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Unique effect of clozapine on adenosine A_{2A}-dopamine D_2 receptor heteromerization

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ABSTRACT

The striatal dopamine D_2 receptor (D_2R) is generally accepted to be involved in positive symptoms of schizophrenia and is a main target for clinically used antipsychotics. D_2R are highly expressed in the striatum, where they form heteromers with the adenosine A_{2A} receptor ($A_{2A}R$). Changes in the density of $A_{2A}R$-D_2R heteromers have been reported in postmortem tissue from patients with schizophrenia, but the degree to which A_2R are involved in schizophrenia and the effect of antipsychotic drugs is unknown. Here, we examine the effect of exposure to three prototypical antipsychotic drugs on $A_{2A}R$-D_2R heteromerization in mammalian cells using a NanoBiT assay. After 16 h of exposure, a significant increase in the density of $A_{2A}R$-D_2R heteromers was found with haloperidol and aripiprazole, but not with clozapine. On the other hand, clozapine, but not haloperidol or aripiprazole, was associated with a significant decrease in $A_{2A}R$-D_2R heteromerization after 2 h of treatment. Computational binding models of these compounds revealed distinctive molecular signatures that explain their different influence on heteromerization. The bulky tricyclic moiety of clozapine displaces TM 5 of D_2R, inducing a clash with $A_{2A}R$, while the extended binding mode of haloperidol and aripiprazole stabilizes a specific conformation of the second extracellular loop of D_2R that enhances the interaction with $A_{2A}R$. It is proposed that an increase in $A_{2A}R$-D_2R heteromerization is involved in the extrapyramidal side effects (EPS) of antipsychotics and that the specific clozapine-mediated destabilization of $A_{2A}R$-D_2R heteromerization can explain its low EPS liability.

1. Introduction

The dopamine D_2 receptor (D_2R) is a G protein-coupled receptor (GPCR) that signals through inhibitory G proteins ($G_{i/o}$) and is crucially involved in several physiological functions, such as control of movement, goal-directed behavior and reward, memory, and behavioral salience, as well as regulation of prolactin release. In the brain, D_2R is highly expressed in the striatum and to a somewhat lower extent in the midbrain, cortex, hypothalamus, amygdala, and hippocampus [1]. Antipsychotic drugs are antagonists or weak partial agonists of D_2R. The first-introduced 'typical' or first-generation antipsychotics are D_2R antagonists or inverse agonists and often associated with a high risk of extrapyramidal side effects (EPS), a broadly defined concept that includes both hypokinetic symptoms (parkinsonism) and

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hyperkinetic/dyskinetic symptoms (akathisia, dyskinesia). Despite this drawback, many 'typical' drugs are still used in the clinic, including haloperidol, which remains a widely prescribed drug more than half a century after its introduction [2].

The 'atypical', or second-generation antipsychotics, are also D_2R antagonists or inverse agonists, but were introduced somewhat later and generally show a lower risk of inducing EPS. Many of these drugs target multiple receptors, mainly serotonin 5-HT_{2A} receptors (5-HT_{2A}Rs), which has been suggested to be responsible for the lower incidence of EPS, at least in part by contributing to antipsychotic efficacy and thus lowering the occupancy of D_2R required for its therapeutic effect [3]. The first antipsychotic in this group, clozapine, was introduced in the 1970 s and, in addition to the very low liability to induce hypokinetic and hyperkinetic EPS, has a higher clinical efficacy compared to many other antipsychotics, particularly for the negative symptoms of schizophrenia and for patients who are refractory to other antipsychotic treatment [4–10]. However, despite these favorable characteristics, clozapine has rare but potentially significant adverse effects, such as agranulocytosis and metabolic side effects that increase the risk of cardiovascular morbidity [7].

The most recent antipsychotics, or third-generation antipsychotics, are partial D_2R agonists and were introduced for clinical use in the late 1990 s with aripiprazole. Aripiprazole is less likely to induce EPS than first-generation antipsychotics, although occupancy of D_2R in clinically effective doses can often reach 90% [11] while first- and second-generation antipsychotics generally induce motor side effects with occupancy of D_2R of about 80% [12]. The partial agonism of aripiprazole could then allow a sufficient level of D_2R activation to provide clinical efficacy by avoiding EPS. Another interpretation that has been leading in the preclinical field is functional selectivity, with the ability of aripiprazole to partially, but selectively, activate G protein-dependent versus β-arrestin-dependent D_2R signaling [13,14]. Therefore, it is proposed that blockade of β-arrestin-dependent D_2R signaling mediates the therapeutic effects of antipsychotics, while blocking G protein-dependent D_2R signaling is mainly responsible for EPS [13–15]. However, although it has lower liability than first-generation antipsychotic drugs, aripiprazole is not devoid of the risk of EPS, including hypokinetic and hyperkinetic EPS [15–17]. Furthermore, according to several meta-analyses, clozapine still represents one of the most effective antipsychotic drugs with the lowest incidence in EPS [5,9], and is yet an antagonist at both G protein-dependent and β-arrestin-dependent D_2R signaling [13,18].

Therefore, identification of the unique molecular and cellular mechanisms of clozapine, as compared to those of other antipsychotics, should allow us to explain its unique therapeutic profile. The targeting of 5HT_{2A}Rs or a rapid D_2R dissociation rate have been claimed as specific properties of clozapine, although they are also shared to a greater or lesser degree by other antipsychotics [2,11,19,20]. Another possible mechanism to consider is the differential effect of clozapine on the immediate environment of D_2R, more specifically on its interactions with other membrane proteins, such as other GPCRs. There is significant experimental evidence indicating that a main population of striatal D_2R forms heteromers with the adenosine A_{2A} receptor ($A_{2A}R$) within a macromolecular complex that includes adenylyl cyclase [21–23], and there is preclinical evidence indicating the involvement of $A_{2A}R$ and $A_{2A}R$-D_2R heteromers in schizophrenia.

In the $A_{2A}R$-D_2R heteromers, activation of the Gi/o-coupled D_2R inhibits adenylyl cyclase activation mediated by the Gs/olf-coupled $A_{2A}R$ and reciprocally activation of $A_{2A}R$ decreases D_2R agonist binding affinity and downstream G protein-dependent signaling [21–23]. Furthermore, recent studies indicate that heteromerization with $A_{2A}R$ increases D_2R-mediated recruitment of β-arrestin [24–27]. This explains why the effects of D_2R ligands depend on the $A_{2A}R$ function. Thus, $A_{2A}R$ knockout mice ($A_{2A}R^{-/-}$), as well as mice administered with $A_{2A}R$ antagonists, are resistant to the cataleptic effects of haloperidol [28–31], and haloperidol reduces spontaneous locomotion to a lesser extent in $A_{2A}R^{-/-}$ animals compared to wild-type counterparts [32]. In fact, $A_{2A}R$ agonists have been proposed as putative antipsychotics based on their ability to counteract hyperlocomotion and sensory gating deficits (specifically, prepulse inhibition or PPI) in rodents induced by the psychotomimetic drug phencyclidine (PCP) [33,34]. Furthermore, $A_{2A}R^{-/-}$ mice show certain behavioral traits reminiscent of psychotic symptoms, including impaired PPI [31,35,36]. Finally, $A_{2A}R$ antagonists counteract clozapine and haloperidol-induced c-Fos induction in the rat striatum [37], but prolonged treatment with haloperidol, and not clozapine, was associated with significant locomotor activation induced by the non-selective adenosine receptor antagonist theophylline [38]. These studies would implicate $A_{2A}R$ and $A_{2A}R$-D_2R heteromers in the actions of antipsychotics and would suggest their differential role in the effects of typical versus atypical antipsychotics.

