Receptor crosstalk: haloperidol treatment enhances A_{2A} adenosine receptor functioning in a transfected cell model

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Abstract A_{2A} adenosine receptors are considered an excellent target for drug development in several neurological and psychiatric disorders. It is noteworthy that the responses evoked by A_{2A} adenosine receptors are regulated by D₂ dopamine receptor ligands. These two receptors are co-expressed at the level of the basal ganglia and interact to form functional heterodimers. In this context, possible changes in A_{2A} adenosine receptor functional responses caused by the chronic blockade/activation of D₂ dopamine receptors should be considered to optimise the therapeutic effectiveness of dopaminergic agents and to reduce any possible side effects. In the present paper, we investigated the regulation of A_{2A} adenosine receptors induced by

antipsychotic drugs, commonly acting as D₂ dopamine receptor antagonists, in a cellular model co-expressing both A_{2A} and D₂ receptors. Our data suggest that the treatment of cells with the classical antipsychotic haloperidol increased both the affinity and responsiveness of the A_{2A} receptor and also affected the degree of A_{2A}–D₂ receptor heterodimerisation. In contrast, an atypical antipsychotic, clozapine, had no effect on A_{2A} adenosine receptor parameters, suggesting that the two classes of drugs have different effects on adenosine–dopamine receptor interaction. Modifications to A_{2A} adenosine receptors may play a significant role in determining cerebral adenosine effects during the chronic administration of antipsychotics in psychiatric diseases and may account for the efficacy of A_{2A} adenosine receptor ligands in pathologies associated with dopaminergic system dysfunction.

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Introduction

The endogenous neuromodulator adenosine controls and integrates a wide range of brain functions [1] and is involved in the etiopathogenesis of several pathologies ranging from epilepsy to neurodegenerative disorders and psychiatric conditions. The adenosine system has thus been recognised as a prime target for the development of new therapeutics to treat these diseases.

From epidemiological to animal studies, it is clear that extracellular adenosine acting at adenosine receptors (ARs) influences the functional outcome of a broad spectrum of brain injuries. In the brain, A_{2A}ARs are mainly expressed at the level of neurons, glial cells [2–7] and peripheral inflammatory

cells, playing a role in both neuroprotection and neurodegeneration [8–10]. The alterations in A_{2A}AR density and functional responsiveness that occurred during pathological conditions (i.e. neurological and psychiatric diseases) represent a crucial aspect in the balance between the protective and toxic effects exerted by this receptor subtype at the central level. Using a rat model of focal ischaemia, it has been demonstrated that increases in A_{2A}AR density may contribute to A_{2A}AR-mediated neuron death [11]. In addition, an increase in A_{2A}AR density and/or functionality has also been detected in psychiatric diseases such as schizophrenia [12] and bipolar disorders [13]: in these pathologies, alterations in A_{2A}AR parameters have been related to the chronic use of antipsychotic drugs, D₂ dopamine receptor (DR) antagonists [14–16], and also the functional crosstalk between the adenosine and dopamine systems.

In recent years, it has been indicated that adenosine and dopamine interact functionally in the brain and that this structural association may have pathophysiological and therapeutic implications [17–19]. In the corpus striatum and mesolimbic areas, where the two receptors mostly co-localise [20], it has been demonstrated that they are structurally associated to form functional heterodimers in which the adenosine and dopamine receptors mutually antagonise their respective responses [21–25]. On the basis of these results, A_{2A}AR agonists have been suggested as possible drugs, in association with antipsychotics, for the treatment of psychotic symptoms in schizophrenia and bipolar disorders [26, 27].

Investigation of the molecular mechanisms involved in the crosstalk between A_{2A}ARs and D₂DRs and the effects of antipsychotic drugs on A_{2A}AR modulation may represent a crucial starting point to (1) clarify the role of the adenosine system in several psychiatric disorders; (2) optimise antipsychotic therapy; and (3) reduce the possible side effects inherent in hyperactivity of the adenosine system induced by the chronic administration of antipsychotic drugs, such as extrapyramidal symptoms.

