Striatal Pre- and Postsynaptic Profile of Adenosine A2A Receptor Antagonists

Marco Orru1*, Jana Bakešová2*, Marc Brugarolas2, César Quiroz1, Vahri Beaumont3, Steven R. Goldberg1, Carme Lluís2, Antoni Cortés2, Rafael Franco2, Vicent Casadó2, Enric I. Canela2, Sergi Ferré1*

1 National Institute on Drug Abuse, IRP, NIH, DHHS, Baltimore, Maryland, United States of America, 2 Centro de Investigación Bioquímica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Faculty of Biology, University of Barcelona, Barcelona, Spain, 3 CHDI Management, CHDI Foundation, Los Angeles, California, United States of America

Abstract

Striatal adenosine A2A receptors (A2ARs) are highly expressed in medium spiny neurons (MSNs) of the indirect efferent pathway, where they heteromerize with dopamine D2 receptors (D2Rs). A2ARs are also localized presynaptically in corticostriatal glutamatergic terminals contacting MSNs of the direct efferent pathway, where they heteromerize with adenosine A1 receptors (A1Rs). It has been hypothesized that postsynaptic A2AR antagonists should be useful in Parkinson’s disease, while presynaptic A2AR antagonists could be beneficial in dyskinetic disorders, such as Huntington’s disease, obsessive-compulsive disorders and drug addiction. The aim of this work was to determine whether selective A2AR antagonists may be subdivided according to a preferential pre- versus postsynaptic mechanism of action. The potency at blocking the motor output and striatal glutamate release induced by cortical electrical stimulation and the potency at inducing locomotor activation were used as in vivo measures of pre- and postsynaptic activities, respectively. SCH-442416 and KW-6002 showed a significant preferential pre- and postsynaptic profile, respectively, while the other tested compounds (MSX-2, SCH-420814, ZM-241385 and SCH-58261) showed no clear preference. Radioligand-binding experiments were performed in cells expressing A2AR-D2R and A1R-A2AR heteromers to determine possible differences in the affinity of these compounds for different A2AR heteromers. Heteromerization played a key role in the presynaptic profile of SCH-442416, since it bound with much less affinity to A2AR when co-expressed with D2R than with A1R. KW-6002 showed the best relative affinity for A2AR co-expressed with D2R than co-expressed with A1R, which can at least partially explain the postsynaptic profile of this compound. Also, the in vitro pharmacological profile of MSX-2, SCH-420814, ZM-241385 and SCH-58261 was in accordance with their mixed pre- and postsynaptic profile. On the basis of their preferential pre- versus postsynaptic actions, SCH-442416 and KW-6002 may be used as lead compounds to obtain more effective antidyskinetic and antiparkinsonian compounds, respectively.

Introduction

The striatum is the major input structure of the basal ganglia [1]. More than ninety five percent of striatal neurons are γ-aminobutyric-acidergic (GABAergic) medium spiny neurons (MSNs). These neurons receive two main inputs: glutamatergic afferents from cortical, thalamic and limbic areas and dopaminergic afferents from the substantia nigra pars compacta and the ventral tegmental area [1]. MSNs are efferent neurons that give rise to the two efferent pathways of the basal ganglia, the ‘direct’ and ‘indirect’ striatal efferent pathways [1]. It is generally accepted that stimulation of the direct and indirect pathways results in motor activation and motor inhibition, respectively, and that smooth motor drive results from the counterbalanced influence of the direct and indirect pathways on the neural activity of the output structures [2,3]. Direct MSNs express dopamine receptors predominantly of the D1 receptor (D1R) subtype, whereas indirect MSNs are known for their high expression of dopamine D2 receptors (D2Rs) and adenosine A2A receptors (A2ARs) [1,4,5].

There is clear evidence for the existence of postsynaptic mechanisms in the control of glutamatergic neurotransmission to the indirect MSN by at least two reciprocal antagonistic interactions between A2AR and D2R [4]. In one type of interaction, A2AR and D2R are forming heteromers and, by means of an allosteric interaction, A2AR counteracts the D2R-mediated inhibitory modulation of the effects of NMDA receptor stimulation in the indirect MSN, which includes Ca2+ influx, transition to the up-state and neuronal firing in the up-state [6,7]. This interaction has been suggested to be mostly responsible for the locomotor depressant and activating effects of A2AR agonist and antagonists, respectively [4]. The second type of interaction

* E-mail: sferre@intra.nida.nih.gov
† These authors contributed equally to this work.

Citation: Orru M, Bakešová J, Brugarolas M, Quiroz C, Beaumont V, et al. (2011) Striatal Pre- and Postsynaptic Profile of Adenosine A2A Receptor Antagonists. PLoS ONE 6(1): e16088. doi:10.1371/journal.pone.0016088

Editor: Alfred Lewin, University of Florida, United States of America

Received October 18, 2010; Accepted December 4, 2010; Published January 11, 2011

This is an open-access article distributed under the terms of the Creative Commons Public Domain declaration which stipulates that, once placed in the public domain, this work may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose.

Funding: Work supported by the National Institute on Drug Abuse, Intramural Research Program, CHDI Foundation and by the Spanish Ministerio de Ciencia y Tecnología (grant numbers SAF2008-00146 and SAF2008-03229-E). The co-author Vahri Beaumont, scientific manager from CHDI Foundation, participated in the design of the experiments. The other funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.
involves A2AR and D2R that do not form heteromers, but most probably homomers [4]. In this interaction, which takes place at the level of adenylyl-cyclase (AC), stimulation of G_{i}-coupled D2R counteracts the effects of G_{s}-coupled A2AR [4]. Due to a strong tonic effect of endogenous dopamine on striatal D2R, this interaction keeps A2AR from signaling through AC. However, under conditions of dopamine depletion or with blockade of D2R, A2AR-mediated AC activation is unleashed. This is biochemically associated with a significant increase in the phosphorylation of PKA-dependent substrates, which increases gene expression and the activity of the indirect MSN, producing locomotor depression (reviewed in ref. [4]). This interaction seems to be the main mechanism responsible for the locomotor depression induced by D2R antagonists. Thus the motor depressant and most biochemical effects induced by genetic or pharmacologic blockade of D2R are counteracted by the genetic or pharmacologic blockade of A2AR [8–10].

Striatal A2ARs are not only localized postsynaptically but also presynaptically, in glutamatergic terminals, where they heteromerize with A1 receptors (A1Rs) and where their stimulation facilitates glutamatergic neurotransmission [5,11]. Interestingly, presynaptic A2ARs are preferentially localized in glutamatergic terminals of cortico-striatal afferents to the direct MSN [5]. According to the widely accepted functional basal circuitry model [2,3], blockade of postsynaptic A2AR localized in the indirect MSN should produce motor activation (by potentiating D2R-mediated effects by means of A2AR-D2R receptor interactions). On the other hand, according to the same model, blockade of presynaptic A2AR localized in the cortico-striatal glutamatergic terminals that make synaptic contact with the direct MSN should decrease motor activity (by inhibiting glutamate release). The preferential locomotor-activating effects of systemically administered A2AR receptor antagonists can be explained by a stronger influence of a tonic adenosine and A2AR receptor-mediated modulation of the indirect pathway versus the direct pathway under basal conditions. In any case, the potency at inducing locomotor activation can be used as an in vivo measure of the ability of an A2AR antagonist to block postsynaptic striatal A2AR.

