



Functional crosstalk and heteromerization of serotonin 5-HT_{2A} and dopamine D₂ receptors

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ABSTRACT

The serotonin 5-HT_{2A} receptor (5-HT_{2A}R) and dopamine D₂ receptor (D₂R) are high-affinity G protein-coupled receptor targets for two different classes of antipsychotic drugs used to treat schizophrenia. Interestingly, the antipsychotic effects are not based on the regulation of same signaling mediators since activation of the 5-HT_{2A}R and of the D₂R regulate G_{q/11} protein and G_{i/o} protein, respectively. Here we use radioligand binding and second messenger production assays to provide evidence for a functional crosstalk between 5-HT_{2A}R and D₂R in brain and in HEK293 cells. D₂R activation increases the hallucinogenic agonist affinity for 5-HT_{2A}R and decreases the 5-HT_{2A}R induced inositol phosphate production. *In vivo*, 5-HT_{2A}R expression is necessary for the full effects of D₂R antagonist on MK-801-induced locomotor activity. Co-immunoprecipitation studies show that the two receptors can physically interact in HEK293 cells and raise the possibility that a receptor heterocomplex mediates the crosstalk observed. The existence of this 5-HT_{2A}R–D₂R heteromer and crosstalk may have implications for diseases involving alterations of serotonin and dopamine systems and for the development of new classes of therapeutic drugs.

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1. Introduction

The brain serotonin and dopamine systems are each strongly associated with mood regulation, cognition, memory, learning, motivation and pleasure (Adayev et al., 2005; Arias-Carrion and Poppel, 2007; Bromberg-Martin et al., 2010; Nichols and Nichols, 2008). Altered serotonin and dopamine neurotransmission has been implicated in various psychiatric disorders including anxiety, depression and schizophrenia (de Almeida et al., 2008; Kienast and Heinz, 2006; Mann et al., 2001; Wong and Licinio, 2001). Both dopamine and serotonin have each been proposed to play a fundamental role in the pathophysiology and/or symptoms of schizophrenia (Iqbal and van Praag, 1995; Joyce, 1993; Laruelle et al., 2003). The dopamine hypothesis of schizophrenia was consistent with the observation that typical antipsychotic drugs, such as chlorpromazine and haloperidol, are antagonists of dopamine D₂ receptor (D₂R) signaling. Excessive dopamine activity in the mesolimbic system

resulting from dysregulated dopamine neurotransmission has been hypothesized to contribute to the symptoms of the disease. The similarity to the symptoms of schizophrenia of some aspects of the responses to serotonergic hallucinogens implicated abnormalities of serotonergic signaling in the disease. LSD (lysergic acid diethylamide) and DOI ((+/-)-2,5-dimethoxy-4-iodoamphetamine), hallucinogenic serotonin receptor agonists, trigger effects comparable to positive symptoms (delusions, hallucinations) of schizophrenic patients (Costa, 1960; Nichols, 2004). The effects of these drugs in humans and animal models depend on the serotonin 5-HT_{2A} receptor (5-HT_{2A}R). Moreover, a second generation of drugs called atypical antipsychotics (e.g. clozapine, risperidone and olanzapine) has been developed. The antipsychotic effects of these dopamine–serotonin antagonists, which have higher affinity for 5-HT_{2A}R, are believed to mediate the blockade of mesolimbic dopamine D₂R and mesocortical serotonin 5-HT_{2A}R (Kapur and Remington, 1996; Meltzer et al., 1989; Nichols, 2004).

However the relationship between classical and atypical antipsychotic drugs remains to be elucidated. We speculated whether a serotonin 5-HT_{2A}R–dopamine D₂R complex might exist that could connect the effects of these drugs. Crosstalk among receptor systems can occur either at the level of downstream signaling or via direct functional interaction between the two receptor subtypes

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(Gurevich and Gurevich, 2008; Rives et al., 2009). Several studies have largely shown that dimerization of G protein-coupled receptors (GPCR) can contribute to regulation of receptor targeting/internalization, pharmacological or signaling properties (Bulenger et al., 2005; Jordan and Devi, 1999; Milligan, 2009; Terrillon and Bouvier, 2004). GPCR dimerization has been identified in native tissues, precluding an artifact due to artificial receptor aggregation (Albizu et al., 2010a). Moreover, its functional relevance in central nervous system and the relationship between altered receptor–receptor interactions and psychiatric diseases have been described (Albizu et al., 2010b; Ferre et al., 2007). Here we report functional crosstalk between serotonin 5-HT_{2A}R and dopamine D₂R, two major antipsychotic drug targets, and show a physical interaction between the two GPCR.

2. Experimental procedures

2.1. Cell culture and in vitro transfection

HEK293 cells were maintained in culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin and streptomycin in an atmosphere of 95% air and 5% CO₂ at 37 °C. Cells were transiently transfected with serotonin 5-HT_{2A}R and/or dopamine D₂R by using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions.

2.2. Membrane preparation

Membranes were prepared from mouse brain or HEK293 cell line. Mouse brain samples were obtained from adult (8–12 weeks old) male 129/SV mice. Mouse brain or culture dishes of HEK293 cells expressing serotonin 5-HT_{2A}R or co-expressing 5-HT_{2A}R and dopamine D₂R were washed twice in PBS without calcium and magnesium, and cold lysis buffer (15 mM Tris–HCl, 2 mM MgCl₂, 0.3 mM EDTA, pH 7.4) was added. HEK293 cells were scraped with a rubber. Then cells or brain samples were homogenized with a polytron and centrifuged at 100 g for 5 min at 4 °C. Supernatants were recovered and centrifuged at 44000 g for 30 min at 4 °C. Pellets were resuspended in a suspension medium (50 mM Tris–HCl, 5 mM MgCl₂, pH 7.4) and centrifuged at 44000 g for 30 min at 4 °C. Pellets were resuspended in an appropriate volume of the same buffer. For each membrane preparation, protein content was evaluated and membranes were then aliquoted and frozen in liquid nitrogen.

