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Allosteric Modulation of Dopamine D_2 Receptors by Homocysteine

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It has been suggested that L-DOPA-induced hyperhomocysteinemia can increase the risk of stroke, heart disease, and dementia and is an additional pathogenetic factor involved in the progression of Parkinson's disease. In Chinese hamster ovary (CHO) cells stably cotransfected with adenosine A_{2A} and dopamine D_2 receptors, homocysteine selectively decreased the ability of D_2 receptor stimulation to internalize adenosine A_{2A}−dopamine D_2 receptor complexes. Radioligand-binding experiments in the same cell line demonstrated that homocysteine acts as an allosteric D_2 receptor antagonist, by selectively reducing the affinity of D_2 receptors for agonists but not for antagonists. Mass spectrometric analysis showed that, by means of an arginine (Arg)−thiol electrostatic interaction, homocysteine forms noncovalent complexes with the two Arg-rich epitopes of the third intracellular loop of the D_2 receptor, one of them involved in A_{2A}−D_2 receptor heteromerization. However, homocysteine was unable to prevent or disrupt A_{2A}−D_2 receptor heteromerization, as demonstrated with Fluorescence Resonance Energy Transfer (FRET) experiments in stably cotransfected HEK cells. The present results could have implications for Parkinson's disease.

Keywords: homocysteine • dopamine D_2 receptor • allosteric modulation • mass spectrometry • Parkinson's disease

1. Introduction

Homocysteine is a sulfur-containing amino acid which is produced by demethylation of methionine. Homocysteine levels are normally kept low by remethylation to methionine in a reaction that requires folate and vitamin B12 ("remethylation pathway"). Furthermore, cysteine is generated by condensation of homocysteine and serine ("transulfuration pathway").[1] It has been shown that elevations in plasma homocysteine levels are a common risk factor for vascular disease, such as coronary artery disease and stroke.[2−4] Furthermore, an association has been established between hyperhomocysteinemia and Alzheimer's disease,[5,6] and an increasing number of epidemiological and experimental data suggest that homocysteine-mediated neurotoxicity is involved in basal ganglia disorders, such as Huntington's disease and Parkinson's disease.[7−15]

L-DOPA, associated with a peripheral DOPA decarboxylase inhibitor, is still the most commonly used symptomatic treatment for Parkinson's disease. When L-DOPA reaches the brain, it is taken up by dopaminergic cell terminals and decarboxilated to dopamine, the endogenous neurotransmitter. However, a significant amount of L-DOPA is also O-methylated to 3-*O*-methyldopa (3-OMD) by the enzyme catechol-*O*-methyl-transferase (COMT). COMT uses *S*-adenosyl-L-methionine (SAM) as a methyl donor, which is converted to *S*-adenosyl-homocysteine (SAH), which is further metabolized to adenosine and homocysteine by the enzyme SAH hydrolase.[7,9,10,14,16] Ultimately, L-DOPA treatment can potentially increase the levels of homocysteine in the brain. In fact, elevated plasma levels of homocysteine have also been reported in Parkinson's disease patients using L-DOPA as a therapeutic agent.[7,9,10,14,15] Importantly, experimental data suggest that dopaminergic cells are more sensitive to the neurotoxic effects of homocysteine.[15,16] Thus, although the possible toxicity of L-DOPA is still a matter of debate, it has been suggested that L-DOPA-induced hyperhomocysteinemia can render patients at increased risk of stroke, heart disease, and dementia and even be an additional pathogenetic factor involved in the progression of Parkinson's disease.[12,13]

Production of reactive oxygen species during auto-oxidation of homocysteine seems to be a main mechanism involved in atherogenic propensity associated with hyperhomocysteinemia.[2] Of particular importance to neurological diseases is that homocysteine is an *N*-methyl-D-aspartate (NMDA) receptor agonist, and it has been suggested that overstimulation of

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NMDA receptors could contribute to its neurotoxic effects.[17] In the present study, we describe a new functional mechanism of homocysteine, which might have implications for basal ganglia disorders. Homocysteine was found to allosterically modulate dopamine D_2 receptor function at concentrations lower than those previously shown to be required to modulate NMDA receptor function.[17] Homocysteine decreased agonist but not antagonist binding and selectively decreased the ability of D_2 receptor stimulation to internalize adenosine A_{2A}−dopamine D_2 receptor complexes in cotransfected cells. On the basis of mass spectrometric analysis of homocysteine−peptide interactions, it is proposed that the allosteric binding site of the D_2 receptor targeted by homocysteine includes an arginine (Arg)-rich domain of the third intracellular loop of the D_2 receptor.