Recently we have established an in vivo model that evaluates the efficacy of cortico-striatal glutamatergic neurotransmission to the direct MSN, by quantifying the correlation between the current delivered into the orofacial premotor cortex and the concomitant electromyographic response elicited in the jaw muscles [5]. In this model, A2AR or D2R antagonists were able to counteract the motor output induced by cortical electrical stimulation, which can only be explained by blockade of striatal presynaptic A2AR or postsynaptic D2R, respectively [5,12].

Receptor heteromer is defined as a macromolecular complex composed by at least two (functional) receptor units with biochemical properties that are demonstrably different from those of its individual components [13]. Specific ligand binding characteristics are one of those properties [13,14]. The aim of the present study was, first, to investigate the possible existence of different pre- and postsynaptic profiles of several A2AR antagonists. The potency at blocking the motor output and striatal glutamate release induced by cortical electrical stimulation and the potency at inducing locomotor activation were used as in vivo measures of pre- and postsynaptic activities, respectively. Second, we wanted to evaluate if the different pre- and postsynaptic profiles could be related to different affinities that A2AR could have for those compounds when forming heteromers with either A1R or D2R. In fact, the results strongly suggest that heteromerization plays a key role in the pre- and postsynaptic profile of A2AR antagonists.

Materials and Methods

All animals used in the study were handled in accordance with the National Institutes of Health Animal care guidelines. The animal research conducted to perform this study was approved by the NIDA IRP Animal Care and Use Committee (under the auspices of protocol 09-BNRB-73) on 12/7/2009.

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing between 300–350 g were used in these experiments. Rats were housed 2 per cage and they maintained at a temperature of 22±2°C on a regular 12-h light-dark cycle. Food and water were available ad libitum.

Adenosine A2AR antagonists

The following A2AR antagonists were used: 2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-c][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH-442416), 2-(2-Furanyl)-7-[2-(2-[4-(2-methoxyethoxy)phenoxy]-1-piperazinyl-1-ethyl]-7H-pyrazolo[4,3-c][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH-58261), 2-(2-Furanyl)-7-[2-[4-[2-(methoxyethoxy)phenoxy]-1-piperazinyl-1-ethyl]-7H-pyrazolo[4,3-c][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH-420814), 4-2-[7-Amino-2-[2-furyl]-1,2,4-triazolo[2,3-a][1,3,5]triazin-5-ylamine]ethylenephonolin (ZM-241385), (E)-1, 3-dithyli(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6-dione (KW-6002), (E)-3-[3-hydroxypropyl]-8-[2-[2-(2-methoxyphenyl)vinyl]-7-methyl-1-prop-2-ynyl]-3,7-dihydropurine-2,6-dione (MSX-2) and its water-soluble phosphate prodrug (E-phosphoric acid mono-[3-[2-[2-(methoxyethoxy)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydropurin-3-yl]propyl] ester diisodium salt (MSX-3). MSX-3 is a water-soluble phosphate pro-drug of MSX-2; in vivo MSX-3 is readily converted to the A2AR antagonist MSX-2 (Sauer et al., 2002). For their systemic administration, the compounds were prepared as follows:

SCH-442416 and SCH 58261 were suspended in a solution of 5% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MI; 5% TWEEN80 (Sigma-Aldrich, St. Louis, MI) and 90% ddH2O; SCH-420814 was suspended in a solution of 20% PEG400, 40% β-cyclodextrin and 40% Lutrol 1% (in ddH2O); ZM-241383 was suspended in a solution of 15% DMSO, 10% TWEEN80 and 75% ddH2O; KW-6002 was suspended in a solution of 8% TWEEN80 and 92% ddH2O; MSX-3 was dissolved in sterile saline (with 3 μl/ml saline of 1 M NaOH solution, final pH 7.4). All drugs but MSX-3 (Sigma-Aldrich, St. Louis, MI) were provided by CHDI Foundation Inc. (Los Angeles, CA, U.S.). SCH-420814 was administered subcutaneously (s.c.) at 1 ml/kg and the other drugs were administered via intraperitoneal (i.p.) injection at volume of 2 ml/kg.

Locomotor Activity

Locomotor activity was measured by placing the animals individually in motility soundproof chambers (50×50 centimeters; Med Associates Inc., VT). Locomotion was measured by counting the number of breaks in the infrared beams of the chambers. The animals were placed in individual acrylic chambers at noon on the day of testing. A lamp inside each chamber remained lit during this period. Following 90 min of habituation, the rats were injected i.p. with different doses of each compound or vehicle and locomotor activity was recorded for 90 min after the drug or vehicle administration. All the animals were tested only once. The effect of different doses of the A2AR antagonists on locomotor activity were analyzed using a one-way analysis of variance (ANOVA), followed by Newman-Keuls’ post-hoc test.
Surgical procedures

Rats were anesthetized with 3 ml/kg of Equithesin (4.44 g of chloral hydrate, 0.972 g of Na pentobarbital, 2.124 g of MgSO4, 44.4 ml of propylene glycol, 12 ml of ethanol and 30 ml of formalin solution; I. N. D. A. Pharmacy, Baltimore, MD) and implanted unilaterally with bipolar stainless steel electrodes, 0.15 mm in diameter (Plastics One, Roanoke, VA), into the orofacial area of the lateral agranular motor cortex (3 mm anterior, 3 and 4 mm lateral, and 4.2 mm below bregma). The electrodes and a head holder (connected to a swivel during stimulation) were fixed on the skull with stainless steel screws and dental acrylic resin. For the experiments with electroneuromyographic (EMG) recording, electrodes were also implanted in mastication muscles (during the same surgical procedure). Two 5 mm-long incisions were made in the skin on the upper and lower jaw areas to expose the masseter and the lateral pterygoid muscles. Two silicon rubber-coated coated stainless steel recording electrodes (Plastics One, Roanoke, VA) were slipped below the skin from the incision in the skin until the tips showed up from the incisions in the jaw. The bare tips of the electrodes were then held in contact with the masseter and the lateral pterygoid muscles and the skin was closed with surgical staples. The other end of the recording electrodes was encased in a molded plastic pedestal with a round threaded post which was attached to an electrical swivel and then to a differential amplifier (Grass Instruments, Warwick, RI). The pedestal was secured to the skull with dental cement together with the stimulation electrodes. For the in vivo microdialysis experiments, concentric microdialysis probes with 2-mm long dialysis membranes (Eicom Corp., Tokio, Japan) were implanted respectively into the striatum ipsilateral to the stimulation electrodes (0.0 mm AP, 4.5 ML and 7.0 mm DV).