2.3. Radioligand binding assay

Competition experiments were performed on membranes expressing serotonin 5-HT_{2A}R or co-expressing 5-HT_{2A}R and dopamine D₂R. [³H]Ketanserin was used to label 5-HT_{2A}R as previously reported (Gonzalez-Maeso et al., 2007). Membranes were incubated for 1 h at 30 °C with [³H]Ketanserin (1–5 nM) and with increasing concentrations of non radioactive ligand ranging from 1 pM to 100 μM. Non specific binding was determined with an excess of unlabeled ketanserin (1 μM). Bound tritiated ketanserin fractions were separated from the free tritiated ketanserin by filtration. We used Whatman GF-C filters preincubated with bovine serum albumin (10 mg/ml). Filtration was performed on a Tomtec harvester. Radioactivity was counted on a beta-counter Tri-carb 2100TR (PerkinElmer). Each assay was performed in triplicate. Data were analyzed with the program Graphpad Prism. The inhibition constants (*K_i*) for ligands were calculated by using the following relation: $K_i = IC_{50}/(1 + [*L]/K_d)$, where *IC*₅₀ is the concentration of ligand leading to a decrease of 50% of the specific binding and [³H] and *K_d* are the concentration and dissociation constant of [³H] Ketanserin, respectively.

2.4. Inositol phosphate production

The accumulation of inositol phosphates was determined by measurement of [³H]inositol polyphosphate production. The inositol phosphate accumulation experiments on HEK293 cells-expressing serotonin 5-HT_{2A}R and/or dopamine D₂R were performed as previously described (Albizu et al., 2007). Briefly, experiments were performed in 12-well plates (7 × 10⁵ cells/well) after overnight labeling with [³H]-myo-inositol (1 μCi/ml/well) in serum-free medium at 37 °C. After washing cells three times with experimental medium (Hank's buffer containing LiCl 20 mM and Hepes 20 mM), cells were preincubated in experimental medium with various concentrations of drugs for 10–30 min at 37 °C. The reaction was stopped by replacing the medium with 10 mM formic acid (4 °C). Plates were maintained at 4 °C for 30 min to extract the accumulated [³H]inositol polyphosphate from cells. Supernatants were recovered, and inositol phosphates were purified by ion exchange chromatography method using Dowex-1 resin X8 (Bio-Rad). Radioactivity was measured on a beta-counter Tri-carb 2100TR (PerkinElmer).

2.5. Behavioral studies

Experiments were performed on adult (8–12 weeks old) male 129S6/SvEv mice. 5-HT_{2A}R-KO mice have been previously described (Gonzalez-Maeso et al., 2007). Animals were housed at 12 h light/dark cycle at 23 °C with food and water *ad libitum*. The Institutional Animal Use and Care Committee approved all experimental procedures at the Mount Sinai School of Medicine. Locomotor behavioral studies were performed as previously described (Gonzalez-Maeso et al., 2008).

Motor function was assessed with a computerized three-dimensional activity monitoring system (AccuScanInstruments). The activity monitor has 32 infrared sensor pairs, with 16 along each side spaced 2.5 cm apart. The system determines motor activity on the basis of the frequency of interruptions to infrared beams traversing the x, y and z planes. Total distance (cm) and horizontal activity were automatically determined from the interruptions of the beams in the horizontal plane.

2.6. Co-immunoprecipitation assays

Assays were performed in HEK293 cells using C-terminally RLuc-tagged D₂R and GFP-tagged D₂R, 5-HT_{2A}R, metabotropic glutamate receptors mGluR2 and mGluR3. Proteins solubilized in lysis buffer (Hepes 50 mM, NaCl 100 mM, glycerol 10%, dodecylmaltoside 0.4% and a protease inhibitor mixture from Roche) were then centrifuged at 14,000 rpm for 30 min at 4 °C. Supernatants were incubated overnight with anti-GFP (Roche Diagnostics) or anti-RLuc (Millipore Corp) antibody at 4 °C on a rotating wheel. Protein G beads were added and incubated 2 h at 4 °C on a rotating wheel. Immunoprecipitated proteins were eluted in Laemli sample buffer, resolved by SDS-polyacrylamide gel electrophoresis, and detected by western blotting. Membranes were incubated overnight at 4 °C with the primary anti-RLuc, anti-GFP or anti-N-Cadherin antibody. Membranes were washed three times for 10 min each in blocking buffer and exposed for 1 h at room temperature with the secondary horseradish peroxidase labeled anti-mouse antibody (Amersham Life Sciences). Detection of proteins was conducted using ECL system.

2.7. Statistical analysis

All data were analyzed using GraphPad Prism 5.0 software. Data are expressed as mean ± SEM. Student's *t*-test or *F* test were used, results were considered statistically significant when *p* < 0.05.

3. Results

3.1. D₂R activation increases the serotonin agonist affinity for 5-HT_{2A}R

Competition binding assays can provide a sensitive measure of functional interactions within a receptor complex (Gonzalez-Maesó et al., 2008). The effect of receptor crosstalk is observed by determining whether the competition binding curve for one of the receptors is altered by a ligand for the other receptor. This assay can detect crosstalk of untagged receptors expressed in transfected cells and of endogenous receptors expressed *in vivo*. When appropriate specific controls are performed, the presence of cross-receptor binding modulation strongly supports the existence of a functional crosstalk between receptor subtypes. Therefore, we initially studied the effect of the D₂R agonist quinpirole on the competition of the serotonergic hallucinogen DOI for antagonist ketanserin binding sites on 5-HT_{2A}R in membranes from HEK293 cells transfected with both receptors (Fig. 1A). Quinpirole caused a marked increase in the affinity of DOI for 5-HT_{2A}R. Notably, the curve corresponding to the DOI displacement of [³H]Ketanserin fits to a one-site model while the curve corresponding to the DOI displacement of [³H]Ketanserin in the presence of quinpirole fits to a two-site model.

