



Pharmacological evidence for different populations of postsynaptic adenosine A_{2A} receptors in the rat striatum

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ABSTRACT

Adenosine A_{2A} receptors (A_{2A}Rs) are highly concentrated in the striatum. Two pharmacological different functional populations of A_{2A}Rs have been recently described based on their different affinities for the A_{2A}R antagonist SCH-442416. This compound has high affinity for A_{2A}Rs not forming heteromers or forming heteromers with adenosine A₁ receptors (A₁Rs) while showing very low affinity for A_{2A}Rs forming heteromers with dopamine D₂ receptors (D₂Rs). It has been widely described that striatal A₁R–A_{2A}R heteromers are preferentially localized presynaptically in the glutamatergic terminals that contact GABAergic dynorphinergic neurons, and that A_{2A}R–D₂R heteromers are localized postsynaptically in GABAergic enkephalinergic neurons. In the present study we provide evidence suggesting that SCH-442416 also targets postsynaptic A_{2A}R not forming heteromers with D₂R, which are involved in the motor depressant effects induced by D₂R antagonists. SCH-442416 counteracted motor depression in rats induced by the D₂R antagonist raclopride at a dose that did not produce motor activation or that blocked motor depression induced by an A_{2A}R agonist. Furthermore, we re-evaluated the recently suggested key role of cannabinoid CB₁ receptors (CB₁Rs) in the effects of A_{2A}R antagonists acting at postsynaptic A_{2A}Rs. By recording locomotor activity and monitoring striatal glutamate release induced by cortical electrical stimulation in rats after administration of A_{2A}R and CB₁R antagonists, we did not find evidence for any significant role of endocannabinoids in the post- or presynaptic effects of A_{2A}R antagonists. The present results further suggest the existence of at least two functionally and pharmacologically different populations of striatal postsynaptic A_{2A}Rs.

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1. Introduction

Adenosine A_{2A} receptors (A_{2A}Rs) are highly abundant in the striatum, where they are preferentially localized postsynaptically in the GABAergic striatopallidal enkephalinergic medium spiny neurons (MSNs) (Quiroz et al., 2009; Rosin et al., 2003; Schiffmann et al., 2007). These neurons also present a high density of dopamine D₂ receptors (D₂Rs) (Schiffmann et al., 2007). The existence of postsynaptic mechanisms in the control of glutamatergic neurotransmission to the enkephalinergic MSN by at least two reciprocal antagonistic interactions between A_{2A}Rs and D₂Rs has been described (Ferré et al., 2008). In one type of these antagonistic interactions, stimulation of A_{2A}R counteracts the D₂R agonist-mediated inhibitory modulation of NMDA receptor-mediated effects (Azdad et al., 2009; Higley and Sabatini, 2010). This A_{2A}R–

D₂R interaction depends on the formation of A_{2A}R–D₂R heteromers (Azdad et al., 2009) and seems to be mostly responsible for the locomotor activating effects of A_{2A}R antagonists (Ferré et al., 2008; Orrú et al., 2011).

In a second type of A_{2A}R–D₂R receptor interaction, D₂R stimulation prevents A_{2A}R to signal through the activation of adenylyl cyclase (AC) pathway (Chen et al., 2001; Håkansson et al., 2006; Hillion et al., 2002; Kull et al., 1999). Under normal conditions, the ability of A_{2A}Rs to activate the AC–PKA cascade is restrained by a tonic inhibitory effect of endogenous dopamine on striatal D₂R, which efficiently inhibits A_{2A}R-mediated AC activation (Svenningsson et al., 1999; Karcz-Kubicha et al., 2003a). Antagonism of D₂R is then associated with a significant activation of the AC–PKA cascade and the motor depressant and most biochemical effects induced by genetic or pharmacologic blockade of D₂Rs are counteracted by genetic or pharmacological blockade of A_{2A}Rs (Bertran-Gonzalez et al., 2009; Chen et al., 2001; Håkansson et al., 2006). The two described reciprocal antagonistic interactions take place simultaneously in the same cell, suggesting that they are most likely mediated by the existence of

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two different populations of postsynaptic striatal A_{2A}R in the enkephalinergic MSN (Ferré et al., 2008). The two proposed populations could be distinguished by their ability of forming or not heteromers with D₂Rs (Fig. 1).

Striatal A_{2A}Rs are also localized presynaptically, in glutamatergic terminals that contact GABAergic striatonigral dynorphinergic MSNs (Rosin et al., 2003; Quiroz et al., 2009), where they heteromerize with A₁ receptors (A₁Rs) (Ciruela et al., 2006; Quiroz et al., 2009) (Fig. 1). Activation of the A₁R in the A₁R–A_{2A}R heteromer produces inhibition of glutamate release, while the additional activation of the A_{2A}R produces the opposite effect (Ciruela et al., 2006). A recent study demonstrated that the affinity of A_{2A}Rs for several A_{2A}R antagonists differs depending on heteromerization with different receptors (Orrú et al., 2011). For instance, the A_{2A}R antagonist SCH-442416 binds with similar affinity to A_{2A}Rs not forming heteromers or forming heteromers with A₁Rs, but binds with much lower affinity to A_{2A}Rs forming heteromers with D₂Rs. On the other hand, from several tested A_{2A}R antagonists, KW-6002 showed the best relative affinity for A_{2A}Rs co-expressed with D₂Rs. Parallel *in vivo* experiments demonstrated that A_{2A}R antagonists with different pharmacological profile could be used to dissect the functional role of presynaptic A_{2A}Rs that form heteromers with A₁R and postsynaptic A_{2A}Rs that form heteromers with D₂Rs (Orrú et al., 2011). In the present study, we have used this pharmacological approach to further understand the functional role of postsynaptic A_{2A}Rs, and distinguish between those forming and those not forming heteromers with D₂Rs. Furthermore, the present study was also aimed at reevaluating the role of endocannabinoids in the functional role of the different populations of pre- and postsynaptic striatal A_{2A}Rs.