For this purpose, we established a cellular model in which these receptors are stably co-expressed at high levels, and we investigated the effects of D₂DR antagonists (haloperidol and clozapine) on A_{2A}AR binding parameters and functional responsiveness. The availability of this cellular model allowed us to overcome the problem of recovering biological samples from patients in quantities sufficient for biochemical analysis.

Materials and methods

Calcium phosphate transfection of D_{2L} receptor in CHO cells

Cell lines co-expressing A_{2A}ARs and D₂DRs were obtained by calcium phosphate transfection. A solution of 100 µl of

2.5 M CaCl₂ and 10 µg of plasmid pcDNA3.1/Hygro(+) containing human D_{2L} (long-form) DR was diluted 1/10 in TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 7.6) to a final volume of 500 µl. One volume of this 2x Ca^H/DNA solution was added quickly to an equal volume of 2X HEPES solution (140 mM NaCl, 1.5 mM Na₂HPO₄, 50 mM HEPES, pH 7.05 at 23°C). The two solutions were mixed quickly and added dropwise to Chinese hamster ovary (CHO) cells stably transfected with A_{2A}AR [28]. After 1 day, the plates were washed twice with NaCl 0.9% and new medium without antibiotics was added. The following day, 0.5 mg/ml Hygromycin was added to the media to initiate selection for stably transfected cells. Selection continued with periodic treatment of cells with different concentrations of antibiotic to a final concentration of 0.2 mg/ml.

Cell culture

CHO cells stably transfected with human A_{2A}ARs and D_{2L}DRs were grown adherently and were maintained in Dulbecco's modified Eagle's medium with nutrient mixture F12 (DMEM/F12) containing 10% foetal bovine serum (FBS), penicillin (200 U/ml), streptomycin (200 µg/ml), and L-glutamine (4 mM) at 37°C, 5% CO₂ and 95% humidity.

Cell treatments

CHO cells co-transfected with A_{2A}ARs and D₂DRs were plated using complete media supplemented with 0.2 mg/ml Hygromycin and 0.2 mg/ml Geneticin (G418) in order to maintain selection pressure for D₂DR and A_{2A}AR, respectively. After 48 h, cells were treated for 1–24 h with 1 nM–10 µM of the antipsychotic haloperidol or clozapine under serum-deprived conditions using DMEM/F12 supplemented with 1% FBS. At the end of the treatments, membrane fractions were prepared and protein concentrations were detected according to the Bio-Rad method [29] using bovine albumin as a reference standard. Control and treated cells were used for A_{2A}AR binding and functional assays.

D₂DR binding assay

Cells were washed twice with a 0.9% NaCl solution, detached with hypotonic buffer (1 mM HEPES, 2 mM EDTA) and incubated for 10 min at 4°C. The suspension was homogenised and centrifuged at 48,000×g for 20 min at 4°C. The pellet was then suspended in binding buffer (50 mM Tris-HCl, 155 mM NaCl, pH 7.4).

D₂DR saturation binding assays were performed by incubating aliquots of CHO cell membranes (30 µg) in 1 ml of binding buffer supplemented with 1.5 mM ascorbic acid in the presence of increasing D₂DR antagonist, *cis-N*-(1-benzyl-2-methylpyrrolidine-3-yl)-5-chloro-2-methoxy-4-methylami-

nobenzamide ($[^3\text{H}]\text{YM 09151-2}$) concentrations. Nonspecific binding was determined in the presence of 1.5 mM dopamine. The cells were incubated for 1 h at 30°C, and the binding reaction was terminated by rapid filtration under vacuum through Whatman GF/C glass fibre filters (Millipore Corporation) pretreated with 0.6% polyethylenimine. The filters were washed three times with 4 ml of filtration buffer (10 mM Tris–HCl, 155 mM NaCl, pH 7.4) and then counted in 4 ml of scintillation cocktail.