To confirm that the effect of quinpirole on 5-HT_{2A}R agonist affinity resulted from its exclusive binding to the D₂R, we repeated the experiment using membranes from HEK293 cells expressing only 5-HT_{2A}R (Fig. 1B). We found that the dopamine agonist did not alter DOI affinity since the two competition curves in presence and absence of quinpirole are very close. We also observed an effect of receptor co-expression on DOI binding in the absence of D₂R

agonist. The competition curve seen with 5-HT_{2A}R expressed alone fits to a two-site model (Fig. 1B, black curve). However, co-expression of 5-HT_{2A}R and D₂R caused one-site binding to be observed (Fig. 1A, black curve) suggesting that D₂R expression is sufficient to alter the binding properties of DOI on 5-HT_{2A}R. D₂R expression decreases the affinity of the hallucinogenic drug for 5-HT_{2A}R and this decrease is counteracted by D₂R activation (in presence of D₂R agonist).

We obtained similar results in membranes from the mouse striatum. D₂R agonist induced a large increase in the affinity of DOI for 5-HT_{2A}R (competition curve best fitted with a two-site model) (Fig. 1C). The specificity of this effect is demonstrated by using D₂R antagonist raclopride (Fig. 1D). While DOI affinity in the presence of raclopride is not altered (data not shown), raclopride prevents the enhanced effect induced by quinpirole on DOI affinity. Thus dopamine D₂R expression and activation alter the serotonin 5-HT_{2A}R ligand binding properties (Table 1). This observation supports the presence of allosteric modulation across 5-HT_{2A}R and D₂R expressed in heterologous system and in native tissue.

3.2. D₂R alters the 5-HT_{2A}R mediated inositol phosphate production

To test whether D₂R expression modulates 5-HT_{2A} mediated G_{q/11} protein signaling, we measured inositol phosphate (IP) production induced by 5-HT_{2A}R activation in the presence and absence of the D₂R (Fig. 2A,B). The response to serotonin (5-HT) was not altered by the presence of D₂R (Fig. 2A). However, efficacy of the hallucinogenic agonist DOI was significantly increased in the presence of the D₂R since we observed a shift of the curve to the left (Fig. 2B). The apparent difference in 5-HT_{2A}R signaling observed for DOI, but not for serotonin, is consonant with data from several studies suggesting

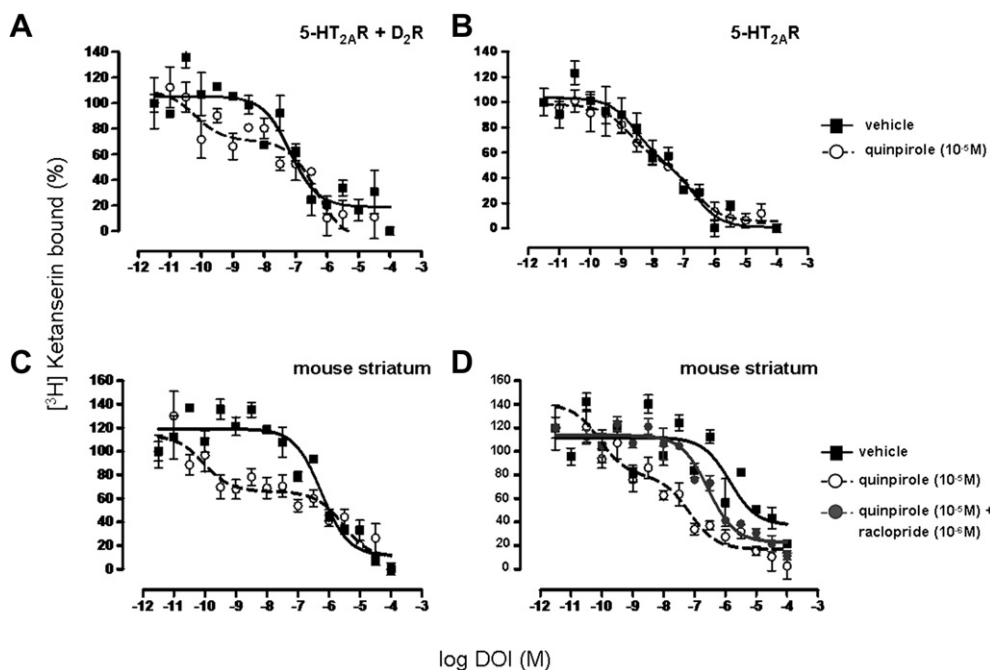


Fig. 1. 5-HT_{2A}R radioligand competitive curves performed in HEK293 cells and mouse striatum. **A**, In HEK293 cells co-expressing 5-HT_{2A}R and D₂R, 5-HT_{2A}R agonist DOI affinity is higher in the presence of D₂R agonist quinpirole (10^{−5} M) (two-site model; $K_{i-high} = 1.1 \times 10^{-11}$ M and $K_{i-low} = 2.8 \times 10^{-8}$ M) (○) from the control (one-site model; $K_i = 1.7 \times 10^{-8}$ M) (■). **B**, In HEK293 cells expressing only 5-HT_{2A}R, DOI affinity does not change in the presence of quinpirole (10^{−5} M) (two-site model; $K_{i-high} = 2.9 \times 10^{-10}$ M, $K_{i-low} = 3.5 \times 10^{-8}$ M) (○) from the control (two-site model; $K_{i-high} = 5.8 \times 10^{-10}$ M, $K_{i-low} = 4.2 \times 10^{-8}$ M) (■). **C**, In mouse striatum, DOI affinity is significantly higher in the presence of quinpirole (10^{−5} M) (two-site model; $K_{i-high} = 1.5 \times 10^{-11}$ M and $K_{i-low} = 4.7 \times 10^{-7}$ M) (○) from the control (one-site model; $K_i = 9.5 \times 10^{-8}$ M) (■). **D**, In mouse striatum, the increased DOI affinity in the presence of quinpirole (10^{−5} M) (two-site model; $K_{i-high} = 8.5 \times 10^{-11}$ M and $K_{i-low} = 6.9 \times 10^{-8}$ M) (○) from the control (one-site model; $K_i = 3.3 \times 10^{-6}$ M) (■) is decreased when D₂R antagonist raclopride is added (one-site model; $K_i = 2.7 \times 10^{-7}$ M) (●). Data are means ± SEM for each experiment performed in triplicate. The K_i values shown are for this single experiment. Averages of K_i values are indicated in Table 1.

