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Antipsychotic-Like Efficacy of Dopamine D_2 Receptor-Biased Ligands is Dependent on Adenosine A_{2A} Receptor Expression

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Abstract Dopamine D_2 receptor (D_2R) activation triggers both G protein- and β-arrestin-dependent signaling. Biased D_2R ligands activating β-arrestin pathway have been proposed as potential antipsychotics. The ability of D_2R to heteromerize with adenosine A_{2A} receptor ($A_{2A}R$) has been associated to D_2R agonist-induced β-arrestin recruitment. Accordingly, here we aimed to demonstrate the $A_{2A}R$ dependence of D_2R/β-arrestin signaling. By combining bioluminescence resonance energy transfer (BRET) between β-arrestin-2 tagged with yellow fluorescent protein and bimolecular luminescence complementation (BiLC) of $D_2R/A_{2A}R$ homomers and heteromers, we demonstrated that the D_2R agonists quinpirole and UNC9994 could promote β-arrestin-2 recruitment only when $A_{2A}R/D_2R$ heteromers were expressed. Subsequently, the role of $A_{2A}R$ in the antipsychotic-like activity of UNC9994 was assessed in wild-type and $A_{2A}R^{-/-}$ mice administered with phencyclidine (PCP) or amphetamine (AMPH). Interestingly, while UNC9994 reduced hyperlocomotion in wild-type animals treated either with PCP or AMPH, in $A_{2A}R^{-/-}$ mice, it failed to reduce PCP-induced hyperlocomotion or produced only a moderate reduction of AMPH-mediated hyperlocomotion. Overall, the results presented here reinforce the notion that $D_2R/A_{2A}R$ heteromerization facilitates D_2R β-arrestin recruitment, and furthermore, reveal a pivotal role for $A_{2A}R$ in the antipsychotic-like activity of the β-arrestin-biased D_2R ligand, UNC9994.

Keywords Functional selectivity, biased ligand, dopamine D_2 receptor · Adenosine A_{2A} receptor · Oligomerization · β-arrestin · BRET

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Introduction

Schizophrenia is a severe mental disorder that affects approximately 1% of the general population [1]. It is characterized by positive (e.g., delusions, hallucinations), negative (e.g., social withdrawal, lack of emotional expression), and cognitive symptoms, which are typically of lifelong duration [2]. Current treatment is based on dopamine D_2 receptor (D_2R) antagonists or weak partial agonists, which may block the excessive dopaminergic activity in the mesolimbic pathway thought to underlie positive symptoms [3]. Antipsychotics are classified into typical and atypical (or first and second generation, respectively) drugs, based on their side-effect profiles. Typical antipsychotics are effective in reducing positive, but not negative, symptoms, but prone to cause severe Parkinson-like motor side effects, so-called extrapyramidal symptoms. Atypical antipsychotics have lower propensity to disturb motor function, but while effective against positive symptoms, still fail to adequately address negative and cognitive symptoms [2, 3].

D_2R is coupled to several intracellular signaling pathways, including the classical $G_{\alpha i/o}$ pathway and the more recently

discovered β-arrestin pathway, each of which can be activated to varying extents by different D_2R ligands [4]. Recent studies found that current antipsychotics are antagonists or partial agonists at both the $G_{\alpha i/o}$- and the β-arrestin pathways [5, 6]. It was further suggested that antipsychotic efficacy might be conferred by modulation of β-arrestin signaling, while a reduction of $G_{\alpha i/o}$-pathway activity would be responsible for extrapyramidal symptoms. Interestingly, by modifying the scaffold of the partial agonist antipsychotic, aripiprazole, a new series of D_2R selective ligands, the "UNC family," was recently developed [7]. These ligands act as partial agonists for β-arrestin recruitment, without eliciting G protein-dependent signaling, and show antipsychotic-like efficacy and low propensity for motor inhibition in preclinical animal models assessing potential antipsychotic activity [8, 9]. Since β-arrestin expression is higher in cortex compared to the striatum, it was suggested that these ligands, by means of their partial agonist activity at β-arrestin recruitment, may exert agonist activity preferentially in cortical areas [9], offering a potential avenue towards simultaneously treating the striatal hyperdopaminergia and cortical hypodopaminergia believed to underlie positive and negative symptoms, respectively.

It is well established that direct receptor-receptor interactions between D_2R and $A_{2A}R$ occur in striatal medium spiny neurons, which modulate the output of striatal circuitry [10, 11]. In addition, $D_2R/A_{2A}R$ oligomerization has been shown to favor β-arrestin recruitment in heterologous systems [12, 13]. Hence, here we aimed to test whether the antipsychotic-like effects of one member of the UNC family, UNC9994, might involve $A_{2A}R$-dependent, D_2R-biased signaling. Accordingly, we first designed a robust methodology, based on bimolecular luminescence complementation (BiLC) and bioluminescence resonance energy transfer (BRET) to test the impact of $A_{2A}R$ expression on D_2R signaling bias. and thereafter, we examined the effects of UNC9994 in pharmacological mouse models of psychosis both in wild-type (WT) and $A_{2A}R$-deficient ($A_{2A}R^{-/-}$) mice.