2. Materials and Methods

Maintenance of Cell Lines. Previously characterized Chinese hamster ovary (CHO) cells stably cotransfected with hemeagglutinin (HA)-tagged dog adenosine A_{2A} receptor cDNA and the human dopamine D_{2L} (long form) receptor were used (A_{2A}-D_2 cell line).[18] A_{2A}-D_2 CHO cells were cultured routinely at 37 °C with 5% CO_2 in MEM alpha medium without nucleosides supplemented with 10% fetal calf serum, 100 U/mL penicillin/streptomycin, 300 μg/mL hygromycin for selection of adenosine HA-A_{2A} receptor cDNA, and 400 μg/mL Geneticin (G-418) for selection of dopamine receptor cDNAs. HEK-293T cells (American Type Tissue Culture) were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and 10% fetal calf serum at 37 °C with 5% CO_2. All cell culture reagents were from Invitrogen.

Double-Immunolabeling Experiments. For immunofluorescence staining, the A_{2A}-D_2 cells were grown on glass slides (Chamber Slide Culture, Labtek/Nunc) coated with poly-L-lysine (Sigma) and exposed or not to 1 mM homocysteine in serum-free medium for 20 h at 37 °C with 5% CO_2. Subsequently, cells were exposed to the A_{2A} receptor agonist CGS21680 (200 nM, Sigma) or the D_2 receptor agonist quinpirole (50 μM, Sigma) in serum-free medium for 3 h at 37 °C. Control cells were exposed to serum-free medium for the same periods. At the end of the treatments, cells were rinsed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde and 0.06 M sucrose for 20 min, washed with PBS containing 20 mM glycine, and subsequently treated with PBS containing 20 mM glycine and 1% BSA for 30 min at room temperature. Double immunostaining was performed with rabbit D_2 receptor antibody (1:800; a kind gift from Dr. Stanley Watson) and mouse anti-HA antibody (1:100; Roche) in PBS, pH 7.4, supplemented with 1% goat serum at 4 °C overnight. Cells were then rinsed several times and incubated with an anti-rabbit biotinylated antibody (1:200; Amersham Pharmacia Biotech) for 1 h at room temperature. After rinsing twice in PBS, the double-immunofluorescence staining was performed with a red fluorolink Cy3-labeled streptavidin antibody (1:100; Amersham Pharmacia Biotech) for D_2 and with a green Alexa fluor 488-labeled goat anti-mouse antibody (1:100; Molecular Probes) for HA-A_{2A} for 1 h at room temperature. Finally, the slides were rinsed three times in PBS and mounted with a medium suitable for immunofluorescence (30% Mowiol, Calbiochem).

Image Analysis. Microscopic observations and analysis of colocalization were made as described in detail elsewhere.[19] Parameters used for statistical analysis were overlap coefficient and *M*-factors (M_{A2A} and M_{D2}). *M*-factor is the amount of immunoreactivity of each fluorochrome within highly correlated pixels as a fraction of its total immunoreactivity (total field area multiplied by the mean intensity of that fluorochrome within the entire image). Overlap coefficient is a measure of colocalization, the overall evaluation of the correspondence of the red and green intensities within the entire image. The coefficient is equal to "1" and to "0" for the perfect match and mismatch between the two fluorochromes, respectively. Each experiment was performed five times, and 20 cells per treatment were evaluated in each experimental session. The mean values of the measured parameters obtained in the treated cell cultures were then compared to the value observed in the corresponding control group by Student's *t*-test (GraphPad Prism 3.03, GraphPad Software, Inc.).

Radioligand Binding Experiments. The cells were lifted from flasks with a cell scraper. Harvested cells were washed twice with ice-cold PBS and centrifuged at 1200*g* for 5 min at 4 °C. The cell pellet was sonicated (30 s) and resuspended in the incubation buffer in the presence of adenosine deaminase (ADA; Boehringer Mannheim; 5 U/mL). The homogenate was centrifuged at 1800*g* for 10 min at 4 °C, the precipitated nuclear fraction was discarded, and the supernatant was preincubated for 30 min at 37 °C and centrifuged at 40 000*g* for 40 min at 4 °C. The membrane pellet was then resuspended by sonication in incubation buffers. For ^{3}H-raclopride binding, the following buffer was used: 50 mM Tris-HCl (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. For ^{3}H-dopamine binding, the following buffer was used: 50 mM Tris-HCl (pH 7.4) containing 5 mM $MgCl_2$ and 1 mM ascorbic acid. The final protein concentration measured with Lowry's standardized protein assay was 0.2 mg/mL. Experiments with ^{3}H-raclopride were carried out with one concentration (2 nM) of ^{3}H-raclopride (87 Ci/mmol; Amersham) by incubation for 30 min at 22 °C, in the presence or absence of homocysteine (10^{-5}−10^{-2} M). Nonspecific binding was defined as the binding in the presence of 100 μM dopamine. Experiments with ^{3}H-dopamine (45 Ci/mmol; Amersham) (range 0.1−10 nM) were performed by incubation for 30 min at 22 °C, in the presence or absence of homocysteine (10^{-5}−10^{-2} M). Nonspecific binding was defined as the binding in the presence of 1 mM apomorphine. The incubation was stopped by fast filtration through glass-fiber filters (GF/B, Whatman) by washing three times with 5 mL of 50 mM ice-cold Tris-HCl (pH 7.4) with an automatic cell harvester (Millipore). The radioactivity content of the filters was detected by liquid scintillation spectrometry (LS 6000, Beckman). Differences of specific binding in the presence or absence of homocysteine were analyzed by ANOVA followed by Dunnett's test. ^{3}H-Dopamine saturation isotherms were analyzed by nonlinear regression using the program GraphPAD Prism (GraphPAD Software, San Diego, CA), and the B_{max} and K_D values for several independent replications were averaged to permit statistical comparisons (Student's *t*-test) between experiments carried out in the absence and presence of homocysteine (1 mM) added to the membrane suspension 15 min before binding.

