-specific binding. The amount of bound [^{3}H]-raclopride was determined by liquid scintillation spectrometry.

cAMP Functional Assay

Intracellular cAMP levels were determined using a cAMP-Glo™ assay detection kit (Promega, Madison, WI, USA). HEK293T cells expressing either D_2R and $mGluR_5$ or $A_{2A}R$, D_2R and $mGluR_5$ 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 $1 \times$ 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 G_i 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-LFP 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 Na_2HPO_4, 1.47 mM KH_2PO_4, 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 $A_{2A}R$ with D_2R and $mGluR_5$ Had a Significant Impact on D_2R-$mGluR_5$ Heteroreceptor Complex Formation

HEK293T cells were transiently transfected with constant amounts of D_2R^{Rluc} and increasing amounts of plasmids encoding for $mGluR_5^{GFP2}$ with/without transient co-expression of $A_{2A}R$. The transient co-expression of $A_{2A}R$ with D_2R^{Rluc} and $mGluR_5^{GFP2}$ had a significant impact on D_2R^{Rluc}-$mGluR_5^{GFP2}$ heteroreceptor complex formation (Fig. 1A). Transient co-expression of $A_{2A}R$ promoted a significant increase of netBRET^{2}max ratio value (0.084 ± 0.003 AU) compared to that found in cells without transient co-expression of $A_{2A}R$ (0.043 ± 0.002 AU) (Fig. 1B). When the $A_{2A}R$ was coexpressed with D_2R^{Rluc} and $mGluR_5^{GFP2}$, 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 BRET50, which represents the acceptor/donor ratio giving 50% of the maximal signal. The netBRET^{2}50 ratio value for D_2R^{Rluc}-$mGluR_5^{GFP2}$ heteromerization was significantly reduced by transient co-expression of $A_{2A}R$ from (1.58 ± 0.09 AU) to (0.94 ± 0.11 AU) (Fig. 1C) indicating increased affinity of the two
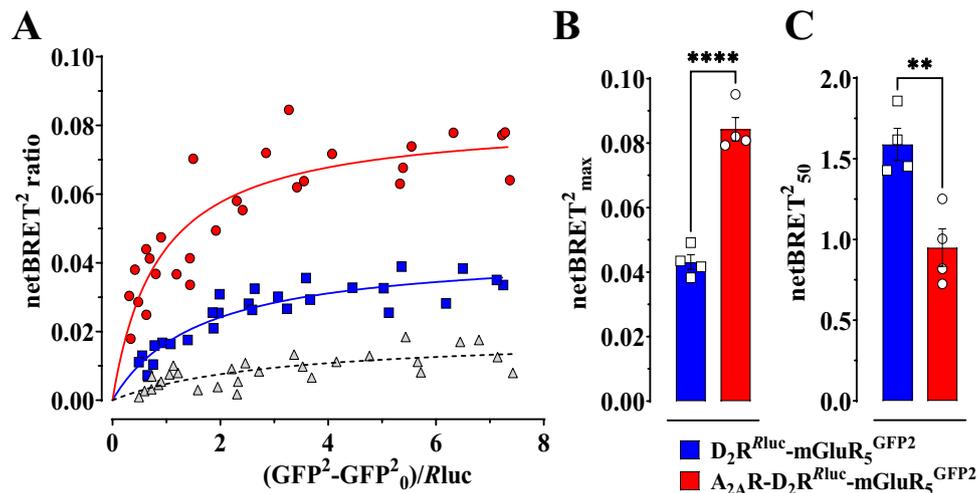
Fig. 1 Effect of $A_{2A}R$ expression in D_2R-mGluR$_5$ heteromer formation assessed by BRET^2 assay in HEK293T cells. Cells were transiently transfected with plasmids encoding the D_2R tagged with Rluc (i.e. D_2R^{Rluc}) and mGluR$_5$ with GFP2 (i.e. mGluR$_5^{GFP2}$) in the absence (blue squares) or presence (red circles) of $A_{2A}R$ expression. The A_1R^{GFP2}–D_2R^{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_2R^{Rluc} and increasing amounts of the mGluR$_5^{GFP2}$ plasmid or A_1R^{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 $\pm$ SEM ($n=4$, each determination performed in quadruplicates). ****$p<0.0001$ and **$p<0.01$, Student's t-test

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_2R-mGluR$_5$ Heteroreceptor Complexes in HEK Cells

The role of $A_{2A}R$ in the dynamics of the D_2R-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_2R-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 D_2R-mGluR$_5$ heteroreceptor complexes as shown by the marked increase in the number of PLA-positive D_2R-mGluR$_5$ complexes, while this was significantly reduced in HEK293T cells without co-expressing $A_{2A}R$ (Fig. 2 B and D). Few and weak PLA clusters were detected in the PLA-negative controls (lack of D_2R antibodies) representing background labelling (Fig. 2C).