In a recent study, using a new antibody-based amplified luminescent proximity homogeneous assay (ALPHA), we found evidence for a significant decrease in the density of $A_{2A}R$-D_2R heteromers in the postmortem caudate nucleus of schizophrenic subjects, although both receptors were upregulated [36]. A significant reduction in striatal $A_{2A}R$-D_2R heteromerization with concomitant up-regulation of D_2R was also found in a mouse model of psychosis based on phencyclidine (PCP) [36]. It could also be shown that subchronic treatment with haloperidol, but not clozapine, counteracted the effect of PCP [36], which could be related to a specific ability of haloperidol to promote increased heteromerization of $A_{2A}R$-D_2R, since a trend for such an increase was observed in mice after subchronic treatment with haloperidol [36]. Using a NanoBiT assay, our objective was to investigate in more detail the dynamics of $A_{2A}R$-D_2R heteromerization in living cells after long exposure to haloperidol, aripiprazole, and clozapine. Computational studies were implemented to structurally rationalize the differential impact of antipsychotics on $A_{2A}R$-D_2R heteromerization. Importantly significant qualitative differences were obtained with clozapine compared to haloperidol and aripiprazole, providing a possible new mechanism involved in the unique clinical profile of clozapine.

2. Materials and methods

2.1. Reagents

Aripiprazole, clozapine, and haloperidol were purchased from Tocris Bioscience (Bristol, UK). DMSO and Tween-80 were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Animals

$A_{2A}R^{-/-}$ mouse generated on a CD-1 genetic background [31,39] and the corresponding littermates $A_{2A}R^{+/+}$ weighing 20–25 g were used. The animal protocol (#7085) was approved by the University of Barcelona Committee on Animal Use and Care. The animals were housed and tested according to the guidelines provided by the Guide for the Care and Use of Laboratory Animals [40] and following the directives of the European Union (2010/63/EU). Mice were housed in groups of five in standard cages with ad libitum access to food and water and maintained in a 12 h dark/light cycle (starting at 7:30 AM), 22 °C temperature, and 66% humidity (standard conditions).

2.3. Generation of $A_{2A}R$ and D_2R NanoBiT-based constructs

The human $A_{2A}R$ and D_2R cDNA were cloned in the pIREShyg3 plasmid vector (Clontech Laboratories, Inc.) containing the sequences for the long subunit (LgBiT) or small subunit (SmBiT) of nanoluciferase [41] within the *Alf*I and *Bstx*I restriction enzyme sites. Additionally, the $mGlu_5$ receptor signal peptide and the HA epitope sequence [42] were also included in 5′ of the MCS to allow for plasma membrane trafficking and detection, respectively. In summary, the cDNA encoding human $A_{2A}R$ and D_2R was amplified using the primers: i) F*Bam*HI 5′-

CGT**GGATCC**CCCATCATGGGCTCCTCGGTGTACATCACG – 3′ and R*Eco*RV 5′- AAACAC**GATATC**GGACACYCCYGCAGGYAGGACCCG – 3′ for $A_{2A}R$; ii) F*Bam*HI 5′- ACAGCG**GGATCC**GATCCACTGAATCTGTCC TGG-3′ and R*Eco*RV 5′- ACAGCG**GATATC**GCAGTGGAGGATCTTC AGGAAGG – 3′) for D_2R; and cloned in *Bam*HI / *Eco*RV of the plasmids $pIRES^{LgBiT}$ or $pIRES^{SmBiT}$, respectively. All constructs were verified by DNA sequencing.

2.4. Cell culture and transfection

Human embryonic kidney 293 T (HEK-293 T) cells obtained from ATCC (American Type Culture Collection, Rockville, MD, USA; CRL-321, RRID: CVCL_0063) were grown in Dulbecco's modified Eagle medium (DMEM) preheated at 37 °C and supplemented with 5% (v/v) fetal bovine serum (previously inactivated at 55 °C for 30 min), 100 U / ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and non-essential amino acids. Manipulation and maintenance were carried out in a Class 1 biological safety cabinet and in an incubator at 37 °C, 5% CO_2 and 90% relative humidity. The absence of mycoplasma was checked regularly; therefore, only cells without mycoplasma were used. HEK-293 T cells were transiently transfected with pIRES-HA-PS-$A_{2A}R^{LgBiT}$ and pIRES-HA-PS-D_2R^{SmBiT} constructs using polyethylenimine (PEI) transfection reagent [43]. Cell medium was replaced with fresh DMEM after 4 h and cells were kept at 37 °C, 5% CO_2 for 24–48 h until use.

2.5. NanoBiT assay

The NanoBiT assay was performed as recently described [44]. HEK-293 T transfected cells were transferred to a white 96-well plate (Corning 3600, Sigma-Aldrich, St. Louis, MO, USA) at a density of 50,000 cells/cm^2. Drugs were diluted in complete cell culture medium and added to cells at different concentrations for 2 h and 16 h. Cells were washed in HBSS (Sigma-Aldrich) and incubated for 2 min in a final volume of 90 μl before adding 10 μl of a 10 μM coelenterazine 400a solution (NanoLight Technologies, Pinetop, AZ, USA) was added to each well. After one minute of incubation, the end-point luminescence was determined using a CLARIOstar Optima plate reader (BMG Labtech GmbH, Ortenberg, Germany) and the output luminescence was reported as the integrated relative light units (RLU).

2.6. Immunofluorescence

For immunocytochemistry, HEK-293 T-$A_{2A}R^{LgBiT}$-D_2R^{SmBiT} and cells were grown in poly-L-ornithine (0.1 mg / ml) coverslips, fixed in 4% paraformaldehyde for 15 min and washed with PBS containing 20 mM glycine (buffer A) to quench aldehyde groups. Cells were then permeabilized with buffer A containing 0.2% Triton X-100 for 10 min. Subsequently, cells were labeled overnight at 4 °C with mouse anti-$A_{2A}R$ (1 μg/ml, Santa Cruz Biotechnology, TX, USA) or rabbit anti-D_2R (1 μg/ml, Frontier Institute Co. Ltd, Hokkaido, Japan). The next day, the cells were washed and stained with Cy3-conjugated donkey anti-mouse IgG antibody (1/200, Jackson ImmunoReserach Laboratories Inc.) and Alexa Fluor 488 Dye donkey anti-rabbit IgG antibody (1/200, Jackson ImmunoReserach Laboratories Inc.) for 2 h. The coverslips were rinsed for 3 min in PBS, mounted with Vectashield immunofluorescence medium (Vector Laboratories) containing DAPI, and examined using a Leica TCS 4D confocal scanning laser microscope (Leica Lasertechnik GmbH).

2.7. Gel electrophoresis and immunoblotting

Total membrane extracts of transiently transfected HEK-293 T cells or mouse striatum were prepared as previously described [45]. The protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA) and 25 μg of protein was used for immunoblotting. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS/PAGE) was performed using 10% polyacrylamide gels. Proteins were transferred to Hybond®-LFP polyvinylidene difluoride (PVDF) membranes (GE Healthcare) using a Trans-Blot® SD semi-dry transfer cell (Bio-Rad, Hercules). PVDF membranes were blocked with 5% dry nonfat milk (wt / vol) in PBS containing 0.05% Tween-20 (PBS-T) for 45 min and immunoblotted using mouse anti-$A_{2A}R$ (1 μg/ml, Santa Cruz Biotechnology, TX, USA), rabbit anti-D_2R (1 μg/ml, Frontier Institute Co. Ltd, Hokkaido, Japan) or mouse anti-α-actinin (1 μg/ml, sc-166524; Santa Cruz Biotechnology Inc., Dallas, TX, USA) antibodies in blocking solution overnight at 4 °C. PVDF membranes were washed with PBS-T three times (5 min each) before incubation with goat anti-mouse IgG (1/10,000; Pierce Biotechnology) and goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (1/30,000; Pierce Biotechnology) in blocking solution at 20 °C for 2 h. After washing the PVDF membranes with PBS-T, immunoreactive bands were detected with an Amersham Imager 600 (GE Healthcare Europe GmbH, Barcelona, Spain) [46].