Peptides. The A_{2A} receptor $_{370}$SAQEpSQGNT$_{378}$ and D_2 receptor epitopes $_{215}$VLRRRRKRVN$_{224}$ and $_{266}$NRRRVEAARR$_{275}$ were synthesized at the John Hopkins School of Medicine Peptide Synthesis Core Facility. Stock solutions were prepared in water at a concentration of 1 mM. Sample mixtures, consisting of VLRRRRKRVN or NRRRVEAARR at 10 μM and the acidic peptide SAQEpSQGNT at 150 μM in water, and/or homocys-

teine at a concentration of 1 mM were employed for mass spectrometric analysis.

Mass Spectrometry. A matrix-assisted laser desorption/ionization time-of-flight-time-of-flight (MALDI-TOF-TOF) 4700 (Applied Biosystems Framingham, MA) was used. All spectra were acquired in reflectron positive ion-mode. The MALDI matrix, 2,5-dihydroxybenzoic acid (DHB), was purchased from Aldrich (Milwaukee, WI) and prepared fresh daily as a saturated solution in water. Volumes of 0.3 μL of peptide mixture and 0.3 μL of matrix (DHB) were applied to the MALDI target and allowed to air-dry prior to introduction into the mass spectrometer.

Fluorescence Resonance Energy Transfer (FRET). HEK-293T cells were transiently transfected with plasmid cDNAs corresponding to the human D_{2L} receptor fused to the fluorescent protein GFP2 (D_{2L}-GFP2, donor) and human A_{2A} receptor fused to the fluorescent protein YFP (A_{2A}-YFP; acceptor) using a ratio of donor/acceptor cDNA of 1:2, as described in detail elsewhere.[20] Fluorescence readings were performed 48 h after transfection as previously described, and linear unmixing of the emission signals was applied to the data.[20] The results are shown as the sensitized emission of the acceptor when cells were excited at 400 nm. Homocysteine (1 mM) was either added 3 h before the FRET assay or 30 min before transfection and kept in culture medium during protein expression and FRET assay.

3. Results

Effect of Homocysteine on Agonist-Induced Internalization of A_{2A}−D_2 Receptor Complexes. A_{2A} and D_2 receptors are coexpressed in one subtype of striatal neurons, the GABAergic enkephalinergic striatopallidal neurons, and form heterodimers when cotransfected in mammalian cells.[20−22] In a previous study, we showed that 3 h-exposure to either the A_{2A} receptor agonist CGS 21680 or the D_2−D_3 receptor agonist quinpirole induced co-internalization of A_{2A}−D_2 receptor complexes in cotransfected CHO cells (A_{2A}-D_2 cells).[18] One goal was to ascertain if long-term exposure to homocysteine (20 h) could modify agonist-mediated modulation of the trafficking of A_{2A}−D_2 receptor complexes. As previously reported, under basal conditions, there was evidence for colocalization of A_{2A} with D_2 receptors.[18] The quantitative analysis indicated that a 3 h-exposure to either the D_2 receptor agonist quinpirole (200 nM) or the A_{2A} receptor agonist CGS 21680 (50 μM) causes a reduction of A_{2A} and D_2 receptor immunoreactivities, a significant reduction of A_{2A}−D_2 receptor colocalization (overlap coefficient: $p < 0.05$ in both cases), and a significant reduction of the fractions of A_{2A} and D_2 receptor immunoreactivities that are colocalized with D_2 and A_{2A} receptors, respectively (M_{A2A} and M_{D2}: $p < 0.05$) (Figure 1). These results suggest that both quinpirole and CGS 21680 induce co-internalization of A_{2A} and D_2 receptors and that the fraction of A_{2A} and D_2 receptors that are colocalized are more likely to internalize upon agonist treatment. Surprisingly, pretreatment with homocysteine (1 mM) completely counteracted the effects of quinpirole, while similar significant effects were still observed with CGS 21680-induced A_{2A}−D_2 receptor co-internalization (Figure 1).