Materials and Methods

Reagents

The ligands used were amphetamine (AMPH), CGS21680, quinpirole, phencyclidine (PCP) from Tocris Bioscience (Bristol, UK), and UNC9994 from Axon Medchem B.V. (Groningen, the Netherlands).

Plasmid Constructs

To perform BiLC, we used two complementary fractions of the *R*luc8 (*Renilla* luciferase 8) protein (L1 and L2) kindly provided by Dr. J.A. Javitch (University of Columbia, NY, USA). Both fractions were extracted from its template by digestion with XhoI and XbaI restriction enzymes, and inserted in a pcDNA3.1 vector (pcDNA3.1-L1 and pcDNA3.1-L2). Subsequently, the complementary DNA (cDNA) encoding D_2R [14] and $A_{2A}R$ [15] were amplified by a polymerase chain reaction using the following primers: FD2RHind (5′-GCCA AGCTTATGGTCCTTCTGTTGATCCTGTCAG-3′) and RD2REco (5′-CCGGAATTCGGCAGTGGAGGATCTTC AG-3′) and FA2AHind (5′-GCCAAGCTTATGGTCCTTCT GTTGATCCTGTCAG-3′) and RA2AXho (5′-GCGC TCGAGAGGACACTCCTGCTCCATCC-3′). Finally, the different PCR products were subcloned into the HindIII/EcoRI or HindIII/XhoI sites (for D_2R and $A_{2A}R$, respectively) of the above-mentioned pcDNA3.1-L1 and pcDNA3.1-L2 vectors. On the other hand, for BRET experiments, we also used G-proteins and β-arrestin-2 constructs containing a yellow fluorescent protein (YFP): $G_{\alpha s}^{YFP}$ and $G_{\alpha i}^{YFP}$, kindly provided by Dr. J.P. Vilardaga (University of Pittsburgh, Pittsburgh, USA), and β-arrestin-2^{YFP} [12].

Cell Culture and Transfection

Human embryonic kidney (HEK)-293 T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL streptomycin, 100 mg/mL penicillin, and 5% (*v*/*v*) fetal bovine serum at 37 °C and in an atmosphere of 5% CO_2. HEK-293 T cells growing in 25-cm^2 flasks or six-well plates containing 18-mm coverslips were used for BRET or fluorescence imaging, respectively. Cells were transiently transfected with the cDNA encoding the specified proteins using polyethylenimine (Polysciences Inc., Warrington, PA, USA).

Bioluminescence Resonance Energy Transfer Measurements

BRET experiments in HEK-293 T cells were performed as previously described [16]. In brief, HEK-293 T expressing the indicated constructs were rapidly washed, detached, and resuspended in HBSS buffer (137 mM NaCl, 5 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.26 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 10 mM HEPES, pH 7.4) containing 10 mM glucose. Cell suspensions (20 μg of protein) were distributed in 96-well microplate plates, incubated with the corresponding ligands, and finally, BRET was determined, after adding 5 μM h-coelenterazine (NanoLight Technology, Pinetop, AZ, USA), in a POLARstar Optima plate reader (BMG Labtech, Durham, NC, USA) as previously described [16].

Animals

CD-1 mice (Charles River Laboratories) and $A_{2A}R^{-/-}$ mice developed in a CD-1 genetic background [17] (animal facility of University of Barcelona) of around 3 months old were used. The University of Barcelona Committee on Animal Use and Care approved the protocol. Animals were housed and tested in compliance with the guidelines described in the Guide for the Care and Use of Laboratory Animals [18] and following the European Union directives (2010/63/EU). All efforts were made to minimize animal suffering and the number of animals used. All animals were housed in groups of five in standard cages with ad libitum access to food and water and maintained under 12-h dark/light cycle (starting at 7:30 AM), 22 °C temperature, and 66% humidity (standard conditions). Behavioral testing was performed in mice aged 2–3 months and between 2 and 7 PM.

Locomotor Activity

Horizontal locomotor activity was studied in an open-field arena measuring 30 × 30 cm, made from plywood, and painted black. Following i.p. injection with UNC9994 dissolved in physiological saline supplemented with 10% Tween-80 and 7.3% DMSO, or vehicle (VEH), animals were placed in the arena for an initial habituation period of 30 min, after which the animals were injected (i.p.) with 6 mg/kg PCP or 3 mg/kg AMPH (both dissolved in saline) and immediately returned to the arena for another 60 min. Locomotion was recorded by a camera placed above the arena and analyzed using ImageJ (National Institutes of Health, Bethesda, MD) together with an automated tracking plug-in (SpotTracker; Ecole Polytechnique Fédérale de Lausanne, Switzerland).

Statistics

The number of samples/animals (n) in each experimental condition is indicated in figure legends. Statistical analysis was performed by Student's t test and by two-way ANOVA followed by Bonferroni's multiple comparison post hoc test. Statistical significance is indicated for each experiment.