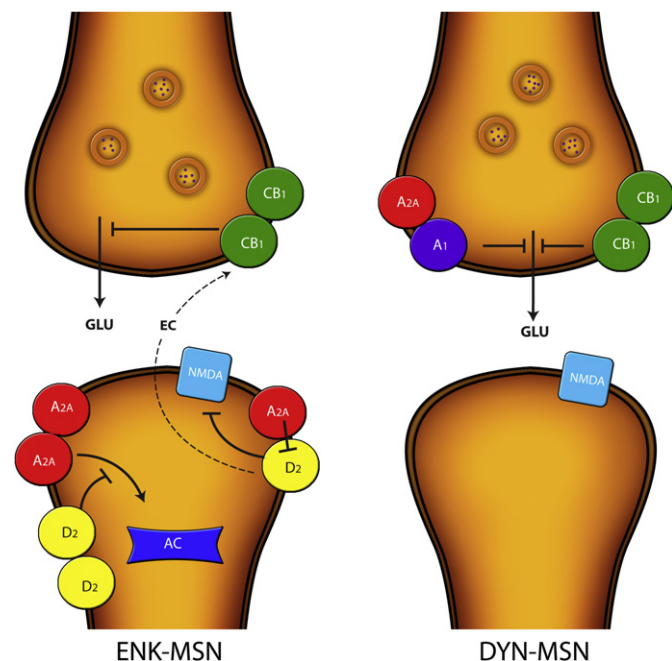


Fig. 1. Scheme of the different subpopulations of striatal A_{2A}Rs. Presynaptic A_{2A}Rs are localized in glutamatergic nerve terminals that contact dynorphinergic medium spiny neurons (DYN-MSNs), while postsynaptic A_{2A}Rs forming or not forming heteromers with D₂Rs are localized in the enkephalinergic medium spiny neurons (ENK-MSNs). An A_{2A}R–D₂R heteromer-mediated modulation of endocannabinoid (EC) has recently been suggested to modulate glutamate (GLU) release to the ENK-MSN (dashed arrow). CB₁: CB₁ receptor; NMDA: NMDA receptor; AC: adenylyl-cyclase; solid arrows: activation; interrupted solid line: inhibition. Receptors not forming heteromers are shown as forming homomers. For further explanations, see text.

2. Material and methods

2.1. Animals and drugs

Male Sprague–Dawley albino rats (362 animals; Charles River Laboratories, Wilmington, MA, USA), weighting 300–350 g, were used in all the experimental procedures. Animals were housed 2 per cage and kept on a 12/12-h dark/light cycle with food and water available *ad libitum*. All animals used in the study were maintained in accordance with the guidelines of National Institutes of Health Animal Care and the animal research conducted to perform this study was approved by the NIDA IRP Animal Care and Use Committee (protocol #:09-BNRR-73). A_{2A}R antagonists SCH-442416 and KW-6002 were provided by CHDI Foundation Inc. (Los Angeles, CA, USA), whereas MSX-3, a soluble phosphate pro-drug of MSX-2, was synthesized at the Pharmaceutical Institute, University of Bonn (Bonn, Germany) (Hockemeyer et al., 2004). A_{2A}R agonist CGS-21860 and D₂R antagonist raclopride tartrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The cannabinoid CB₁ receptor (CB₁R) antagonist SR-141716A and the CB₁R agonist Δ⁹-tetrahydrocannabinol (THC) were provided by the National Institute on Drug Abuse (Baltimore, MD, USA). SCH-442416 was suspended in a solution of 5% dimethyl- sulfoxide (DMSO) (Sigma–Aldrich, St. Louis, MO, USA), 5% TWEEN80 (Sigma-Aldrich, St. Louis, MO, USA) and 90% ddH₂O; KW-6002 was suspended in a solution of 8% TWEEN80 and 92% ddH₂O; SR-141716A was dissolved in vehicle containing 2% ethanol and 2% TWEEN80 and saline. Raclopride and CGS-21860 were dissolved in saline solution. All drugs were administered in an injection volume of 2 ml/kg body weight (i.p.). MSX-3 was used in microdialysis experiments, since is an A_{2A}R antagonist that can be dissolved in artificial CSF and, therefore, perfused in the striatum through the microdialysis probe (reverse dialysis).

2.2. Locomotor activity

Locomotor activity was measured by placing the animals individually in open field motility soundproof chambers (50 × 50 cm) (Med Associates Inc., VT, USA). Locomotion was measured by counting the number of breaks in the infrared beams of the chambers during consecutive periods of 10 min. A lamp inside each chamber remained lit during this period. Rats were moved into the experimental room and allowed to habituate to the testing room for 2 h before being introduced in the chamber. The animals were not habituated to the experimental cages and the recording of the locomotor activity started immediately after placing the animals in the boxes and lasted for 90 min. Data were analyzed as the average of all transformed values (square root) of accumulated counts per 10 min during the first hour. This period corresponds to the highest exploratory activity during exposure to a new test environment. Four sets of experiments were performed. In the first set of experiments, rats were injected i.p. with increasing doses of vehicle, KW-6002 (0.1–3 mg/kg) or SCH-442416 (0.1–3 mg/kg) 40 min before placing them in the experimental boxes (*n* = 6–8 animals per group). In the second set of experiments rats were placed in the motility cages 40 min after an injection of vehicle, KW-6002 (1 mg/kg) or SCH-442416 (1 mg/kg) and 10 min after vehicle or CGS-21860 (0.2 mg/kg) (*n* = 8–10 animals per group). In the third set of experiments, animals were injected with vehicle, KW-6002 (1 mg/kg) or SCH-442416 (1 mg/kg) 40 min before being placed in the motility cages and 70 min before vehicle or raclopride (0.1 mg/kg) (*n* = 8–10 animals per group). In the fourth group of experiments, animals were injected with vehicle, KW-6002 (1 mg/kg) or SCH-442416 (3 mg/kg) 40 min before being placed in the motility boxes and 90 min before vehicle or SR-141716A (*n* = 8–10 animals per group). The timing and doses were selected based on previous experiments in which they elicited fully significant effects in the behavioral parameters tested, under our experimental conditions (Karcz-Kubicha et al., 2003b; Orrú et al., 2011; Solinas et al., 2010). All animals were tested only once.