EMG recording and power correlation analysis

Rats were placed in individual bowl chambers. Both stimulation electrodes and recording electrodes were attached using flexible shielded cabling to a four channel electrical swivel. Stimulation electrodes were connected to two-coupled constant current isolation units (PSIU6X, Grass Instruments West Warwick, RI) driven by an electrical stimulator (Grass S88X; Grass Instruments). The recording electrodes were connected to a differential amplifier (Grass LP511, West Warwick, RI). This configuration allows the rat to move freely while the stimulation and EMG recordings are taking place. After 60 min of habituation, biphasic current pulse trains (pulse of 0.1 ms at 120–200 μA; 100 Hz, 160 ms trains repeating once per 2 seconds) were delivered. The current intensity was adjusted to the threshold level, defined as the minimal level of current intensity allowing at least 95% of the stimulation pulses to elicit a positive EMG response. Positive EMG response was defined as at least 100% increase of the peak to peak amplitude respect to the background tonic EMG activity lasting more than 100 ms or at least 70% increase in the power of the EMG signal respect to the baseline. Positive EMG responses always matched observable small jaw movements. The threshold level was different for each animal but it was very stable and reproducible once established. The threshold level was in the 100 to 150 μA range for most cases and it reached 200 μA in a few (6) animals. Animals that failed to show a positive EMG response with electrical cortical stimulation intensities of 200 μA were discarded from the experimental procedure (less than 10%). Both stimulator monitoring and the amplified and filtered EMG signal (20,000 times gain, bandwidth from 10 to 1,000 Hz with a notch filter set at 60 Hz) were directed to analog-to-digital converter for recording (Lab-Trax-4, World Precision Instruments, Sarasota, FL) and backup (NI 9215, National Instruments, Austin, TX) and digitized at a sampling rate of 10,000 samples/second. Recordings of the digitized data were made using the software Data Trax2 software (World Precision Instruments) and LabVIEW SignalExpress (National Instruments). A power correlation analysis was used to quantify the correlation between the stimulation pulses of current delivered into the orofacial motor cortex (input signal; μA) and the elicited EMG response in the jaw muscles (output signal; μV). Decrease in the power correlation coefficient (PCC) between these two signals is meant to describe a decrease in the efficacy of the transmission in the neural circuit. Off-line, both signals were rectified and the root mean square (RMS) over each period of the stimulation pulses was calculated in the recorded signals using Data Trax2 software. The transformed data (RMS) from the stimulator monitor and the EMG were then exported with a time resolution of 100 samples/second to a spreadsheet file. The stimulation signal values were used as a reference to select data in a time window of 320 ms starting at the beginning of each train of pulses. This time window was chosen to ensure the analysis of any EMG response whose occurrence or length was delayed from the onset of the stimulation trains and to maximize the exclusion from the analysis of spontaneous jaw movements not associated with the stimulation. Pearson’s correlation between the RMS values from the stimulation and EMG signals was then calculated for each experimental subject. PCC was calculated using the data recorded 40 min after the administration of the dose of any compound or vehicle. The effects of the different doses of A2AR antagonists on PCC were analyzed by a one-way ANOVA, followed by Dunnett’s post-hoc test.

In vivo microdialysis

The experiments were performed on freely moving rats 24 h after probe implantation. An artificial cerebrospinal solution of (in mM): 144 NaCl, 4.8 KCl, 1.7 CaCl2 and 1.2 MgCl2 was pumped through the microdialysis probe at a constant rate of 1 μl/min. After a washout period of 90 min, dialysate samples were collected at 20-min intervals. After 60 min of collecting samples for baseline, the rats were injected either with the A2AR antagonists KW-6002 or SCH-442416. Both compounds were compared to vehicle controls (5% DMSO, 5% of TWEEN80 and 90% of ddH2O). After 20 min from drug or vehicle injection, electrical stimulation pulses were applied through the electrodes implanted in the orofacial motor cortex for 20 min (pulse of 0.1 ms at 50–150 μA; 100 Hz, 160 ms trains repeating once per second) and samples were collected for 2 additional hours. Glutamate content was measured by reverse-phase HPLC coupled to a fluorimetric detector (Shimadzu Inc., Tokio, Japan) [15]. Glutamate values were transformed as percentage of the mean of the three values before the drug or vehicle injection and transformed values were statistically analyzed. The effect of KW-6002, SCH-442416 and vehicle were analyzed using a one-way ANOVA for repeated measures followed by a Tukey’s post-hoc test.

Cell clones

To obtain CHO cells expressing single receptors or co-expressing A2A R and A1R or A2AR and D2R, the human cDNAs for A2AR or D2R cloned in pcDNA3.1 vector (containing a geneticin resistance gene) were used. The human A2AR was cloned into a pcDNA3.1/Hyg vector with a hygromycin resistance gene. For single transfections, CHO cells were transfected with the cDNA corresponding to A2AR, A1R or D2R using lipofectamine (Invitrogen, Carlsbad, USA) method following the instructions of the supplier. 24 h after transfection the selection antibiotic was added at a concentration that was previously determined by a selection antibiotic test. Antibiotic resistant clones were isolated in the presence of the selection antibiotic (1200 μg/ml geneticin or

Pre- and Postsynaptic A2A Receptor Antagonists
1000 µg/ml hygromycin). After an appropriate number of days/ passes, several stable lines were selected and cultured in the presence of the selection antibiotic (600 µg/ml gentamicin or 500 µg/ml hygromycin). To obtain clones co-expressing A2AR and A1R or A2AR and D3R, CHO cells expressing high affinity A2AR (obtained as above described) were transfected with the human cDNAs for A1R, D3R cloned in pcDNA3.1 vector using lipofectamine. After an appropriate number of days/passes stable lines were selected and cultured in the presence of the selection antibiotic. The receptor(s) expression in the cell clones was first detected by dot-blot of cell lysates using commercial available antibodies and wild-type CHO cells lysates as negative basal staining. Positively moderated stained clones were grown to obtain membranes in which the receptor expression was quantified by radioligand-binding experiments (see Results).

**Bioluminescence Resonance Energy Transfer (BRET) assays**

The fusion proteins A2AR-REnilla Luciferase (A2AR-RLuc), A1R-Yellow Fluorescence Protein (A1R-YFP) and D3R-YFP were prepared and characterized as described elsewhere [16]. The cDNA encoding serotonin 5HT2B-YFP receptor was kindly prepared and characterized as described elsewhere [16]. The human cDNAs for A1Ro rD2R cloned in pcDNA3.1 vector using the indicated free concentration of the A1R, A2AR, or D3R antagonist [3H]DPCPX (GE Healthcare, UK), [3H]YM-241385, or [3H]YM-09151-2, respectively (NEN Perkin Elmer, Wellesley, MA, USA) or the A1R agonist [3H]R-(PIA) (Moravek Biochemicals Inc., Brea, CA, USA) and increasing concentrations of DPCPX, YM-241385, YM-09151-2, the A2AR agonist CGS-21680 or the tested A2AR antagonist (all provided by CHDI Foundation Inc.). Non-specific binding was determined in the presence of 11 µM of the corresponding non-radiolabeled ligand. Free and membrane-bound ligand were separated by rapid filtration of 500 µl aliquots in a cell harvester (Brandel, Gaithersburg, MD, USA) through Whatman GF/C filters embedded in 0.3% polyethylenimine that were subsequently washed for 5 s with 5 ml of ice-cold 50 mM Tris-HCl buffer. The filters were incubated with 10 ml of Ecoscint H scintillation cocktail (National Diagnostics, Atlanta, GA, USA) overnight at room temperature and radioactivity counts were determined using a Tri-Carb 1600 scintillation counter (PerkinElmer, Boston, MA, USA) with an efficiency of 62% [17]. All displacers were dissolved in DMSO and diluted in the binding medium. The DMSO concentration in the binding incubates was less than 0.5% and, at this concentration, it did not affect agonist or antagonist affinity for their respective receptors.

