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Hallucinogenic 5-HT2AR agonists LSD and DOI enhance dopamine D2R protomer recognition and signaling of D2-5-HT2A heteroreceptor complexes

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ABSTRACT

Dopamine $D2_LR$-serotonin 5-HT2AR heteromers were demonstrated in HEK293 cells after cotransfection of the two receptors and shown to have bidirectional receptor–receptor interactions. In the current study the existence of $D2_L$-5-HT2A heteroreceptor complexes was demonstrated also in discrete regions of the ventral and dorsal striatum with *in situ* proximity ligation assays (PLA). The hallucinogenic 5-HT2AR agonists LSD and DOI but not the standard 5-HT2AR agonist TCB2 and 5-HT significantly increased the density of D2like antagonist ^{3}H-raclopride binding sites and significantly reduced the pK_{iH} values of the high affinity D2R agonist binding sites in ^{3}H-raclopride/DA competition experiments. Similar results were obtained in HEK293 cells and in ventral striatum. The effects of the hallucinogenic 5-HT2AR agonists on D2R density and affinity were blocked by the 5-HT2A antagonist ketanserin. In a forskolin-induced CRE-luciferase reporter gene assay using cotransfected but not D2R singly transfected HEK293 cells DOI and LSD but not TCB2 significantly enhanced the $D2_LR$ agonist quinpirole induced inhibition of CRE-luciferase activity. Haloperidol blocked the effects of both quinpirole alone and the enhancing actions of DOI and LSD while ketanserin only blocked the enhancing actions of DOI and LSD. The mechanism for the allosteric enhancement of the D2R protomer recognition and signalling observed is likely mediated by a biased agonist action of the hallucinogenic 5-HT2AR agonists at the orthosteric site of the 5-HT2AR protomer. This mechanism may contribute to the psychotic actions of LSD and DOI and the D2-5-HT2A heteroreceptor complex may thus be a target for the psychotic actions of hallunicogenic 5-HT2A agonists.

1. Introduction

Biophysical methods demonstrated dopamine $D2_LR$-serotonin 5-HT2AR heteromers in cellular models after cotransfection of the two receptors with bidirectional receptor–receptor interactions [1,2]. The 5-HT2AR-mediated phospholipase C activation by 5-HT was synergistically enhanced by the concomitant activation of the $D2_LR$ protomer by the D2R agonist quinpirole as shown in a NFAT-luciferase reporter gene assay. A specific and significant elevation of the intracellular calcium levels was also observed when both receptor protomers were simultaneously activated. Conversely, when the $D2_LR$-mediated adenylyl cyclase inhibition by the D2R agonist quinpirole was assayed coagonist stimulation of $D2_LR$ and 5-HT2AR protomers by quinpirole and 5-HT, respectively, led to a reduction of the $D2_LR$ protomer signaling. These results suggested the existence of a 5-HT2AR-mediated $D2_LR$ trans-inhibition phenomenon [1]. The $D2_LR$-5-HT2AR heteromer represents a novel target for antipsychotic drugs since such drugs are well known to exert their therapeutic actions at least with regard to positive symptoms via blockade of D2Rs [3].

Albizu et al. [4] recently observed that activation of D2Rs by quinpirole increases the affinity of the hallucinogenic 5-HT2AR agonist DOI ((±)-2,5-dimethoxy-4-iodoamphetamine) for the 5-HT2AR and reduces the DOI induced $G_{q/11}$ coupling to the 5-HT2AR as seen from the diminished production of inositol phosphate by DOI. This is different from the case when the endogenous ligand 5-HT was used to activate the 5-HT2AR [1]. These findings have led us to study if the observed ability of 5-HT to produce an

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allosteric counteraction of D2R agonist induced D2R signaling via the 5-HT2AR protomer [1] is altered when using the known hallucinogenic high affinity 5-HT2AR agonists lysergic acid diethylamide (LSD) and DOI [5,6]. The 5-HT receptor agonist activity of LSD in the CNS was first described in 1968 [7]. Such an alteration if enhancing D2R protomer function may contribute to their psychotic actions. The current study tested this hypothesis in HEK293 cells and ventral striatum studying 5-HT2AR-D2R interactions using LSD and DOI and a standard 5-HT2AR agonist TCB2 [8] to modulate D2R binding and signaling. Indications were obtained for the existence of D2R-5-HT2AR heteroreceptor complexes in ventral and dorsal striatum with proximity ligation assays (PLA) [9].

2. Materials and methods

Detailed descriptions are available in Supplementary Material and Methods on: chemicals, reagents and drugs; antibodies; cell culture and transfection; membrane preparation and immunofluorescence microscopy.

2.1. Animals

All studies involving animals were performed in accordance with the Swedish National Board for Laboratory Animal and European Communities Council Directive (Cons 123/2006/3) guidelines for accommodation and care of Laboratory Animals. Male Sprague–Dawley rats, 10 weeks old, weighing 310–350 g were obtained from Charles River Laboratories (Germany). The animals were housed one week before experiments under a 12-h light/dark cycle, with ambient temperature of 21 ± 2 °C, relative humidity of 50 ± 5%. Food and water available *ad libitum*.