2.8. Computational methods

The model of the D_2R monomer was constructed from the inactive structure of D_2R (6CM4) [47] and the model of the $A_{2A}R$-D_2R heteromer was built from the TM 4/5 dimeric interface observed in the β_1-adrenergic receptor (PDB code 4GPO) [48], using crystal structures of $A_{2A}R$ (5IU4) [49] and D_2R with ECL 2 in the helical (6CM4) [50] and extended (7DFP) [51] conformations. Fusion proteins were removed, and stabilizing mutations were mutated to the native sequence. Antipsychotics were modelled in the orthosteric binding cavity of D_2R using as a reference the structures of D_2R bound to haloperidol (6LUQ) [47] for haloperidol, $5HT_{2A}R$ bound to zotepine (6A94) [52] for clozapine, and D_3R bound to eticlopride (3PBL) [53] for aripiprazole. These structures were embedded in a lipid bilayer box, constructed using PACKMOL-memgen [54], containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, water molecules, and monoatomic Na^+ and Cl^- ions. MD simulation of these systems was performed with GROMACS 2019 [55] using the protocol previously reported [56]. The analysis of the trajectories was performed with MDAnalysis [57] and GetContacts (https://getcontacts.github.io/), while the energies of interaction of protein-protein complexes were calculated with PRODIGY [58].

2.9. Statistics

Data are represented as mean ± standard error of mean (SEM) with statistical significance set at $p < 0.05$. The number of samples (n) in each experimental condition is indicated in the corresponding figure legend. Outliers were evaluated using the ROUT method [59] assuming a Q value of 1% on GraphPad Prism 9 (San Diego, CA, USA). No outliers were found. Data normality was assessed using the Shapiro-Wilk normality test ($p < 0.05$). Comparisons between experimental groups were made using Student's unpaired t test or analysis of variance (ANOVA), followed by Tukey's post hoc multiple comparison test using GraphPad Prism 9, as indicated.

3. Results

3.1. Antipsychotics differentially alter the dynamics of $A_{2A}R$-D_2R heteromerization in living cells

We engineered a NanoLuc Binary Technology (NanoBiT)-based assay [41] to monitor the dynamics of $A_{2A}R$-D_2R heteromerization in HEK-293 T cells. To this end, $A_{2A}R$ and D_2R were fused to a long portion (LgBiT) and a short portion (SmBiT) of nanoluciferase (NL) (Fig. 1A). Upon expression in HEK-293 T cells, $A_{2A}R^{LgBiT}$ and D_2R^{SmBiT} showed a high degree of co-distribution both intracellularly and at the plasma membrane level (Fig. 1B). The ability of NL to reconstitute after heteromerization in HEK293T cells expressing $A_{2A}R^{LgBiT}$ and D_2R^{SmBiT} was

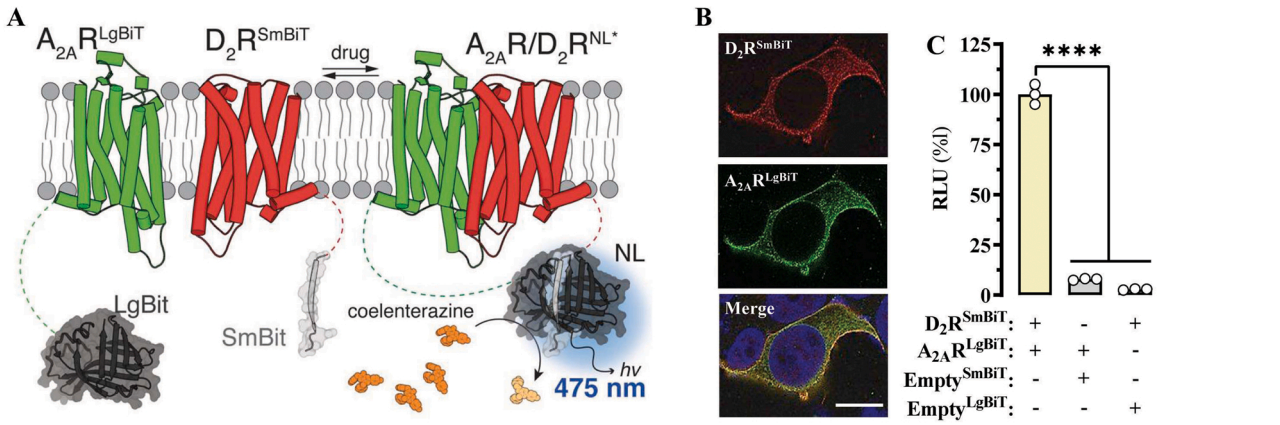

Fig. 1. NanoBiT-based $A_{2A}R/D_2R$ heteromer detection in living cells. (A) Schematic representation of the NanoBiT-based receptor-receptor interaction detection system. The $A_{2A}R$ and D_2R tagged with LgBiT and SmBiT (i.e., $A_{2A}R^{LgBiT}$ and D_2R^{SmBiT}, respectively) and its potential drug-dependent dynamic modulation is shown. Upon $A_{2A}R/D_2R$ heteromerization, the proximity of the receptors may allow the NanoBiT fragments to reconstitute a functional nanoluciferase (NL), thus able to metabolize the coelenterazine 400a substrate leading to 475 nm light emission. (B). Immunofluorescence detection of $A_{2A}R$ and D_2R in living cells. HEK-293 T cells were transiently transfected with the cDNA encoding $A_{2A}R^{LgBiT}$ and D_2R^{SmBiT} and processed for immunofluorescence detection using specific anti-$A_{2A}R$ and anti-D_2R antibodies (*see* Materials and Methods). (C) $A_{2A}R/D_2R$ heteromer-mediated NL complementation. HEK-293 T cells transiently transfected with $A_{2A}R^{LgBiT}$ and D_2R^{SmBiT} were incubated with coelenterazine 400a (10 μM) and the luminescence recorded. Results from three independent experiments performed in triplicate were expressed as percentages (mean ± SEM) of the relative luminescence signal (RLU). * ** *$p < 0.0001$ one-way ANOVA with Dunnett's *post-hoc* test when compared to $A_{2A}R^{LgBiT}$ plus D_2R^{SmBiT} expressing cells.

evaluated by recording NL-mediated luminescence (Fig. 1C). Collectively, these results validate our NanoBiT-based approach to monitor the density of $A_{2A}R$-D_2R heteromers in living cells.