A_{2A}AR binding assay

Cells were washed twice with cold PBS (9.1 mM NaH₂PO₄, 1.7 mM Na₂HPO₄, 150 mM NaCl, pH 7.4) and detached with lysis buffer (50 mM Tris–HCl, 2 mM EDTA, pH 7.4). Cells were harvested, homogenised and centrifuged at 48,000×g for 20 min at 4°C before the pellet was suspended in binding buffer (50 mM Tris–HCl, 2 mM MgCl₂, pH 7.4).

Competitive binding assays (cold analysis) were performed by incubating 30 µg of CHO membranes with 2 U/ml adenosine deaminase (ADA) in 0.5 ml of binding buffer containing 10 nM of the AR agonist $[^3\text{H}]\text{5}'\text{-}N\text{-ethylcarboxamide adenosine}$ (NECA) and increasing concentrations of unlabeled NECA (0.1 nM–1 µM). Nonspecific binding was determined in the presence of 100 µM N⁶-(R)-1-methyl-2-phenyl-ethyl]adenosine. Cells were incubated for 3 h at 25°C, and the binding reaction was terminated by rapid filtration under vacuum through Whatman GF/C glass fibre filters (Millipore Corporation). Filters were washed three times with 4 ml of binding buffer and counted in 4 ml of scintillation cocktail.

A_{2A}AR and D₂DR immunoprecipitation and immunoblotting

CHO cells, treated without (control) or with 1 µM haloperidol or 1 µM clozapine for 24 h, were lysed for 60 min at 4°C by the addition of 200 µl RIPA buffer (9.1 mM NaH₂PO₄, 1.7 mM Na₂HPO₄, 150 mM NaCl, pH 7.4, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS, protease inhibitor cocktail).

Extracts were then equalised by protein assay, and A_{2A}ARs and D₂DRs were immunoprecipitated from cell lysates using A_{2A}AR (Alpha Diagnostic cat no. A_{2A}R12-A) or D₂DR (Alpha Diagnostic cat no. D₂R12-A) antibodies, respectively. Cell lysates (1 mg) were pre-cleared with protein A Sepharose (1 h at 4°C) to precipitate and eliminate IgG. Samples were then centrifuged for 10 min at 4°C (14,000×g); the supernatants were incubated with anti-A_{2A}AR or anti-D₂DR receptor antibody (3 µg/sample) overnight at 4°C under constant rotation and then immunoprecipitated with protein A Sepharose (2 h at 4°C).

Immunocomplexes, after being washed, were resuspended in Laemmli solution and boiled for 5 min and were then resolved by SDS-PAGE (10%), transferred to nitrocellulose membranes and probed overnight at 4°C with primary antibody anti-A_{2A}AR (1:500). Membranes, after incubation with the secondary antibody, were developed using Millipore chemiluminescent reagents. The specificity of A_{2A}AR immunoreactive bands were evaluated using wild-type CHO cells as a negative control.

AC activity at A_{2A}AR

Cells were washed twice with cold PBS and detached with ice-cold hypotonic buffer (5 mM Tris, 2 mM EDTA, pH 7.4). The cell suspension was homogenised on ice (Ultra-Turrax, 2×15 s at full speed) and the homogenate was centrifuged for 10 min at 1,000×g at 4°C. The supernatant was then centrifuged for 45 min at 100,000×g, and the membrane pellet was resuspended in 50 mM Tris–HCl buffer, pH 7.4, and immediately used in functional assays.

The potency of the agonist NECA against A_{2A}AR was determined by assessing the ability of increasing agonist concentrations to stimulate cAMP accumulation. The procedure was carried out as previously described [30], with minor modifications. A total volume of 100 µl of incubation medium contained 50 mM Tris–HCl, pH 7.4, approximately 200,000 cpm of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, 100 µM unlabeled ATP, 10 µM GTP, 100 µM cyclic AMP, 1 mM MgCl₂, 500 µM Ro 20-1724, 0.4 mg/ml creatine kinase, 5 mM creatine phosphate, 2 mg/ml bovine serum albumin and 0.2 U/ml ADA.