2.2. Proximity ligation in situ assay

HEK293T cells transiently co-expressing $D2_LR$ and 5-HT2AR or rat striatal floating sections (30 μm) were employed for *in situ* proximity ligation assay (PLA) [10–12]. *In situ* PLA was performed according to manufacturer's instructions (Duolink *in situ* PLA detection kit, Olink, Sweden). The primary antibodies of different species, monoclonal anti-D2R produced in mouse (WH00018113M1, 5 μg/ml, Sigma–Aldrich, Sweden) and polyclonal anti-5-HT2AR produced in rabbit (ab16028, 5 μg/ml, Abcam, Sweden), were used. Control experiments employed only one primary antibody or HEK293T cells transfected with cDNAs encoding only one type of receptor. The products were visualized using a Leica SP2 confocal microscope (Leica, USA). Each image represents a single Z-scan. and was taken using the following acquisition parameters: *Nuclei:* DAPI, ex.405 em.windows 440/500 (35% intensity, gain 468, offset −1); *PLA clusters/blobs:* Texa Red ex.543 em.windows 600/670 (50% intensity, gain 650, offset −1).

2.3. Luciferase reporter gene assay

We used a dual luciferase reporter assay to indirectly detect variations of cAMP levels in transiently transfected cell lines treated with different compounds in a range of concentrations (typically 25 nM to 1 μM). Stimulation of Gi/o-coupled GPCRs decreases intracellular cAMP reducing CRE-linked reporter-gene transcription. In order to study this inhibitory effect, assays are usually performed in the presence of forskolin, a stimulator of adenylyl cyclase, to raise the cAMP level so that the inhibition is easier to detect [13,14]. For luciferase assays, 24 h before transfection cells were seeded at a density of 1×10^6 cells/well in 75 cm^2 flasks and transfected with Fugene® HD transfection reagent (Promega, Sweden). Cells were co-transfected with plasmids corresponding to three constructs as follows: 2 μg firefly luciferase-encoding experimental plasmid (pGL4-CRE-luc2p; Promega, Sweden), 2 μg of wild type 5-HT2A or D2LR expression vectors and 1 μg *Renilla* luciferase-encoding internal control plasmid (phRG-B; Promega). Approximately 36 h post transfection, cells were treated for 30 min with appropriate ligands, including forskolin, washed and incubated for 4 h before harvested with passive lysis buffer (Promega). Then the luciferase activity of cell extracts was determined using a luciferase assay system according to the manufacturer's protocol in a POLARstar Optima plate reader (BMG Labtech) using a 30-nm bandwidth excitation filter at 535 nm. Firefly luciferase was measured as firefly luciferase luminescence over a 15 s reaction period. The luciferase values were normalized against *Renilla* luciferase luminescence values. Transfection experiments were performed in quadruplicate and repeated at least three times.

2.4. [^{3}H]-raclopride binding experiments

Saturation binding experiments with the D2-likeR antagonist [^{3}H]-raclopride (specific activity 82.8 Ci/mmol, PerkinElmer Life Sciences, USA) was performed essentially as described earlier [15,16]. Briefly, membrane homogenates from HEK cells expressing 5-HT2AR and D2R or ventral striatal rat membrane preparations (100 μg protein/ml) were incubated with increasing concentrations of [^{3}H]-raclopride (ranging from 0.1 to 10 nM) in 250 μl of incubation buffer (IB; 50 mM Tris–HCl pH 7.4, containing 100 mM NaCl, 7 mM MgCl2, 1 mM EDTA, 0.05% BSA and 1 mM dithiothreitol) for 60 min at 30 °C, in the presence or absence of different concentrations of hallucinogenic 5-HT2AR agonists LSD and DOI, a standard 5-HT2AR agonist TCB2 or 5-HT. Nonspecific binding was defined as the binding in the presence of 1 mM dopamine hydrochloride (Sigma–Aldrich, Sweden). The incubation was terminated by rapid filtration through Whatman GF/B filters (Maidstone, Kent, UK) using a MultiScreen™ Vacuum Manifold 96-well (Millipore Corp, Sweden), followed by five washes (~250 μl per wash) with ice-cold washing buffer (50 mM Tris–HCl pH 7.4). The filters were dried, 5 ml of scintillation cocktail was added and the bound ligand was determined after 12 h in each sample by liquid scintillation spectrometry (4 min). [^{3}H]-raclopride (2.0–3.0 nM) binding was displaced by dopamine in competition experiments to determine pK_{iH} and pK_{iL} values from the competition curves obtained. Briefly, membrane homogenates from HEK cells expressing 5-HT2A and D2 receptors or ventral striatal rat membrane preparations (100 μg protein/ml) were incubated with increasing concentrations of dopamine (ranging from 0.1 to 1 mM) in 250 μl of IB for 60 min at 30 °C, in the presence or absence of different nanomolar concentrations of the hallucinogenic high affinity 5-HT2A agonists LSD and DOI [5,6], a standard 5-HT2AR antagonist TCB2 or 5-HT. The incubation was terminated as described above and the radioactivity content of the filters was detected by liquid scintillation spectrometry. Nonspecific binding was defined by radioligand binding in the presence of 1 mM dopamine hydrochloride (Sigma Aldrich, Sweden).

2.5. Statistical analysis

The number of samples (n) in each experimental condition is indicated in figure legends. Data from saturation and competition experiments were analyzed by nonlinear regression analysis using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). The density of the binding sites (B_{max}), dissociation constant (K_D) and inhibition constants of the high and low affinity state of the receptor (pK_{iH}, pK_{iL}) from several independent replications were aver-

aged allowing statistical comparisons using a one way analysis of variance (ANOVA). Group differences after ANOVAs were measured by post hoc Turkey's Multiple Comparison Test. The *P* value 0.05 and lower was considered significant.