The impact of antipsychotics on the temporal dynamics $A_{2A}R$-D_2R heteromerization was evaluated by treating $A_{2A}R^{LgBiT}$ and D_2R^{SmBiT} expressing HEK-293 T cells with haloperidol, clozapine, or aripiprazole for 2 h or 16 h. To this end, concentration-response curves were constructed after incubation of $A_{2A}R^{LgBiT}$ and D_2R^{SmBiT} expressing HEK-293 T cells with increasing concentrations of haloperidol, clozapine, and aripiprazole for 2 h or 16 h (Fig. 2A). Interestingly, while 2 h of incubation with haloperidol or aripiprazole did not alter the heteromer content, the same treatment with clozapine significantly reduced the amount of heteromer in a concentration dependent manner (pEC_{50} =6.1 ± 0.3) (Fig. 2A). However, 16 h of exposure resulted in a significant concentration-dependent increase in $A_{2A}R$-D_2R heteromers with haloperidol or aripiprazole (pEC_{50} =7.8 ± 0.4 and pEC_{50} =6.8 ± 0.4, respectively), while clozapine did not show differences compared to controls (Fig. 2A). In general, these results demonstrate a differential effect of clozapine compared to other antipsychotics on the dynamics of $A_{2A}R$-D_2R heteromerization. Both haloperidol and aripiprazole promoted an increase in $A_{2A}R$-D_2R heteromerization upon long-term exposure, while clozapine shows a unique temporal modulation, with an initial decrease followed by normalization and a lack of long-term-induced increase in $A_{2A}R$-D_2R heteromerization.

Subsequently, the impact of antipsychotic treatment on the density of $A_{2A}R$ and D_2R was evaluated by immunoblotting. Therefore, $A_{2A}R^{LgBiT}$ and D_2R^{SmBiT} expressing HEK-293 T cells were treated with 1 μM of haloperidol, clozapine, or aripiprazole for 2 h or 16 h before analyzing cell membrane extracts by immunoblotting (Fig. 3A). The 1 μM concentration was chosen based on the concentration-response experiments on the heteromerization of $A_{2A}R$ and D2R, where this concentration was significantly effective for the three compounds (Fig. 2A). Interestingly, this concentration also falls within the proposed therapeutic reference range of these antipsychotics [60]. The specificity of the antibodies used (i.e., mouse anti-$A_{2A}R$ and rabbit anti-D_2R) was validated in HEK-293 T cells transiently transfected and not transfected with $A_{2A}R$ or D_2R (Supplementary Fig. S1A). Furthermore, the mouse anti-$A_{2A}R$ antibody was also validated using striatal extracts of the $A_{2A}R$ knockout mouse (Supplementary Fig. S1B) as we did for the rabbit anti-D_2R antibody [31]. Interestingly, a three-way ANOVA (antipsychotic x receptor x time) confirmed a significant main effect of antipsychotic treatment ($F_{(3, 32)} = 3.5$, $p = 0.03$), time of treatment ($F_{(1, 32)} = 7.4$, $p = 0.01$) but not type of receptor ($F_{(1, 32)} = 1.2$, $p = 0.28$) [the interaction between antipsychotic x receptor ($F_{(3, 32)} = 0.3$, $p = 0.85$), antipsychotic x time ($F_{(3, 32)} = 1.1$, $p = 0.35$), receptor x time ($F_{(1, 32)} = 0.01$, $p = 0.91$) and antipsychotic x receptor x time ($F_{(3, 32)} = 0.05$, $p = 0.99$) were not statistically significant]. Bonferroni's post hoc test revealed a significant increase ($p = 0.0048$) in D_2R density in cells treated with haloperidol for 16 h, but not with clozapine or aripiprazole (Fig. 3B). Although immunoblotting may not be as sensitive as other techniques, such as radioligand binding, in detecting slight changes in receptor density, the results obtained with this approach have been found to be comparable to those shown in *ex vivo* studies in rodents and non-human primates after prolonged exposure to antipsychotics [61–65], as well as recent in vitro studies in HEK293 cells [66].

3.2. *Different structural conformations of D_2R upon binding to antipsychotics*

GPCRs are dynamic proteins that permit rapid ligand-dependent small-scale structural fluctuations [67]. We have proposed that these fluctuations promote allosteric interactions in GPCR heteromers through their transmembrane (TM) interface [23], which we recently applied to the analysis of the effect of clozapine on the signaling of the CB_1R-$5HT_{2A}R$ heteromer [68]. Thus, to understand the distinctive molecular signature of clozapine, relative to haloperidol or aripiprazole, we first performed computational simulations of these three antipsychotics (Fig. 2B) bound to the D_2R monomer. Our initial simulations revealed key structural differences, despite those all three antipsychotics bind to the same orthosteric pocket (Fig. 2C). Specifically, the aromatic ring of clozapine occupies an additional volume near TM 5, while both haloperidol and aripiprazole, with a more compact and elongated structure, extend towards the extracellular environment (Fig. 2C). Therefore, the bulky aromatic ring, present only in clozapine, could alter TM 5 of D_2R, disturbing $A_{2A}R$-D_2R heteromerization; whereas the extended binding modes of haloperidol and aripiprazole to the extracellular domain, absent in clozapine, could influence the conformation of extracellular loop (ECL) 2, favouring $A_{2A}R$-D_2R heteromerization (Fig. 2C). To test these hypotheses, we first performed three replicate runs of unbiased 1 μs molecular dynamics (MD) simulations (*see* Methods) of the D_2R