Table 15-HT_{2A}R agonist DOI affinity calculated from [³H]Ketanserin competition curves in mouse striatum and HEK293 cells expressing 5-HT_{2A}R and D₂R or 5-HT_{2A}R alone.

Receptor system	Ligand	K _i (M)		% High
		K _{i-high}	K _{i-low}	
HEK-5HT _{2A} R-D ₂ R	Vehicle	NA	$7.7 \times 10^{-9} \pm 0.25$	NA
	Quinpirole (10^{-5} M)	$6.2 \times 10^{-11} \pm 0.22$	$1.7 \times 10^{-8} \pm 0.32$	46 ± 3
HEK-5HT _{2A} R	Vehicle	$3.2 \times 10^{-9} \pm 0.18$	$6.1 \times 10^{-7} \pm 0.4$	41 ± 5
	Quinpirole (10^{-5} M)	$1.5 \times 10^{-9} \pm 0.45$	$1.3 \times 10^{-7} \pm 0.33$	48
Mouse striatum	Vehicle	NA	$2.5 \times 10^{-7} \pm 0.26$	NA
	Quinpirole (10^{-5} M)	$6.5 \times 10^{-11} \pm 0.81$	$5.1 \times 10^{-7} \pm 0.89$	44 ± 2
	Raclopride (10^{-6} M)	NA	$6.6 \times 10^{-7} \pm 0.24$	NA
	Quinpirole (10^{-5} M) + raclopride (10^{-6} M)	NA	$6.9 \times 10^{-7} \pm 0.1$	NA

DOI competition of [³H]Ketanserin binding was performed in the absence (vehicle) or in the presence of D₂R agonist quinpirole (10^{-5} M) and/or D₂R antagonist raclopride (10^{-6} M). Competition curves were analyzed by nonlinear regression to derive dissociation constants for the high- (K_{i-high}) and low- (K_{i-low}) affinity states of the receptor. % High refers to the percentage of high-affinity binding sites as calculated from nonlinear fitting. One-site model or two-site model as a better description of the data was determined by *F* test. Two-site model, *p* < 0.05. NA, two-site model is not applicable (*p* > 0.05). K_i; averages are expressed as means ± SEM of at least three separate experiments.

DOI displacement curve of [³H]Ketanserin with quinpirole (10^{-5} M) compared to the one with vehicle: *F*[2,43] = 3.42, **p* < 0.05 in HEK-5-HT_{2A}R-D₂R; *F*[2,43] = 7, ***p* < 0.01 in HEK-5-HT_{2A}R and *F*[2,43] = 12.88, ***p* < 0.001 in mouse striatum. DOI displacement curve of [³H]Ketanserin with quinpirole (10^{-5} M) + raclopride (10^{-6} M) compared to the one with vehicle: *F*[2,40] = 3.2, **p* < 0.05 in mouse striatum.

that hallucinogenic serotonergic agonists stabilize a different 5-HT_{2A}R conformation than do non-hallucinogenic ligands (Gonzalez-Maeso et al., 2007, 2003).

We also determined the consequences of D₂R activation by two different agonists (quinpirole and ropinirole) on IP levels induced by 5-HT or DOI in cells co-expressing 5-HT_{2A}R and D₂R (Fig. 2C,D). No effect of either dopamine agonist was detected on serotonin induced IP production (Fig. 2C). In contrast, the promoting effect of D₂R expression on DOI-induced IP production was abolished by D₂R activation. 5-HT_{2A}R activation is less efficient in presence of D₂R agonists (the concentration–response curves shifted to the right) (Fig. 2D). This decrease of efficacy results strictly from 5-HT_{2A}R stimulation since stimulation of D₂R

alone does not induce G_{q/11} protein signaling pathway activation. Thus dopamine D₂R expression and activation alter the hallucinogenic drug-stimulated 5-HT_{2A}R second messenger production (Table 2).

3.3. Role of the 5-HT_{2A}R in behavioral effects of antipsychotic D₂R antagonist

The identification of functional crosstalk between the 5-HT_{2A}R and D₂R in transfected cells and in mouse brain striatal membranes raised the question of whether evidence for crosstalk could be obtained *in vivo*. The potent and selective non-competitive NMDA receptor antagonist MK-801 (dizocilpine) is used as a pharmacological model