3. Results

3.1. PLA and immunofluorescence experiments

PLA demonstrated the presence of red clusters (blobs) of D2R and 5-HT2AR, representing D2-5-HT2A heteroreceptor complexes in discrete regions of the neuropil of the dorsal striatum and nucleus accumbens core but not in the corpus callosum and the anterior limb of the anterior commissure (Fig. 1). Also no PLA positive clusters were observed in the cortical regions analized at the Bregma level −1.7. These results were validated in HEK293 cells transiently cotransfected with human $D2_LR$ and 5-HT2AR as seen from colocalization and generation of PLA positive signals (red blobs) found after cotransfection but not after single $D2_LR$ transfections (Supplementary Fig. S1).

Using double immunofluorescence procedures with green and red immunofluorescence for D2Rs and 5-HT2ARs, respectively, it was possible to observe a widespread appearance of punctate yellowish fluorescence in the dorsal striatum and the nucleus accumbens. It likely mainly represents a dendritic colocalization of the D2 and 5-HT2A receptors (Supplementary Fig. S2) since the main part of the D2 and 5-HT2A receptors shows a postjunctional location in the forebrain [17,18]. Yellowish immunofluorescence was also observed in discrete nerve cell body populations in the striatal regions.

3.2. CRE-luciferase reporter gene experiments

A forskolin-induced CRE-luciferase reporter gene assay in cotransfected HEK293 cells was used to study the modulation of the $D2_LR$ protomer responses by the hallucinogenic 5-HT2AR agonists DOI and LSD and the standard 5-HT2AR agonist TCB2. As seen in Fig. 2, 60 and 10 nM of DOI and LSD, respectively (three times the K_i values) significantly enhanced the quinpirole induced inhibition of CRE-luciferase activity. These effects could not be observed in cells singly transfected with $D2_LR$ (Fig. 2B). The D2R antagonist

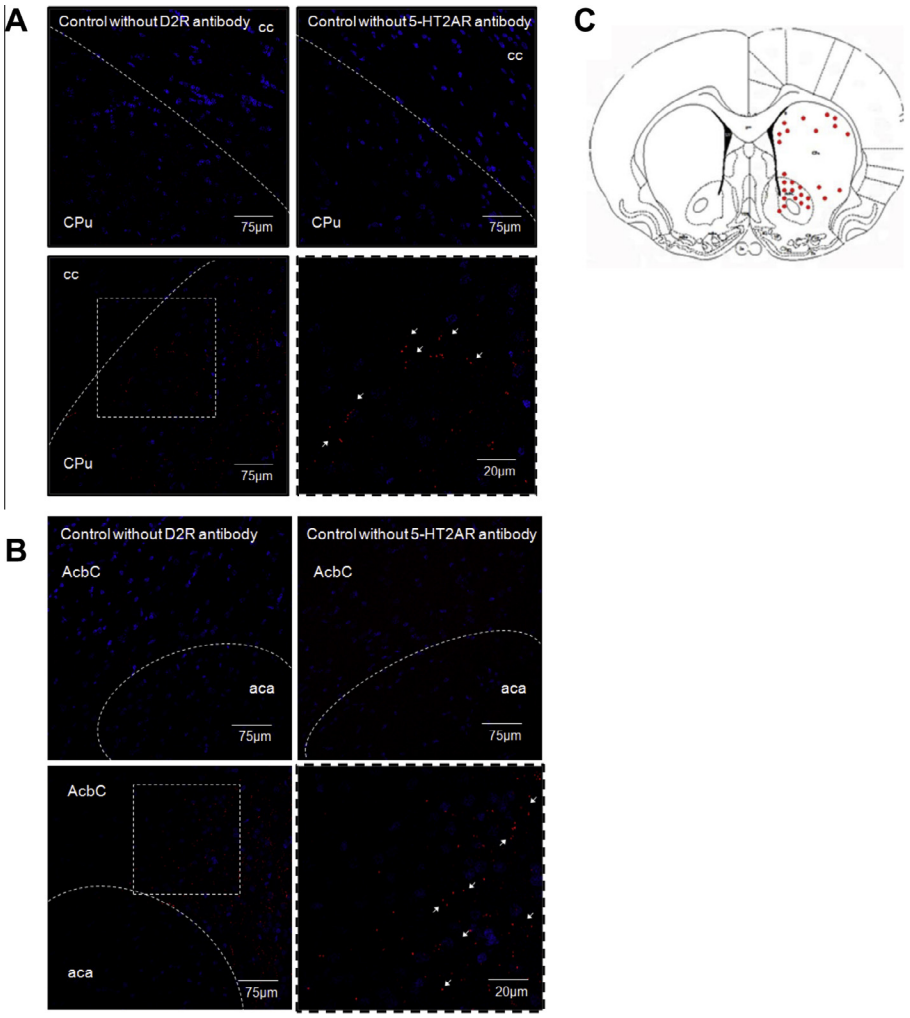

Fig. 1. D2-5-HT2A heteroreceptor complexes in striatal sections of rat detected by PLA. *In situ* PLA was performed using primary antibodies of different species directed to D2R and 5-HT2AR (see Section 2). The detected heteroreceptor complexes are seen as red clusters indicated by arrows. Specific D2R-5-HT2AR clusters are visualized within discrete regions of the dorsal striatum (A: CPu) and nucleus accumbens, especially the core (B: AcbC). They were almost absent in cortical regions, the corpus callosum (cc) and the anterior limb of the anterior commissure (aca). In "C" the relative densities of PLA cluster distributions are schematically illustrated by the density of red puncta. Nuclei are shown in blue (DAPI). Scale bars are shown in the right low part of each panel. Three independent experiments (three rats) were performed. Bregma level −1.7. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