Results

A number of strategies have been designed to study biased GPCR signaling [19]. Here, we developed a novel approach to assay $G_{\alpha i/o}$- and β-arrestin signaling from the $D_2R/A_{2A}R$ oligomer. Accordingly, we used a BiLC/BRET assay, in which a BRET process between G-proteins/β-arrestin and receptors takes place only when receptors interact in close proximity (Fig. 1). Noteworthy, this kind of approach was first described as complemented donor-acceptor resonance energy transfer (CODA-RET), in which BRET was engaged after complementation of Rluc (the two complementary halves of Rluc separately fused to two different receptors) and YFP fused to a G_α subunit [20]. We first assessed bicomplementation of the donor molecules by determining luminescence after transfection of the corresponding D_2R and/or $A_{2A}R$ forms containing complementary *R*Luc fragments. Thus, we transfected increasing concentrations of *R*Luc1 and *R*Luc2-containing proteins ($A_{2A}R^{L1}/A_{2A}R^{L2}$, D_2R^{L1}/D_2R^{L2}, $A_{2A}R^{L1}/D_2R^{L2}$) in a 1:1 ratio and observed a BRET saturation bell-shaped curve in all cases (Supplementary Fig. 1a). Similarly, we examined acceptor molecules ($G_{\alpha s}{}^{YFP}$, $G_{\alpha i}{}^{YFP}$, β-arrestin-2^{YFP}) to achieve similar fluorescence levels (Supplementary Fig. 1b). Once donor and acceptor molecules had been characterized, we performed the BiLC/BRET assay by co-transfecting the constructs and challenging transfected cells with selective agonists (Fig. 1). First, we examined the ability of $A_{2A}R/A_{2A}R$ and D_2R/D_2R homodimers to interact with $G_{\alpha s}$ and $G_{\alpha i}$ proteins, respectively. Both the adenosine $A_{2A}R$ agonist CGS21680 (100 nM) and D_2R agonist quinpirole (100 nM) could recruit $G_{\alpha s}$ and $G_{\alpha i}$ proteins to $A_{2A}R^{L1}/A_{2A}R^{L2}$ and D_2R^{L1}/D_2R^{L2} homodimers, respectively (Fig. 2a, b). Of note, the former single doses of CGS21680 and quinpirole were chosen as those eliciting similar emission levels (Supplementary Fig. 2), and used for subsequent experiments. Next, we assessed whether the effects of selective agonists were affected upon $D_2R/A_{2A}R$ oligomerization. We observed that CGS21680-induced $G_{\alpha s}{}^{YFP}$ and quinpirole-induced $G_{\alpha i}{}^{YFP}$ recruitment produced emission levels like those obtained with the respective receptor homodimers (Fig. 2a, b), indicating that G-protein signaling was not modified when receptors heterodimerized. We then followed the same approach to evaluate the interaction of both homodimers and heterodimers with β-arrestin-2. Notably, both the $A_{2A}R^{L1}/A_{2A}R^{L2}$ homodimer and the $A_{2A}R^{L1}/D_2R^{L2}$ heterodimer recruited β-arrestin-2 when challenged with CGS21680, inducing a comparable BRET signal (Fig. 2c). Thus, although it cannot be excluded that the ability of $A_{2A}R$ to recruit β-arrestin-2 could be affected upon D_2R expression, we did not observe significant changes in the present conditions. Conversely, only the $A_{2A}R^{L1}/D_2R^{L2}$ oligomer but not the D_2R^{L1}/D_2R^{L2} homodimer interacted with β-arrestin-2 when challenged with quinpirole (Fig. 2d). Overall, these results indicate that $A_{2A}R$ co-expression increased the ability of D_2R to signal via the β-arrestin pathway.

As described above, a series of β-arrestin-biased D_2R ligands, namely, the UNC family, has been shown to have antipsychotic-like activity [8, 9]. Therefore, we aimed to elucidate whether the activity of these UNC compounds is $A_{2A}R$ dependent. To this end, we selected UNC9994 as it was recently demonstrated to be the most arrestin-selective drug, both in terms of its incapacity to antagonize G protein-dependent D_2R signaling in vitro, and its antipsychotic-like inefficacy in mice lacking β-arrestin-2 in D_2R-expressing neurons [7]. Accordingly, we evaluated the UNC9994 ability to selectively recruit β-arrestin-2 upon $A_{2A}R$ expression by