2.3. Surgical procedures

Rats were deeply 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 distilled H₂O up to 100 ml of final solution (NIDA Pharmacy, Baltimore, MD, USA) and implanted unilaterally with a bipolar stainless steel electrodes, 0.15 mm in diameter (Plastics One, Roanoke, VA, USA), into the orofacial area of the lateral agranular motor cortex (coordinates in mm: anterior, 3.0; lateral, 3.0 and 4.0, and vertical –4.2 from bregma) and a microdialysis probe with a 2 mm-long H69 membrane in the lateral striatum (coordinates in mm: anterior, 0.0, lateral, 4.5 and vertical –7.0 from bregma), as described previously (Orrú et al., 2011). After surgery, rats were allowed to recover in hemispherical CMA-120 cages (CMA Microdialysis AB, Solna, Sweden) equipped with a two-channel electrical swivel (Plastics One, Roanoke, VA, USA) and two-channel overhead fluid swivels (Instech, Plymouth Meeting, PA, USA) connected to a sample collector (CMA 470, CMA, Solna, Sweden).

2.4. Cortical electrical stimulation and *in vivo* microdialysis

Twenty-four hours after implanting the electrode and the probe, experiments were performed on freely moving rats in the same hemispherical home cages in

which they recovered overnight from surgery. An artificial cerebrospinal solution containing (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 and the electrodes were attached to the electrical swivel with shielded flexible cabling. Cortical electrical stimulation (biphasic pulses of 0.1 ms, 80–150 µA, 100 Hz, 160-ms trains repeating once per second) was applied using a square pulse generator (Grass S88X, Grass Instruments, West Warwick, RI, USA) attached to two-coupled constant current units (Grass PSIU6, Grass Instruments, West Warwick, RI, USA). Current intensity for each rat was established before the start of the microdialysate sample collection by briefly testing the minimal values of current producing distinctive jaw movements paired with the stimulation trains in at least 90% of the cases. Current values were in the 80–150 µA range with few cases reaching 200 µA. Animals who failed to show observable jaw movements at 200 µA were discarded of the experiment (6 out of 88). After a washout period of 90 min, a total of 11 microdialysate samples were collected at 20-min intervals with a refrigerated fraction collector (CMA 470, CMA, Solna, Sweden). After 60 min of collecting samples for baseline, animals received either an intraperitoneal injection of 1 mg/kg of SR-141716A or vehicle followed after 50 min of an injection of THC (3 mg/kg) or vehicle. In a second set of experiments, rats were injected intraperitoneally either with 1 mg/kg of SR-141716A or vehicle followed after 50 min for reverse dialysis of either 1 µM of MSX-3, 1 µM of raclopride or the combination of both dissolved in ACSF during 40 additional minutes before applying electrical cortical stimulation, matching the timing of the treatments in the locomotor activity experiments described above. In all groups, 90 min after the first injection of SR-141716A or vehicle, trains of current were applied to the orofacial motor cortex during 20 min. The microdialysate samples were collected for additional 60 min, loaded in a refrigerated autosampler and analyzed by HPLC equipped with an immobilized glutamate oxidase reactor and electrochemical detection (Eicom Corporation, Kyoto, Japan). The amount of glutamate in the samples was determined by a five-point calibration curve and data was normalized against the mean value of the three samples collected prior stimulation. At the end of the experiment, rats were given an overdose of equithesin, the brains were extracted, fixed in formaldehyde, and probe and electrode placement were verified using cresyl violet staining.

2.5. Statistical analysis

Prism GraphPad 5 was used for all statistical analyses. All data are presented as mean ± S.E.M. For the locomotor activity experiments, the significance of differences between groups was determined by one- or two-way analysis of variance (ANOVA) followed by Newman–Keuls's or Bonferroni–Dunn's tests for multiple comparisons, respectively. Statistical significance was accepted at the $p < 0.05$ level. For the microdialysis experiments, data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison tests to compare between the effects of different drugs on the release of glutamate induced by cortical electrical stimulation. Only the values of the samples obtained after cortical stimulation were used for the analysis.

3. Results

3.1. Differential effect of A_{2A}R antagonists on the locomotor depression induced by a selective A_{2A}R agonist and by a selective D₂R antagonist

The comparison of the dose-response effects of KW-6002 and SCH-442416 on locomotion in rats habituated to the test environment has recently been reported (Orrú et al., 2011). Under these experimental conditions, basal locomotor activity is very low, making difficult to interpret the effects of locomotor-depressant drugs. Therefore, we performed our experiments in rats not habituated to the testing environment. The same potency than in previous experiments (Orrú et al., 2011) in habituated rats was obtained, with KW-6002 being already effective at 0.1 mg/kg and inducing a high locomotor response at 1 mg/kg (one-way ANOVA: $F_{(4,31)} = 6.8$, $p < 0.001$; Fig. 2a and c). Newman–Keuls's test showed significant effect compared with the vehicle-treated group with the doses of 0.1 to 3 mg/kg (see Fig. 2c for degree of significance). SCH-442416 was effective at 3 mg/kg but did not alter significantly the locomotor activity at lower doses (one-way ANOVA: $F_{(4,30)} = 6.9$, $p < 0.001$; Fig. 2b and c). Newman–Keuls's test only showed significant effect compared with the vehicle-treated group with the dose of 3 mg/kg (see Fig. 2c for degree of significance).