**Binding data analysis**

Radioligand competition curves were analyzed by nonlinear regression using the commercial Grafit curve-fitting software (Erithacus Software, Surrey, UK), by fitting the binding data to the mechanistic two-state dimer receptor model [18,19]. Since there is now abundant evidence for GPCR oligomerization, including A1R, A2AR and D3R [20–23] and the minimal functional unit of GPCRs in biological tissues seems to imply dimerization [23], this model considers a homodimer as the minimal structural unit of the receptor. Here, we also consider the possibility of a homodimer as the minimal structural unit of a receptor forming homomers or forming heteromers with another receptor. To calculate the macroscopic equilibrium dissociation constants the following equation for a competition binding experiment deduced previously [19,24] was considered:

\[
A_{\text{total bound}} = (K_{\text{DA2}}A + 2A^2 + K_{\text{DA2AB}} / K_{\text{DAB}}) R_T / (K_{\text{DA1}}K_{\text{DA2}} + K_{\text{DA2A}} + A^2 + \\
K_{\text{DA2 AB}} / K_{\text{DAB}} + K_{\text{DA1}}K_{\text{DA2B}} / K_{\text{DDB}}) + A_{\text{non-specific bound}}
\]

where \(A\) represents free radioligand (the adenosine A1R or A2AR or dopamine D3R antagonist [3H]DPCPX, [3H]YM-241385 or [3H]YM-09151-2, respectively or the A1R agonist [3H]R-(PIA) concentration, \(R_T\) is the total amount of receptor dimers and \(K_{\text{DA1}}\) and \(K_{\text{DA2}}\) are the macroscopic equilibrium dissociation constants describing the binding of the first and the second radioligand molecule (A) to the dimeric receptor; B represents the assayed...
competing compound concentration, and $K_{DB1}$ and $K_{DB2}$ are, respectively, the macroscopic equilibrium dissociation constants for the binding of the second ligand molecule (B) to the dimer and for the binding of the second ligand molecule (B) to the semi-occupied dimer; $K_{DAB}$ is the hybrid equilibrium radioligand/competitor binding of the second ligand molecule (B) to the semi-occupied receptor dimer, whereas $K_{D1}$ and $K_{D2}$ represent the “high-affinity” and the “low-affinity” binding sites, respectively. On the other hand, for positive cooperativity, $K_{D2}/K_{D1} < 4$ and then $K_{D2}$ represents the “high-affinity” and $K_{D1}$ represents the “low-affinity” binding sites [25]. The two-state dimer model also introduces a cooperativity index ($D_{CB}$). The dimer cooperativity index for the competing ligand B is calculated as [19,25]:

$$D_{CB} = \log \left( \frac{4K_{DB1}}{K_{DB2}} \right)$$

The way the index is defined is such that its value is “0” for non-cooperative binding, positive values of $D_{CB}$ indicate positive cooperativity, whereas negative values imply negative cooperativity [14,19].

In experimental conditions when both the radioligand A and the competitor B (i.e., most adenosine A2A receptor antagonist tested in the present study) show non-cooperativity, it results that $K_{DAA2} = 4K_{DAA1}$ [19,25] and, therefore, $K_{DAA1}$ is enough to characterize the binding of the radioligand A:

$$A_{total\ bound} = (4K_{DAA1}A + 2A^2 + 4K_{DAA1}AB / K_{DAB})R_T / (4K_{DAA1}^2 + 4K_{DAA1}A + A^2 + 4K_{DAA1}AB / K_{DAA1} + 4K_{DAA1}^2B / K_{DB1} + 4K_{DAA1}^2B^2 / (K_{DB1}K_{DB2})) + A_{non-specific\ bound}$$

When both the radioligand A and the competitor B (DPCPX, ZM241384, SCH 23390 or YM-09151-2) are the same compound and the binding is non-cooperative, eq. (3) simplifies to:

$$A_{total\ bound} = (4K_{DAA1}A + 2A^2 + AB)R_T / (4K_{DAA1}^2 + 4K_{DAA1}A + A^2 + AB + 4K_{DAA1}B + B^2) + A_{non-specific\ bound}$$

Goodness of fit was tested according to reduced $\chi^2$ value given by the nonlinear regression program. The test of significance for two different population variances was based upon the F-distribution [25] for details. Using this F test, a probability greater than 95% ($p<0.05$) was considered the criterion to select a more complex equation to fit binding data over the simplest one. In all cases, a probability of less than 70% ($p>0.30$) resulted when one equation to fit binding data was not significantly better than the other. Results are given as parameter values ± S.E.M. of three-four independent experiments.

## Results

### Striatal pre- versus postsynaptic profile of A$_{2A}$ receptor antagonists

Dose-response experiments with the six A$_{2A}$R antagonists indicated that four compounds (SCH-420814, SCH-58261, MSX-3 and ZM-241385) had a similar potency (similar minimal significant effective doses) at inducing locomotor activation (Fig. 1) and at reducing PCC (Fig. 2). The other two compounds had a very different profile: KW-6002 produced a strong locomotor activation already at the dose of 0.3 mg/kg i.p., while it did not reduce PCC at the highest tested dose (10 mg/kg i.p.). On the other hand, SCH-442416 produced a very weak locomotor activation, only significant at doses higher than 3 mg/kg i.p., while it significantly decreased PCC already at the dose of 0.1 mg/kg i.p.

In vivo microdialysis with cortical electrical stimulation was used as an additional in vivo evaluation of the preferential pre- and postsynaptic activity of SCH-442416 and KW-6002, respectively. SCH-442416 significantly counteracted striatal glutamate release induced by cortical stimulation at a dose that strongly reduced PCC but did not induce locomotor activation (1 mg/kg i.p.; Fig. 3). On the other hand, KW-6002 did not modify striatal glutamate release induced by cortical stimulation at a dose that produced a pronounced locomotor activation but did not reduce PCC (1 mg/kg i.p.; Fig. 3).

### Development of CHO cell-lines expressing A$_1$-A$_{2A}$ or A$_{2A}$-D$_2$ receptor heteromers

Cell clones expressing A$_2$AR, A$_1$R-A$_{2A}$R heteromers or A$_{2A}$R-D$_2$R heteromers and control clones expressing A$_1$R or D$_2$R were generated (see Materials and Methods). First of all, the ability of A$_2$AR to form heteromers with A$_1$R or D$_2$R in CHO cells was demonstrated by BRET experiments in cells transiently coexpressing A$_{2A}$AR-Rluc and A$_1$R-YFP or A$_{2A}$AR-Rluc and D$_2$R-YFP. A positive BREIT signal for energy transfer was obtained (Fig. 4). The BREIT signal increased as a hyperbolic function of the concentration of the YFP-fusion construct added reaching an asymptote. As a negative control the BRET pair formed by A$_{2A}$AR-Rluc and 5-HT$_{3B}$R-YFP was used. As shown in Figure 4, the negative control gave a linear non-specific BRET signal. The significant and hyperbolic BREIT signal found for these fusion proteins indicates that the intermolecular interaction between A$_2$AR and A$_1$R or A$_{2A}$R and D$_2$R in CHO cells is specific.