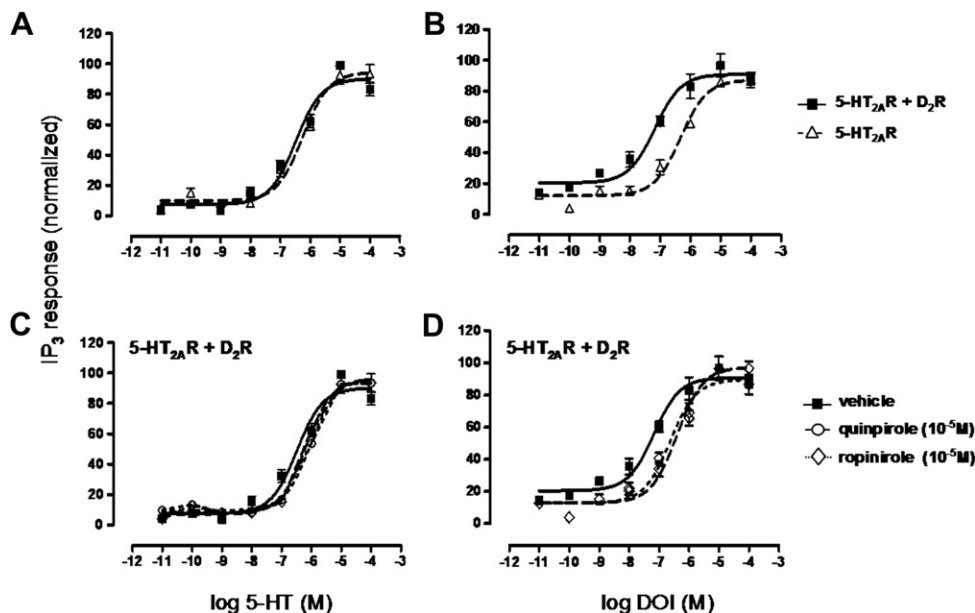


Fig. 2. Inositol Phosphate (IP) production induced by 5-HT_{2A} activation with natural agonist 5-HT or hallucinogenic drug DOI. A, Serotonin (5-HT)-stimulated 5-HT_{2A}R in HEK293 cells expressing 5-HT_{2A}R (Δ) and HEK293 cells co-expressing 5-HT_{2A}R and D₂R (■). D₂R expression does not affect 5-HT-induced 5-HT_{2A}R-IP₃ production (EC₅₀ = 5.5×10^{-7} M). B, DOI-stimulated 5-HT_{2A}R in HEK293 cells expressing 5-HT_{2A}R (Δ) (EC₅₀ = 3.8×10^{-6} M) and HEK293 cells co-expressing 5-HT_{2A}R and D₂R (■) (EC₅₀ = 6.7×10^{-8} M). D₂R expression increases DOI-induced 5-HT_{2A}R-IP₃ production. C, D₂R agonists (10^{-5} M), quinpirole (○) (EC₅₀ = 6.6×10^{-7} M) and ropinirole (◇) (EC₅₀ = 8×10^{-7} M), have no effect on 5-HT-induced 5-HT_{2A}R-IP₃ production (vehicle; EC₅₀ = 5.5×10^{-7} M). D, D₂R agonists (10^{-5} M), quinpirole (○) (EC₅₀ = 3.9×10^{-7} M) and ropinirole (◇) (EC₅₀ = 2.4×10^{-7} M) decrease DOI-induced 5-HT_{2A}R-IP₃ production (vehicle; EC₅₀ = 6.7×10^{-8} M). Data are means ± SEM for each experiment performed in triplicate. The EC₅₀ values shown are for this single experiment. Averages of EC₅₀ values over all experiments can be found in Table 2.

Table 2Efficacy of 5-HT_{2A}R agonists (5-HT and DOI) to induce inositol phosphate production in HEK293 cells expressing 5-HT_{2A}R or 5-HT_{2A}R and D₂R.

Receptor system	Ligands	5-HT		DOI	
		EC ₅₀ (M)	E _{max} (cpm)	EC ₅₀ (M)	E _{max} (cpm)
HEK-5HT _{2A} R	Vehicle	$6.1 \times 10^{-7} \pm 0.27$	647 ± 74	$2.6 \times 10^{-6} \pm 0.33$	329 ± 50
HEK-5HT _{2A} R-D ₂ R	Vehicle	$6.1 \times 10^{-7} \pm 0.12$	608 ± 12	$8.9 \times 10^{-8} \pm 0.31$	332 ± 9
	Quinpirole (10^{-5} M)	$7.2 \times 10^{-7} \pm 0.15$	589 ± 22	$2.3 \times 10^{-7} \pm 0.35$	332 ± 20
	Ropinirole (10^{-5} M)	$3.9 \times 10^{-7} \pm 0.42$	564 ± 47	$1.8 \times 10^{-7} \pm 0.1$	338 ± 61

Dose-responses: cells were stimulated by increasing concentrations (10^{-11} M– 10^{-4} M) of serotonin 5-HT_{2A}R agonist (serotonin 5-HT or hallucinogen DOI) in presence or not of dopamine D₂R agonist (quinpirole or ropinirole) at 10^{-5} M. Efficient concentrations 50% (EC₅₀) measured reflect the 5-HT/DOI efficacy to activate the 5-HT_{2A}R mediated G_{q/11} protein signaling pathway and inositol phosphate production. E_{max} represents the maximum possible effect for 5-HT/DOI. EC₅₀ averages and E_{max} are expressed as means \pm SEM of at least three separate experiments.

for schizophrenia in mice (Reimherr et al., 1986). MK-801 increases the locomotor activity of mice, a behavior that is suppressed by the antipsychotic haloperidol which acting at D₂R (Gattaz et al., 1994). In order to probe for 5-HT_{2A}R and D₂R crosstalk *in vivo*, we compared the effects of haloperidol on MK-801-stimulated locomotion in wild-type and 5-HT_{2A}R-KO mice.

The increased locomotor activity elicited by MK-801 in wild-type and 5-HT_{2A}R-KO mice was similar (Fig. 3A). In the wild-type mice, the effect of MK-801 was largely eliminated in animals pre-treated with dopamine D₂R antagonist haloperidol. Interestingly, the effect of haloperidol was significantly reduced in the 5-HT_{2A}R-KO mice ($p < 0.01$) (Fig. 3B). These findings suggest that the haloperidol antipsychotic-like behavioral response requires the expression of 5-HT_{2A}R.