The specificity of the PLA-positive D_2R-mGluR$_5$ complexes, shown as red blobs in the mouse dorsal striatum (Fig. 3A), was demonstrated using $D_2R^{-/-}$ mice (Fig. 3C).
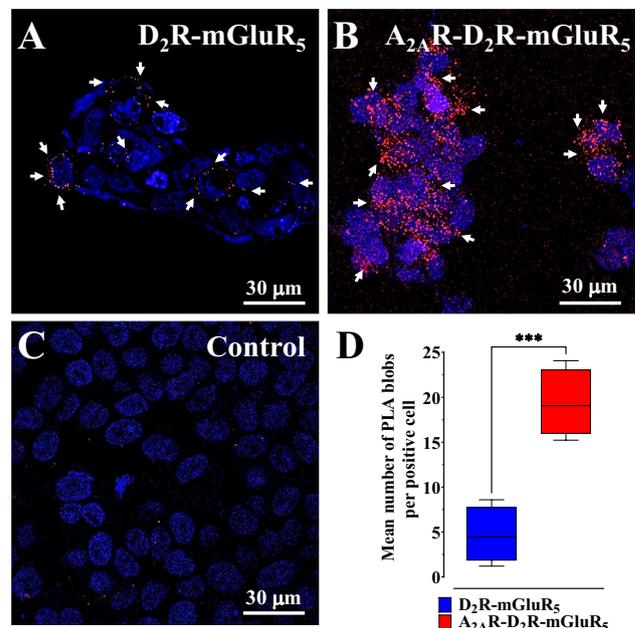

Fig. 2 In situ PLA assessment of D_2R-mGluR$_5$ heteromer formation in the absence (**A**) or presence (**B**) of $A_{2A}R$ (see "Methods"). The in situ PLA-positive D_2R-mGluR$_5$ heteroreceptor complexes were shown as red blobs (arrows) and nuclei in blue (DAPI staining). A negative in situ PLA control (**C**) was included by incubating the cells in the absence of the primary anti-D_2R antibody. **D** Quantification of D_2R-mGluR$_5$ complexes. The number of PLA blobs (red clusters) per positive cell ($n=4\times50$ cells) was assessed as described in Methods. Results were expressed as mean $\pm$ 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 D_2R-$GluR_5$ complexes, shown as mean number of red blobs/Nucleus, was markedly and highly significantly reduced (Fig. 3D). Furthermore, the loss of the red D_2R-$mGluR_5$ blobs to the same high degree in the $A_{2A}R^{-/-}$ mice (Fig. 3 B, D) likely reflects the requirement of D_2R-$mGluR_5$ heterocomplexes to be part of an $A_{2A}R$-D_2R-$mGluR_5$ to be expressed in the mouse striatum, probably by dorsal striatal-pallidal GABAergic neurons. In this way, it forms D_2R-$mGluR_5$ complexes that are close enough to be visualized by PLA.

[.^{3}H]-Raclopride/Quinpirole Competition Experiments: the $A_{2A}R$ and $mGluR_5$ Protomers Interact and Modulate D_2R Protomer Recognition

In HEK293T cells expressing D_2R and $mGluR_5$, the $mGluR_5$ agonist CHPG (500 nM) reduced the affinity of the high-affinity state ($K_{i, High}$) of the D_2R for the agonist quinpirole with no effects on its low-affinity state ($K_{i, Low}$). Co-treatment with $A_{2A}R$ agonist CGS-21680 (100 nM) did not