Effect of Homocysteine on D_2 Receptor Agonist and Antagonist Binding. The selective counteracting effect of homocysteine on the pharmacological effects of quinpirole suggested that homocysteine is a D_2 receptor antagonist. However, different concentrations of homocysteine (0.01−10 mM) did not displace the specific binding of the selective D_2 receptor

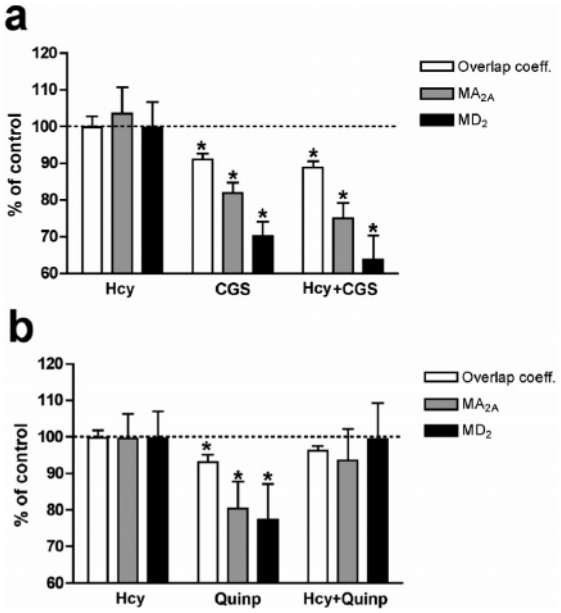

Figure 1. Quantitative evaluation of colocalization between A_{2A} and D_2 receptors in stably cotransfected CHO cells after treatment (3 h) with either (a) the A_{2A} receptor agonist CGS 21680 (50 μM) or (b) the D_2 receptor agonist quinpirole (200 nM) with or without previous exposure (20 h) to homocysteine (Hcy; 1 mM). The effect of Hcy alone was also analyzed. The overlap coefficient is a measure of colocalization, and *M*-factor (M_{A2A} or M_{D2}) is the amount of immunoreactivity of each fluorochrome within the highly correlated pixels (see Materials and Methods). Both quinpirole and CGS 21680 induce co-internalization of A_{2A} and D_2 receptors with the fraction of A_{2A} and D_2 receptors that are colocalized being more susceptible to internalize upon agonist treatment. Homocysteine completely counteracts quinpirole-induced, but not CGS 21680-induced, internalization of A_{2A} and D_2 receptors. Results are shown as means ± SEM; $n = 5$; (*) significantly different compared to control (Student's *t*-test: $p < 0.05$).

antagonist ^{3}H-raclopride (2 nM) in membrane preparations of A_{2A}−D_2 cotransfected CHO cells (Figure 2a). These results are in agreement with a recent screening of homocysteine and some acidic homocysteine derivatives with various radioligands,[23] which included the D_2 receptor antagonist ^{3}H-*N*-methylspiperone. Nevertheless, homocysteine was found to efficaciously and potently displace the specific binding of ^{3}H-dopamine (4 nM), with 0.01 and 10 mM inducing a decrease of about 20% and 60% of D_2 receptor agonist binding (Figure 2b). Saturation experiments with ^{3}H-dopamine at concentrations that bind to the high-affinity state of the D_2 receptor (0.1−10 nM),[24] showed that the K_D value is significantly increased by 2- to 3-fold (Student's *t*-test: $p < 0.01$) and B_{max} value is slightly but significantly decreased (Student's *t*-test: $p < 0.05$) in the presence of homocysteine (1 mM) (Figure 3). Since raclopride (as it is the rule for most competitive antagonists) binds with the same affinity to both high and low states of affinity of the D_2 receptor for agonists, these radioligand binding data indicate that homocysteine is an allosteric antagonist of the D_2 receptor.