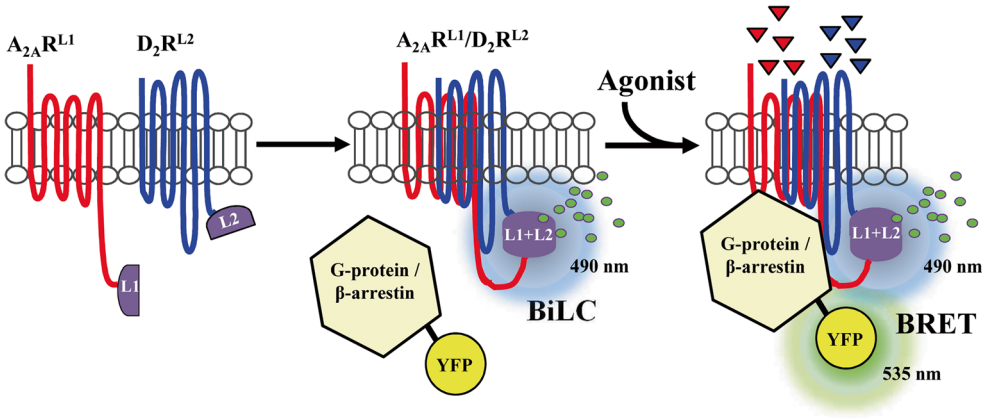

Fig. 1 Schematic representation of the BiLC/BRET assay. Agonist (*blue triangles*) binding to $A_{2A}R$/D_2R heterodimer complementing two halves of the *R*Luc8 protein (L1 and L2) prompted YFP-tagged G-protein or β-arrestin recruitment. The $A_{2A}R$/D_2R heterodimer interaction with either G-protein or β-arrestin was monitored by BiLC/BRET. *Red circles* indicate the luciferase substrate coelenterazine

using our BiLC/BRET assay. Interestingly, UNC9994 (300 nM) [7] produced a significant ($P < 0.05$) BRET signal between receptors and β-arrestin-2 only upon D_2R-$A_{2A}R$ heteromerization (Fig. 3). On the other hand, no BRET signal was observed between D_2R containing homodimers and heterodimers with $G_{\alpha i}^{YFP}$ (Fig. 3), as previously reported [7, 9]. Overall, UNC9994 β-arrestin-2-biased signaling was also shown to depend on $A_{2A}R$ expression in our heterologous system. Subsequently, we next aimed to correlate the observed in vitro functional selectivity with potential in vivo therapeutic effects in pharmacological mouse models of psychosis. To this end, we examined the ability of UNC9994 to reduce hyperlocomotion induced by PCP (6 mg/kg) or AMPH (3 mg/kg) in WT and $A_{2A}R^{-/-}$ mice. Of note, PCP- and AMPH-induced hyperactivity have become frequently used rodent models of psychosis, and its reversal by drugs is considered a useful measure for predicting clinical antipsychotic activity [21–23]. Interestingly, while 10 mg/kg UNC9994 (i.p.) robustly reduced PCP-induced hyperlocomotion in WT animals (Fig. 4a), it was ineffective in $A_{2A}R^{-/-}$ mice (Fig. 4b). Thus, when examining the cumulative distance traveled (Fig. 4c), the two-way ANOVA analysis revealed a significant effect of UNC9994 treatment [$F_{(1, 37)} = 17.54$, $P < 0.001$], but not of genotype, and a significant interaction between genotype and drug treatment [$F_{(1, 37)} = 6.63$, $P < 0.05$]. Also, Bonferroni-corrected pairwise comparisons detected a significant difference between UNC9994-pretreated WT and $A_{2A}R^{-/-}$ animals (*$P < 0.05$), whereas no differences were observed between vehicle (VEH)-pretreated WT and $A_{2A}R^{-/-}$ animals. Overall, only upon $A_{2A}R$ expression, an effect of UNC9994 on reducing PCP-induced hyperlocomotion was observed. On the other hand, we also assessed the effects of AMPH on hyperlocomotion. Interestingly, UNC9994 (10 mg/kg, i.p.) significantly reduced AMPH-induced hyperlocomotion both in WT and $A_{2A}R^{-/-}$ mice (Fig. 5a, b). However, a close view of