The dose of 1 mg/kg for each compound was chosen then to evaluate their possible antagonistic effect on the motor depressant effect of equipotent doses (as assessed in pilot experiments) of the

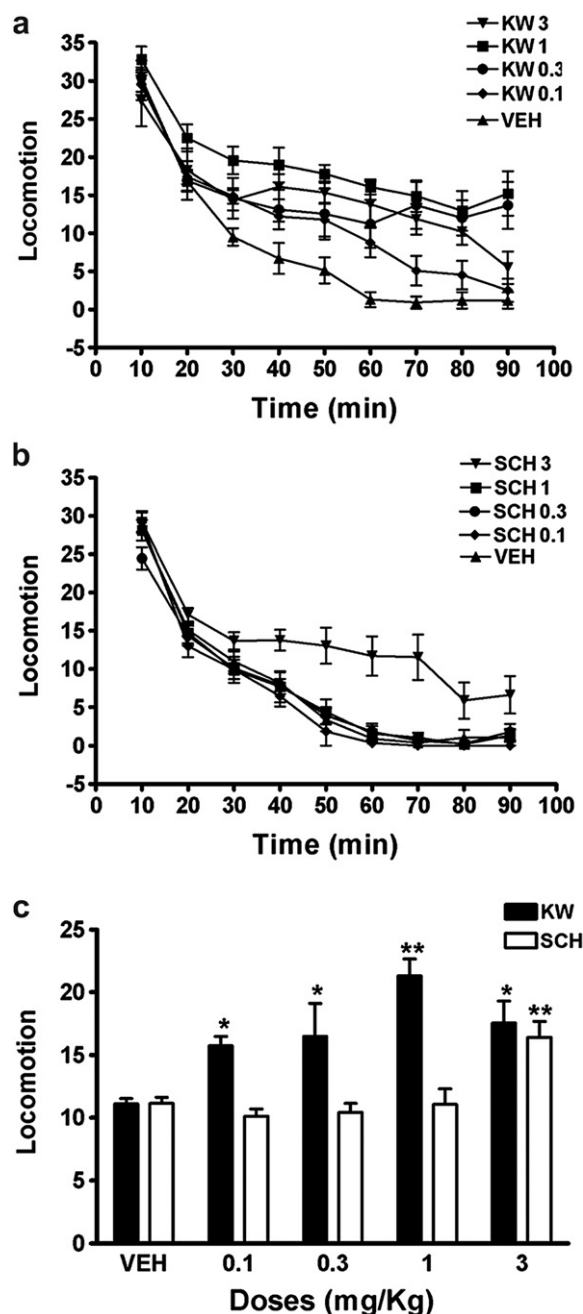


Fig. 2. Locomotor activation induced by the A_{2A}R antagonists KW-6002 and SCH-442416. Dose-response of the locomotor-activating effects of KW-6002 (KW) and SCH-442416 (SCH) in non-habituated rats (60 min) after 40 min following drug administration. In (a) and (b), time course of locomotion per 10-min periods. Data represent means ± S.E.M. of transformed data (square root) of accumulated counts per period ($n = 6-8$ per group). In (c), average (means ± S.E.M.) of the 10-min period transformed values during the first 60 min of recording. * and **: $p < 0.05$ and $p < 0.01$, respectively, compared to vehicle-treated animals (VEH); one-way ANOVA with *post-hoc* Newman–Keuls's comparisons.

A_{2A}R agonist CGS-21680 (0.2 mg/kg) and the D₂R antagonist raclopride (0.1 mg/kg). KW-6002 produced the same locomotor activation with or without co-administration of CGS 21680 (one-way ANOVA: $F_{(3,27)} = 26.5$, $p < 0.001$; Fig. 3a and c). Newman–Keuls's test showed a significant locomotor activation compared with the vehicle-treated group with KW-6002, alone or co-administered with CGS-21680, and a significant locomotor depression compared with the vehicle-treated group with CGS-