Data analysis

For data analysis and graphic presentation, the nonlinear multipurpose curve fitting computer programme Graph-Pad Prism (GraphPad, San Diego, CA) was used. For saturation and competition binding studies, Scatchard analysis was performed, whilst sigmoidal dose–response curve fitting was used for functional data. Statistical analyses of binding and functional data (expressed as means ± SEM) were performed using Student's *t* test. Results were considered significant at *P*<0.05.

Results

A_{2A}AR and D₂DR equilibrium binding parameters in CHO cells

A_{2A}AR and D₂DR equilibrium binding parameters were evaluated in CHO cells expressing only a single or both receptor subtypes. The results obtained by Scatchard analysis of saturation (for D₂DRs) and competition (for A_{2A}ARs) binding data are reported in Table 1. A

representative Scatchard plot of binding data is also shown in Electronic supplementary material (ESM) Fig. 1. The results demonstrated that co-expression of both A_{2A}ARs and D₂DRs induced significant changes in receptor binding parameters in terms of $B_{\max}$ and/or K_d values.

Co-immunoprecipitation of A_{2A}ARs and D₂DRs in CHO cells

The presence of a structural interaction between A_{2A}ARs and D₂DRs in co-transfected CHO cells was evaluated by immunoprecipitation and immunoblotting assay. CHO cells treated with 1 μ M haloperidol or 1 μ M clozapine for 24 h and the respective controls were immunoprecipitated using an anti-D₂DR and then immunoblotted with an A_{2A}AR antibody. In parallel, lysates from control cells were also immunoprecipitated using A_{2A}AR antibody and immunoblotted with the same antibody. In A_{2A}AR immunoprecipitates obtained from control cells, the anti-A_{2A} AR antibody recognised a 45-kDa protein corresponding to the A_{2A}AR subtype [31]. In parallel, no significant labelling was detected in wild-type CHO cells not expressing the A_{2A}AR subtype, demonstrating the specificity of antibody immunoreactivity. The A_{2A}AR immunoblotting performed on immunoprecipitated D₂DR from control cells revealed the same major band of approximately 45 kDa corresponding to A_{2A}AR (Fig. 1a), demonstrating that A_{2A}ARs co-immunoprecipitated with D₂DRs and suggesting that these receptors form a heteromeric complex. Cell treatment with haloperidol caused a significant increase in immunoprecipitated A_{2A} AR, as demonstrated by the densitometric analysis of the A_{2A}AR immunoreactive band (1.19 ± 0.02 increase in optical density vs. control, set to 1, $P < 0.01$; Fig. 1b). In contrast, no significant change in the levels of immunoprecipitated A_{2A}AR was detected following cell treatment with clozapine. These results suggest that only the classical drug affected the heteromeric association between A_{2A}ARs and D₂DRs, increasing the levels of co-immunoprecipitated A_{2A}ARs–D₂DRs.

Effect of antipsychotic drugs on A_{2A}AR equilibrium binding parameters

Because antipsychotic cell treatments were performed under serum deprivation, we first evaluated the effect of

24-h exposure to low serum levels (1% FBS) on A_{2A}AR binding. A_{2A}AR Scatchard analysis showed [³H]NECA K_d values of 26.2 ± 1.34 nM and a $B_{\max}$ of $9,740 \pm 1,210$ fmol/mg of proteins (Fig. 3a). These values were not significantly different from those obtained in control cells (10% serum, $P > 0.05$), suggesting that serum limitation did not affect A_{2A} AR binding parameters in CHO cells.