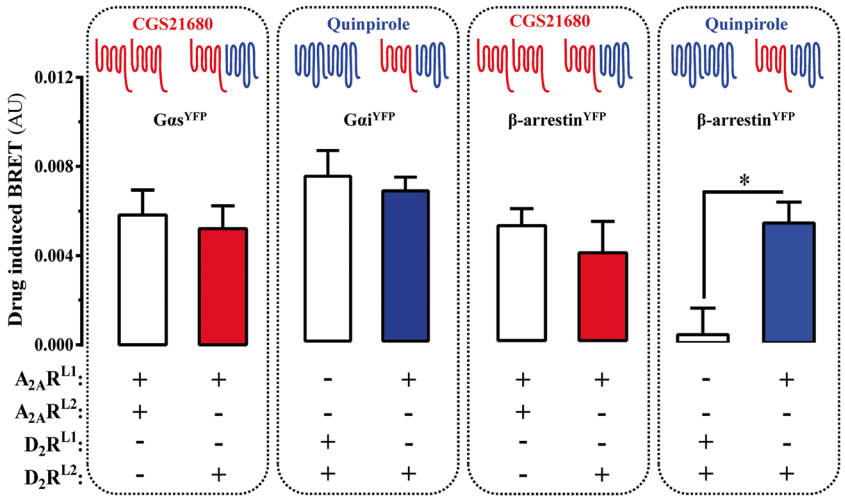

Fig. 2 $A_{2A}R$-dependent biased activation of the D_2R. **a**, **c** BRET was measured in HEK293T cells co-expressing either $A_{2A}R^{L1}/A_{2A}R^{L2}$ homodimers or $A_{2A}R^{L1}/D_2R^{L2}$ heterodimers as donors and $G_{\alpha s}^{YFP}$ or β-arrestin-2^{YFP} proteins as acceptors (as indicated in each panel), and challenged with the selective $A_{2A}R$ agonist CGS21680 (100 nM). **b**, **d** BRET was measured in HEK293T cells co-expressing either D_2R^{L1}/D_2R^{L2} homodimers or $A_{2A}R^{L1}/D_2R^{L2}$ heterodimers as donors and $G_{\alpha i}^{YFP}$ or β-arrestin-2^{YFP} proteins as acceptors (as indicated in each panel), and challenged with the selective D_2R agonist quinpirole (100 nM). Data (expressed as arbitrary units (AUs)) represent the mean ± SEM of at least three independent experiments. Statistical significance was assessed using a paired Student's *t* test (*$P < 0.05$)

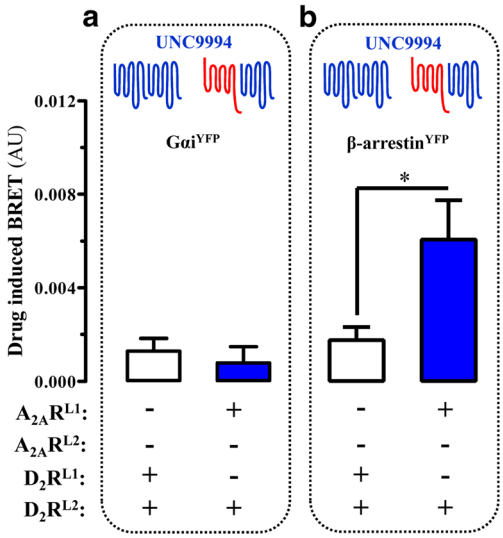

Fig. 3 UNC9994 as a β-arrestin-2-biased D_2R ligand in the BiLC/BRET assay. BRET was measured in HEK293T cells co-expressing either D_2R^{L1}/D_2R^{L2} homodimers or $A_{2A}R^{L1}/D_2R^{L2}$ heterodimers as donors and $G_{\alpha i}{}^{YFP}$ or β-arrestin-2^{YFP} proteins as acceptors (as indicated in each panel), and challenged with UNC9994 (300 nM). Data (expressed as arbitrary units (AUs)) represent the mean ± SEM of at least three independent experiments. Statistical significance was assessed using a paired Student's *t* test (*$P < 0.05$)

the results obtained showed that the effect of UNC9994 in $A_{2A}R^{-/-}$ mice was lower than in WT mice. Accordingly, when analyzing the cumulative distance traveled (Fig. 5c), the two-way ANOVA revealed significant main effects of UNC9994 treatment ($F_{(1,\ 36)} = 32.25$, $P < 0.001$) and genotype ($F_{(1,\ 36)} = 7.74$, $P < 0.01$), but no significant interaction between genotype and drug treatment. Also, Bonferroni-corrected pairwise comparisons detected a significant difference between UNC9994-pretreated WT and $A_{2A}R^{-/-}$ animals (*$P < 0.05$), whereas no such difference was observed between VEH-pretreated WT and $A_{2A}R^{-/-}$ animals. Overall, the ability of UNC9994 to block AMPH-induced hyperlocomotion was reduced in the absence of $A_{2A}R$ expression, thus supporting the notion that $A_{2A}R$ may lead to the biased activity of the D_2R ligand.

Discussion

The development of drugs displaying antipsychotic efficacy without extrapyramidal side effects is a major goal in drug discovery. Biased D_2R agonists triggering β-arrestin activation without G-protein coupling have been postulated to be potential antipsychotic candidates [7–9]. Interestingly, the concept of functional selectivity or biased signaling has emerged in recent years as a novel mechanism to optimize drug therapeutic actions. Thus, functionally selective ligands, by promoting distinct conformational rearrangements and preferential activation of signaling pathways, may lead to different receptors' signaling outcomes (for review, see [24–26]). Accordingly, these kinds of drugs, by discriminating mechanisms leading to therapeutic or undesired effects, may potentially permit to achieve better benefit/risk balances. Here, we assessed the impact of $A_{2A}R$ in the antipsychotic-mediated effects of UNC9994, a D_2R/β-arrestin bias compound. Our hypothesis was based on previous results indicating that striatal allosteric D_2R-$A_{2A}R$ interactions favor D_2R β-arrestin-2 recruitment [12, 13].