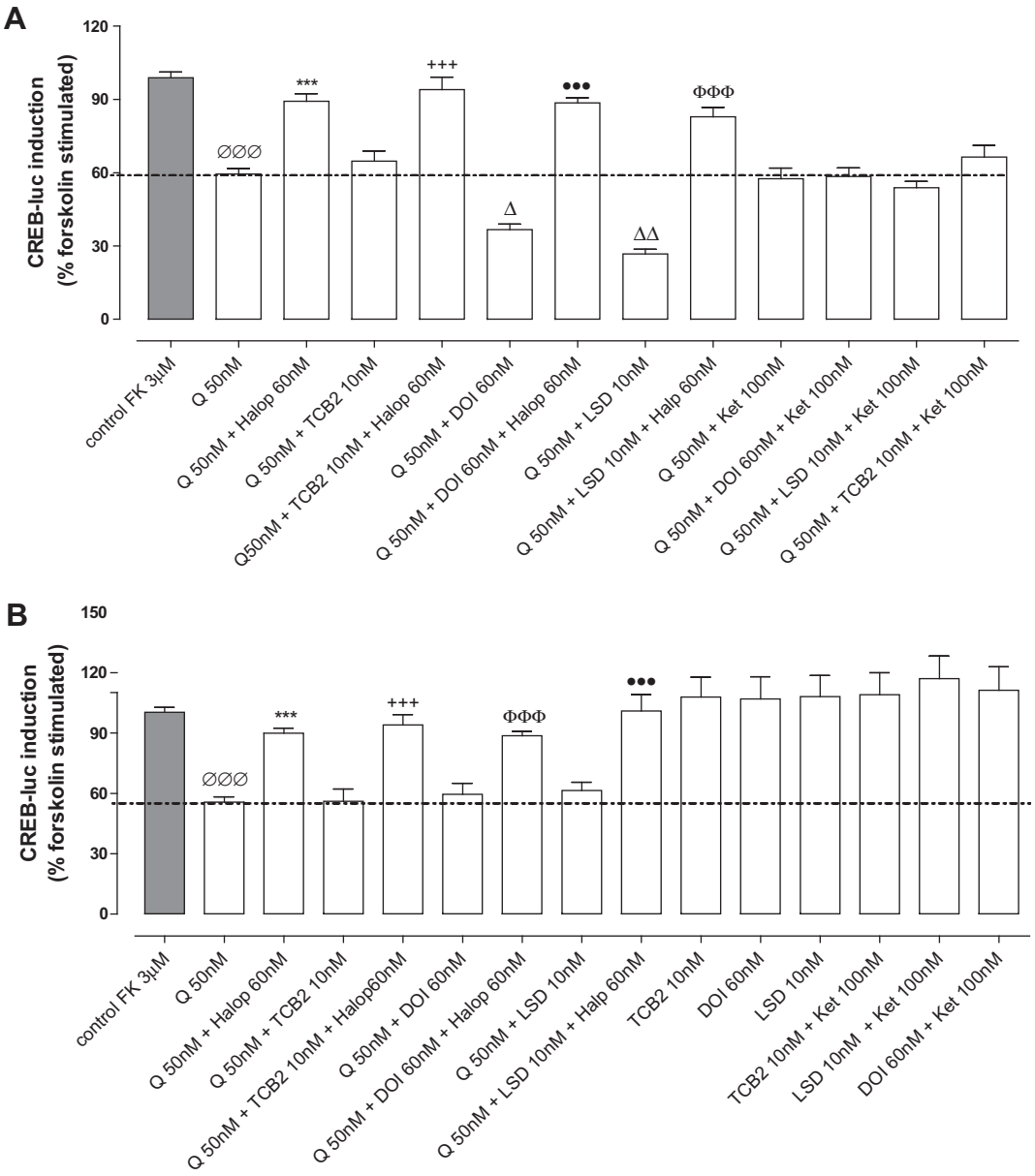

Fig. 2. (A) Demonstration that LSD and DOI but not TCB2 can enhance the $D_{2L}R$ agonist inhibition of the forskolin-induced CRE-luciferase reporter gene expression. The counteraction exerted by haloperidol and ketanserin on these actions is also shown. HEK293T cells were transiently co-transfected with 1 μg firefly luciferase-encoding experimental plasmid (pGL4-CRE-luc2p), 1 μg of both (5-HT2AR and $D2_LR$) expression vectors and 50 ng *Renilla* luciferase-encoding internal control plasmid (phRG-B). After cell incubation with respective agonists and/or antagonists (in presence of 3 μM forskolin, sub-maximal concentration value) luciferase activity was measured. Light emission is expressed as a percentage of the control forskolin-induced value. The data represent the means ± S.E.M. of three independent experiments performed in triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The *P* value 0.05 and lower was considered significant. ^{ØØØ}: Significantly different compared to control ($P < 0.001$); ***: Significantly different compared to quinpirole 50 nM ($P < 0.001$); $^{+++}$: Significantly different compared to quinpirole 50 nM + TCB2 10 nM ($P < 0.001$); $^{●●●}$: Significantly different compared to quinpirole 50 nM + DOI 60 nM ($P < 0.001$); $^{ΔΔ \text{ and } Δ}$: Significantly different compared to quinpirole 50 nM, quinpirole 50 nM + Ket 100 nM, quinpirole 50 nM + DOI 60 nM + Ket 100 nM and quinpirole 50 nM + LSD 10 nM + Ket 100 nM ($P < 0.01$ and $P < 0.05$); ^{ΦΦΦ}: Significantly different compared to quinpirole 50 nM + LSD 10 nM ($P < 0.001$). (B) Demonstration that LSD, DOI and TCB2 cannot enhance the $D2_LR$ agonist inhibition of the forskolin-induced CRE-luciferase reporter gene expression in HEK293 cells singly transfected with $D2_LR$. The experimental conditions are the same as described above (A) with the only difference being that HEK293T cells were transiently transfected only with 1 μg firefly luciferase-encoding experimental plasmid (pGL4-CRE-luc2p), 1 μg of $D2_LR$ expression vector and 50 ng *Renilla* luciferase-encoding internal control plasmid (phRG-B). The data represent the means ± S.E.M. of three independent experiments performed in triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The *P* value 0.05 and lower was considered significant. ^{ØØØ}: Significantly different compared to control ($P < 0.001$); ***: Significantly different compared to quinpirole 50 nM ($P < 0.001$); $^{+++}$: Significantly different compared to quinpirole 50 nM + TCB2 10 nM ($P < 0.001$); ^{ΦΦΦ}: Significantly different compared to quinpirole 50 nM + DOI 60 nM ($P < 0.001$); $^{●●●}$: Significantly different compared to quinpirole 50 nM + LSD 10 nM ($P < 0.001$). Forskolin (3 μM); Q, quinpirole (50 nM); halop, haloperidol (60 nM), TCB2, 4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide (10 nM), DOI, 1-(2,5-dimethoxy-4-iodophenyl)-propan-2-amine (60 nM), LSD, (6aR,9R)-*N*,*N*-diethyl-7-methyl-4,6,6a,7,8,9-hexahydroindolo-[4,3-fg]quinoline-9-carboxamide (Lysergic acid diethylamide) (10 nM) and Ket, ketanserin (100 nM).