Fig. 3 Assessment of D_2R-$mGluR_5$ heteromer formation in mouse dorsal striatum by in situ PLA. Photomicrographs showing PLA recognition of D_2R-$mGluR_5$ heteromers in the dorsal striatum of wild type (**A**), $A_{2A}R^{-/-}$ (**B**) and $D_2R^{-/-}$ (**C**) mice. The in situ PLA-positive D_2R-$mGluR_5$ heteroreceptor complexes are shown as red blobs (arrows) and nuclei in blue (DAPI staining). **D** Quantification of D_2R-$mGluR_5$ complexes showing a highly significant reduction of D_2R-$mGluR_5$-positive red blobs in the absence of $A_{2A}R^{-/-}$ or $D_2R^{-/-}$. The number of PLA blobs (red clusters) per nucleus was assessed as described in Methods. Results were expressed as mean ± SEM ($n = 5$ animals). ***$p < 0.001$, one-way ANOVA followed by Dunnett's post hoc test when compared with wild-type animals

significantly alter the D_2R $K_{i, High}$ 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 $A_{2A}R$, D_2R and $mGluR_5$, $mGluR_5$ agonist stimulation also reduced the affinity of the high-affinity state ($K_{i, High}$) of the D_2R 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 $A_{2A}R$ by itself (without agonist stimulation) potentiates $mGluR_5$ agonist effects on the high-affinity D_2R agonist binding sites (Fig. 4B, Tables 1 and 2). Finally, the co-stimulation of $A_{2A}R$ and $mGluR_5$ synergistically increased in the $K_{i, High}$ values of the D_2R protomer upon co-expression of the $A_{2A}R$ (Table 2). Nevertheless, in cells expressing $A_{2A}R$, D_2R and $mGluR_5$, further analysis should be performed to test the effect of combine treatment of $A_{2A}R$ (ZM-241385) and $mGluR_5$ (CHPG) to figure out if the expression of $A_{2A}R$, without agonist stimulation and its corresponding constitutive activity, is responsible for increased in the $K_{i, High}$ values of the D_2R protomer upon co-expression of the $A_{2A}R$.

In both HEK293T cells expressing D_2R and $mGluR_5$ or $A_{2A}R$, D_2R and $mGluR_5$, the incubation with $A_{2A}R$ antagonist ZM-241385 (1 µM) and $mGluR_5$ antagonist MPEP (300 µM) alone or in combination resulted in an almost complete blockade of the $mGluR_5$ increase of the D_2R $K_{i, High}$ values and $A_{2A}R$ agonist-induced increase of $mGluR_5$ agonist effects on the high-affinity D_2R agonist binding sites (Tables 1 and 2).

cAMP Functional Experiments: the $A_{2A}R$ and $mGluR_5$ Protomers Interact and Modulate D_2R Protomer Signalling

In cells expressing D_2R and $mGluR_5$ forming D_2R-$mGluR_5$ heterocomplexes (Fig. 2), the D_2R 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 D_2R antagonist raclopride (1 µM). In these cells, the $mGluR_5$ agonist CHPG stimulation significantly counteracted the D_2R agonist-induced reduction of cAMP accumulation (Fig. 5A). The significant effect of CHPG (500 nM) was significantly reduced by the $mGluR_5$ antagonist MPEP (300 µM). The co-treatment with the $A_{2A}R$ agonist did not enhance the counteraction of the inhibitory D_2R signalling by CHPG (Fig. 5A).