3.4. Interaction between serotonin 5-HT_{2A}R and dopamine D₂R

We then examined the possible existence of a physical interaction between serotonin 5-HT_{2A}R and dopamine D₂R which could contribute to the receptor crosstalk observed. We performed co-immunoprecipitation assays to test whether 5-HT_{2A}R and D₂R could form heteromeric complexes. Receptor co-immunoprecipitation was performed in HEK293 cells co-expressing RLuciferase (RLuc)-tagged

D₂R and green fluorescent protein (GFP)-tagged 5-HT_{2A}R (Fig. 4A). In control cells expressing D₂R-RLuc alone, the RLuc form was immunoprecipitated with the anti-RLuc antibody and D₂R-RLuc homomers were detected. No GFP immunoreactivity was detected. In control cells expressing 5-HT_{2A}R-GFP alone, no bands were detected by probing with the anti-RLuc antibody, whether immunoprecipitation was done with the anti-GFP antibody or the anti-RLuc antibody, demonstrating the selectivity of the anti-RLuc antibody used. In cells co-expressing both receptors, immunoprecipitation with the anti-GFP antibody led to immunoreactivity detected by the anti-RLuc antibody, supporting the presence of heteroreceptor complexes. Signal was detected at ~ 170 kDa, which are consistent with the presence of heteromers. Larger receptor heterocomplexes were also observed. As a negative control, membranes were probed for N-Cadherin immunoreactivity, a protein which is highly expressed in the plasma membrane. The absence of N-Cadherin signal in the immunoprecipitated samples indicates the specificity of the assay and supports a physical association between 5-HT_{2A}R and D₂R in cotransfected cells.

To confirm the specificity of the 5-HT_{2A}R-D₂R interaction, D₂R-RLuc was co-expressed with metabotropic glutamate receptors mGluR2-GFP or with mGluR3-GFP. No co-immunoprecipitation was detected in either case (Fig. 4B,C).

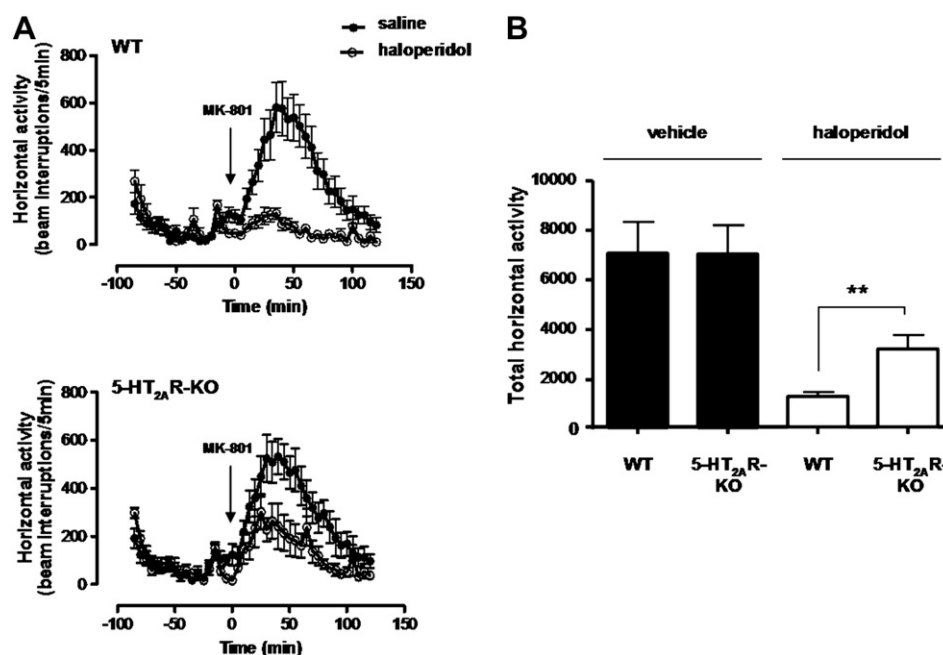


Fig. 3. 5-HT_{2A}R is necessary to induce D₂R ligand dependent behavioral effects in mice. Expression of 5-HT_{2A}R is necessary for the inhibition of MK-801-induced locomotor activity by D₂R antagonist haloperidol. **A**, The time course of MK-801-induced locomotion is measured in 5 min blocks. Time of injections of MK-801 is indicated by arrows. Horizontal activity is measured in wild-type (WT) and 5-HT_{2A}R-KO mice. **B**, Corresponding bar graph of the total MK-801-induced locomotion as a summation of horizontal activity from $t = 10$ min to $t = 120$ min. Mice were administered haloperidol (1 mg/kg) or vehicle followed by MK-801 (0.5 mg/kg; i.p.) ($n = 6$; $**p < 0.01$; error bars shows SEM).