Binding of Homocysteine to Arg-Rich Epitopes of the D_2 Receptor. We have recently demonstrated that heteromerization of A_{2A} with D_2 receptors and glutamate NMDA receptors (NR1-1 subunit) with dopamine D_1 receptors depend on epitope−epitope electrostatic interactions and suggested that

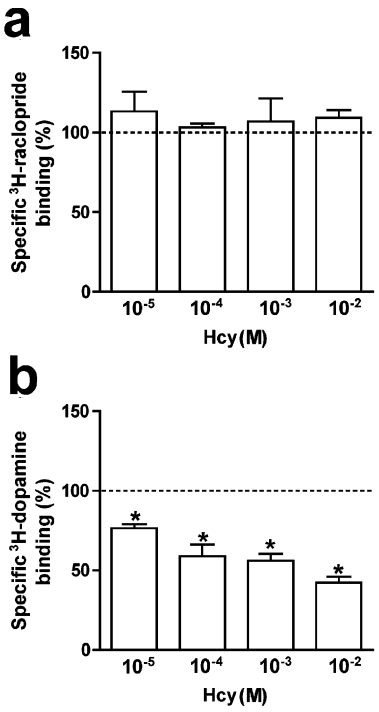

Figure 2. Effect of homocysteine (Hcy; 10^{-5}−10^{-2} M) on the specific binding of (a) the D_2 receptor antagonist ^{3}H-raclopride (2 nM) and (b) ^{3}H-dopamine (4 nM) in membrane preparations from stably cotransfected CHO cells. Homocysteine produced a selective concentration-dependent displacement of ^{3}H-dopamine binding. Results are shown as means ± SEM; n = 4; (*) significantly different compared to the binding in the absence of homocysteine (ANOVA with Dunnett's test; n = 4−6/treatment).

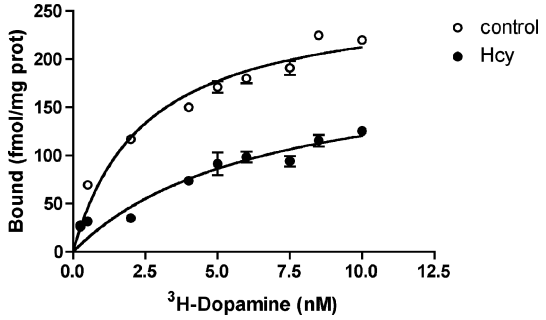

Figure 3. Saturation experiments with ^{3}H-dopamine (0.1−10 nM) in the presence and absence of Hcy (1 mM) in membrane preparations from stably cotransfected CHO cells. K_D and B_{max} values in the absence of homocysteine (means ± SEM; n = 4) were 2.5 ± 0.2 nM and 263.4 ± 11.3 fmol/mg prot, respectively (n = 4); K_D and B_{max} values in the presence of homocysteine (means ± SEM; n = 4) were 6.6 ± 0.3 nM and 201.6 ± 10.8 fmol/mg prot, respectively.

this is a general mechanism for receptor heteromerization.[25] These electrostatic interactions involve a basic epitope containing adjacent Arg residues (D_2 and NMDA receptors) and an acidic epitope containing a phosphorylated serine (A_{2A} and D_1 receptors). The Arg-rich domain ($_{215}$VLRRRRKRVN$_{224}$) of the D_2 receptor is localized in the N-terminal portion of the third intracellular loop (3IL). Furthermore, in the D_{2L} isoform, there is an additional Arg-rich epitope in the middle of the 3IL ($_{266}$NRRRVEAARR$_{275}$). Although this additional epitope could potentially interact with the A_{2A} receptor acidic epitope (a

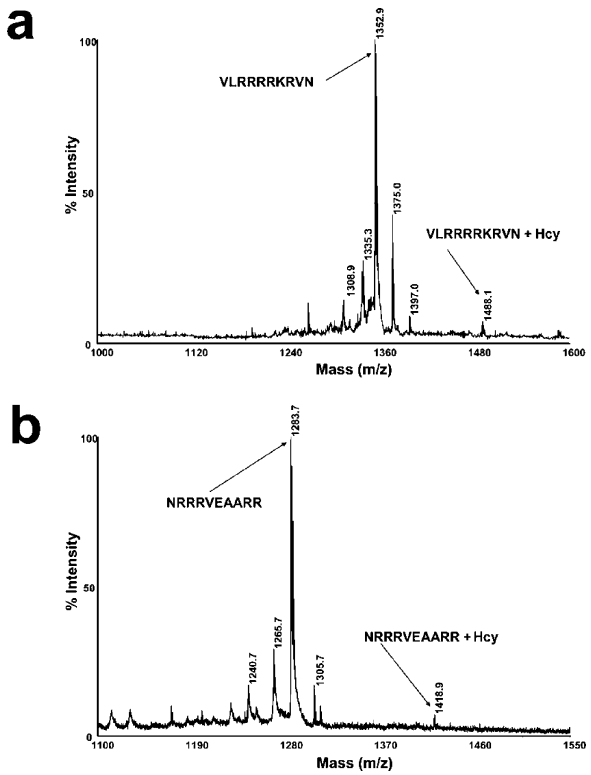

Figure 4. Spectrum of a mixture of the D_2 receptor epitopes VLRRRRKRVN (a) or NRRRVEAARR (b) with homocysteine (Hcy).