Likewise, quinpirole significantly reduced the cAMP level in cells expressing $A_{2A}R$, D_2R and $mGluR_5$ (Fig. 5B). The $mGluR_5$ agonist CHPG had an improved ability to counteract the adenylyl cyclase inhibition produced by the D_2R agonist in these cells, yielding cAMP levels similar to those obtained after blocking D_2R signalling with raclopride (Fig. 5B). Upon $A_{2A}R$ and $mGluR_5$ agonist co-activation, a larger counteraction of the D_2R agonist action was found compared to that obtained with such a co-treatment performed in cells expressing only D_2R
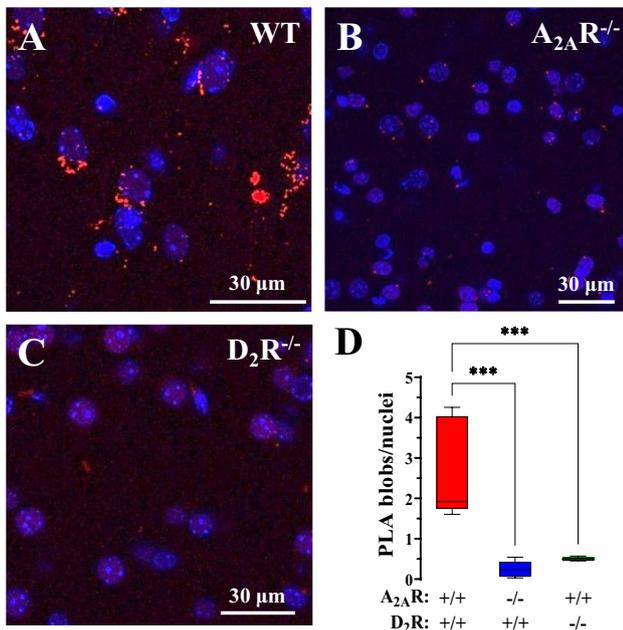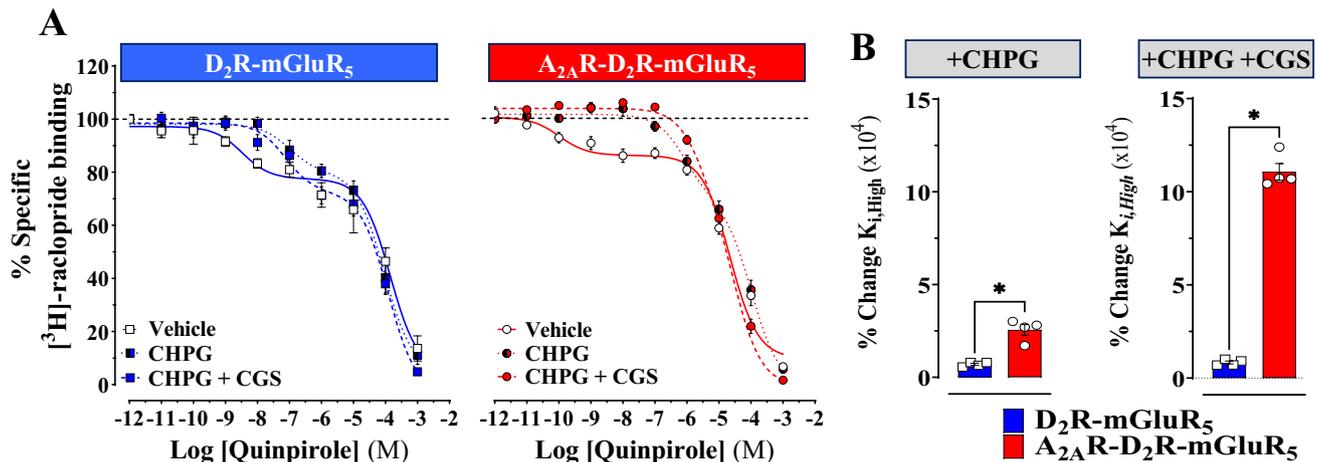
Fig. 4 Assessing $A_{2A}R$-dependent allosteric modulation of D_2R-GluR$_5$ heteromer by [^{3}H]-raclopride/quinprole competition binding experiments. **A** Competition assays were performed in HEK293T cells transiently expressing D_2R and mGluR$_5$ (blue squares) or $A_{2A}R$, D_2R and mGluR$_5$ (red circles) with/without agonist(s)/antagonist(s) for adenosine $A_{2A}R$ or mGluR$_5$ either alone or in combination as indicated. (+)-Butaclamol (100 µM) was used to determine the non-specific binding, and the specific binding at the lowest concentration of the [^{3}H]-raclopride employed was defined as 100%. Results are expressed as percentage of specific binding (mean ± SEM; $n = 4$ independent experiments performed in triplicate). **B** Percentage of change comparing CHPG alone or CHPG plus CGS-21680-induced changes in the D_2R high affinity values (($K_{i, High}$ (nM)) with/without transient co-expression of $A_{2A}R$. Results are expressed as means ± S.E.M.; $n = 4$, each determination performed in triplicate. *$p < 0.05$, Mann–Whitney U-test

Table 1 Values for quinprole binding site affinities to the D_2-likeR by [.^{3}H]-raclopride/quinprole competition assays in HEK293T cells transiently expressing D_2R and mGluR$_5$ incubated with agonist(s) or/and 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 + ZM-241385	16 ± 5†††	63 ± 19
+ CGS-21680	4.3 ± 2.2	127 ± 62
+ CGS-21680 + ZM-241385	5.7 ± 0.9	21 ± 62

$K_{i, High}$, D_2R high-affinity value and $K_{i, Low}$, D_2R 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. §§§($p < 0.001$); *significant reduced* compared to cells incubated with CHPG. †††($p < 0.001$); *significant reduced* compared to cells incubated with CHPG and CGS-21680

Table 2 Values for quinprole binding site affinities to the D_2-likeR by [.^{3}H]-raclopride/quinprole competition assays in HEK293T cells transiently expressing $A_{2A}R$, D_2R and mGluR$_5$ incubated with agonist(s) or/and 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