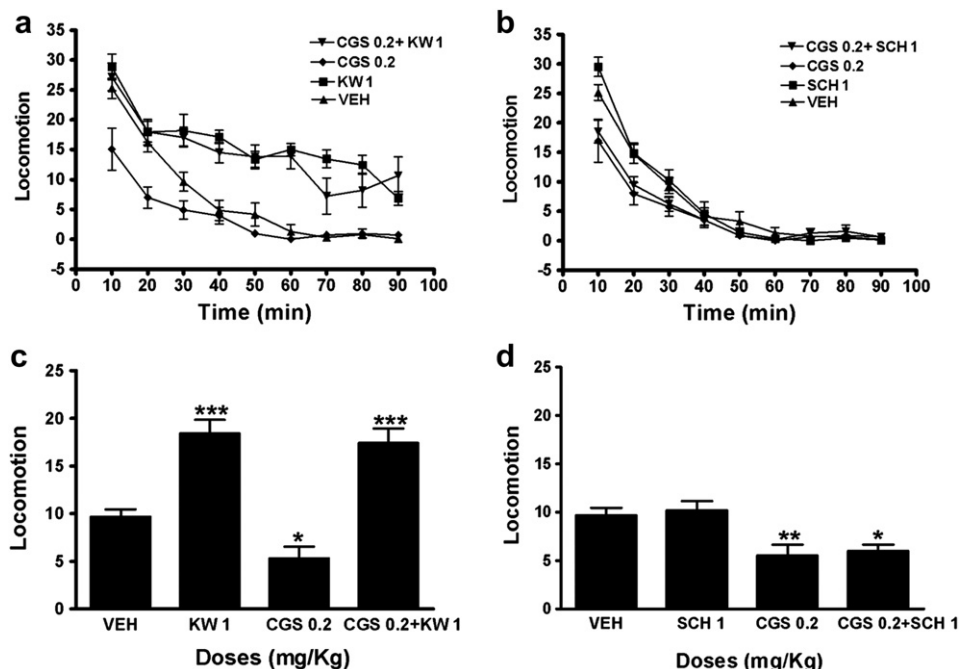


Fig. 3. Effect of the A_{2A} R antagonists KW-6002 and SCH-442416 on the motor depression induced by the A_{2A} R agonist CGS-21680. Animals were placed in the motility cages 40 min after vehicle (VEH), KW-6002 (KW, 1 mg/kg) or SCH-442416 (SCH, 1 mg/kg) administration and 10 min after VEH or CGS-21680 (CGS, 0.2 mg/kg). In (a) and (b), time course of locomotion per 10-min periods. Data represent means \pm S.E.M. of transformed data (square root) of accumulated counts per period ($n = 8-10$ per group). In (c) and (d), average (means \pm S.E.M.) of the 10-min period transformed values during the first 60 min of recording. *, ** and ***: $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, compared to VEH-treated animals; one-way ANOVA with *post-hoc* Newman–Keuls’s comparisons.

21680 administered alone (see Fig. 3c for degree of significance). Similarly, KW-6002 produced the same locomotor activation with or without co-administration of raclopride (one-way ANOVA: $F_{(3,29)} = 89.2$, $p < 0.001$; Fig. 4a and c). Newman–Keuls’s test showed a significant locomotor activation compared with the vehicle-treated group with KW-6002, alone or co-administered

with raclopride, and a significant locomotor depression compared with the vehicle-treated group with raclopride administered alone (see Fig. 4c for degree of significance). On the other hand, SCH-442416 did not produce locomotor activation and did not counteract the motor depression induced by CGS 21680 (one-way ANOVA: $F_{(3,34)} = 7.4$, $p < 0.001$; Fig. 3b and d). Newman–Keuls’s

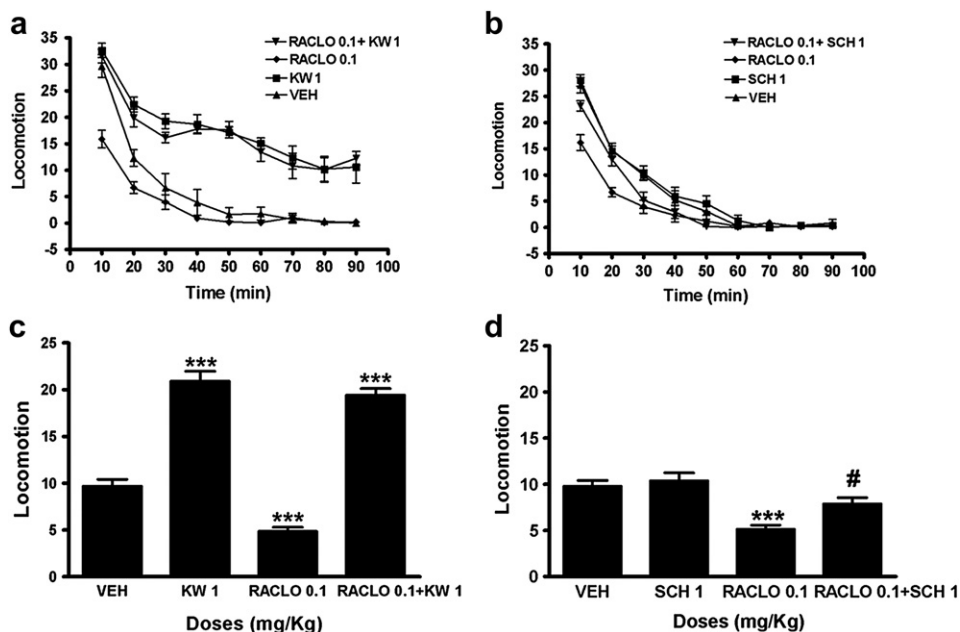


Fig. 4. Effect of the A_{2A} R antagonists KW-6002 and SCH-442416 on the motor depression induced by the D_2 R antagonist raclopride. Animals were placed in the motility cages 40 min after vehicle (VEH), KW-6002 (KW, 1 mg/kg) or SCH-442416 (SCH, 1 mg/kg) administration and 70 min after VEH or raclopride (RACLO, 0.1 mg/kg). In (a) and (b), time course of locomotion per 10-min periods. Data represent means \pm S.E.M. of transformed data (square root) of accumulated counts per period ($n = 8-10$ per group). In (c) and (d), average (means \pm S.E.M.) of the 10-min period transformed values during the first 60 min of recording. *, ** and ***: $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, compared to VEH-treated animals; #: $p < 0.05$ compared to RACLO; one-way ANOVA with *post-hoc* Newman–Keuls’s comparisons.

test did not show a significant difference between the group treated with CGS-21680 alone and the group also treated with SCH-442416 (see Fig. 3d for degree of significance). On the other hand, SCH-442416 counteracted the motor depression induced by raclopride (one-way ANOVA: $F_{(3,37)} = 12.4$, $p < 0.001$; Fig. 4b and d). Newman–Keuls's test showed a significant difference between the group treated with raclopride alone and the group also treated with SCH-442416 (see Fig. 4d for degree of significance).