haloperidol blocked not only the inhibitory action of the D2R like agonist quinpirole alone but also the enhancement by DOI and LSD of the inhibition of CRE-luciferase activity (Fig. 2A). The 5-HT2AR antagonist ketanserin (100 nM) only blocked the enhancement of $D2_LR$ receptor signaling produced by DOI and LSD but not the action of quinpirole (Fig. 2A).

Table 1
Effects of the hallucinogenic 5-HT2A agonists LSD and DOI, the standard 5-HT2A agonist TCB2 and 5-HT on the binding characteristics of D2like receptors in membrane preparations from the ventral striatum.^{3}H-raclopride binding saturation and ^{3}H-raclopride/DA competition experiments. Rat membrane preparations from the ventral striatum were incubated with increasing concentrations of [^{3}H]-raclopride for 60 min at 30 °C in the presence or absence of the hallucinogenic 5-HT2AR agonists LSD and DOI, a standard 5-HT2AR agonist TCB-2 and 5-HT as indicated. The 5-HT2AR antagonist ketanserin in the concentration indicated blocked the action of LSD and DOI. Nonspecific binding was defined as the binding in the presence of dopamine hydrochloride (1 mM). Nonlinear curve-fitting using GraphPad PRISM 5.0 determined maximal binding capacities (B_{max}) and dissociation constants (K_D). Data are presented as means ± S.E.M. of three independent experiments, each one performed in triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The P value 0.05 and lower was considered significant. $^{**}(P < 0.01)$: Significant difference compared with respective control B_{max} value; $^{\#}(p < 0.05)$ and $^{\&\&}(p < 0.01)$: Significant difference compared with LSD and DOI B_{max} value, respectively.

Receptors 5-HT2AR-D2R	[^{3}H]-raclopride binding saturation		[^{3}H]-raclopride competition with dopamine	
	*B*max (fmol/mg protein)	K_D (nM)	pK_{iH}	pK_{iL}
Control	182 ± 13	1.29 ± 0.31	−7.10 ± 0.18	−5.30 ± 0.16
5-HT (0.5 μM)	189 ± 12	1.36 ± 0.19	−7.36 ± 0.19	−5.50 ± 0.12
5-HT (0.5 μM) + Ketanserin (1 μM)	196 ± 14	1.57 ± 0.35	−7.22 ± 0.11	−5.17 ± 0.10
Control	179 ± 10	1.21 ± 0.23	−7.73 ± 0.18	−5.33 ± 0.11
DOI (50 nM)	235 ± 11**	1.54 ± 0.24	−8.31 ± 0.24*	−5.38 ± 0.14
DOI (50 nM) + Ketanserin (50 nM)	174 ± 10 &&	1.51 ± 0.26	−7.95 ± 0.11$^{\#}$	−5.32 ± 0.06
Control	192 ± 11	1.33 ± 0.26	−7.85 ± 0.25	−5.76 ± 0.13
LSD (10 nM)	258 ± 19**	1.74 ± 0.39	−8.50 ± 0.26**	−5.58 ± 0.12
LSD(10 nM) + Ketanserin (50 nM)	216 ± 10$^{\#}$	1.59 ± 0.21	−7.95 ± 0.15$^{\#}$	−5.66 ± 0.17
Control	190 ± 12	1.37 ± 0.30	−7.56 ± 0.16	−5.56 ± 0.10
TCB-2 (10 nM)	194 ± 11	1.53 ± 0.27	−7.41 ± 0.15	−5.50 ± 0.11
TCB-2 (10 nM) + Ketanserin (50 nM)	194 ± 12	1.27 ± 0.28	−7.24 ± 0.14	−5.38 ± 0.12

Competition experiments with the dopamine D2-like receptor antagonist [^{3}H] raclopride (2–3 nM) versus increasing concentrations of dopamine in ventral striatum were also performed in the same four groups as above. Means ± S.E.M are given for the negative logarithm of the K_i values (K_i is the inhibition constant) of the high affinity DA binding (pK_{iH}) and low affinity DA binding (pK_{iL}) sites from three independent experiments, each one performed in triplicate. The higher the value the higher the affinity of DA. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The P value 0.05 and lower was considered significant. $^{*}(P < 0.05)$ and $^{**}(P < 0.01)$: Significantly different compared to the respective control pK_{iH} value; $^{\#}(P < 0.05)$: Significantly different compared to the LSD pK_{iH} value and the DOI pK_{iH} value, respectively.