A$_{2A}$R-D$_2$R and A$_1$R-A$_{2A}$R heteromerization in stably transfected CHO cells was shown by ligand binding experiments. This is an indirect approach for the identification of a receptor heteromer in native tissues or cells [13]. In the A$_2$AR-D$_2$R heteromer, an allosteric interaction between both receptors in the heteromer has been described, in which the dopamine D$_2$R agonist asfinit decreases in the presence of an A$_2$AR agonist [14]. In CHO cells stably expressing A$_2$AR and D$_2$R, the affinity of the D$_2$R for dopamine was determined by competition experiments of the D$_2$R agonist $[^{3}H]$YM-09151-2 versus dopamine in the presence (Fig. 5a) or in the absence (Fig. 5b) of the A$_2$AR agonist.
CGS-21680 (200 nM). By fitting data obtained in the absence of CGS-21680 to eq. 3 (Methods; considering \( K_{DA1} = 2.9 \text{nM} \) see below) the calculated \( K_{DB1} \) was \( 9 \pm 2 \mu\text{M} \). In the presence of CGS-21680, 5 \mu\text{M} of dopamine was unable to decrease the radioligand bound and more than 50% of radioligand bound was found in the presence of 100 \mu\text{M} of dopamine (Fig. 5b). A \( K_{DB1} > 30 \mu\text{M} \) was

![Figure 1. Locomotor activation in rats induced by A2AR antagonists.](image)

Data represent means \( \pm \) S.E.M. of the locomotor activity (distance traveled, in cm, of total accumulated counts) in habituated rats (90 min) during 90 min following the drug administration \( n = 6-8 \) per group. * and **: \( p < 0.05 \) and \( p < 0.01 \), respectively in comparison to vehicle-treated animals (0 mg/kg); ANOVA with post-hoc Newman–Keuls' comparisons, \( p < 0.05 \) and \( p < 0.01 \), respectively).

doi:10.1371/journal.pone.0016088.g001

![Figure 2. Blockade by A2AR antagonists of the motor output induced by cortical electrical stimulation.](image)

Dose-dependent decrease in the Power Correlation Coefficient (PCC) induced by the administration of different A2AR antagonists. Results represent means \( \pm \) S.E.M. \( n = 5-6 \) per group. * and **: \( p < 0.05 \) and \( p < 0.01 \), respectively in comparison to vehicle-treated animals (0 mg/kg); ANOVA with post-hoc Dunnett' comparisons, \( p < 0.05 \) and \( p < 0.01 \), respectively.)

doi:10.1371/journal.pone.0016088.g002
estimated and it was shown that CGS-21680 induced a decrease in the dopamine affinity for D2R. An allosteric interaction in the A1R-A2AR heteromer has also been described, in which the A1R agonist affinity decreases in the presence of an A2AR agonist [11]. As shown in Figure 6a, the displacement of the A1R agonist [3H]R-PIA by CGS21680 was significantly (p<0.001) better fitted by a biphasic than by a monophasic curve. At low CGS21680 concentrations, when it binds preferentially to A2AR (at concentrations of CGS-21680 <500 nM, the direct binding of CGS-21680 to A1R is <1%, according to the calculated affinity of A1R for CGS-21680), CGS-21680 decreased the binding of [3H]R-PIA to the A1R with an IC50 value of 386±35 nM (n = 3). At high CGS21680 concentrations (>10 μM), the [3H]R-PIA binding displacement reflects the binding of CGS-21680 directly to the A1R and the competition between CGS-21680 and R-PIA for the binding to the A1R. In fact, in the control clone expressing only A1R, the displacement by CGS-21680 of [3H]R-PIA only occurred at CGS-21680 concentrations higher than 10 μM (Fig. 6b).

A pharmacological characterization of selected cell clones was performed with competition experiments of radio-labeled agonists of A1, A2A and D2 receptors versus selective agonists or antagonists. In all cases, the competition curves of the A2AR antagonist [3H]ZM-241385 (2 nM) versus ZM-241385 (0.1 nM to 11 μM), the D2R antagonist [3H]YM-09151-2 (0.2 nM) versus YM-09151-2 (0.01 nM to 11 μM) or the A1R antagonist [3H]DPCPX (2 nM) versus DPCPX (0.1 nM to 11 μM) were monophasic, indicating the absence of cooperativity (see Materials and Methods). By fitting the binding data to eq. 4 (Materials and Methods), the KD (K0) values obtained for the antagonists ZM-241385 or YM-09151-2 were 8.2±0.3 nM and 2.9±0.3 nM, respectively, for the chosen A2AR-D2R clone, the KD values obtained for the A1R and A2AR antagonists were 8.2±2 nM (DPCPX) and 1.8±0.4 nM (ZM-241385), respectively, for the chosen A1R-A2AR cell clone and the KD value obtained for A2AR antagonist (ZM-241385) was 0.9±0.3 nM for the chosen A2AR cell clone. Also by fitting the binding data to eq. 4 (Materials and Methods), the KD value obtained for the A1R antagonist (DPCPX) was 8.6±0.9 nM for the A1R cell clone and the K0 value obtained for the D2R antagonist (YM-09151-2) was 0.23±0.08 nM for the D2R cell clone. These values were then used to determine the affinity constants showed in Tables 1 and 2. The agonists affinity in each selected clone was determined by competition experiments using the A2AR antagonist [3H]ZM-241385 (2 nM) versus the agonist CGS-21680 (1 nM to 50 μM), the D2R antagonist [3H]YM-09151-2 (0.2 nM) versus the agonist quinpirole (0.1 nM to 30 μM), or the A1R antagonist [3H]DPCPX (2 nM) versus the agonist R-PIA (1 nM to 50 μM). As it is shown in Tables 1 and 2, the agonist affinity for A2AR in A2AR, A2AR-D2R or in A2AR-A1R cells is in the same range as that reported for brain striatum or for cells expressing human A2AR (between 30 and 250 nM) [7]. Nevertheless, the affinity of the A2AR for the selective agonist CGS-21680 was slightly but significantly lower when co-expressed with D2R (see Table 2). A1R but not A2AR or D2R agonist binding showed negative cooperativity (negative D CB values, see Materials and Methods), both in cells expressing A1R and in cells co-expressing A1R and A2AR (Tables 1 and 2).

Screening of A2AR antagonists on cells expressing A1-A2A or A2A-D2 receptor heteromers

To test if selected A2AR antagonists display different selectivity for A1R-A2AR or A2AR-D2R heteromers, competition experiments with these ligands were performed using CHO cells expressing A2AR-A1R, A2AR-A2AR or A2AR-D2R. We found that none of the six A2AR antagonists first tested in the in vivo models were able to bind with moderate affinity to A1R or to D2R in CHO cells expressing A1R or D2R (data not shown), indicating that these compounds are specific ligands for A2AR. Competition experiments of [3H]ZM-241385 (2 nM) binding versus increasing concentrations of each A2AR antagonist (1 nM to 100 μM) were performed as indicated in Methods and binding data from competition experiments were fitted assuming that receptors are dimers and statistically (F test, see Materials and Methods) testing whether the competitor (A2AR antagonists) binding was cooperative (biphasic competition curves; fitting to eq. 2) or non-cooperative (monophasic competition curves;
fitting to eq. 3). Since the screened compounds are A2AR antagonists, competition curves were expected to be monophasic, assuming that antagonist binding is not cooperative. In fact, in all cell clones, MSX-2, KW-6002, SCH-420814, ZM-241385 and SCH-58261 gave monophasic competition curves (fitting binding data to eq. 2 was not better than fitting to eq. 3; see Methods and Fig. 7 a–c as an example). Accordingly, the pharmacological characterization for these compounds gave DCB = 0 and KDB2 = 4KDB1 (see Table 3). For all compounds, co-transfection with A1R did not significantly modify their affinity for A2AR. On the other hand, co-transfection with D2R significantly reduced the affinity of A2AR for MSX-2, SCH-420814, SCH-58261 and ZM-241385, from two to about nine times, and did not significantly modify the affinity of A2AR for KW-6002 (Table 3).