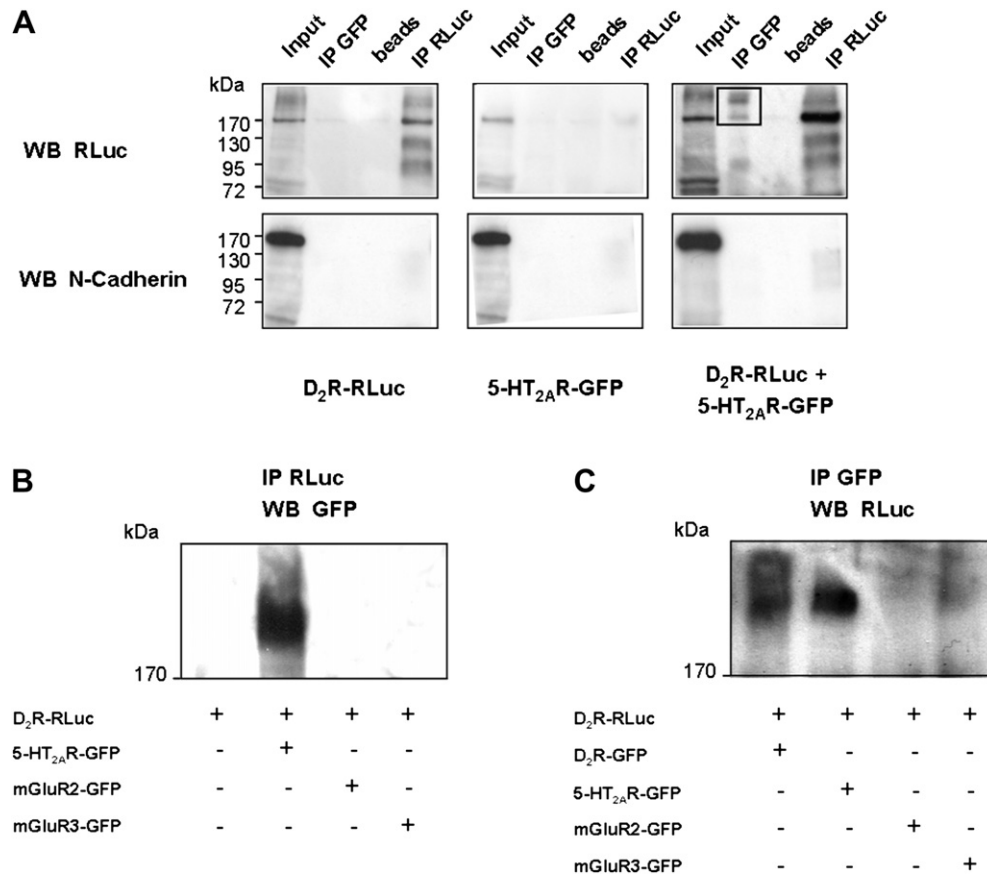


Fig. 4. 5-HT_{2A}R and D₂R co-immunoprecipitate. **A**, Specific co-immunoprecipitation of 5-HT_{2A}R and D₂R in HEK293 cells. RLuc-tagged D₂R and GFP-tagged 5-HT_{2A}R were immunoprecipitated using anti-GFP or RLuc antibodies. Membranes were probed with either anti-RLuc antibody or anti-N-Cadherin antibody. The co-immunoprecipitated receptor complex is indicated by the box in the upper right panel. **B**, RLuc-tagged D₂R and GFP-tagged 5-HT_{2A}R, mGluR2 or mGluR3 were immunoprecipitated using anti-RLuc antibody and membranes were probed with anti-GFP antibody. A co-immunoprecipitated receptor is detected only for D₂R with 5-HT_{2A}R. **C**, RLuc-tagged D₂R and GFP-tagged D₂R, 5-HT_{2A}R, mGluR2 or mGluR3 were immunoprecipitated using anti-GFP antibody and membranes were probed with anti-RLuc antibody. Complex formation was observed only for D₂R homomers and D₂R-5-HT_{2A}R heteromers. Panels are representative of three independent experiments. WB, western blot. IP, immunoprecipitation.

4. Discussion

The related effects of serotonin 5-HT_{2A}R and dopamine D₂R antagonists on the symptoms of psychosis in humans and animal models and the colocalization of the two receptors in dopaminergic neurons motivated the study of their heteromerization. Studies showed 5-HT_{2A}R and D₂R are both expressed in dopaminergic cells in the ventral tegmental area of the midbrain, the substantia nigra pars compacta and the arcuate nucleus of the hypothalamus (Doherty and Pickel, 2000; Nocjar et al., 2002; Pazos et al., 1985). 5-HT_{2A}R activation by serotonin leads to the dopaminergic neuron activation (Bortolozzi et al., 2005). Radioligand assays confirmed that both receptors are co-expressed in mouse striatal membranes (data not shown). In this study, we found evidence for functional crosstalk between 5-HT_{2A}R and D₂R involving allosteric modulation, receptor signaling and drug-induced behavior. Moreover, we provided first evidence supporting the existence of 5-HT_{2A}R-D₂R heteromers expressed in transfected cells and in brain.

5-HT_{2A}R is mainly coupled to the G_{q/11} protein and activates inositol phosphate production (Conn and Sanders-Bush, 1984) while D₂R is mainly coupled to the G_{i/o} protein and inhibits cAMP production (Montmayeur et al., 1993). We found that D₂R expression increases the efficacy of DOI to activate the phosphoinositide G_{q/11} signaling pathway downstream of the 5-HT_{2A}R. Addition of D₂R agonists eliminates the effect of D₂R expression on 5-HT_{2A}R signaling. This effect on 5-HT_{2A}R signaling is seen only for the

hallucinogenic partial agonist DOI and not for serotonin. Notably, some studies suggest that hallucinogenic 5-HT_{2A}R agonists stabilize a different receptor conformation than do non-hallucinogenic serotonin agonists (Gonzalez-Maeso et al., 2007, 2003). Thus our results suggest that the allosteric crosstalk observed between the 5-HT_{2A}R and D₂R may be influenced by the conformational selectivity of the ligands complexing with the receptors. It is also interesting that the effects of D₂R expression and activation on 5-HT_{2A}R signaling properties appear opposite to the effects on 5-HT_{2A}R ligand affinity. D₂R expression eliminates the high-affinity DOI binding site and D₂R agonist restores it. Finally, the 5-HT_{2A}R-D₂R crosstalk might be associated to a decrease in the 5-HT_{2A}R agonist binding potency and an increase in the 5-HT_{2A}R agonist functional potency. When D₂R is activated, opposite effects are observed (Fig. 5). It is difficult to compare directly the allosteric effects obtained by antagonist-displacement competition binding in membrane preparations and by intact cell signaling assays. However, the two effects can be reconciled if we assume that the high-affinity site in the membrane binding assay corresponds to a receptor that is uncoupled from G_{q/11} protein. In addition, the involvement of other interacting proteins cannot be excluded.