We developed a new BiLC/BRET assay allowing the study of $D_2R/A_{2A}R$ heteromer signaling. Thus, by using our BiLC/BRET approach, we unequivocally demonstrated that D_2R agonist-mediated β-arrestin-2 recruitment was $A_{2A}R$ dependent. While our results are in agreement to those showing $D_2R/A_{2A}R$ heteromer-dependent, D_2R agonist-mediated β-arrestin-2 recruitment [12, 13], we further demonstrated for the first time the $D_2R/A_{2A}R$/β-arrestin-2 trimeric formation upon agonist challenge. Indeed, it has been reported that upon

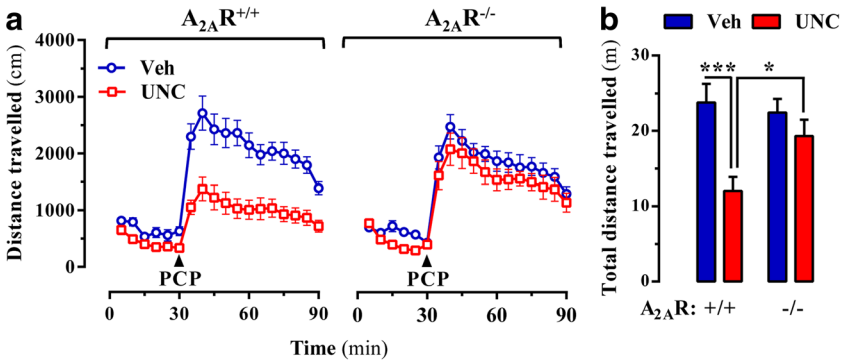

Fig. 4 Effects of the β-arrestin-2-biased D_2R ligand, UNC9994, on PCP-induced hyperlocomotion in WT and $A_{2A}R^{-/-}$ mice. **a**, **b** PCP-induced locomotor activity was assessed in WT and $A_{2A}R^{-/-}$ mice (n = 10–12) either pretreated with vehicle (VEH) or UNC9994 (UNC). VEH or UNC (10 mg/kg, i.p.) was administered immediately prior to introducing the animals to the arena, whereas PCP (6 mg/kg, i.p.) was given at 30 min. **c** Quantified locomotor activity in WT and $A_{2A}R^{-/-}$ animals pretreated with VEH or UNC after PCP administration. Significant differences were found between UNC-pretreated WT and $A_{2A}R^{-/-}$ animals (*P;0.05, two-way ANOVA followed by Bonferroni-corrected pairwise comparisons)

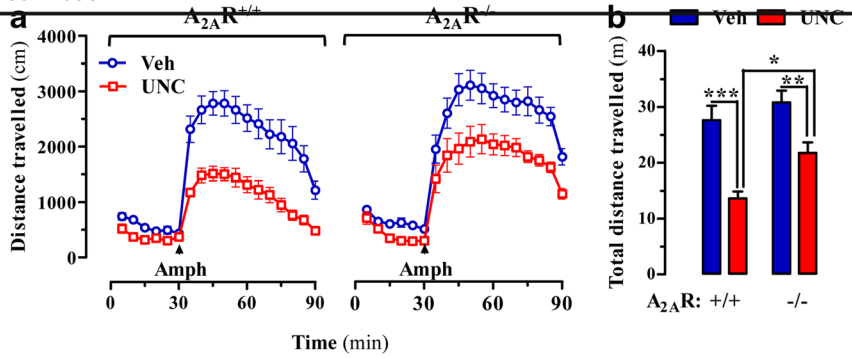

Fig. 5 Effects of the β-arrestin-biased D_2R ligand, UNC9994, on AMPH-induced hyperlocomotion in WT and $A_{2A}R^{-/-}$ mice. **a**, **b** AMPH-induced locomotor activity was assessed in WT and $A_{2A}R^{-/-}$ mice (n = 10) pretreated with vehicle (VEH) or UNC9994 (UNC). VEH or UNC (10 mg/kg, i.p.) was administered immediately prior to introducing the animals to the arena, whereas AMPH (3 mg/kg, i.p.) was given at 30 min. **c** Quantified locomotor activity in WT and $A_{2A}R^{-/-}$ animals pretreated with UNC or VEH after AMPH administration. Significant differences were found between UNC-pretreated WT and $A_{2A}R^{-/-}$ animals (*P < 0.05, two-way ANOVA followed by Bonferroni-corrected pairwise comparisons)

certain experimental conditions (e.g., high D_2R agonist concentrations or different D_2R/β-arrestin-2 ratios), the D_2R is able to signal through β-arrestin in an $A_{2A}R$-independent manner [9]. Nevertheless, the $A_{2A}R$ dependencies of D_2R/β-arrestin-signaling under physiological conditions are yet unexplored. Thus, we assessed the impact of $A_{2A}R$ in D_2R/β-arrestin signaling in vivo by evaluating the UNC9994-mediated antipsychotic-like effect in $A_{2A}R$-deficient mice. Interestingly, our behavioral data point to a scenario in which the striatal D_2R/$A_{2A}R$-β-arrestin-2 module would be involved in the antipsychotic-like effects of UNC9994. Needless to say, although $A_{2A}R$ has a very restrictive localization in the brain, thus being mainly expressed in the striatum and olfactory bulb [27], it cannot be excluded the possibility that allosteric $A_{2A}R$-D_2R interactions in other brain areas may participate in modulating the effects of UNC9994. In the striatum, the $A_{2A}R$ exerts a fine-tuning regulation of D_2R activity [28], which among other mechanisms may involve the enhancement of β-arrestin-2 recruitment. In order to ascertain whether the in vivo actions of a β-arrestin-biased ligand (i.e., UNC9994) was effectively dependent on $A_{2A}R$ expression, we evaluated the effects of UNC9994 on PCP- and AMPH-induced hyperlocomotion in WT and $A_{2A}R^{-/-}$ mice. In agreement with previous reports [7, 9], UNC9994 significantly reduced both PCP- and AMPH-induced hyperactivity in WT mice. Conversely, in $A_{2A}R^{-/-}$ mice, UNC9994 failed to block PCP-mediated locomotor effects and partially inhibited AMPH-induced hyperlocomotion when compared to WT animals.