phosphorylated serine localized in the C-terminus of the receptor), computerized modeling suggested a predominant involvement of the $_{215}$VLRRRRKRVN$_{224}$ in A_{2A}−D_2 receptor heteromerization.[20] Homocysteine contains a thiol (SH) group, with an available electron pair that can potentially interact with the delocalized positive charges on the Arg-rich motifs. A mixture of homocysteine (1 mM) and the Arg-rich epitopes of the D_2 receptor (10 μM) was analyzed with mass spectrometry. The formation of noncovalent complexes (NCXs) between either of the D_2 receptor epitopes and homocysteine was seen at 1488.1 and 1418.9 amu, respectively (Figure 4). Mixing homocysteine with different control peptides that did not contain adjacent Arg residues showed no formation of NCXs (data not shown). As previously shown with the Arg−phosphate electrostatic interaction and the Arg−aromatic interaction,[25,26] more than one adjacent Arg's were required for the Arg−thiol electrostatic interaction (data not shown). As previously reported, the D_2 receptor epitope $_{215}$VLRRRRKRVN$_{224}$ (10 μM) formed NCXs with the A_{2A} receptor epitope containing pSer$_{374}$ ($_{266}$NRRRVEAARR$_{275}$) (150 μM) (Figure 5a). The peptide VLRRRRKRVN could even bind two molecules of the peptide SAQEpSQGNT (Figure 5a). Similarly, the D_2 receptor epitope $_{266}$NRRRVEAARR$_{275}$ (10 μM) formed NCXs with one or two molecules of the A_{2A} receptor epitope $_{370}$SAQEpSQGNT$_{378}$ (150 μM) (Figure 6a).

When homocysteine (1 mM) was added to the mixtures of A_{2A} and D_2 receptor epitopes, NCXs of either of the two D_2 receptor epitopes with homocysteine or with the A_{2A} receptor epitope were also observed, although the relative abundance of the NCXs of A_{2A} and D_2 receptor epitopes was substantially reduced (Figures 5b and 6b). This would suggest that homocysteine competes with the A_{2A} receptor epitope for the binding to the D_2 receptor epitopes. However, higher quantities of homocysteine than the A_{2A} receptor epitope were needed to

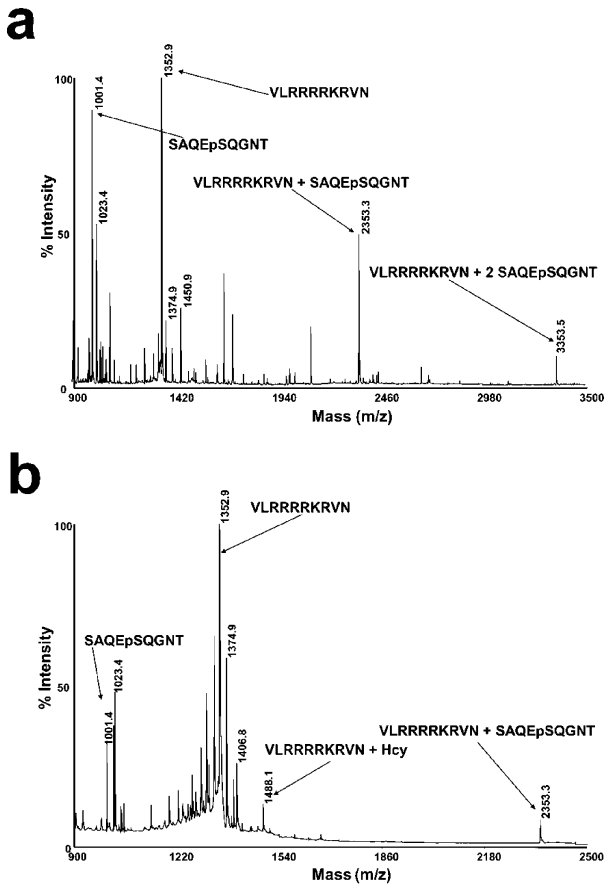

Figure 5. Spectrum of a mixture of the D_2 receptor epitope VLRRRRKRVN and the A_{2A} receptor epitope SAQEpSGNT in the absence (a) and presence (b) of homocysteine (Hcy). Normalized relative abundance of the NCXs formed by the A_{2A} and D_2 receptor epitopes in the absence and presence of homocysteine was 72% and 18%, respectively. Normalization was obtained by dividing the relative abundance of the MH^+ of the NCXs by the relative abundance of the MH^+ of the A_{2A} receptor epitope.