For SCH-442416, a careful statistically-based analysis of the monophasic or biphasic nature of the competition curves led to an unexpected finding: in A2AR-D2R cells, competition curves of [3H]ZM-241385 (2 nM) binding versus increasing concentrations of SCH-442416 were biphasic (fitting to eq. 2 improves the fitting to eq. 3; see Methods) (Fig. 7d). Table 4 shows the deduced pharmacological parameters from competition experiments of [3H]ZM-241385 versus SCH-442416 in cells expressing A2AR, A1R-A2AR and A2AR-D2R. In A2AR and A1R-A2AR cells the curves were monophasic. Accordingly, the pharmacological characterization gave a DCB values of 0 and a KDB2 = 4KDB1. In contrast, as mentioned above, in cells expressing A2AR-D2R, competition curves were biphasic, and binding data were then fitted to eq. 2 (Methods) and robust parameters were obtained (Table 4). Thus, in A2AR-D2R cells, SCH-442416 binding showed a strong negative cooperativity and, consequently, with a marked loss of affinity (an increase of 600 times in KDB2) respect to cells expressing A2AR. This is reflected by the B50 value (concentration competing 50% of radioligand binding), which was more than 40 times higher in A2AR-D2R cells than in A1R-A2AR cells or A2AR cells.

Figure 4. Identification of receptor heteromers in CHO cells by BRET saturation curve. BRET experiments were performed with CHO cells co-expressing A2AR-RLuc and A1R-YFP (A) or A2AR-RLuc and D2R-YFP (B). Co-transfections were performed with increasing amounts of plasmid-YFP (0.25 to 4 µg cDNA corresponding to A1R-YFP and 0.5 to 8 µg corresponding to D2R-YFP) whereas the A2AR-RLuc construct was maintained constant (0.5 µg cDNA). Both fluorescence and luminescence of each sample were measured before every experiment to confirm similar donor expressions (about 100,000 luminescent units) while monitoring the increase acceptor expression (10,000–25,000 fluorescent units). As a negative control, linear BRET was obtained in cells expressing equivalent luminescence and fluorescence amounts corresponding to A2AR-RLuc (0.5 µg transfected cDNA) and serotonin SHT2A-YFP (0.5 to 8 µg transfected cDNA) receptors. The relative amount of acceptor is given as the ratio between the fluorescence of the acceptor minus the fluorescence value of cells expressing the donor alone (YFP) and the luciferase activity of the donor (Rluc). BRET data are expressed as means ± S.D. of 4–6 different experiments grouped as a function of the amount of BRET acceptor.
doi:10.1371/journal.pone.0016088.g004

Figure 5. Allosteric interaction between A2AR and D2R in A2AR-D2R CHO cells. Competition experiments were performed in membrane preparations from CHO cells expressing A2AR and D2R with 0.5 nM [3H]YM-09151-2 and increasing concentrations of dopamine (from 0.1 nM to 30 µM) in the absence (a) or in the presence (b) of 200 nM CGS-21680 as indicated in Methods. Data represent means ± S.E.M. of a representative experiment performed with triplicates.
doi:10.1371/journal.pone.0016088.g005
compounds already known as selective A2AR antagonists were present different striatal pre- and postsynaptic profiles. Six antagonists previously thought as being pharmacologically similar to A2ARs with agonist CGS-21680 as indicated in Methods. Data represent means ± S.E.M. of a representative experiment performed with triplicates.

Discussion

An important finding of the present study is that several A2AR antagonists previously thought as being pharmacologically similar present different striatal pre- and postsynaptic profiles. Six compounds already known as selective A2AR antagonists were first screened for their ability to block striatal pre- and postsynaptic A2ARs with in vivo models. Locomotor activation was used to evaluate postsynaptic activity while PCC reduction was used to determine presynaptic activity (see Introduction). Two compounds, SCH-420814, SCH-58261 and ZM-241385, showed preferential presynaptic profiles, respectively, and four compounds, MSX-3, SCH-420814, SCH-58261 and ZM-241385, showed mixed pre-postsynaptic profiles. Combining in vivo microdialysis with cortical electrical stimulation was used as an additional in vivo evaluation of presynaptic activity of SCH-420814 and KW-6002. In agreement with its preferential presynaptic profile, SCH-420814 significantly counteracted striatal glutamate release induced by cortical stimulation at a dose (1 mg/kg i.p.) that strongly reduced PCC but did not induce locomotor activation. On the other hand, according to its preferential postsynaptic profile, KW-6002 did not modify striatal glutamate release induced by cortical stimulation at a dose (1 mg/kg i.p.) that produced a pronounced locomotor activation but did not counteract PCC. In a previous study, we reported that intrastriatal perfusion of MSX-3 almost completely counteracted striatal glutamate release induced by cortical electrical stimulation [5], which agrees with its very effective reduction of PCC shown in the present study.

Another important finding of the present study is that at least part of these pharmacological differences between A2AR antagonists can be explained by the ability of pre- and postsynaptic A2ARs to form different receptor heteromers, with A1R and D2R, respectively [4-6,11,14]. Radioligand-binding experiments were performed in CHO cells stably expressing A2AR, A2AR-D2R heteromers or A1R-A2AR heteromers to determine possible differences in the affinity of these compounds for different A2AR heteromers. Co-expression with A1R did not significantly modify the affinity of A2AR for the different ligands, but co-expression with D2R decreased the affinity of all compounds, with the exception of KW-6002. The structural changes in the A2AR induced by heteromerization with the D2R could be detected not only by antagonists but also by agonists. Indeed, the affinity of the selective A2AR agonist CGS-21680 was reduced in cells co-

Table 1. Pharmacological parameters for agonist binding to A1R, A2AR and D2R in A1R, A2AR and D2R CHO cells.

| Parameters | A2AR cells | A1R cells | D2R cells |
|------------|------------|-----------|-----------|
| KDB1  | 90±10 nM | 13±3 nM | 120±60 nM |
| KDB2  | 360±120 nM | 1±0.3 nM | 480±240 nM |
| B0  | 0 | -1.3 | 0 |
| B20 | 180±60 nM | 110±30 nM | 240±120 nM |

Binding data from competition experiments were fitted assuming that receptors form homodimers, and cooperativity (DCB = 0, fitting to eq. 2; Materials and Methods) or non-cooperativity (DCB = 0, fitting to eq. 3; Materials and Methods) in competitor ligand binding was statistically tested (F test). $K_{DB1}$ and $K_{DB2}$ are, respectively, the equilibrium dissociation constants of the first and second binding of B (the A1R, A2AR, or D2R agonists: R-PIA, CGS-21680 or quinpirole, respectively) to the dimer. DCB is the "dimer cooperativity" index for the binding of the ligand B, and $B_0$ is the concentration providing half saturation for B. Data are mean ± S.E.M. values of three experiments.

Table 2. Pharmacological parameters for agonist binding to A1R, A2AR and A2AR-D2R CHO cells.

| Parameters | A2AR-D2R cells | A2AR-A1R cells |
|------------|----------------|----------------|
| A2AR  | D2R  | A2AR  | A1R  |
| KDB1  | 200±40 nM* | 1.2±0.6 μM | 70±10 nM | 0.7±0.3 nM |
| KDB2  | 0.8±0.4 μM | 4.8±2.4 μM | 280±40 nM | 1.1±0.5 μM |
| DCB  | 0  | 0  | 0  | 2.6 |
| B20  | 0.4±0.08 μM | 2.4±1.2 μM | 140±20 nM | 30±10 nM |

Binding data from competition experiments were fitted assuming that receptors form homodimers, and cooperativity (DCB = 0, fitting to eq. 2; Materials and Methods) or non-cooperativity (DCB = 0, fitting to eq. 3; Materials and Methods) in competitor ligand binding was statistically tested (F test). $K_{DB1}$ and $K_{DB2}$ are, respectively, the equilibrium dissociation constants of the first and second binding of B (the A1R, A2AR, and D2R agonists: R-PIA, CGS-21680 or quinpirole, respectively) to the dimer. DCB is the "dimer cooperativity" index for the binding of the ligand B, and $B_0$ is the concentration providing half saturation for B. Data are mean ± S.E.M. values of three experiments.