$K_{i, High}$, D_2R high-affinity value and $K_{i, Low}$, D_2R 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. §§§($p < 0.001$); *significant differences* compared to cells incubated with CHPG. †††($p < 0.001$); *significant reduced* compared to cells incubated with CHPG and CGS-21680

and mGluR$_5$. These results suggest a synergistic and significant counteraction by $A_{2A}R$ and mGluR$_5$ agonists of the D_2R agonist-induced decrease of cAMP accumulation (Fig. 5B). Such effects of the combined agonist treatment were only weakly reduced by the $A_{2A}R$ antagonist (ZM-241385). In cells not expressing the $A_{2A}R$, the $A_{2A}R$ antagonist failed to produce any changes in the cAMP accumulation under such co-agonist treatments.

It should be noted that CHPG agonist produces similar increases in cAMP levels as found after the $A_{2A}R$ agonist GGS in HEK293T cells co-expressing D_2R, $A_{2A}R$ and mGluR$_5$ (Fig. 5C). Therefore, we should consider also that mGluR$_5$ might simply activate Gs, inducing cAMP accumulation, independently of D2-Gi-induced inhibition of adenylate cyclase (Fig. 5 A−C).
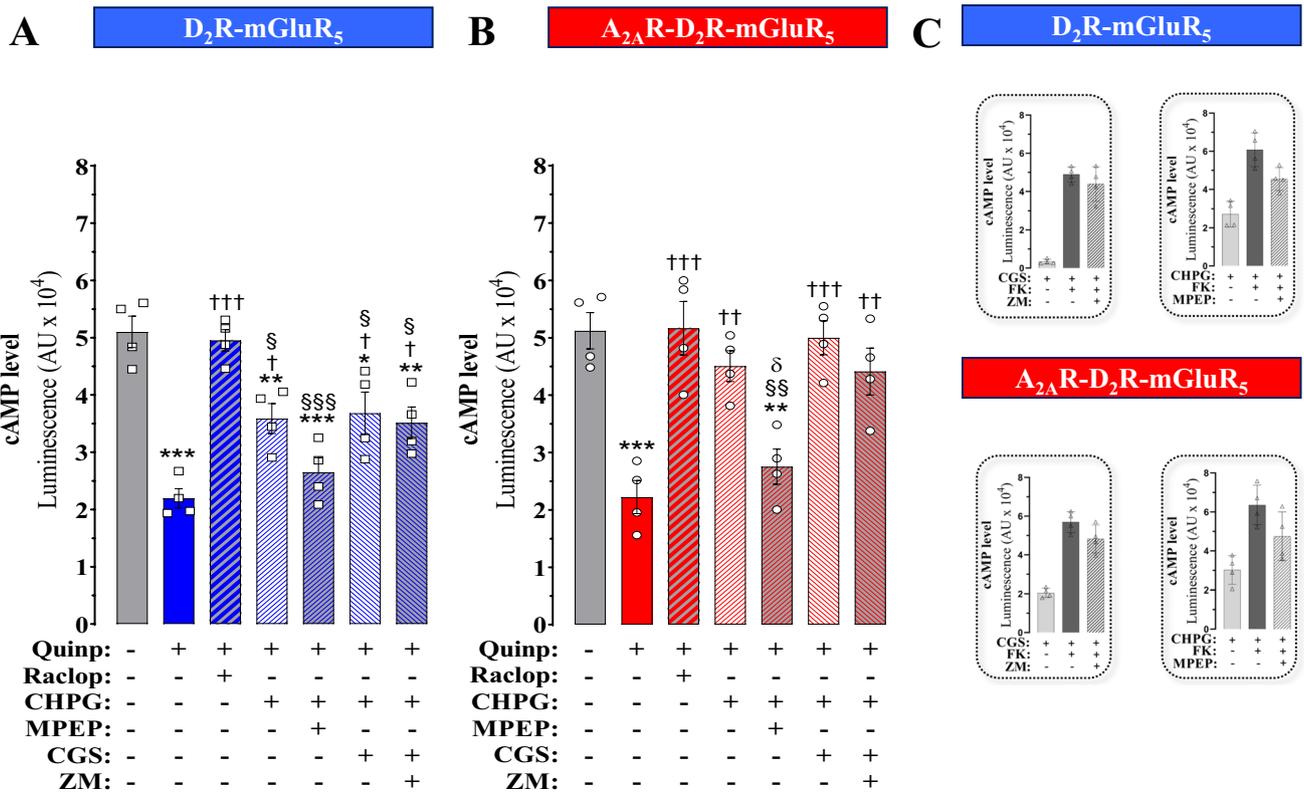
Fig. 5 Functional evaluation of A_{2A}R-mediated modulation of D_2R-GluR$_5$ heteromer. HEK293T cells transiently expressing D_2R and mGluR$_5$ (**A**) or A_{2A}R, D_2R and mGluR$_5$ (**B**) were treated with forskolin before incubation with quinpirole (Quinp, X nM), raclopride (Raclop, X nM), CHPG (X nM), MPEP (X nM), CGS-21680 (CGS, X nM) and ZM-241385 (ZM, X nM). The cAMP levels were determined using a cAMP-Glo™ Assay detection kit (see Methods). Intracellular cAMP levels are given in luminescence intensity (AU, arbitrary units) after subtracting background basal luminescence (cAMP levels measured in non-transfected, non-treated cells). Results are expressed as means ± SEM; $n = 4$ independent experiments, each determination performed in quadruplicates. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$, one-way ANOVA followed by Tukey's post hoc test compared with cells treated only with forskolin (control). †††$p < 0.001$, ††$p < 0.01$ and †$p < 0.05$, one-way ANOVA followed by Tukey's post hoc test when compared with cells treated only with quinpirole; §§§$p < 0.001$, §§$p < 0.01$ and §$p < 0.05$, when compared with cells treated only with quinpirole plus raclopride;