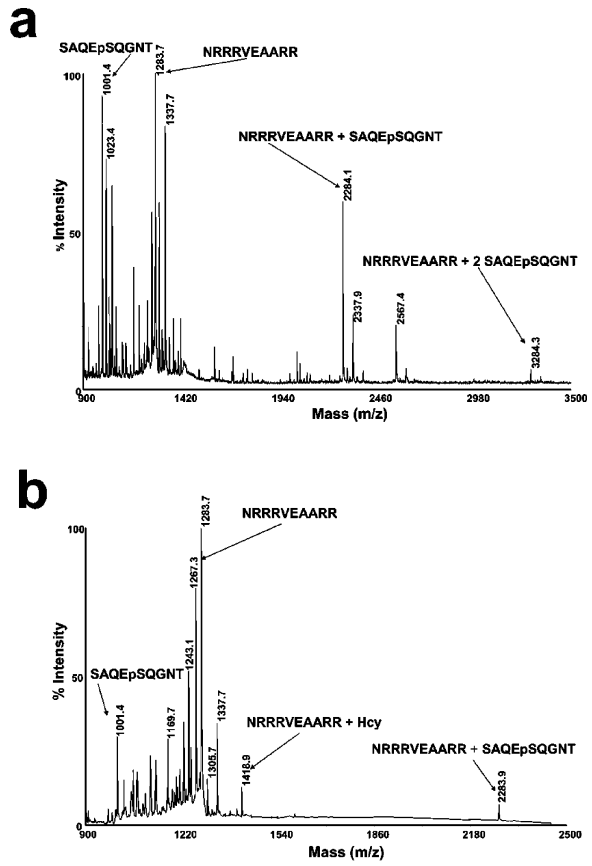

Figure 6. Spectrum of a mixture of the D_2 receptor epitope NRRRVEAARR and the A_{2A} receptor epitope SAQEpSGNT in the absence (a) and presence (b) of homocysteine (Hcy). Normalized relative abundance of the NCXs formed by the A_{2A} and D_2 receptor epitopes in the absence and presence of homocysteine was 65% and 18%, respectively. Normalization was obtained by dividing the relative abundance of the MH^+ of the NCXs by the relative abundance of the MH^+ of the A_{2A} receptor epitope.

form NCXs. Thus, the relative abundance of NCXs obtained with 150 pmol of the A_{2A} receptor epitope and 10 pmol of either of the D_2 receptor epitopes (Figures 5a and 6a) was much higher than that obtained in separate experiments with 1 nmol of homocysteine and 10 pmol of the D_2 receptor epitope (Figure 4). Altogether, the results of mass spectrometry experiments suggest that homocysteine can bind and, therefore, exert an allosteric modulation of the D_2 receptor by means of electrostatic interactions with the Arg-rich motifs of the 3IL.

Lack of Interference of A_{2A}−D_2 Receptor Heteromerization by Homocysteine. The ability of homocysteine to bind to the D_2 receptor epitope involved in A_{2A}−D_2 receptor heteromerization and to apparently compete with $pSer_{374}$-containing A_{2A} receptor epitope prompted us to investigate if homocysteine could alter A_{2A}−D_2 receptor heteromerization, if the A_{2A} receptor and homocysteine would compete for their binding to the D_2 receptor. The formation of $A_{2A}R$−D_2R heterodimers was shown, as previously reported[21] by FRET technique in HEK cells transiently cotransfected with the fusion proteins A_{2A}-YFP and D_{2L}-GFP2. No change in FRET efficiency was observed in transfected cells after exposure for 3 h to homocysteine (1 mM), or in cells constantly exposed to homocysteine (1 mM) before and after transfection (Figure 7). These results show that homocysteine at a concentration of 1 mM neither disrupts nor

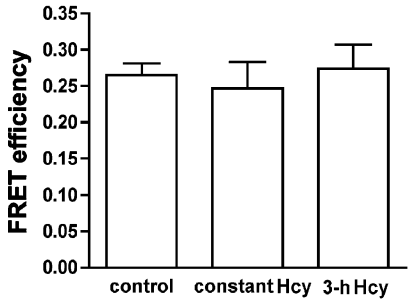

Figure 7. FRET efficiency of the A_{2A}-YFP and the D_{2L}-GFP2 pair by sensitized emission in transiently cotransfected living HEK cells after exposure for 3 h to 1 mM of homocysteine (3-h Hcy) or in cells constantly exposed to homocysteine (1 mM) before and after transfection (constant Hcy). Neither homocysteine treatment produced a significant change (ANOVA followed by Dunnett's test) in FRET efficiency. The results (means ± SEM; $n = 3$) are shown as the sensitized emission of the acceptor when cells were excited at 400 nm.

prevents A_{2A}−D_2 receptor heteromerization. Therefore, the mass spectrometric data showing a decrease in the Arg−phosphate interaction is most probably not related to real competition between homocysteine and the A_{2A} receptor epitope, and it could be due to the fact that homocysteine at

a millimolar concentration lowers the pH of the unbuffered mixture solution (down to about 4.5), thus, causing some interference with complex formation or even causing disruption of the already formed NCXs. However, a buffered salted solution was used for binding experiments, where pH (7.4) was not modified by homocysteine. Of importance for the present experiments, both CHO and HEK-293T cells have been previously reported to express cystine−cysteine−glutamate transport systems,[27,28] which can also transport homocysteine inside the cells and interact with the 3IL of the D_2 receptor.[29]