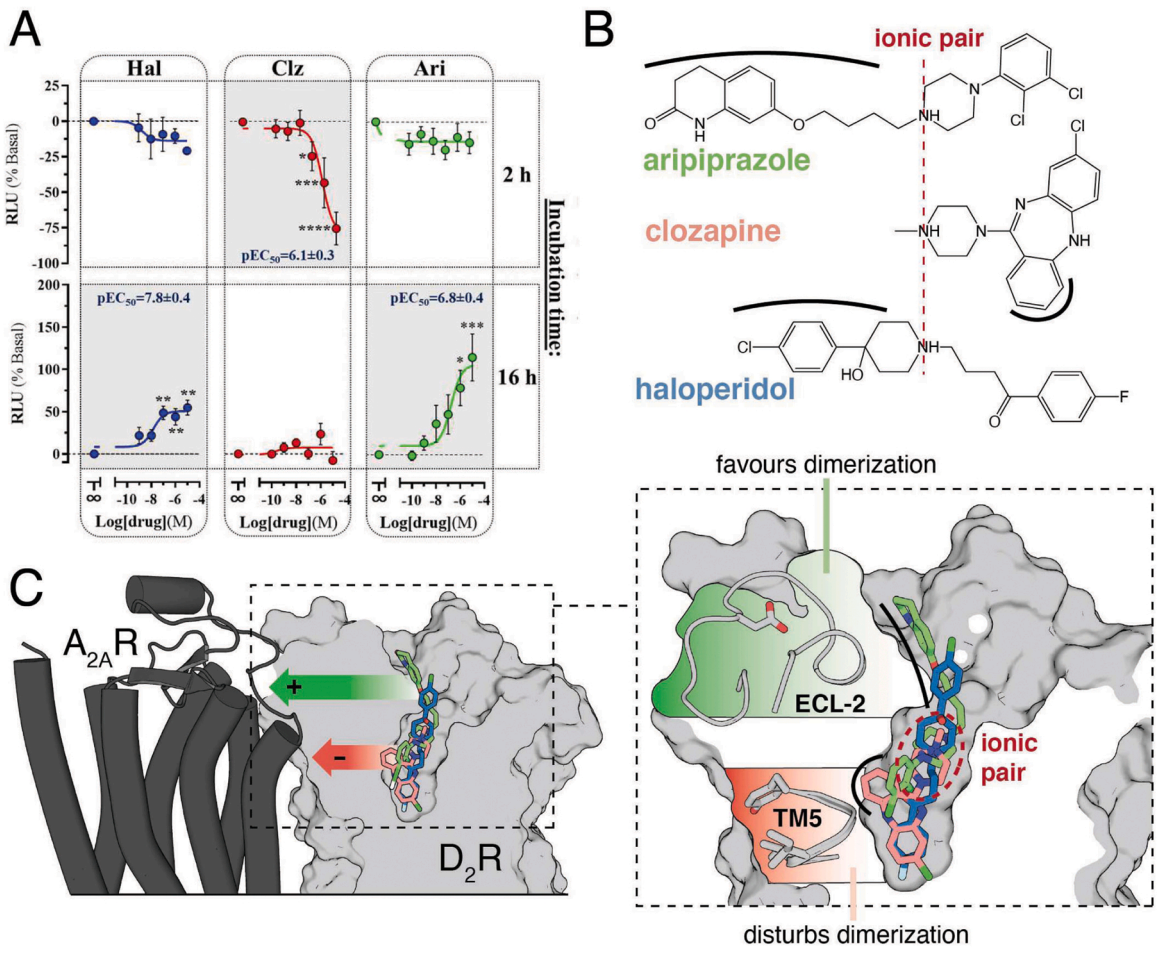

Fig. 2. Dynamic modulation of $A_{2A}R$-D_2R heteromerization by antipsychotics. (A) HEK-293 T cells transiently expressing $A_{2A}R^{LgBiT}$ and D_2R^{SmBiT} were incubated with increasing concentrations of haloperidol (Hal), clozapine (Clz), and aripiprazole (Ari) for 2 h or 16 h and NanoBiT concentration-response curves were constructed. Results are expressed as percentages (mean ± SEM) of the relative luminescence signal (RLU) of vehicle (∞) treated cells from five independent experiments performed in triplicate. *p < 0.0, * *p < 0.01, * **p < 0.001, one-way ANOVA with Dunnett's post hoc test when compared to vehicle treated cells. (B, C) Chemical structures and molecular docking of haloperidol (blue), clozapine (pink), and aripiprazole (green) into the D_2R monomer (PDB id 6CM4). The protonated amine of the ligands (red dashed line) that forms an ionic pair with D3.32 of D_2R is highlighted (red circle). The bulky aromatic ring of clozapine, absent in the other compounds, that occupies an additional volume near TM 5 is highlighted (black line); and the groups of haloperidol and aripiprazole, absent in clozapine, that extend toward ECL 2 are also highlighted (black line). The position of $A_{2A}R$ (5IU4, grey cylinders) in the $A_{2A}R$-D_2R heteromer, constructed from the TM 4/5 dimeric interface observed in the β_1-adrenergic receptor (4GPO), is shown as a reference but not included in the docking procedure.

monomer in the absence and presence of these antipsychotics.

Simulations suggest that neither haloperidol nor aripiprazole alters the conformation of TM 5 with respect to apo-D_2R, while clozapine displaces TM 5 (Y5.41 and I5.44) 1.2 Å toward the membrane (supposedly toward the $A_{2A}R$ interface, *see* below), relative to apo-D_2R ($p = 0.023$, Table 1) (Fig. 4A). On the other hand, ECL 2 of D_2R, between TMs 4 and 5, also involved in the $A_{2A}R$ interface (*see* below), has adopted two different conformations in the known structures: helical in inactive D_2R bound to haloperidol [47] and risperone [50] or extended in inactive D_2R bound to spiperone [51] and active D_2R bound to bromocriptine [69,70] (Fig. 5A). Previous MD simulations by others have shown that this ECL 2 is highly dynamic, with a spontaneous transition between both conformations [71]. However, the extended conformation positions ECL 2 in the binding pocket, limiting its size [51], so bitopic ligands with extended binding modes to the extracellular domain, such as haloperidol and aripiprazole, could favour the helical conformation. The flexibility of ECL 2 in our MD simulations, modelled in the helical conformation (*see* Methods), was characterized by the root-mean-square fluctuation (RMSF) and associated B-factor values in the absence and presence of these antipsychotics (Fig. 5B). As expected, the lack of the extended binding mode of clozapine makes ECL 2 of D_2R more flexible (comparable to the values of apo-D_2R) than when haloperidol or aripiprazole are bound.

3.3. *Influence of the different conformations of D_2R on the $A_{2A}R$-D_2R heteromeric interface*

Here, we want to address the influence of the outward movement of TM 5 of D_2R, triggered by the bulky aromatic ring of clozapine; and the helical conformation of ECL 2 of D_2R, stabilized by the extended binding mode of haloperidol and aripiprazole, on the experimentally observed ligand-induced changes of $A_{2A}R$-D_2R heteromerization. Consequently, we performed similar simulations to those with the D_2R monomer (*see* above) on the $A_{2A}R$-D_2R heteromer, which, in this case, required computational modelling of the $A_{2A}R$-D_2R interface. Previously reported bimolecular fluorescence complementation (BiFC) experiments, in the presence of synthetic peptides corresponding to different TM domains of $A_{2A}R$ and D_2R, revealed TM 4 and 5 to form the $A_{2A}R$-D_2R heteromer interface (TM 4/5 interface) [22].

Our simulations of the $A_{2A}R$-D_2R heteromer confirmed that the voluminous aromatic ring of clozapine displaces TM 5 of D_2R 2 Å towards $A_{2A}R$, relative to apo-D_2R ($p = 0.025$, Fig. 4B and Table 1). This

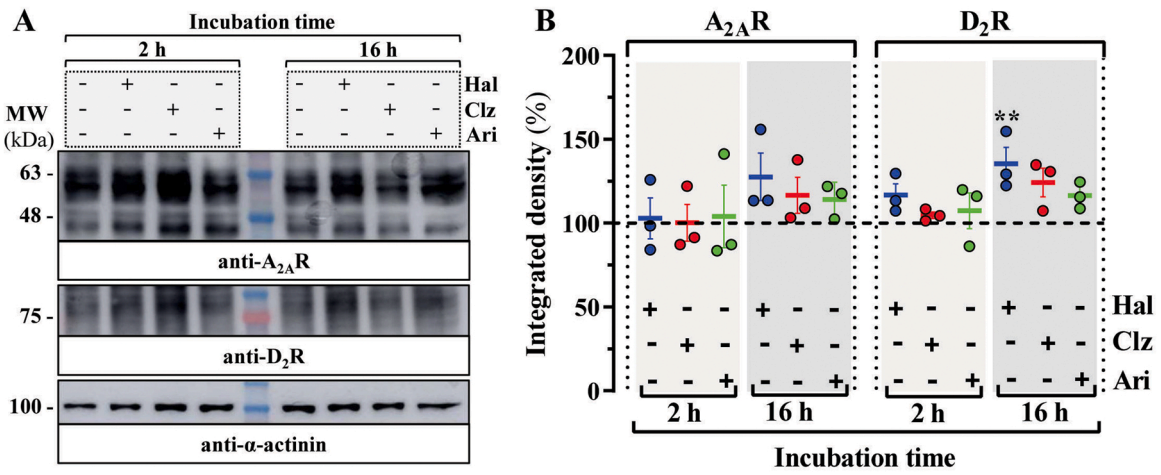

Fig. 3. Effect of antipsychotics on $A_{2A}R$ and D_2R density in living cells. (A) Immunoblots showing $A_{2A}R$ and D_2R density in HEK-293 T cells. HEK-293 T cells transiently transfected with $A_{2A}R^{LgBiT}$ and D_2R^{SmBiT} were treated with 1 μM of haloperidol (Hal), clozapine (Clz) or aripiprazole (Ari) for 2 h and 16 h. Cell extracts were analyzed by SDS-PAGE (20 μg of protein/lane) and immunoblotted using mouse anti-$A_{2A}R$, rabbit anti-D_2R, and mouse anti-α-actinin antibodies. The immunoblots shown are representative examples from a total of three independent experiments. (B) Relative quantification of $A_{2A}R$ and D_2R density. The immunoblot protein bands corresponding to $A_{2A}R$, D_2R and α-actinin were quantified by densitometric scanning. Density values were normalized by the respective density of the α-actinin band in each lane to correct for protein loading. Results from three independent experiments are expressed as percentages (mean ± SEM) of the receptor densities of vehicle treated cells. * *p < 0.01, three-way ANOVA with Bonferroni's *post-hoc* test when compared to vehicle treated cells (dashed line).