$\delta p < 0.05$, when compared to cells treated with quinpirole and CHPG. **C** CHPG and CGS21680-induced cAMP levels. HEK293T cells transiently expressing D_2R and mGluR$_5$ (top inset box) or A_{2A}R, D_2R and mGluR$_5$ (Bottom inset box) were treated with and without forskolin before incubation with ligands. Results are expressed as means ± S.E.M.; $n = 4$ independent experiments, each determination performed in quadruplicates. Statistical analysis performed using one-way ANOVA followed by Tukey's post hoc test (top inset box). CHPG versus CHPG + forskolin ($p < 0.001$), CHPG versus CHPG + forskolin + MPEP ($p < 0.05$); CGS-21680 versus CGS-21680 + forskolin ($p < 0.001$), CGS-21680 versus CGS-21680 + forskolin + ZM-241385 ($p < 0.001$) (bottom inset box). CHPG versus CHPG + forskolin ($p < 0.01$), CHPG versus CHPG + forskolin + MPEP (*ns*); CGS-21680 versus CGS-21680 + forskolin ($p < 0.001$), CGS-21680 versus CGS-21680 + forskolin + ZM-241385 ($p < 0.001$). Concentrations for ligands used: quinpirole (100 nM), forskolin (5 μM), raclopride (1 μM), CHPG (500 nM), MPEP (300 μM), CGS-21680 (100 nM) and ZM-241385(1 μM)

Experiments on Haloperidol-Induced Catalepsy

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 D_2R agonist-induced activation of the

D_2R homo- and heterocomplexes like the D_2R-A_{2A}R or the D_2R-mGluR$_5$ heterocomplexes [24, 47–50]. The D_2R activation of the dorsal striatal-pallidal GABA neurons is also essential for maintenance of normal locomotor activity.