Cells were treated for 24 h with the classical and atypical antipsychotics haloperidol and clozapine at different concentrations (ranging from 1 nM to 10 μ M), and then [³H]NECA-specific binding to A_{2A}ARs was evaluated. The results (Fig. 2) showed that haloperidol, but not clozapine, was able to significantly increase [³H]NECA binding when used at high concentrations of 1 and 10 μ M. A_{2A} AR binding parameters were evaluated following treatment with haloperidol and clozapine for short (1 h) and long time periods (24 h). Treatment of cells with haloperidol for 1 and 24 h induced a significant increase in [³H]NECA affinity for A_{2A}AR (K_d : 1 h, 15 ± 1.41 nM; 24 h, 18.1 ± 1.36 nM, $P < 0.01$ vs. control cells) without significant changes in A_{2A}AR density ($B_{\max}$: 1 h, $7,100 \pm 720$ fmol/mg proteins; 24 h, $6,640 \pm 1,580$ fmol/mg of proteins, $P > 0.05$ vs. control cells; Fig. 3a). Treatment with 1 μ M clozapine (Fig. 3b) for either 1 or 24 h did not change the affinity or density of A_{2A}AR compared to controls (K_d : 1 h, 24.3 ± 1.45 nM; 24 h, 27.7 ± 0.68 nM, $P > 0.05$ vs. control cells; $B_{\max}$: 1 h, $8,720 \pm 635$ fmol/mg proteins; 24 h, $8,460 \pm 1,190$ fmol/mg proteins, $P > 0.05$ vs. control cells).

In addition, we evaluated the levels of D₂DR following treatment with the antipsychotics by means of radioligand binding. We demonstrated that following treatment with 1 μ M haloperidol for 24 h, 59% of the receptors were labelled with respect to untreated control cells (specific binding, 198.1 vs. 334.9 fmol/mg proteins, $P < 0.001$). In contrast, when the cells were treated with 1 μ M clozapine, no significant change in D₂ DR-specific binding was detected (378.2 fmol/mg proteins, $P > 0.05$ vs. control cells), suggesting that following 24-h treatment with this drug, D₂DRs were not affected.

Effect of antipsychotic drugs on A_{2A}AR functional responsiveness

The effect of treatment with the antipsychotics for 24 h on A_{2A}AR functional responsiveness was evaluated by assessing NECA-stimulated activation of adenylyl cyclase (AC).

Table 1 [³H]NECA (for A_{2A}AR) and [³H]YM 09151-2 (for D₂DR) equilibrium binding parameters in transfected CHO cells

	[³ H]YM09151-2		[³ H]NECA	
	CHO D ₂	CHO A _{2A} /D ₂	CHO A _{2A}	CHO A _{2A} /D ₂
K_d (nM)	0.049 ± 0.01	0.060 ± 0.01	12.91 ± 1.10	$26.2 \pm 1.34^{**}$
$B_{\max}$ (fmol/mg)	841 ± 87.1	$1600 \pm 173^*$	2712 ± 184	$9740 \pm 1210^{**}$

* $P < 0.01$; ** $P < 0.001$

Fig. 1 Co-immunoprecipitation (IP) and immunoblotting (IB) of A_{2A}AR. CHO cells were treated with medium alone (control) or with 1 μ M haloperidol or 1 μ M clozapine for 24 h. One milligram of protein was immunoprecipitated using a polyclonal antibody against D₂DR and resolved by SDS electrophoresis. Proteins were transferred to nitrocellulose membranes and A_{2A}AR IB was performed using a polyclonal antibody against A_{2A}AR. In control A_{2A}AR-transfected cells, but not in wild-type cells, A_{2A}ARs were detected following immunoprecipitation with the A_{2A} AR antibody. Immunoreactive proteins were visualised by chemiluminescence (a). Densitometric analysis of A_{2A} AR immunoreactive bands (b)

In control cells, NECA activated AC with an EC₅₀ value of 5.09 ± 0.67 nM (Fig. 4a). After treatment with 1 μ M haloperidol (Fig. 4b), a significant increase in A_{2A}AR responsiveness compared to the control was observed (haloperidol: EC₅₀ = 3.64 ± 0.57 nM, $P < 0.01$ vs. control cells). Cells treated with 1 μ M clozapine (Fig. 4c) did not show any significant change in NECA-mediated AC activation (clozapine: EC₅₀ = 4.82 ± 1.26 nM, $P > 0.05$ vs. control cells). The maximal efficacy of the agonist NECA to stimulate cAMP accumulation was not affected following treatment with both tested antipsychotic compounds (maximal effect: $P > 0.05$ vs. control cells).