We also found that functional 5-HT_{2A}R-D₂R crosstalk occurs at the level of the animal behavior. 5-HT_{2A}R expression is necessary for the full antipsychotic effect of drug acting at the D₂R. The haloperidol induced inhibitory effect on animal locomotion is reduced in 5-HT_{2A}R-KO mice. This raises the possibility that the effects of

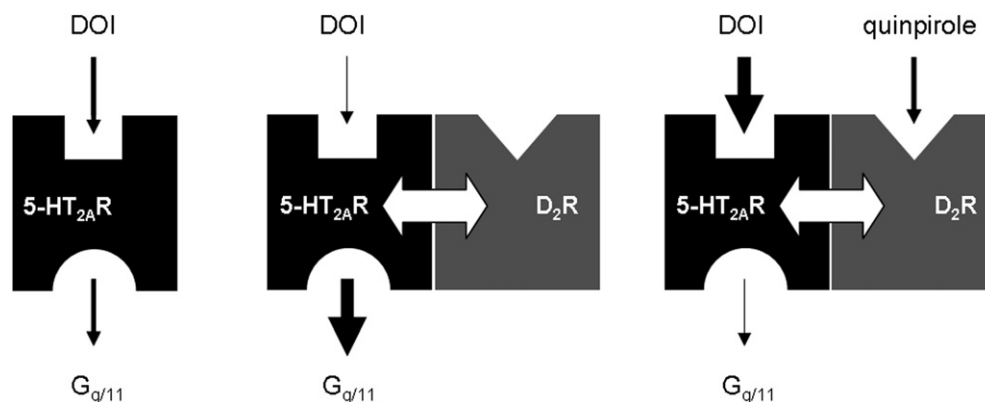


Fig. 5. 5-HT_{2A}R-D₂R heteromer crosstalk hypothesis: effects of D₂R expression and activation on 5-HT_{2A}R binding and signaling properties. On the left, serotonin 5-HT_{2A}R is illustrated as binding its agonist DOI and being coupled to G_{q/11} protein. In the middle, dopamine D₂R expression might have an allosteric effect (illustrated with a white arrow) on 5-HT_{2A}R by decreasing DOI agonist affinity to 5-HT_{2A}R and increasing 5-HT_{2A}R coupling to G_{q/11} protein. In contrast, on the right, D₂R activation (induced by its agonist quinpirole) leads to an increased affinity of 5-HT_{2A}R for DOI and a decreased G_{q/11} protein coupling to 5-HT_{2A}R.

classical antipsychotics are mediated, at least in part, through a 5-HT_{2A}R-D₂R interaction. Notably, the 5-HT_{2A}R is expressed in midbrain dopaminergic neurons that express D₂R and that project to striatal and limbic areas. It will be interesting to determine if both 5-HT_{2A}R and D₂R co-localize presynaptically on dopaminergic neurons where they would significantly influence the patterns of dopamine release in these behaviorally important circuits.

The functional crosstalk observed could result either from the direct physical interactions of the two receptors in a heterocomplex or from interactions of pathways downstream of each receptor. Our results do not distinguish between these two possibilities, which are not mutually exclusive. Both the 5-HT_{2A}R and D₂R have been identified as forming heteromers with other GPCR with implications in neurological disorders. 5-HT_{2A}R forms a functional interaction in cortical neurons with mGluR2 (Gonzalez-Maeso et al., 2008) while D₂R has been reported to form heteromers with dopamine D₁R, D₃R, adenosine A_{2A}R, cannabinoid CB₁R, histamine H₃Rs and metabotropic glutamate receptor 5 (mGluR5) (Albizu et al., 2010b). D₂R homomers have been identified in living cells (Armstrong and Strange, 2001). Recently, 5-HT_{2A}R homomers and 5-HT_{2A}R-D₂R heteromers have been detected by biophysical approaches in HEK293 cells cotransfected with tagged receptors (Borrito-Escuela et al., 2010; Lukasiewicz et al., 2010). While many receptors have been reported to form heteromers in transfected cells, the evidence for functional interactions and for complex formation *in vivo* is more limited (Albizu et al., 2010a; Barki-Harrington et al., 2003; Damak et al., 2003; Zhao et al., 2003). Here we found that the D₂R itself has an allosteric effect on the binding of an agonist to the 5-HT_{2A}R. Moreover, a high concentration of D₂R agonist increases hallucinogenic agonist DOI binding on 5-HT_{2A}R. The monophasic competition curve of [³H]ketanserin by DOI changed to become biphasic when D₂R is activated by quinpirole. The detection of a high-affinity binding site revealed the positive cooperative effect between the serotonergic and dopaminergic binding sites. Several studies showed the link between ligand dependent allosteric modulations and GPCR oligomer existence (Durroux, 2005; Giraldo, 2008; Mattera et al., 1985; Springael et al., 2006; Urizar et al., 2005; Wreggett and Wells, 1995). In agreement with previous work (Albizu et al., 2006), the positive cooperative binding can be explained by considering a multivalent complex in which the binding of a ligand on one protomer enhances the binding of a second ligand on the second binding site. Associated with co-immunoprecipitation data, these results provide evidence showing that 5-HT_{2A}R and D₂R form functional heterocomplexes both in cell lines and in brain.

5. Conclusion

Understanding the mechanism by which hallucinogens induce their effects and antipsychotics block them is essential in the treatment of psychosis. Such mechanism helps to explain communication between two different G_{q/11} and G_{i/o} protein-coupled receptors involved in the altered brain processes of schizophrenia. Because schizophrenia can be treated by medications that act on the 5-HT_{2A}R and/or the D₂R, mutual influences of their signaling pathways would improve the understanding of antipsychotic drug interactions and the development of better therapeutics (Rozenfeld and Devi, 2010). The 5-HT_{2A}R-D₂R functional crosstalk provides a new window into novel mechanisms for signal integration across serotonin and dopamine systems with relevance for normal brain physiology and for psychopharmacology. The discovery of a 5-HT_{2A}R-D₂R heterocomplex represents a potential opportunity as a new target for antipsychotics. Future investigations are necessary to confirm the role of the 5-HT_{2A}R-D₂R heteromer in mediating the receptor crosstalk observed.

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