The catalepsy induced by the D_2R 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 mGluR$_5$-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 A_{2A}R$^{-/-}$ mice. Furthermore, in vehicle-treated A_{2A}R$^{-/-}$ animals,
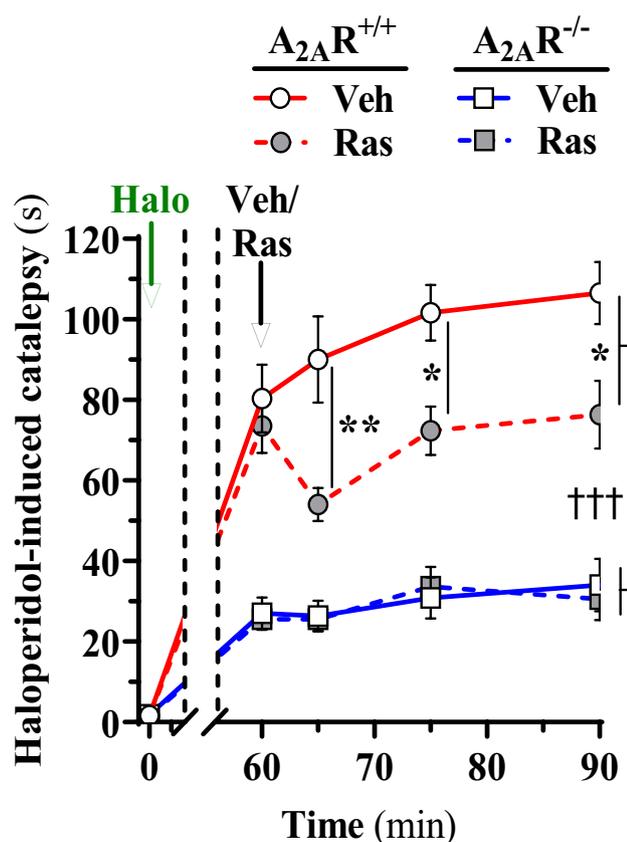
Fig. 6 $A_{2A}R$ expression is needed for $mGluR_5$ modulation of D_2R-dependent behaviour in mice. Raseglurant reverses haloperidol-induced catalepsy. Haloperidol-induced cataleptic behaviour was measured as the time spent with both front paws resting on the bar (see Methods). Wild-type (circles), $A_{2A}R.^{-/-}$ (squares) animals were pretreated with haloperidol (i.p., 1 mg/kg, i.p.) at time 0 (green arrow). Subsequently, 1 h later, animals were administered (i.p.; black arrow) with either vehicle (20% DMSO in saline) or raseglurant (Ras, 1 mg/kg). The time spent in a cataleptic position was measured after 5, 15 and 30 min after raseglurant (or vehicle) administration. Results are expressed as the mean time spent cataleptic $\pm$ SEM over a period of 120-s measurement ($n = 6$ animals per group). The cataleptic behaviour was calculated and compared within groups by a multiple t-test statistical analysis. $*p < 0.05$ and $**p < 0.01$, two-way ANOVA followed by Šídák's post hoc test. $\dagger\dagger\dagger\dagger p < 0.0001$, three-way ANOVA (phenotype, $F_{(1, 100)} = 279.6$)

the haloperidol-induced catalepsy was markedly reduced compared to that obtained in vehicle treated wild-type mice (Fig. 6).

Discussion

The field of dopamine D_2Rs changed markedly with the discovery of many types of D_2R homo- and heteroreceptor complexes in subcortical limbic areas as well as the dorsal striatum [4, 16, 40]. The results indicate that the D_2R 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 D_2R heteroreceptor complexes may contribute to the development of brain disorders through changes in the balance of diverse D_2R 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 $A_{2A}R$-D_2R and $A_{2A}R$-$mGluR_5$ heteroreceptor complexes in native tissue [4, 16, 40, 55, 56]. Furthermore, the existence of the D_2R-$mGluR_5$ 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, D_2R did not associate with $mGluR_5$ [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 $A_{2A}R$-D_2R-$mGluR_5$ heteroreceptor complexes was observed in living cells. Furthermore, by co-immunoprecipitation, experiments validated the existence of an association of $mGluR_5$, D_2R and $A_{2A}R$ in rat striatum homogenates [21].

Herein, we present new findings that further expand the understanding of $A_{2A}R$-D_2R-$mGluR_5$ heteroreceptor complexes. Also, strong evidences which support that the expression of the $A_{2A}R$ is necessary to facilitate the association of D_2R and $mGluR_5$ in a complex.

Our new findings are that transient co-expression of $A_{2A}R$ in HEK293T cells together with D_2R^{Rluc} and $mGluR_5^{GFP2}$ resulted in a significant and marked increase in the formation of the D_2R-$mGluR_5$ heterodimer, a component of the $A_{2A}R$-D_2R-$mGluR_5$ heterocomplex, based on the increase in the $BRET^2$ max values. Such an increase could be related to the development of an increased affinity of the two D_2R and $mGluR_5$ protomers for each other due to allosteric changes related to the formation of the $A_{2A}R$-D_2R-$mGluR_5$ complex. In line with this hypothesis, the $BRET^2 50$ values were significantly reduced for the D_2R-$mGluR_5$ heteromeric component of this trimeric heteroreceptor complex.