*: p<0.05 compared to $K_{DB1}$ values in A1R-A2AR and A2AR cells (Table 1); one-way ANOVA, followed by Newman-Keuls test.

doi:10.1371/journal.pone.0016088.t001

doi:10.1371/journal.pone.0016088.t002
Figure 7. Binding of the A2AR antagonists KW-6002 and SCH-442416 to A1R-A2AR and A2AR-D2R CHO cells. Competition experiments of [3H]ZM-241385 (2 nM) versus increasing concentrations of KW-6002 (a and c) or SCH-442416 (b and d) were performed as indicated in Methods in membrane preparations from CHO cells expressing A1R and A2AR (a and b) or A2AR and D2R (c and d). Data are means ± S.E.M. of a representative experiment performed with triplicates. doi:10.1371/journal.pone.0016088.g007

Table 3. Pharmacological parameters for A2AR antagonist binding to A2AR, A1R-A2AR and A2AR-D2R CHO cells.

| Parameter   | A2AR cells | A1R-A2AR cells | A2AR-D2R cells |
|-------------|------------|----------------|----------------|
| KD1 (nM)    | 0.9±0.3    | 1.8±0.4        | 8±3*           |
| ZM241385    | 3.3±0.3    | 4.7±0.6        | 23±8*          |
| SCH58261    | 3.2±0.2    | 4.2±0.3        | 7±2*           |
| KW6002      | 100±10     | 100±20         | 160±70         |
| SCH420814   | 0.5±0.1    | 1.1±0.1        | 2.7±0.8*       |

Competition experiments of [3H]ZM-241385 (2 nM) binding versus increasing concentrations of A2A receptor antagonists were performed as indicated in Methods in membrane preparations from CHO cells expressing A2AR or A1R and A2AR or A1R and D2R. Binding data were fitted assuming that receptors (also when heteromerizing) form homodimers, and cooperativity (DCB ≠ 0, fitting to eq. 2; Materials and Methods) or non-cooperativity (DCB=0, fitting to eq. 3; Materials and Methods) for competitor ligand binding was statistically tested (F test). Only KD1 values (equilibrium dissociation constant of the first binding of B: ZM-241385, MSX-2, SCH-58261, SCH-420814 or KW-6002) are shown, since the analysis demonstrated non-cooperativity for the five A2AR antagonists. Data are mean ± S.E.M. values of three experiments.  

*: p<0.05 compared to KD1 values in A2AR cells; one-way ANOVA, followed by Newman-Keuls test.  
doi:10.1371/journal.pone.0016088.t003

Table 4. Pharmacological parameters for SCH-442416 binding to A2AR, A1R-A2AR and A2AR-D2R CHO cells.

| Parameter   | A2AR cells | A1R-A2AR cells | A2AR-D2R cells |
|-------------|------------|----------------|----------------|
| KD1 (nM)    | 2.0±0.3 nM | 2.4±0.4 nM     | 7±4 nM         |
| KD2 (nM)    | 8±2 nM     | 10±2 nM        | 5±2 μM**       |
| DCB         | 0          | 0              | −2.3           |
| B50 (nM)    | 4.0±0.6 nM | 4.8±0.8 nM     | 190±80 nM**    |

Competition experiments of [3H]ZM-241385 (2 nM) binding versus increasing concentrations of SCH-442416 were performed as indicated in Methods in membrane preparations from CHO cells expressing A2AR or A1R and A2AR or A2AR and D2R. Results were fitted assuming that receptors (also when heteromerizing) form homodimers, and cooperativity (DCB ≠ 0, fitting to eq. 2; Materials and Methods) or non-cooperativity (DCB=0, fitting to eq. 3; Materials and Methods) of SCH-442416 binding was statistically tested (F test). KD1 and KD2 are, respectively, the equilibrium dissociation constants of the first and second binding of B (SCH-442416) to the dimer. DCB is the “dimer cooperativity” index for the binding of the ligand B, and B50 is the concentration providing half saturation for B. Data are mean ± S.E.M. values of three experiments.  

**: p<0.01, respectively compared to the KD2 and B50 values in A2AR and A1R-A2AR cells; Kruskal-Wallis, followed by Dunn’s test.  
doi:10.1371/journal.pone.0016088.t004
transfected with the D2R. When trying to explain the differential action of SCH-442416 observed in vivo, it is interesting to note that SCH-442416 showed a much higher affinity for the A2AR in a presynaptic-like than in a postsynaptic-like context. The binding of SCH-442416 to the A2AR-D2R heteromer displayed a strong negative cooperativity, phenomenon that was not observed for the binding of SCH-442416 to the A1R-A2AR heteromer. This negative cooperativity explains the pronounced decrease in affinity of A2AR in cells expressing A2AR-D2R heteromers (B50 values 40 times higher in cells expressing A2AR-D2R than A1R-A2AR heteromers).

The loss of affinity of A2AR upon co-expression of D2R was much less pronounced for ZM-241385, SCH-58261, MSX2 or SCH-420814, for which the affinity was reduced from two to about nine fold. Taking into account that these A2AR antagonists behave similarly than the A2AR agonist CGS-21680 in terms of binding to A1R-A2AR and A2AR-D2R heteromers, it is expected that these four compounds compete equally for the binding of the endogenous agonist at pre- and at postsynaptic sites. This would fit with the in vivo data, which shows that these compounds have a non-preferred pre-postsynaptic profile. Yet, KW-6002 was the only antagonist whose affinity was not significantly different in cells expressing A2AR, A1R-A2AR heteromers or A2AR-D2R heteromers. Thus, KW-6002 showed the best relative affinity for A2AR-D2R heteromers of all compounds, which can at least partially explain its preferential postsynaptic profile.

The present results support the notion that receptor heteromers may be used as selective targets for drug development. Main reasons are the very specific neuronal localization of receptor heteromers (even more specific than for receptor subtypes), and a differential ligand affinity of a receptor depending on its partner (or partners) in the receptor heteromer. In the striatum, A2AR provides a particularly interesting target, eventually useful for a variety of neuropsychiatric disorders. A2AR-D2R and A1R-A2AR heteromers are segregated in different striatal neuronal elements. While A2AR-D2R heteromers are located postsynaptically in the dendritic spines of the indirect MSNs [4–6,14], A1R-A2AR receptor heteromers are located presynaptically in glutamatergic terminals contacting the MSNs of the direct pathway [3,11,14]. Blocking postsynaptic A2AR in the indirect MSN should potentiate D2R-mediated motor activation, which is a strategy already used in the development of anti-parkinsonian drugs [26–28]. However, blocking A2AR in glutamatergic terminals to the direct MSN could potentially be useful in dyskinetic disorders such as Huntington’s disease and maybe in obsessive-compulsive disorders and drug addiction [5]. The present results give a mechanistic explanation to the already reported antiparkinsonian activity of KW-6002 [27,28] and suggest that SCH-442416 could be useful in dyskinetic disorders, obsessive-compulsive disorders and in drug addiction. Medicinal chemistry and computerized modeling should help understanding the molecular properties that determine the particular pharmacological profile of SCH-442416 and KW-6002, which may be used as lead compounds to obtain more effective antidysskinetic and antiparkinsonian compounds, respectively. It will also be of importance to take into account potential changes in the expression of pre- and postsynaptic A2ARs and in their respective heteromers which can occur in those mentioned neuropsychiatric disorders. For instance, dopamine denervation seems to differentially modify the expression of striatal A2AR, A1R and D2R [28–31]. This could be addressed by applying the in vivo methodology here described to animal models.