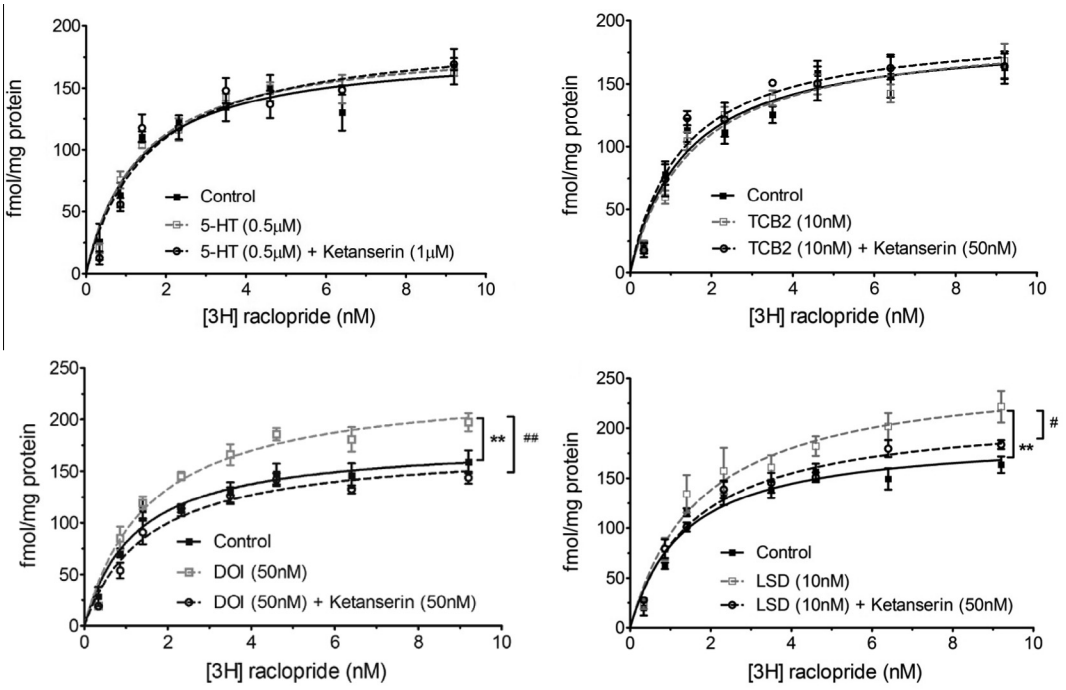

Fig. 3. Effects of LSD, DOI, TCB2 and 5-HT on D2R [^{3}H]-raclopride binding in saturation experiments in ventral striatal membrane preparations. Ventral striatal rat membrane preparations were incubated with increasing concentrations of [^{3}H]-raclopride for 60 min at 30 °C in the presence or absence of the two hallucinogenic 5-HT2AR agonists LSD and DOI, the standard 5-HT2A receptor agonist TCB2 and 5-HT as indicated. Nonspecific binding was defined as the binding in the presence of dopamine hydrochloride (1 mM). Maximal binding capacities (B_{max}) and dissociation constants (K_D) were determined fitting a nonlinear curve using GraphPad PRISM 5.0 software. Data are presented as means ± S.E.M of three independent experiments, each one performed in triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The P value 0.05 and lower was considered significant. $^{**}(P < 0.01)$: Significant difference in B_{max} values compared to respective control group. $^{\#}(P < 0.05)$: Significantly different compared to LSD B_{max} value. $^{\#\#}(P < 0.01)$: Significantly different compared to DOI B_{max} value.

3.3. ^{3}H-raclopride binding experiments

The modulation by DOI, LSD and TCB2 of the [^{3}H]-raclopride binding characteristics was studied in saturation and competition experiments in ventral striatal membranes and in membranes from cotransfected HEK293 cells. Both DOI and LSD but not TCB-2 in equivalent nanomolar concentrations significantly increased the B_{max} values of [^{3}H]-raclopride binding sites in several

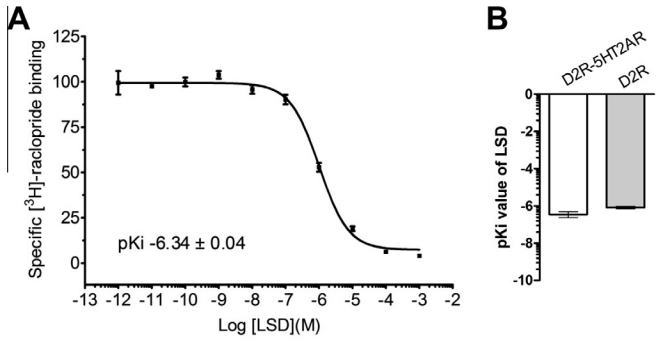

Fig. 4. Displacement of D2-likeR antagonist [^{3}H]-raclopride binding by LSD. Competition experiments with the dopamine D2-like receptor antagonist [^{3}H] raclopride (2–3 nM) versus increasing concentrations of LSD: (A) in ventral striatal and (B): HEK cell membrane preparations from transiently cotransfected 5-HT2AR-D2R cells or singly transfected D2R cells. Nonspecific binding was defined as the binding in the presence of dopamine hydrochloride (1 mM). Data are presented as means ± S.E.M of three independent experiments, each one performed in triplicate. The negative logarithm of the K_i value is given (pK_i).