3.2. Independent effects of a CB₁R antagonist on the locomotor activation induced by the A_{2A}R antagonist KW-6002

The CB₁R antagonist SR-141716A (1 mg/kg) significantly decreased the locomotor effects induced by the A_{2A}R antagonist KW-6002 (1 mg/kg) (Fig. 5a and c) and also by a locomotor activating dose of SCH-442416 (3 mg/kg) (Fig. 5b and d). However, the CB₁R antagonist also produced a significant decrease in locomotion as compared to vehicle-treated animals (Fig. 5a and c). In the experiments with KW-6002 (Fig. 5a and c), a two-way ANOVA showed a significant depressant effect of the CB₁R antagonist ($F_{(1,28)} = 18.4$, $p < 0.001$) and a significant activating effect of the A_{2A}R antagonists ($F_{(1,28)} = 68.8$, $p < 0.0001$) without significant interaction ($F_{(1,28)} = 0.03$). In the experiments with SCH-442416 (Fig. 5b and d), a two-way ANOVA showed a significant depressant effect of the CB₁R antagonist ($F_{(1,27)} = 19.9$, $p < 0.001$) and a significant activating effect of the A_{2A}R antagonists ($F_{(1,27)} = 32.4$, $p < 0.0001$) without significant interaction ($F_{(1,27)} = 0.05$).

3.3. Lack of effect of a D₂R antagonist and a CB₁R antagonist on A_{2A}R-mediated modulation of cortico-striatal glutamate release

In vivo microdialysis was used to study striatal glutamate release induced by cortical electrical stimulation as previously described (Orrú et al., 2011). Whenever hydrosoluble compounds were

available, they were chosen for intrastriatal perfusion (reverse dialysis). We first compared the effect of systemic administration of THC (3 mg/kg), SR-141716A (1 mg/kg) alone and combination with that of the vehicle on glutamate release induced by cortical stimulation (Fig. 6a). One-way ANOVA showed an overall significant effect ($F_{(3,31)} = 3.2$, $p < 0.04$) and the post-hoc analysis showed a significant reduction compared to vehicle only with the THC group (Dunnett's tests: $p < 0.05$). The data therefore indicate that THC blocks glutamate release induced by cortical electrical stimulation and that this effect is counteracted by SR-141716A, which by itself does not produce any significant effect (Fig. 6a). Second, we compared the effect of intrastriatal perfusion of the A_{2A}R antagonist MSX-3 (1 μ M) or raclopride (1 μ M) alone or in combination with that of the vehicle, as well as the effect of combined intrastriatal administration of MSX-3 and systemic administration of SR-141716A (1 mg/kg). significantly ($p < 0.05$) counteracted glutamate release induced by cortical electrical stimulation (Fig. 6b). One-way ANOVA showed an overall significant effect ($F_{(4,45)} = 3.6$, $p < 0.01$) and the post-hoc analysis showed a significant reduction compared to vehicle with the MSX-3, MSX-3 plus raclopride and MSX-3 plus SR-141716A groups (Dunnett's tests: $p < 0.05$). The results therefore indicate that the effect of the A_{2A}R antagonist was not significantly modified by previous administration of SR-141716A (Fig. 6b). Furthermore, intrastriatal perfusion of raclopride did not modify basal striatal extracellular levels of glutamate, as previously reported (You et al., 2001; Marti et al., 2005) or glutamate release induced by cortical electrical stimulation or the counteracting effect of MSX-3 (Fig. 6b).

4. Discussion

Previous studies have shown that striatal presynaptic A_{2A}Rs localized in glutamatergic terminals and postsynaptic A_{2A}Rs localized in GABAergic enkephalinergic terminals form heteromers with

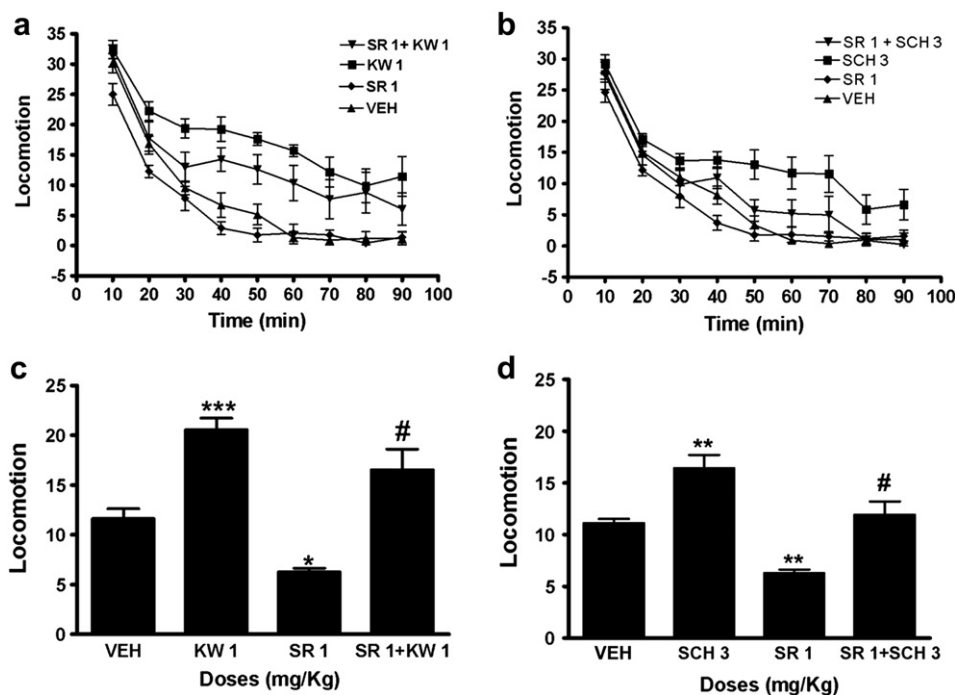


Fig. 5. Effect of the A_{2A}R antagonists KW-6002 and SCH-442416 on the motor depression induced by the CB₁ receptor antagonist SR-141716A. Animals were placed in the motility cages 40 min after vehicle (VEH), KW-6002 (KW, 1 mg/kg) or SCH-442416 (SCH, 1 mg/kg) administration and 90 min after VEH or SR-141716A (SR, 1 mg/kg). In (a) and (b), time course of locomotion per 10-min periods. Data represent means \pm S.E.M. of transformed data (square root) of accumulated counts per period ($n = 8-10$ per group). In (c) and (d), average (means \pm S.E.M.) of the 10-min period transformed values during the first 60 min of recording. *, ** and ***: $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, compared to VEH-treated animals; #: $p < 0.05$ compared to KW or SCH; two-way ANOVA with post-hoc Bonferroni–Dunn's comparisons.