Table 1
Computational simulations of antipsychotic compounds bound to the D_2R monomer or $A_{2A}R$-D_2R heteromer. Average values (in Å) of key properties in three replicas of unbiased 1 μs MD simulations.

	ligand	property	replica 1[a]	replica 2[a]	replica 3[a]	p-value[b]
	—	(x,y) TM 5 D_2R[c]	(9.4, −3.6)	(9.3, −3.4)	(10.0, −3.9)	
	Aripiprazole		(9.9, −3.9)	(10.0, −3.9)	(9.7, −3.6)	(0.188, 0.173)
D_2R	Haloperidol		(9.2, −3.3)	(9.6, −4.1)	(9.6, −3.7)	(0.412,0.413)
	Raclopride		(9.5, −3.8)	(9.5, −4.7)	(9.8, −4.3)	(0.253,0.063)
	Clozapine		(10.1, −5.3)	(9.8, −4.6)	(10.3, −4.6)	(0.063,0.023)
$A_{2A}R$-D_2R	—	(x,y) TM 4 D_2R[d]	(3.4, −13.5)	(3.7, −13.6)	(3.0, −13.4)	
	Clozapine		(3.4, −13.5)	(3.6, −13.8)	(3.7, −13.8)	(0.250,0.089)
	—	(x,y) TM 5 D_2R[e]	(9.8, −3.2)	(9.6, −3.4)	(9.9, −3.6)	
	Clozapine		(10.4, −5.6)	(10.4, −5.8)	(10.8, −5.0)	(0.023,0.025)
	—	(x,y) TM 4 $A_{2A}R$[f]	(15.9, −12.6)	(15.9, −12.6)	(17.7, −11.6)	
	Clozapine		(15.4, −16.6)	(17.0, −15.1)	(16.7, −14.4)	(0.412,0.023)
	—	(x,y) TM 5 $A_{2A}R$[g]	(7.9, −20.9)	(8.2, −21.0)	(10.8, −20.7)	
	Clozapine		(6.9, −23.8)	(8.7, −23.1)	(8.0, −21.6)	(0.206,0.025)
	—	distance between centers of mass[h]	24.4	25.1	23.2	
	Clozapine		28.4	27.4	27.5	0.025

[a]Average values of 100 structures collected every 10 ns
[b]Statistical significant was calculated by Mann−Whitney test, one-tailed, n1 = n2 = 3, relative to unliganded receptor. P-values < 0.05 are shown in bold
[c-g]Center of mass of amino acids [c]Y5.41-I5.44 of D_2R, and [d]T4.55-P4.59 and [e]Y5.41-V5.45 of D_2R and [f]I4.56-P4.60 [g]Y5.40-F5.44 of $A_{2A}R$ in the $A_{2A}R$-D_2R heteromer
[h]Distance between the centers of mass of D_2R and $A_{2A}R$ in the $A_{2A}R$-D_2R heteromer.

clozapine-induced change in TM 5 of D_2R triggers the displacement of TM 4 (3.1 Å, p = 0.023) and TM 5 (2 Å, p = 0.025) of $A_{2A}R$, the TM domains that form the heteromic interface (Fig. 4B and Table 1). Consequently, $A_{2A}R$ moves 3.5 Å apart from D_2R (measured by the distance between the centers of mass of $A_{2A}R$ and D_2R, p = 0.025, Fig. 4B and Table 1), in a rigid body movement in which the deformation of $A_{2A}R$ is negligible. In summary, these MD simulations indicate that the bulky aromatic ring of clozapine destabilizes the heteromerization of $A_{2A}R$-D_2R.

ECL 2 of D_2R in the extended conformation folds over the binding pocket, whereas ECL 2 in the helical conformation, stabilized by haloperidol and aripiprazole binding, orients the G4.63-Q179^{ECL2} stretch of amino acids toward $A_{2A}R$ (Fig. 5A), which could influence $A_{2A}R$-D_2R heteromerization in this extracellular part. Thus, to understand the role of these amino acids in the formation of the TM 4/5 interface of $A_{2A}R$-D_2R, we performed MD simulations of $A_{2A}R$-$D_2R^{extended}$ (5 ×1 μs) in which ECL 2 was modelled in the extended conformation and $A_{2A}R$-$D_2R^{helical}$ (5 ×1 μs) in which ECL 2 was modelled in the helical conformation without ligand bound to them (*see* Methods). The calculated energy of interaction of full TM 4/5 interface shows no significant differences between extended and helical conformations (p = 0.71), but when this analysis is performed only at the extracellular part of the TM 4/5 interface, ECL 2 in the helical conformation forms a stronger interaction with $A_{2A}R$ than in the extended conformation (p = 0.001) (Fig. 5C). In summary, these simulations indicate that, contrary to clozapine, haloperidol and aripiprazole stabilize $A_{2A}R$-D_2R heteromerization by stabilizing the helical conformation of ECL 2 of D_2R.

4. Discussion

Using a NanoBiT-based live cell assay, the present study found evidence for a differential effect of clozapine, compared to haloperidol and aripiprazole, on the dynamics of $A_{2A}R$-D_2R heteromerization. Clozapine disrupted $A_{2A}R$-D_2R heterodimer after 2 h of treatment, while haloperidol and aripiprazole promoted its formation after 16 h of incubation. MD simulations provided mechanistic explanations for the heteromer-