Our findings may be considered within the framework of the recent Urs and collaborator's work [7]. Importantly, these authors demonstrated that the action of UNC9994 was dependent on β-arrestin-2 expression both in cortical and striatal regions. Thus, although UNC9994 efficacy against PCP-induced hyperlocomotion persisted in mice where β-arrestin-2 had been selectively deleted in $A_{2A}R$-expressing neurons, a loss of efficacy against AMPH-induced hyperlocomotion was observed in such animals, seemingly conflicting with the present findings. However, these authors further demonstrated a dual involvement of striatal and cortical mechanisms in mediating the behavioral effects of both PCP and UNC9994. Thus, PCP-induced hyperlocomotion was itself reduced when D_2R was deleted in $A_{2A}R$-expressing neurons, and UNC9994 activity against PCP-induced hyperlocomotion persisted also when β-arrestin-2 was selectively deleted in prefrontal cortex. Hence, some of the discrepancies observed between the present study and that of Urs and collaborators may be explained by the fact that several different brain regions (including cortical and striatal) are involved in mediating the behavioral output. On the other hand, it should be noted that although from our data it might be inferred that the effects of UNC9994 regulating locomotor activity are dependent both on $A_{2A}R$ and β-arrestin-2 expressed at GABAergic striatopallidal neurons, $A_{2A}R$ expression is not only circumscribed to the former neurons. In such way, $A_{2A}R$s are also located at corticostriatal terminals and astroglia tightly regulating glutamate transport activity; thus, they may be involved on the control of psychostimulant or psychomimetic-induced effects, as previously shown [29, 30].

In conclusion, the present study extended previous findings regarding the influence of the $A_{2A}R$ over D_2R signaling, and found evidence for an important role of the $A_{2A}R$ in enabling the arrestin-biased D_2R ligand, UNC9994, to mediate antipsychotic-like effects in two pharmacological mouse models of psychosis. Indeed, these findings increase our understanding of the role of D_2R-$A_{2A}R$ interactions and may be valuable for future drug development efforts targeting these receptors.

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Author Contributions FC and VF-D designed the research; KS, MG-S, MV, VF-D, ML-C, and JJT performed the research; KS MG-S, VF-D, and FC analyzed the data; and VF-D, KS, and FC wrote the paper.