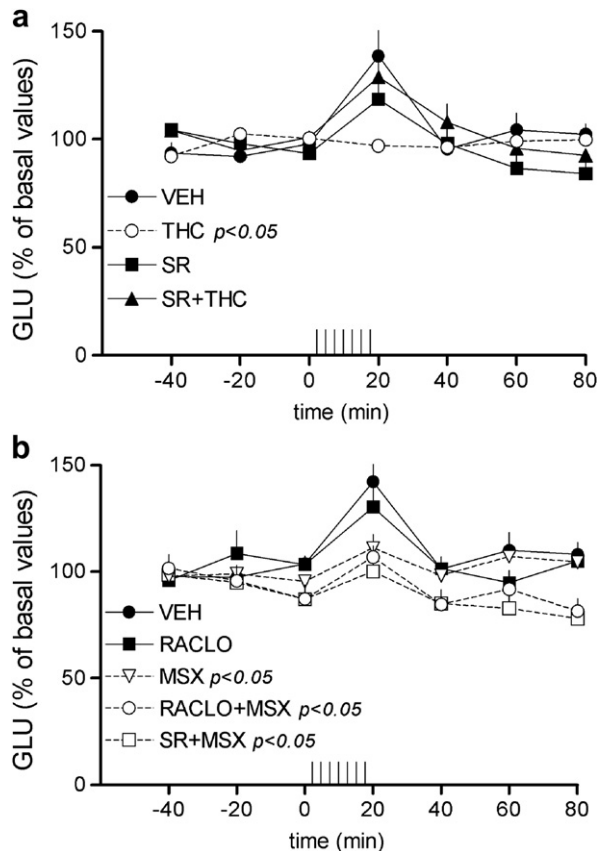


Fig. 6. Role of D₂R and CB₁R on the A_{2A}R-mediated modulation of cortico-striatal glutamate release. In (a), effect of the systemic administration of the CB₁R agonist THC (3 mg/kg) and the CB₁R antagonist SR-141716A (SR, 1 mg/kg), alone and in combination, on the increase in the extracellular levels of glutamate in the lateral striatum induced by cortical electrical stimulation. In (b), effect of the intra-striatal perfusion (reverse dialysis) of the D₂R antagonist raclopride (RACLO, 1 μM) and A_{2A}R antagonist MSX-3 (MSX, 1 μM), alone and in combination, and effect of the combined intra-striatal administration of MSX (1 μM) and systemic administration of SR (1 mg/kg) on the increase in the extracellular levels of glutamate in the lateral striatum induced by cortical electrical stimulation. Results are expressed as (means ± S.E.M.) of percentage of the average of the three values before the stimulation ($n = 6–18$ per group). Time '0' represents the values of the samples previous to the stimulation. The train of vertical lines represents the period of cortical stimulation. When shown, $p < 0.05$, represents the statistical significance of the respective group versus the vehicle group (VEH) (one-way ANOVA followed by Dunnett's test).

A₁Rs and D₂Rs, respectively (Ciruela et al., 2006; Azdad et al., 2009). We have recently shown that the A_{2A}R antagonists SCH-442416 and KW-6002 are able to pharmacologically differentiate between striatal pre- and postsynaptic A_{2A}Rs, respectively, based on their differential potencies with *in vivo* models, and also on their different *in vitro* affinities for binding to A_{2A}Rs that heteromerize with either A₁R or D₂Rs (Orrú et al., 2011). Thus, in cells expressing A_{2A}Rs and D₂Rs, SCH-442416 showed a marked loss of affinity with respect to cells expressing A_{2A}Rs and A₁Rs (an increase of about 40 times in the B₅₀ value; concentration competing 50% of radioligand binding) (Orrú et al., 2011). However, the affinity of SCH-442416 for A_{2A}Rs was the same in cells only expressing A_{2A}Rs as in cells expressing both A_{2A}Rs and A₁Rs (Orrú et al., 2011). The present work aimed at dissecting pharmacologically two subpopulations of striatal postsynaptic A_{2A}Rs. One population is forming heteromers with D₂Rs and seems to be the main responsible for the locomotor depressant and activating effects of A_{2A}R agonists and antagonists, respectively. The other population is not forming heteromers with D₂Rs and seems to be the main responsible for the motor-depressant effects of D₂R antagonists. Taking into account the

different affinity of SCH-442416 for A_{2A}Rs when forming or not heteromers with D₂Rs in transfected cells, it was predicted that SCH-442416 should also be a good ligand for the striatal post-synaptic A_{2A}Rs not forming heteromers with D₂Rs. In fact, SCH-442416, at a dose that does not produce motor activation, but that selectively blocks presynaptic A_{2A}Rs, significantly counteracted the motor depression induced by the D₂R antagonist raclopride. It is improbable that binding to presynaptic A_{2A}Rs could explain these effects, since they are predominantly localized in glutamatergic terminals that contact the dynorphinergic MSN (Quiroz et al., 2009). As shown in a previous study, using identified MSNs from the direct and indirect striatal pathways (dynorphinergic and enkephalinergic MSNs, respectively), A_{2A}R blockade selectively decreases glutamatergic neurotransmission through the dynorphinergic MSNs (Quiroz et al., 2009), which could not counteract raclopride-induced motor depression. If anything, and according to the most accept model of basal ganglia function (DeLong and Wichmann, 2007), presynaptic A_{2A}R blockade should further decrease motor activity. Therefore, the results clearly suggest that SCH-442416 binds to the subpopulation of post-synaptic A_{2A}Rs not forming heteromers with D₂Rs.