Discussion

For the first time, we have demonstrated that the classical antipsychotic haloperidol affected A_{2A}AR-mediated responses in CHO cells co-expressing high levels of A_{2A}ARs and D₂DRs. In particular, haloperidol favoured the structural interaction between A_{2A}–D₂ receptors, caus-

ing an increase in A_{2A}AR binding affinity and functional responsiveness.

These results suggest that during chronic treatment with classical antipsychotic drugs, A_{2A}AR underwent subtle regulatory changes affecting its receptor binding properties and functional responsiveness. These receptor changes represent a crucial aspect in further clarifying the pathophysiological role of adenosine in psychiatric diseases and should be taken into account (1) in developing new drugs as well as (2) in preventing possible side effects of drugs indirectly modulating the adenosine system. In our specific context, the underlying regulation of A_{2A}AR during antipsychotic administration represents an important target for the optimisation of pharmacological therapy in pathologies associated with psychotic symptoms.

To investigate this issue, we developed a CHO cell line that stably co-expressed both A_{2A} and D₂ receptors at high levels. In this clone, a monomeric form of A_{2A}AR [32] was detected both in A_{2A}AR and D₂DR immunoprecipitates, suggesting that A_{2A}AR and D₂DR aggregate to form a heteromeric complex in basal conditions. The treatment with haloperidol caused an increase in immunoprecipitated A_{2A}AR, suggesting that blocking D₂DR favours the structural A_{2A}/D₂ receptor interaction. These data are in accordance with Vidi et al. [25] who demonstrated the ligand-dependent heterodimerisation of D₂ and A_{2A} receptors in living neuronal cells. In particular, they have demonstrated that the formation of A_{2A}/D₂ heteromers is increased in living neuronal cells following treatment with sulpiride, a D₂DR antagonist, for 18 h. In addition, we were not able to detect any significant changes in the levels of A_{2A}–D₂ receptor interaction following treatment with clozapine, suggesting that the two classes of drugs regulate the structural interaction of the two receptor proteins differently at the membrane level.

Fig. 2 Effect of different haloperidol or clozapine concentrations on [³H] NECA-specific binding of A_{2A}AR. CHO cells were treated for 24 h with medium alone or haloperidol or clozapine (1 nM–10 μ M) and then [³H]NECA binding assays were performed. Data are expressed as the percentage of [³H]NECA-specific binding with respect to untreated control cells (100%) and represent the mean $\pm$ SEM of three different experiments

Fig. 3 Effect of antipsychotic treatment on [^3H] NECA binding parameters of $\text{A}_{2\text{A}}\text{AR}$. CHO cells were treated for 1–24 h with medium alone (**a**), 1 μM haloperidol (**b**) or 1 μM clozapine (**c**), and then [^3H]NECA competitive binding experiments were performed. Graphs show a representative Scatchard plot of [^3H] NECA competitive binding data obtained from three experiments yielding similar results

We then investigated the effects of short- and long-term cell treatment with classical and atypical antipsychotics on $\text{A}_{2\text{A}}\text{AR}$ binding parameters and functional responsiveness. The co-expression of $\text{A}_{2\text{A}}\text{AR}$ s and D_2DR s in the same cells induced a significant increase in [^3H]NECA K_{d} and B_{max} values, suggesting that $\text{A}_{2\text{A}}\text{AR}$ are modulated by the presence of D_2DR at the level of the plasma membrane in the absence of D_2DR receptor ligands. When the cells were treated with haloperidol for 1 or 24 h, a significant increase in $\text{A}_{2\text{A}}$ agonist affinity was detected without any changes in receptor density. A significant increase in $\text{A}_{2\text{A}}\text{AR}$ functional responsiveness was also observed. In a previous report [13], we demonstrated that in platelets of human patients with bipolar disorders, chronic administration of classical antipsychotics induced an increase in $\text{A}_{2\text{A}}\text{AR}$ affinity and responsiveness associated with receptor up-regulation. The difference in the regulation of $\text{A}_{2\text{A}}\text{AR}$ density induced by haloperidol in platelets and in transfected cells could be ascribed to the in vivo and in vitro experimental conditions; in fact, the treatment of CHO cells with the drug for 24 h is probably not representative of chronic treatment in patients.