concentrations without affecting the K_D values in ventral striatal membranes (Table 1; Fig. 3 and Supplementary Table S1). The effects were blocked by the 5-HT2AR antagonist ketanserin (50 nM). In [^{3}H]-raclopride/DA competition experiments DOI and LSD but not TCB-2 in concentrations from 10 to 100 nM) produced a significant reduction in the pK_{iH} values for high affinity D2R agonist binding sites but not in their pK_{iL} values (Table 1, Supplementary Table S2). These effects were all blocked by ketanserin treatment. The binding results obtained in ventral striatal membranes were validated in cotransfected but not in singly transfected $D2_LR$ HEK293 cells in both saturation and competition experiments (Supplementary Tables S3 and S4). Only in higher concentrations, with K_i values in the range of 500–1000 nM, did the LSD directly effect the orthosteric site of the D2R protomer as seen by the displacement of [^{3}H]-raclopride in competition experiments in ventral striatal and in HEK293 membrane preparations (Fig. 4).

4. Discussion

Experimental evidence was obtained that the hallucinogenic 5-HT2AR agonists LSD and DOI but not the standard 5-HT2AR agonist TCB2 in the nanomolar range produced an enhancement of the D2R agonist induced D2R protomer recognition and signaling. In contrast, the endogenous ligand 5-HT was previously shown to exert an allosteric antagonistic action on D2R signalling in D2LR-5-HT2AR cotransfected HEK293 cells [1]. TCB2 in the range of 10–100 nM which can fully activate the 5-HT2A signaling pathways [19] lacked the ability to modulate the D2R protomer recognition and signaling in ^{3}H-raclopride binding and CRE reporter gene assays, respectively. Therefore, the enhancement of D2R signaling over the AC-PKA-CRE pathway produced by LSD and DOI cannot be produced by crosstalk in the signaling cascades of the D2 and 5-HT2A receptors. The LSD and DOI enhancement of the agonist-induced D2R protomer signalling observed and blocked by ketanserin is instead mediated by a biased agonist action of DOI and LSD at the orthosteric site of the 5-HT2AR protomer. This leads to an allosteric facilitatory receptor–receptor interaction in the D2-5-HT2A heteroreceptor complex enhancing the D2 protomer signaling over Gi/o.

This allosteric mechanism probably also underlies the ability of DOI and LSD to increase the density of the D2like receptors in membranes from D2R and 5-HT2AR cotransfected HEK cells and from the ventral striatum, an action blocked by ketanserin. Thus, there may exist cryptic D2R protomers of the D2-5-HT2A heteroreceptor complex in the membranes which cannot bind the D2like receptor antagonist. However, after the biased agonist action of DOI and LSD at the 5-HT2AR protomer an allosteric change develops in the cryptic D2R protomers and their orthosteric binding sites become available to binding by ^{3}H-raclopride. In the ^{3}H-raclopride/DA competition experiments LSD and DOI but not TCB2 also significantly enhanced the affinity of the high affinity but not of the low affinity D2R agonist binding sites which was blocked by ketanserin. Thus, the biased 5-HT2AR actions of LSD and DOI at the 5-HT2AR leads both to increased D2R density and increased D2 agonist affinity at the high affinity agonist binding site.

Taken together, the hallucinogenic 5-HT2AR agonists LSD and DOI induce pathological facilitatory allosteric receptor–receptor interactions enhancing D2R protomer recognition and signaling in D2-5-HT2A heteroreceptor complexes demonstrated in discrete regions of the nucleus accumbens core and the dorsal striatum. Thus, the psychotic actions of the 5-HT2AR hallucinogens [20] can involve enhancement of D2R protomer signaling in the D2-5-HT2A heteroreceptor complex in the ventral striatum. This gives also a novel understanding of the molecular mechanism for antipsychotic actions of atypical antipsychotic drugs. Thus, risperidone and clozapine are inter alia characterized by their higher affinity for 5-HT2A than for D2 receptors resulting in their higher potency to block 5-HT2AR than D2Rs [20–22]. One advantage of many atypical antipsychotics with such properties may be that they can counteract the D2 receptor signaling at low doses in the D2-5-HT2A heteroreceptor complex through their combined blockade of the D2 and 5-HT2A protomers. This may lead to antipsychotic effects against positive symptoms of schizophrenia in doses that will not fully block several other D2 receptor populations in the CNS which can be involved in producing cognitive and extrapyramidal side-effects of antipychotics [21,23].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.104.

References

[1] D.O. Borroto-Escuela, W. Romero-Fernandez, A.O. Tarakanov, D. Marcellino, F. Ciruela, L.F. Agnati, K. Fuxe, Dopamine D2 and 5-hydroxytryptamine 5-HT((2)A) receptors assemble into functionally interacting heteromers, Biochem. Biophys. Res. Commun. 401 (2010) 605–610.
[2] S. Lukasiewicz, A. Polit, S. Kedracka-Krok, K. Wedzony, M. Mackowiak, M. Dziedzicka-Wasylewska, Hetero-dimerization of serotonin 5-HT(2A) and dopamine D(2) receptors, Biochim. Biophys. Acta 2010 (1803) 1347–1358.
[3] A. Carlsson, M.L. Carlsson, Adaptive properties and heterogeneity of dopamine D(2) receptors – pharmacological implications, Brain Res. Rev. 58 (2008) 374–378.
[4] L. Albizu, T. Holloway, J. Gonzalez-Maeso, S.C. Sealfon, Functional crosstalk and heteromerization of serotonin 5-HT2A and dopamine D2 receptors, Neuropharmacology 61 (2011) 770–777.
[5] R.A. Glennon, M. Titeler, M.R. Seggel, R.A. Lyon, N-methyl derivatives of the 5-HT2 agonist 1-(4-bromo-2,5-dimethoxyphenyl)-2-aminopropane, J. Med. Chem. 30 (1987) 930–932.
[6] M. Titeler, R.A. Lyon, R.A. Glennon, Radioligand binding evidence implicates the brain 5-HT2 receptor as a site of action for LSD and phenylisopropylamine hallucinogens, Psychopharmacology 94 (1988) 213–216.