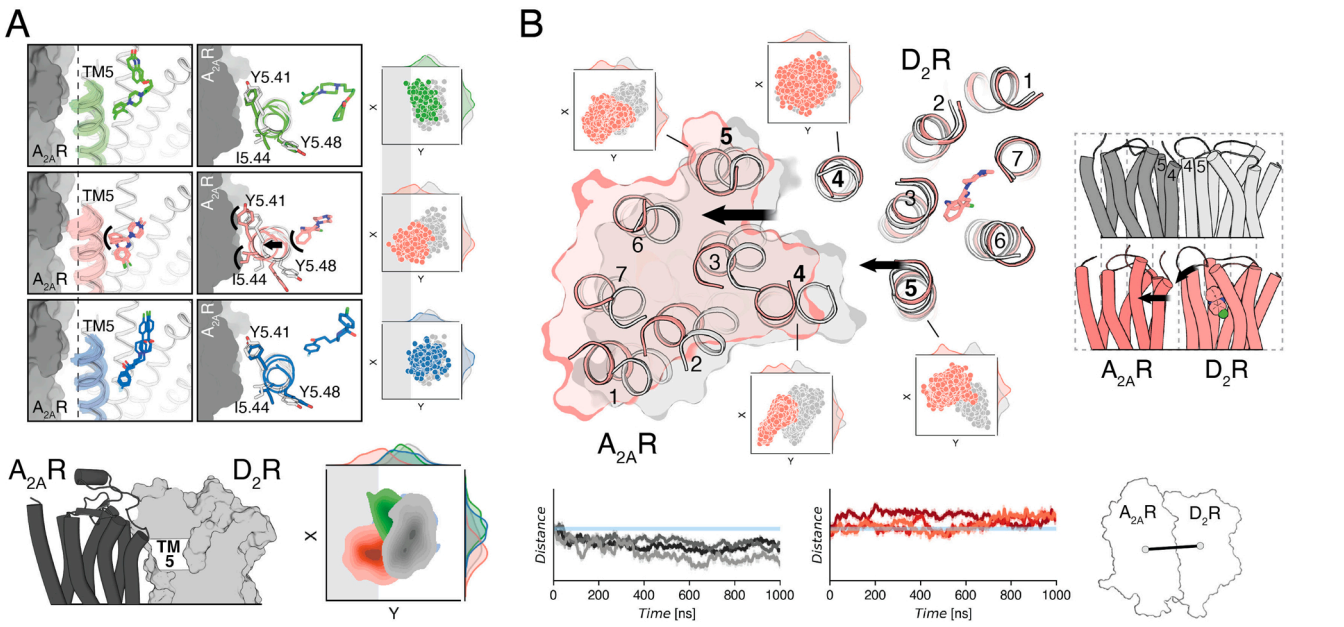

Fig. 4. Influence of clozapine binding to D_2R on the $A_{2A}R$-D_2R heteromer. (A) Representative side (left panels) and upper (middle panels) views of TM 5 and the Y5.41, I5.44, and Y5.48 side chains obtained in MD simulations of the D_2R monomer in the absence (in grey) and presence of haloperidol, clozapine, or aripiprazole (color code as in Fig. 3). The surface of $A_{2A}R$ in grey is shown as a reference but is not included in the simulations. Grey broken line represents the position of TM 5 in the simulation of apo-D_2R, black arrow represents the movement of TM 5 in the presence of clozapine relative to apo-D_2R, and black lines represent the aromatic ring of clozapine and the amino acids of D_2R that would clash with $A_{2A}R$. The evolution of the center of mass of amino acids Y5.41-I5.44 in TM 5 during three replicates of unbiased 1 μs MD simulations (100 structures collected every 10 ns in each replicate) is shown in right panels. The xy plane is as defined by the Orientations of Proteins in Membranes (OPM) [81]. Distributions of the x and y values are shown on the right x-axis and top y-axis, respectively. Grey rectangle represents the area in the y axis that is not occupied by the amino acids of TM5 in the apo simulations. Contour plots and distributions of X,Y values (*see* Table S1) illustrated above for comparison purposes. (B) Representative structures obtained in MD simulations of the $A_{2A}R$-D_2R heteromer in the absence (grey) and presence of clozapine (pink). Evolution of the center of mass of amino acids T4.55-P4.59 in TM 4 and Y5.41-V5.45 in TM 5 of D_2R and I4.56-P4.60 in TM 4 and Y5.40-F5.44 in TM 5 of $A_{2A}R$ during three replicas of unbiased 1 μs MD simulations (100 structures collected every 10 ns in each replica) and their distributions. The xy plane is defined as above. Black arrows represent the movement of TM helices in the presence of clozapine relative to apo-D_2R. The side view on the $A_{2A}R$-D_2R heteromer (right panels) displays a grid to evaluate the movement of $A_{2A}R$ relative to D_2R. The proximity between $A_{2A}R$ and D_2R within the heteromer was monitored by the distance between centers of mass of $A_{2A}R$ and D_2R in the absence (grey) and presence of clozapine (pink). Blue line shows the initial distance that decreases in apo-D_2R and increases in the presence of clozapine.

destabilizing effect of clozapine and the heteromer-stabilizing properties of haloperidol and aripiprazole. The bulky tricyclic moiety of clozapine displaces TM 5 of D_2R, altering the $A_{2A}R$-D_2R heteromeric interface, increasing the mean distance between the $A_{2A}R$ and D_2R protomers in the heteromer. In contrast, the extended binding mode of haloperidol and aripiprazole toward the extracellular domain stabilizes the helical conformation of ECL 2 of D_2R, enhancing the interaction with $A_{2A}R$.

Interestingly, our results are complementary to those of a recent study showing a reduced pharmacological chaperone activity (pharmacoperone) of clozapine, compared to other antipsychotics, including haloperidol and aripiprazole [66]. In this study, clozapine showed the lowest pharmacoperone efficacy to translocate D_2R to the plasma membrane of transfected cells. Furthermore, clozapine was the only antipsychotic for which the cell surface D_2R normalized to the total receptor significantly decreased (~ 60% of vehicle) after 24 h of treatment with 10 μM of clozapine [66]. In fact, this differential effect could then represent an explanatory mechanism for the well-established up-regulation of striatal D_2R after prolonged treatment with all antipsychotics other than clozapine [61–65]. Upregulated D_2R has been assumed to be reflected as a sensitization to the effects of endogenous dopamine, which could explain hyperkinetic/dyskinetic EPS, such as tardive dyskinesia, but also iatrogenic psychoses and resistance of schizophrenia patients to pharmacotherapy over time [72–74]. However, as discussed in the same study, no effective activation of D_2R with dopamine could be detected during exposure to antipsychotics at the concentrations necessary to promote up-regulation of the cell surface of D_2R, since, in fact, these concentrations are associated with a high occupancy of the receptor with D_2R antagonists [66].

The present results circumvent this conundrum by suggesting that it is not simply the increase in the number of D_2Rs in the plasma membrane, but the increase in the number of D_2Rs that form heteromers with $A_{2A}R$, or a relative decrease in the number of D_2R or $A_{2A}R$ not forming heteromers, what facilitates the appearance of EPS upon long exposure to antipsychotics. Indirect evidence for the differential effect of haloperidol and clozapine on $A_{2A}R$-D_2R heteromerization had previously been described by their differential ability to increase the locomotor activating effect of a non-selective adenosine receptor antagonist theophylline [38,75], which, as with caffeine, should depend on allosteric interactions within the striatal $A_{2A}R$-D_2R heteromer [21,76]. Clozapine would then be protective against EPS due to its specific ability to destabilize $A_{2A}R$-D_2R heteromerization, while haloperidol and aripiprazole would facilitate $A_{2A}R$-D_2R heteromerization and, therefore, favour EPS. Indeed, this adds an additional layer of complexity to our initial interpretation of the role of $A_{2A}R$-D_2R heteromerization in schizophrenia, where we claimed that an increase in $A_{2A}R$-D_2R heteromers may be of therapeutic importance [36]. We base this assumption on results showing a decrease in the density of striatal $A_{2A}R$-D_2R heteromers in both schizophrenic patients and in a mouse PCP model of psychosis; as well as in the ability of haloperidol, but not clozapine, to significantly counteract the PCP-induced decrease in $A_{2A}R$-D_2R heteromerization [36]. However, in the same study, the density of D_2R and $A_{2A}R$ in the striatum of schizophrenia patients also increased, indicating a change in stoichiometry in favour of receptors that do not form heteromers [36]. As a result, it could then be possible that the density of $A_{2A}R$s that form and do not form heteromers determines the behavioral/clinical output of D_2R blockade, where A2ARs do not form