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

References

1. Janoutová J, Janácková P, Serý O et al (2016) Epidemiology and risk factors of schizophrenia. Neuro Endocrinol Lett 37:1–8
2. Sanger DJ (2004) The search for novel antipsychotics: pharmacological and molecular targets. Expert Opin Ther Targets. doi:10.1517/14728222.8.6.631
3. Miyamoto S, Miyake N, Jarskog LF et al (2012) Pharmacological treatment of schizophrenia: a critical review of the pharmacology and clinical effects of current and future therapeutic agents. Mol Psychiatry 17:1206–1227. doi:10.1038/mp.2012.47
4. Brust TF, Hayes MP, Roman DL et al (2015) Bias analyses of preclinical and clinical D2 dopamine ligands: studies with immediate and complex signaling pathways. J Pharmacol Exp Ther 352:480–493. doi:10.1124/jpet.114.220293
5. Masri B, Salahpour A, Didriksen M et al (2008) Antagonism of dopamine D2 receptor/beta-arrestin 2 interaction is a common property of clinically effective antipsychotics. Proc Natl Acad Sci U S A. doi:10.1073/pnas.0803522105
6. Klewe IV, Nielsen SM, Tarpø L et al (2008) Recruitment of beta-arrestin2 to the dopamine D2 receptor: insights into anti-psychotic and anti-parkinsonian drug receptor signaling. Neuropharmacology 54:1215–1222. doi:10.1016/j.neuropharm.2008.03.015
7. Urs NM, Gee SM, Pack TF et al (2016) Distinct cortical and striatal actions of a β-arrestin-biased dopamine D2 receptor ligand reveal unique antipsychotic-like properties. Proc Natl Acad Sci U S A. doi:10.1073/pnas.1614347113
8. Park SM, Chen M, Schmerberg CM et al (2016) Effects of β-arrestin-biased dopamine D2 receptor ligands on schizophrenia-like behavior in hypoglutamatergic mice. Neuropsychopharmacology 41:704–715. doi:10.1038/npp.2015.196
9. Allen JA, Yost JM, Setola V et al (2011) Discovery of β-arrestin-biased dopamine D2 ligands for probing signal transduction pathways essential for antipsychotic efficacy. Proc Natl Acad Sci U S A 108:18488–18493. doi:10.1073/pnas.1104807108
10. Fuxe K, Ferre S, Zoli M, Agnati LF (1998) Integrated events in central dopamine transmission as analyzed at multiple levels. Evidence for intramembrane adenosine A2A/dopamine D2 and adenosine A1/dopamine D1 receptor interactions in the basal ganglia. Brain Res Res Rev 26:258–273
11. Fernández-Dueñas V, Taura JJ, Cottet M et al (2015) Untangling dopamine-adenosine receptor-receptor assembly in experimental parkinsonism in rats. Dis Model Mech. doi:10.1242/dmm.018143
12. Borroto-Escuela DO, Romero-Fernandez W, Tarakanov AO et al (2011) On the existence of a possible A2A-D2-beta-arrestin2 complex: A2A agonist modulation of D2 agonist-induced beta-arrestin2 recruitment. J Mol Biol 406:687–699. doi:10.1016/j.jmb.2011.01.022
13. Huang L, Wu D, Zhang L, Feng L (2013) Modulation of A2a receptor antagonist on D2 receptor internalization and ERK phosphorylation. Acta Pharmacol Sin 34:1292–1300. doi:10.1038/aps.2013.87
14. Ciruela F, Burgueno J, Casado V et al (2004) Combining mass spectrometry and pull-down techniques for the study of receptor heteromerization. Direct epitope-epitope electrostatic interactions between adenosine A2A and dopamine D2 receptors. Anal Chem 76:5354–5363. doi:10.1021/ac049295f
15. Cabello N, Gandia J, Bertarelli DC et al (2009) Metabotropic glutamate type 5, dopamine D(2) and adenosine A(2a) receptors form higher-order oligomers in living cells. J Neurochem 109:1497–1507. doi:10.1111/j.1471-4159.2009.06078.x
16. Ciruela F, Fernández-Dueñas V (2015) GPCR oligomerization analysis by means of BRET and dFRAP. Methods Mol Biol 1272:133–144
17. Ledent C, Vaugeois JM, Schiffmann SN et al (1997) Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. Nature 388:674–678. doi:10.1038/41771
18. Clark JD, Gebhart GF, Gonder JC et al (1997) Special report: the 1996 guide for the care and use of laboratory animals. ILAR J 38:41–48
19. Denis C, Saulière A, Galandrin S et al (2012) Probing heterotrimeric G protein activation: applications to biased ligands. Curr Pharm Des 18:128–144
20. Urizar E, Yano H, Kolster R et al (2011) CODA-RET reveals functional selectivity as a result of GPCR heteromerization. Nat Chem Biol 7:624–630. doi:10.1038/nchembio.623
21. Nordquist RE, Risterucci C, Moreau JL et al (2008) Effects of aripiprazole/OPC-14597 on motor activity, pharmacological models of psychosis, and brain activity in rats. Neuropharmacology 54:405–416. doi:10.1016/j.neuropharm.2007.10.010
22. Large CH (2007) Do NMDA receptor antagonist models of schizophrenia predict the clinical efficacy of antipsychotic drugs? J Psychopharmacol 21:283–301. doi:10.1177/0269881107077712
23. Powell SB, Geyer Ma (2007) Overview of animal models of schizophrenia. Curr Protoc Neurosci Chapter 9:Unit 9.24. doi: 10.1002/0471142301.ns0924s39
24. Urban JD, Clarke WP, von Zastrow M et al (2006) Functional selectivity and classical concepts of quantitative pharmacology. J Pharmacol Exp Ther 320:1–13. doi:10.1124/jpet.106.104463
25. Rozenfeld R, Devi LA (2007) Receptor heterodimerization leads to a switch in signaling: beta-arrestin2-mediated ERK activation by mu-delta opioid receptor heterodimers. FASEB J 21:2455–2465. doi:10.1096/fj.06-7793com
26. Guitart X, Navarro G, Moreno E et al (2014) Functional selectivity of allosteric interactions within G protein-coupled receptor oligomers: the dopamine D1–D3 receptor heterotetramer. Mol Pharmacol 86:417–429. doi:10.1124/mol.114.093096
27. Fredholm BB, IJ AP, Jacobson KA et al (2011) International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors—an update. Pharmacol Rev 63:1–34. doi:10.1124/pr.110.003285
28. Fuxe K, Borroto-Escuela DO, Romero-Fernandez W et al (2014) Moonlighting proteins and protein-protein interactions as neurotherapeutic targets in the G protein-coupled receptor field. Neuropsychopharmacology 39:131–155. doi:10.1038/npp.2013.242
29. Matos M, Shen HY, Augusto E et al (2015) Deletion of adenosine A2A receptors from astrocytes disrupts glutamate homeostasis leading to psychomotor and cognitive impairment: relevance to schizophrenia. Biol Psychiatry 78:763–774. doi:10.1016/j.biopsych.2015.02.026
30. Shen HY, Canas PM, Garcia-Sanz P et al (2013) Adenosine a2A receptors in striatal glutamatergic terminals and GABAergic neurons oppositely modulate psychostimulant action and DARPP-32 phosphorylation. PLoS One. doi:10.1371/journal.pone.0080902