In parallel, we demonstrated that the atypical antipsychotic clozapine did not have any effect on $\text{A}_{2\text{A}}\text{AR}$

binding and functional parameters. Despite their common therapeutic usage, the differences in $\text{A}_{2\text{A}}\text{AR}$ regulation induced by these two classes of antipsychotics confirmed that these drugs have different mechanisms of action at the molecular level. Indeed, a crucial point that distinguishes antipsychotics is the degree of dopamine D_2DR occupancy and the kinetics of drug–receptor dissociation, on which the drug response and some unpleasant side effects depend. Classical antipsychotics, such as haloper-

Fig. 4 Effect of antipsychotic treatment on $\text{A}_{2\text{A}}\text{AR}$ functional responsiveness. CHO cells were treated for 24 h with medium alone (**a**), with 1 μM haloperidol (**b**) or with 1 μM clozapine (**c**), and then the ability of different NECA concentrations to stimulate cAMP accumulation was evaluated. Data are expressed as picomoles per milligram proteins per minute and represent the mean $\pm$ SEM of four different experiments

idol, have high D₂DR occupancy and slow dissociation rates compared to atypical antipsychotics [33]; occupancy increases above 80% and is connected with increased extrapyramidal side effects, elevation of prolactin levels [34, 35] and dyskinesias. Clozapine, an atypical drug, shows a much lower occupancy (16%–68%) and a fast dissociation rate; this may explain its lack of extrapyramidal side effects and prolactin elevation [36, 37]. These differences in D₂DR occupancy were confirmed in our experimental conditions. In fact, we showed that following treatment with haloperidol for 24 h and the subsequent washing of the cells, only 59% of D₂DR binding sites are labelled by the D₂DR antagonist [³H]YM 09151-2. These results could be explained by supposing that (1) the drug remained bound to the receptor, causing receptor occupancy of about 41%, and (2) the long time block of D₂DR by the antagonist, haloperidol, caused receptor down-regulation. Only in “in vivo” experiments has it been demonstrated that the chronic administration of haloperidol to rats for 14 days or 8 months induced dysregulation of D₂DR expression levels in different brain regions [38, 39]. Otherwise, in striatal and astroglial cell cultures, treatment with antipsychotics for 12 h is not enough to induce changes in D₂DR expression levels [40]. These data lead us to speculate that the effects of 24-h haloperidol treatment on D₂DR binding in CHO cells are presumably due to receptor occupation rather than to receptor down-regulation.

Clozapine is completely removed from D₂DR binding sites and did not affect D₂DR-specific binding, probably as a consequence of its rapid dissociation constant. These differences in D₂ receptor occupancy may be at the root of specific effects observed in A_{2A}AR regulatory mechanisms, and it is possible to speculate that the degree of D₂DR occupancy is important in the regulation of A_{2A}AR responses. This hypothesis might also be supported by data obtained in immunoprecipitation experiments demonstrating that only haloperidol, but not clozapine, was able to increase A_{2A}/D₂ heteromeric association. Further studies are in progress to investigate the role of this heteromeric association in the control of A_{2A} receptor responsiveness induced by antipsychotics.

The main conclusion of this study is that blockade of D₂DRs induced changes in A_{2A}AR responses, confirming the existence of functional crosstalk between the adenosine and dopamine systems. As a consequence, the hyperresponsiveness of A_{2A}ARs, induced by haloperidol treatment, could justify the proposed use of A_{2A}AR agonists in association with classical antipsychotic therapy [17, 26, 27, 41, 42] in several psychiatric diseases associated with a dopamine system hyperactivity.

On the other hand, one should not forget the neurodegenerative and toxic effects that A_{2A}ARs exert at the

central level [43–49]. In this context, A_{2A}AR hyperactivity may have important pathophysiological implications and might contribute to, induce or exacerbate neurodegenerative effects in several psychiatric diseases. In this scenario, the choice of the antipsychotic, and particularly its dosage, should always follow an investigation of the individual's proneness to other diseases.

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