Acknowledgments
We acknowledge the technical help obtained from Jasmina Jiménez (Molecular Neurobiology laboratory, Barcelona University).

Author Contributions
Conceived and designed the experiments: MO JB MB CQ VB SRG CL AC RC VC EIC SF. Performed the experiments: MO JB MB CQ AC VC. Analyzed the data: MO CQ CL AC VC SF. Wrote the paper: MO CL AC VC SF.

References
1. Gerfen CR (2004) Basal Ganglia. In: Paxinos G, ed. The Rat Nervous System. Amsterdam: Elsevier Academic Press. pp 445–508.
2. Obeso JA, Rodríguez-Oroz MC, Rodríguez M, Arbizu J, Giménez-Amaya JM (2002) The basal ganglia and disorders of movement: pathophysiological mechanisms. News Physiol Sci 17: 51–55.
3. DeLong MR, Wichmann T (2007) Circuits and circuit disorders of the basal ganglia. Arch Neurol 64: 20–24.
4. Ferre S, Quirós C, Woods AS, Chandra R, Popoli P, et al. (2008) An update on adenosine A2A-dopamine D2 receptor interactions. Implications for the function of G protein-coupled receptors. Curr Pharm Des 14: 1468–1474.
5. Quirós C, Luján R, Uchigashima M, Simoes AP, Lerner TN, et al. (2009) Key Modulatory Role of Presynaptic Adenosine A2A Receptors in Cortical Neurotransmission to the Striatal Direct Pathway. TheScientificWorldJournal 9: 1321–1344.
6. Azad K, Gaff D, Woods AS, Ledent C, Ferre S, et al. (2009) Dopamine D2 and adenosine A2A receptors regulate NMDA-excitation in accumbens neurons through A2A-D2 receptor heterorimerization. Neuropsychopharmacology 34: 972–986.
7. Highley MJ, Sahabini BL (2010) Competitive regulation of synaptic Ca2+ influx by D2 dopamine and A2A adenosine receptors. Nat Neurosci 13: 958–966.
8. Svenningson P, Lundskog M, Ledent C, Parmentier M, Greengard P, et al. (2009) Regulation of the phosphorylation of the GnrH1 AMPA receptor by dopamine D2 receptors. J Neurochem 96: 462–474.
9. Chen JF, Moratalla R, Impagnatiello F, Grandy DK, Cuellar B, et al. (2003) The role of the D2/D2 dopamine receptor (D2R/2R) in A2A/2A adenosine receptor (A2AR)-mediated behavioral and cellular responses as revealed by A2A/2A and D2R/2R receptor knockout mice. Proc Natl Acad Sci U S A 98: 1970–1975.
10. Ciruela F, Casado V, Rodriguez RJ, Luján R, Burgueno J, et al. (2006) Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1-A2A receptor heteromers. J Neurosci 26: 2089–2097.
11. Quirós C, Pearson V, Gulyani S, Allen R, Earley C, et al. (2010) Up-regulation of striatal A2A2 receptors with iron deficiency in rats. Effects on locomotion and cortico-striatal neurotransmission. Exp Neurol 224: 292–298.
12. Ferré S, Baler R, Bourier M, Caron MG, Devi LA, et al. (2009) Building a new conceptual framework for receptor heteromers. Nat Chem Biol 5: 131–134.
13. Ferré S, Ciruela F, Woods AS, Lüscher C, Franco R (2007) Functional relevance of neurotransmitter receptor heteromers in the central nervous system. Trends Neurosci 30: 440–446.
14. Quarta D, Ferré S, Solinas M, You Z-B, Hockemeyer J, et al. (2004) Adenosine receptor-mediated modulation of dopamine release in the nucleus accumbens depends on glutamate neurotransmission and NMDA receptor stimulation. J Neurochem 91: 873–880.
15. Navarro G, Aymerich MS, Marcellino D, Cortés A, Casado V, et al. (2009) Interactions between calmodulin, adenosine A2A and dopamine D2 receptors. J Biol Chem 284: 29050–29061.
16. Sarrió S, Casado V, Escriva M, Ciruela F, Mallo J, et al. (2000) The heat shock cognate protein hsc73 assembles with A1 adenosine receptors to form functional modules in the cell membrane. Mol Cell Biol 20: 5164–5174.
17. Franco R, Casado V, Mallo J, Ferré S, Fuxe K, et al. (2005) Dimer-based model for heptaspanning membrane receptors. Trends Biochem Sci 26: 221–232.
18. Casado V, Cortés A, Mallo J, Pérez-Capote K, Ferré S, et al. (2009) GPCR homomers and heteromers: A better choice as targets for drug development than GPCR monomers? Pharmacol Ther 124: 249–257.
19. Ciruela F, Casado V, Mallo J, Canela EI, Lüscher C, et al. (1995) Immunological identification of A1 adenosine receptors in brain cortex. J Neurosci Res 42: 810–826.
20. Canah M, Burgueno J, Marcellino D, Cabello N, Canela EL, et al. (2004) Homodimerization of adenosine A2A receptors: qualitative and quantitative...
assessments by fluorescence and bioluminescence energy transfer. J Neurochem
88: 726–734.
22. Guo W, Urizar E, Kralikova M, Mobarec JC, Shi L, et al. (2008) Dopamine D2
receptors form higher order oligomers at physiological expression levels. EMBO J
27: 2293–2304.
23. Han Y, Moreira IS, Urizar E, Weinstein H, Javitch JA (2008) Allosteric
Communications between protomers of dopamine class A GPCR dimers
modulates activation. Nat Chem Biol 9: 688–695.
24. Casado V, Cortes A, Ciruela F, Mallol J, et al. (2007) Old and new ways to
calculate the affinity of agonists and antagonists interacting with G-protein-
coupled monomeric and dimeric receptors: The receptor-dimer cooperativity
index. Pharmacol Ther 116: 343–354.
25. Casado V, Martí T, Franco R, Lisán C, Mallol J, et al. (1990) A method for
binding parameters estimation of A1 adenosine receptor subtype: a practical
approach. Anal Biochem 184: 117–123.
26. Schwarzschild MA, Agnati L, Fuxe K, Chen JF, Morelli M (2007) Targeting
adenosine A2A receptors in Parkinson’s disease. Trends Neurosci 29: 647–654.
27. Jenner P (2003) A2A antagonists as novel non-dopaminergic therapy for motor
dysfunction in PD. Neurology 61: S32–S8.
28. Stacy M, Silver D, Mendis T, Sutton J, Mori A, et al. (2008) A 12-week, placebo-
controlled study (6002-US-006) of istradefylline in Parkinson disease. Neurology
70: 2235–2240.
29. Kasunen V, Ruottinen HM, Nägren K, Leikonen I, Oikonen V, et al. (2000)
Upregulation of putaminal dopamine D2 receptors in early Parkinson’s disease:
a comparative PET study with [11C] raclopride and [11C]N-methylspiperone.
J Nucl Med 41: 63–70.
30. Pappa A, Cores C, Carta AR, Valentini V, Pedata F, et al. (2002) Modification of
adenosine extracellular levels and adenosine A2A receptor mRNA by
dopamine denervation. Eur J Pharmacol 446: 75–82.
31. Varani K, Vincenzi F, Tosi A, Gesi S, Casetta I, et al. (2010) A2A adenosine
receptor overexpression and functionality, as well as TNF-alpha levels, correlate
with motor symptoms in Parkinson’s disease. FASEB J 24: 587–598.