4. Discussion

The concept of receptor−receptor interactions is now widely accepted.[30−32] Treatments for neuropsychiatric disorders should consider that the target of centrally acting drugs is not any longer the single receptor (monomer), but rather "receptor mosaics" (i.e., oligomers and hetero-oligomers).[33−35] The present study is based on the previous demonstration of the existence of A_{2A}−D_2 receptor heteromers and of their importance for the optimal treatment of Parkinsonian patients.[36−38] In this context, the results obtained can be of paramount importance. In fact, the present study demonstrates that homocysteine acts as an allosteric D_2 receptor antagonist, by selectively reducing the affinity of D_2 receptors for agonists but not for antagonists. Striatal postsynaptic D_2 receptors are a main target for the treatment of basal ganglia disorders and schizophrenia. Those receptors are mostly localized in the GABAergic enkephalinergic striatopallidal neurons, where they form heteromeric complexes with A_{2A} receptors.[21,32]

Stimulation of the A_{2A} receptor in the A_{2A}−D_2 heteromeric complex induces a very similar allosteric effect on D_2 receptor binding properties to that induced by homocysteine, a decrease in the affinity of the D_2 receptor for agonists, and no change in the affinity for antagonists.[24,39] In the present study, we found that homocysteine binds to the Arg-rich epitope of the D_2 receptor involved in A_{2A}−D_2 receptor heteromerization.[25,36,40] This binding depends on an Arg−thiol electrostatic interaction that requires two adjacent Arg guanidinium groups, as in the Arg−phosphate interaction involved in A_{2A}−D_2 receptor heteromerization.[25] Therefore, it would be possible that the same Arg-rich epitope of the D_2 receptor ($_{215}$VLRRRRKRVN$_{224}$) could be involved in A_{2A}−D_2 receptor heteromerization and homocysteine-mediated allosteric modulation. In fact, mass spectrometry showed that homocysteine could impair formation of NCXs between the Arg-rich and the pSer$_{374}$-containing epitopes involved in A_{2A}−D_2 receptor heteromerization. However, higher concentrations of homocysteine than the pSer$_{374}$-containing A_{2A} receptor epitope were required to establish an electrostatic interaction with the Arg-rich domain of the D_2 receptor. Furthermore, a high concentration of homocysteine (1 mM) was unable to disrupt or avoid A_{2A}−D_2 receptor heteromerization, as demonstrated with FRET experiments in cotransfected cells. Therefore, if the D_2 receptor epitope involved in A_{2A}−D_2 receptor heteromerization corresponds to the allosteric site targeted by homocysteine, no modulation seems to be possible if the D_2 receptor is bound to the A_{2A} receptor. In fact, the internalization experiments showed that the fraction of A_{2A} and D_2 receptors that are colocalized is more likely to internalize upon agonist treatment, and such internalization is completely counteracted by homocysteine. Therefore, homocysteine-induced modulation of D_2 receptor binding might depend on its ability to bind the Arg-rich domain localized in the middle portion of the 3IL ($_{266}$NRRRVEAARR$_{275}$), although we cannot rule out the involvement of the Arg-rich epitope found in the N-terminal part of the 3IL. Homocysteine can potentially bind to any series of adjacent Arg, which can also be found in proteins other than the D_2 receptor, such as the C-terminus of the NR1-1 subunit of the NMDA receptor.[25] Implications of those interactions need to be determined.

The present results could have implications for basal ganglia disorders. In particular, they might provide an additional mechanism responsible for the secondary effects of L-DOPA treatment in Parkinson's disease. As explained in the Introduction, L-DOPA methylation generates homocysteine and adenosine by the action of COMT. Thus, hyperhomocysteinemia might contribute to the loss of the therapeutic effect of L-DOPA, due to the allosteric D_2 receptor antagonist properties of homocysteine. Although, to our knowledge, L-DOPA-induced increased formation of adenosine has not been reported, this could be an additional factor involved in the loss its therapeutic effect. Control of homocysteine and maybe adenosine levels adds a possible additional explanation for the positive effects of COMT inhibitors associated with L-DOPA in Parkinson's disease.[41] In fact, it has recently been reported that COMT inhibitors counteract hyperhomocysteinemia in Parkinson's disease.[42] Also, the already reported clinical benefits of the combined treatment of L-DOPA and A_{2A} receptor antagonists[43,44] could be reduced by hyperhomocysteinemia.

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