KW-6002 produced the same locomotor activation with or without co-administration of raclopride. Since, different from SCH-442416, KW-6002 showed the same B₅₀ values for A_{2A}Rs in cells expressing A_{2A}Rs and D₂Rs than in cells only expressing A_{2A}Rs (Orrú et al., 2011), the results suggest that KW-6002 blocks both subpopulations of postsynaptic A_{2A}Rs. Furthermore, KW-6002 also produced the same locomotor activation when co-administered with the A_{2A}R agonist CGS-21680, while SCH-442416, at the same dose that counteracted the depressant effect of raclopride, did not significantly counteract the depressant effect of CGS-21680. These results, further suggest that the subpopulation of postsynaptic A_{2A}R forming heteromers with D₂R are responsible for both the locomotor activation and depression induced by A_{2A}R antagonists and agonists, respectively.

The existence of at least one more subpopulation of postsynaptic A_{2A}Rs forming heteromers with CB₁Rs has been described (Carriba et al., 2007). In the A_{2A}R-CB₁R heteromer, activation of A_{2A}R is necessary to allow CB₁R-mediated signaling (Yao et al., 2003; Andersson et al., 2005; Carriba et al., 2007). Accordingly, Tebano et al. (2009) reported that the depression of synaptic transmission induced by a CB₁R agonist in cortico-striatal slices was prevented by A_{2A}R antagonists by postsynaptic mechanisms. These results would suggest that A_{2A}R antagonists should produce similar behavioral effects than CB₁R antagonists. In fact, pharmacological or genetic inactivation of A_{2A}Rs reduces the motor depressant, cataleptic and rewarding effects of CB₁R agonists (Andersson et al., 2005; Carriba et al., 2007; Justinova et al., 2010; Soria et al., 2004). Although all these studies point out that the motor (depressant) effects of CB₁R agonists might depend on adenosine A_{2A}R signaling, a recent study by Lerner et al. (2010) suggested quite the opposite. In this study, pharmacological inactivation of CB₁Rs reduced the locomotor activation induced by an A_{2A}R antagonist in mice habituated to the testing environment (Lerner et al., 2010). It was suggested that postsynaptic A_{2A}Rs modulate striatal glutamate release by involving endocannabinoids (Lerner et al., 2010). According to this hypothesis, an A_{2A}R antagonist produces locomotor activation by disinhibiting a tonic A_{2A}R-mediated inhibition of D₂R-mediated endocannabinoid release in the enkephalinergic MSN (Fig. 1). The mechanistic explanation of this interaction is related to the previously reported D₂R agonist-mediated endocannabinoid release by the enkephalinergic MSN, which by retrograde signaling would inhibit glutamate release by stimulating CB₁R localized in glutamatergic terminals. This would lead to a decreased stimulation of the enkephalinergic MSN, which would produce locomotor

activation (Kreitzer and Malenka, 2007). However, this interpretation would predict that CB₁R blockade should counteract the motor effects of D₂ receptor agonists and previous studies have reported otherwise (for a recent review, see Ferré et al., 2010). In the present study, and using non-habituated rats, we also show that CB₁R antagonists significantly decrease the locomotor effects induced by an A_{2A}R antagonist. However, we found that CB₁R antagonists produce a comparable decrease in locomotion in vehicle-treated animals. Altogether this suggests that the use of habituated animals (which display very low locomotor activity in the testing environment) could have masked the depressant effect of CB₁R antagonist in the study by Lerner et al. (2010).

With *in vivo* microdialysis experiments we found that the CB₁R agonist THC produced a complete inhibition of striatal glutamate release upon cortical electrical stimulation, which is in agreement with the suggested existence of importantly functional CB₁Rs in glutamatergic inputs to both enkephalinergic and dynorphinergic MSN (Kreitzer and Malenka, 2007; Tozzi et al., 2011) (Fig. 1). As previously reported, a significant decrease in glutamate release was found by the direct perfusion of the A_{2A} antagonist MSX-3 in the striatum (Quiroz et al., 2009). But, in disagreement with the possible involvement of D₂Rs, endocannabinoids and CB₁Rs, the effect of MSX-3 was not modified either by raclopride or by the CB₁R antagonist SR-141716A. It may still be postulated that the blockade of presynaptic CB₁Rs in glutamatergic terminals that contact enkephalinergic MSNs could be responsible for the low but significant locomotor depression induced by the CB₁R antagonist. Nevertheless, administration of SR-141716A, at a dose that completely blocked the effects of THC, did not produce a significant increase in striatal glutamate release upon cortical electrical stimulation. In summary, we suggest as a possible hypothesis that, *in vivo*, A_{2A}R-mediated modulation of glutamate release depends mostly on presynaptic A_{2A}Rs predominantly localized in the cortico-striatal terminals that contact dynorphinergic MSN (Fig. 1). Although we recently postulated the existence of A_{2A}R–CB₁R heteromers in striatal glutamatergic terminals that could mediate the reinforcing effects of cannabinoids (Ferré et al., 2010; Justinova et al., 2010), a recent study by Martire et al. (2011) indicates that these A_{2A}R–CB₁R interactions result from an interaction at the second messenger level, by which A_{2A}R activation prevents CB₁R-mediated inhibition of glutamate release.

In summary, we believe that the present study adds important new information about the existence of different functional and pharmacological populations of striatal A_{2A}Rs. The existence of these populations would allow better understand previous apparent discrepancies in the literature about the role of these receptors and their interactions with other receptors. Our work also emphasizes the possibility of targeting different populations of receptors in different pathological conditions, as recently suggested elsewhere (Orrú et al., 2011).

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Disclosure/conflict of interest

The authors declare that except for income received from the primary employer no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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