[7] N.E. Anden, H. Corrodi, K. Fuxe, T. Hokfelt, Evidence for a central 5-hydroxytryptamine receptor stimulation by lysergic acid diethylamide, Br. J. Pharmacol. 34 (1968) 1–7.

[8] M.A. Fox, H.T. French, J.L. Laporte, A.R. Blackler, D.L. Murphy, The serotonin 5-HT(2A) receptor agonist TCB-2: a behavioral and neurophysiological analysis, Psychopharmacology 212 (2010) 13–23.

[9] D.O. Borroto-Escuela, W. Romero-Fernandez, P. Garriga, F. Ciruela, M. Narvaez, A.O. Tarakanov, M. Palkovits, L.F. Agnati, K. Fuxe, G protein-coupled receptor heterodimerization in the brain, Methods Enzymol. 521 (2013) 281–294.

[10] D.O. Borroto-Escuela, K. Van Craenenbroeck, W. Romero-Fernandez, D. Guidolin, A.S. Woods, A. Rivera, G. Haegeman, L.F. Agnati, A.O. Tarakanov, K. Fuxe, Dopamine D2 and D4 receptor heteromerization and its allosteric receptor–receptor interactions, Biochem. Biophys. Res. Commun. 404 (2011) 928–934.

[11] P. Trifilieff, M.L. Rives, E. Urizar, R.A. Piskorowski, H.D. Vishwasrao, J. Castrillon, C. Schmauss, M. Slattman, M. Gullberg, J.A. Javitch, Detection of antigen interactions ex vivo by proximity ligation assay: endogenous dopamine D2-adenosine A2A receptor complexes in the striatum, Biotechniques 51 (2011) 111–118.

[12] D.O. Borroto-Escuela, W. Romero-Fernandez, G. Mudo, M. Perez-Alea, F. Ciruela, A.O. Tarakanov, M. Narvaez, V. Di Liberto, L.F. Agnati, N. Belluardo, K. Fuxe, Fibroblast growth factor receptor 1–5-hydroxytryptamine 1A heteroreceptor complexes and their enhancement of hippocampal plasticity, Biol. Psychiatry 71 (2012) 84–91.

[13] S.J. Hill, J.G. Baker, S. Rees, Reporter-gene systems for the study of G-protein-coupled receptors, Curr. Opin. Pharmacol. 1 (2001) 526–532.

[14] S.J. Hill, C. Williams, L.T. May, Insights into GPCR pharmacology from the measurement of changes in intracellular cyclic AMP; advantages and pitfalls of differing methodologies, Br. J. Pharmacol. 161 (2010) 1266–1275.

[15] M. Torvinen, L.B. Kozell, K.A. Neve, L.F. Agnati, K. Fuxe, Biochemical identification of the dopamine D2 receptor domains interacting with the adenosine A2A receptor, J. Mol. Neurosci. 24 (2004) 173–180.

[16] S. Dasgupta, S. Ferre, B. Kull, P.B. Hedlund, U.B. Finnman, S. Ahlberg, E. Arenas, B.B. Fredholm, K. Fuxe, Adenosine A2A receptors modulate the binding characteristics of dopamine D2 receptors in stably cotransfected fibroblast cells, Eur. J. Pharmacol. 316 (1996) 325–331.

[17] A. Jansson, B. Tinner, M. Bancila, D. Verge, H.W. Steinbusch, L.F. Agnati, K. Fuxe, Relationships of 5-hydroxytryptamine immunoreactive terminal-like varicosities to 5-hydroxytryptamine-2A receptor-immunoreactive neuronal processes in the rat forebrain, J. Chem. Neuroanat. 22 (2001) 185–203.

[18] R.L. Jakab, P.S. Goldman-Rakic, 5-Hydroxytryptamine2A serotonin receptors in the primate cerebral cortex: possible site of action of hallucinogenic and antipsychotic drugs in pyramidal cell apical dendrites, Proc. Natl. Acad Sci. U.S.A. 95 (1998) 735–740.

[19] T.H. Mclean, J.C. Parrish, M.R. Braden, D. Marona-Lewicka, A. Gallardo-Godoy, D.E. Nichols, 1-Aminomethylbenzocycloalkanes: conformationally restricted hallucinogenic phenethylamine analogues as functionally selective 5-HT2A receptor agonists, J. Med. Chem. 49 (2006) 5794–5803.

[20] F.C. Colpaert, Discovering risperidone: the LSD model of psychopathology, Nat. Rev. Drug Discovery 2 (2003) 315–320.

[21] S. Miyamoto, G.E. Duncan, C.E. Marx, J.A. Lieberman, Treatments for schizophrenia: a critical review of pharmacology and mechanisms of action of antipsychotic drugs, Mol. Psychiatry 10 (2005) 79–104.

[22] H.Y. Meltzer, S. Matsubara, J.C. Lee, Classification of typical and atypical antipsychotic drugs on the basis of dopamine D-1, D-2 and serotonin2 pK_i values, J. Pharmacol. Exp. Ther. 251 (1989) 238–246.

[23] H.Y. Meltzer, S.M. Stahl, The dopamine hypothesis of schizophrenia: a review, Schizophr. Bull. 2 (1976) 19–76.