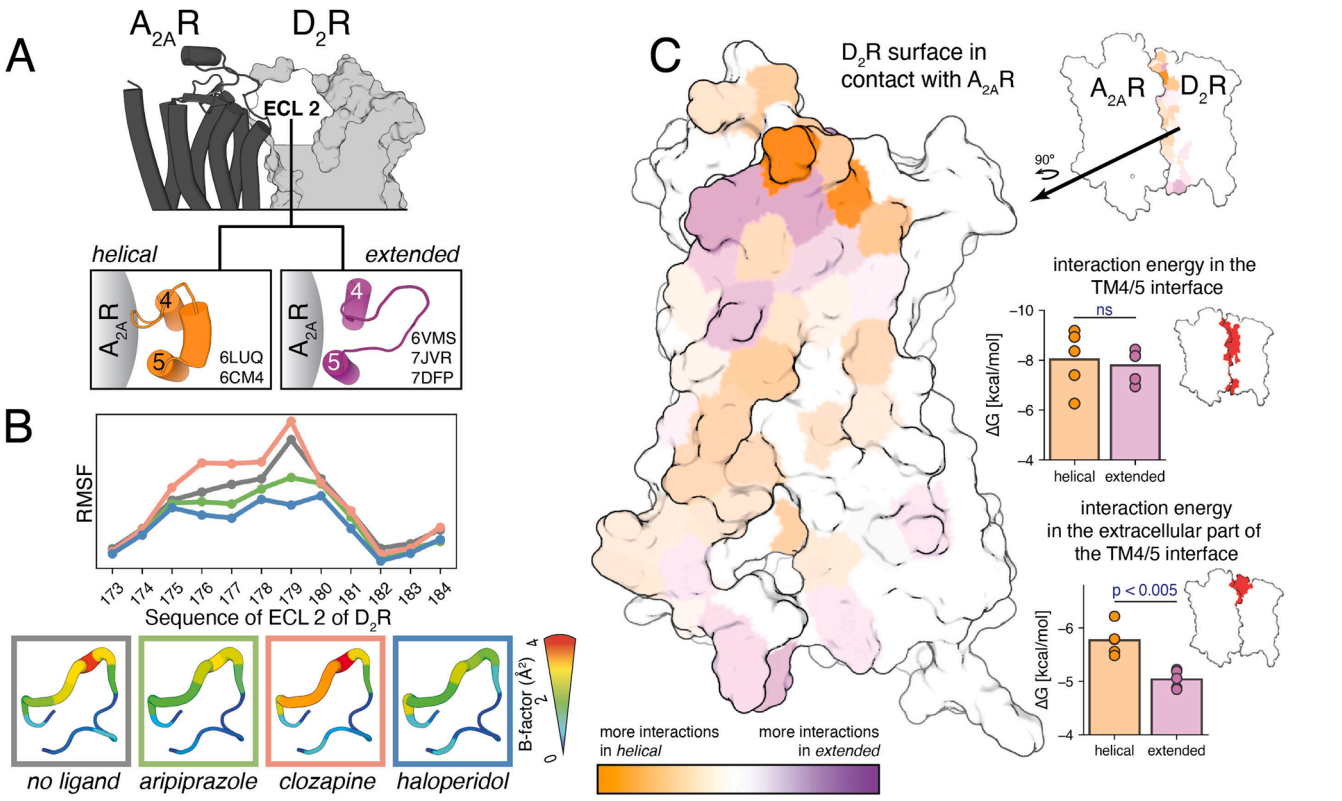

Fig. 5. Influence of haloperidol and aripiprazole binding to D_2R on the $A_{2A}R$-D_2R heteromer. (A) ECL 2 of D_2R, between TMs 4 and 5, adopts a helical conformation in the 6LUQ and 6CM4 structures and an extended conformation in the 6VMS, 7JVR, and 7DFP structures. ECL 2 is oriented toward $A_{2A}R$ in the helical conformation and folds over the binding pocket in the extended conformation. (B) Average root-mean-square fluctuation (RMSF) during three replicates of unbiased 1 μs MD simulations (100 structures collected every 10 ns in each replicate) of ECL 2 of D_2R, in the helical conformation, in the absence and presence of haloperidol, clozapine, or aripiprazole (color code as in Fig. 3). Detailed view of ECL 2 of D_2R and associated B-factor values. (C) Surface representation of D_2R in contact with $A_{2A}R$ in the $A_{2A}R$-D_2R heteromer, color coded by the difference in the number of interactions in the helical or extended conformation of ECL 2 of D_2R, as calculated during five replicates of unbiased 1 μs MD simulations (100 structures collected every 10 ns in each replicate) of $A_{2A}R$-$D_2R^{extended}$ and $A_{2A}R$-$D_2R^{helical}$ in which ECL 2 was modelled in the extended and helical conformation, respectively, without ligand bound to them (*see* Methods). Calculated energies of interaction, with PRODIGY [58], between $A_{2A}R$ and $D_2R^{helical}$ and $A_{2A}R$ and $D_2R^{extended}$ by the amino acids (in red) forming the TM 4/5 interface (top) and the extracellular part of the TM4/5 interface (bottom). Statistical significance was calculated by non-parametric Mann−Whitney test.

heteromers protecting against EPS. A striking functional difference between both $A_{2A}R$ populations is the disappearance of the strong constitutive activity of $A_{2A}R$ when forming heteromers with D_2R [77]. This constitutive activity could then be responsible for the protective effect of $A_{2A}R$ not forming heteromers against EPS, and would be reduced by haloperidol and aripiprazole, but not by clozapine treatment.

Another mechanism by which an increase in $A_{2A}R$-D_2R heteromerization could increase EPS liability could be the $A_{2A}R$-D_2R heteromer-dependent increase in D_2R-mediated recruitment of β-arrestin (*see* Introduction) [24–27]. This would apparently disagree with the generalized hypothesis that advocates that blocking of β-arrestin-dependent and G protein-dependent D_2R signaling mediates therapeutic effects and EPS, respectively [13,14,18], implying that β-arrestin-dependent D_2R signaling is not involved in EPS, a hypothesis based on preclinical results in animal models. However, it is not even entirely clear what the functional role of D_2R-mediated recruitment of β-arrestin in animal models is. In these preclinical studies, biased ligands that preferentially engage D_2R-mediated β-arrestin-dependent signaling were shown to inhibit psychostimulant-induced locomotion in mice, suggesting that β-arrestin signaling inhibits locomotion [13,14,18]. However, a recent study showed clear evidence indicating that β-arrestin recruitment can drive locomotion even in the absence of D_2R-mediated G protein signaling. Therefore, induction of a D_2R mutant recruiting β-arrestin but not G-protein in the ventral striatum of mice lacking D2R restored basal locomotor activity and cocaine-induced locomotor activation, while it did not increase incentive motivation [78].

Several studies indicate that GPCR heteromers should be considered as targets for drug development (for review, *see* [79]). In particular, the $A_{2A}R$-D_2R heteromer is a target of new approaches to the treatment of Parkinson's disease, using $A_{2A}R$ antagonists such as istradephylline [80]. As with caffeine and theophylline, the therapeutic efficacy of $A_{2A}R$ antagonists is based on allosteric interactions within the heteromer, by which $A_{2A}R$ blockade increases the effect of L-DOPA or selective D_2R agonists [21,23,76]. Overall, the present study opens a new rationale for considering GPCR heteromers as therapeutic targets in psychosis: the possibility of changing the density and stability of GPCR heteromers.

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CRediT authorship contribution statement

Marta Valle-León: Data curation, Methodology. **Nil Casajuana-**

Martin: Data curation, Methodology. **Claudia Llinas del Torrent:** Data curation, Methodology. **Josep Argerich:** Methodology. **Laura Gómez-Acero:** Methodology. **Kristoffer Sahlholm:** Writing – review & editing. **Sergi Ferré:** Writing – review & editing. **Leonardo Pardo:** Writing – review & editing. **Francisco Ciruela:** Conceptualization, Writing – review & editing.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114327.

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