These results are also supported by the demonstration with PLA that an increased density of PLA-positive D_2R-$mGluR_5$ clusters was observed when $A_{2A}R$ expression had been added to the cells compared to cells only expressing D_2R and $mGluR_5$. In agreement, in the mouse dorsal striatum, the D_2R-$mGluR_5$ complexes were significantly reduced in the $A_{2A}R^{-/-}$ mice. Thus, it becomes clear that the expression of the $A_{2A}R$ in the mouse dorsal striatum is necessary to facilitate that the D_2R and $mGluR_5$ 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 $A_{2A}R^{-/-}$ mice are of substantial interest since they can indicate a functional role of the $A_{2A}R$-D_2R-$mGluR_5$ heteroreceptor complexes in the dorsal striatum as previously discussed [8, 17]. There was a marked reduction in the haloperidol-induced catalepsy in the $A_{2A}R^{-/-}$ mice compared to wild-type mice. Thus, in the absence of the $A_{2A}R$, the D_2R antagonist haloperidol appears to have a substantially reduced potency to block the D_2R which can be caused by the loss of the antagonistic $A_{2A}R$-D_2R interaction [9, 58]. According to the current findings in cell lines, the D_2R-$mGluR_5$ heterocomplexes should be also formed to a much lower degree in the absence of $A_{2A}R$ in view of their dependency of $A_{2A}R$ according to the PLA experiments performed. The counteraction of the D_2R-mediated inhibitory actions on cAMP signalling by CHPG, a $mGluR_5$ agonist, was in our cell line also more effective in cells co-expressing beside D_2R and $mGluR_5$, also $A_{2A}R$.

It seems likely that the formation of the $A_{2A}R$-D_2R-$mGluR_5$ complex enhances the affinity of the D_2R and $mGluR_5$ protomers for each other in this complex. It is of high interest that the biochemical binding experiments reveal that the $mGluR_5$ CHPG agonist-induced increase in D_2R $K_{i, High}$ values becomes significantly higher in the $A_{2A}R$-D_2R-$mGluR_5$ complex compared to the D_2R-$mGluR_5$ complex despite the absence of $A_{2A}R$ agonist exposure. Thus, although agonist activation of the $A_{2A}R$ seems necessary to exert negative allosteric modulation of the D_2R protomer agonist binding via heteroreceptor complexes, an increased constitutive activity of the $A_{2A}R$ protomer could explain the above results.

As expected, the combined incubation with CHPG and CGS-21680 led to an even stronger increase in the D_2R $K_{i, High}$ values of the $A_{2A}R$-D_2R-$mGluR_5$ complex, demonstrating the impact of the $A_{2A}R$ protomer on the D_2R-$mGluR_5$ allosteric interactions, which can involve both constitutive and $A_{2A}R$ agonist-induced inhibition of D_2R agonist binding. Our findings represent one of the first examples of integrative activity within a higher-order heteroreceptor complex and show how one receptor ($A_{2A}R$) can substantially modulate the structure and recognition of a participating receptor heterodimer (D_2R-$mGluR_5$) in such a trimeric receptor complex.

The pharmacological analysis of the $A_{2A}R$-D_2R-$mGluR_5$ complex and its impact on cAMP levels indicated that the $A_{2A}R$ can modulate the effects of the D_2R-$mGluR_5$ interactions on cAMP signalling. It was found that when the $A_{2A}R$-D_2R-$mGluR_5$ complex was likely to be formed through the expression also of the $A_{2A}R$, the $mGluR_5$ agonist had an increased ability to counteract the D_2 agonist-induced $G_{i/o}$-mediated inhibition of the cAMP levels in comparison with the counteraction observed in the absence of $A_{2A}R$ expression. The same was also true for the combined treatment with the $mGluR_5$ agonist CHPG and the $A_{2A}R$ agonist CGS-21680 when the $A_{2A}R$ was coexpressed. A stronger counteraction of the D_2R-induced inhibition of the cAMP levels was observed when $A_{2A}R$ expression was present.

Taken together, our work on cell lines gives strong indications that, in the $A_{2A}R$-D_2R-$mGluR_5$ complex, the $A_{2A}R$ protomer enhances the formation of the D_2R-$mGluR_5$ component of the complex with enhanced inhibition of D_2 agonist binding recognition and its $G_{i/o}$-mediated cAMP signalling. The inhibitory effects by $A_{2A}R$ and $mGluR_5$ on D_2R recognition and signalling reveal a significant molecular integration in $A_{2A}R$-D_2R-$mGluR_5$ complexes, likely formed also in the dorsal striatum. The $A_{2A}R$ and $mGluR_5$ antagonists targeting the $A_{2A}R$-D_2R-$mGluR_5$ complexes in dorsal striatum may reduce the haloperidol-induced catalepsy by removal of the $A_{2A}R$ and $mGluR_5$ protomer-mediated allosteric inhibition of the D_2R 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.

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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