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Hetero-dimerization of serotonin 5-HT_{2A} and dopamine D_2 receptors

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ABSTRACT

In the present study, detailed information is presented on the hetero-dimerization of the serotonin 5-HT_{2A} receptor and the dopamine D_2 receptor. Biophysical approaches (fluorescence spectroscopy as well as fluorescence lifetime microscopy) were used to determine the degree of fluorescence resonance energy transfer (FRET) between cyan and yellow fluorescent protein labeled receptor variants co-expressed in human embryonic kidney 293 cells (HEK293). Recorded data demonstrate the existence of energy transfer between the wild-type forms of 5-HT_{2A}R and D_2R, pointing toward the formation of hetero-5-HT_{2A}R/D_2R dimers and homo-5-HT_{2A}R/5-HT_{2A}R dimers. Moreover, the present study investigates the role of specific motifs (one containing adjacent arginine residues (217RRRRKR222) in the third intracellular loop (ic3) of D_2R, and the other consisting of acidic glutamate residues (454EE455) in the C-tail of (5-HT_{2A}R) in the formation of noncovalent complexes between these receptors. Our results suggest that these regions of 5-HT_{2A}R and D_2R may be involved in the interaction between these two proteins. On the other hand, the above-mentioned motifs do not play an important role in the homo-dimerization of these receptors. Furthermore, we estimated the influence of specific receptor ligands on the dimerization processes. Agonists (DOI and quinpirole) and antagonists (ketanserin and butaclamol) cause different effects on FRET efficiency depending on whether homo- or hetero-complexes are present. These data may have therapeutic implications, since (using the immunofluorescence double labeling protocols) the co-localization of these two receptors was demonstrated in the medial prefrontal cortex and pars reticulate of the substantia nigra of the rat brain.

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1. Introduction

In recent years, various evidence points to the existence of both homo-dimers, hetero-dimers or higher-order oligomers of G protein-coupled receptors (GPCRs, also known as heptahelical receptors) on the plasma membrane [1–4]. The capacity of these plasma membrane receptors to interact with other GPCRs superfamily members is important for their function and biogenesis [5–8]. The association of these proteins plays a key role in processes such as receptor maturation, protein folding, cell surface delivery [9–13] and G protein binding [14–18] as well as receptor activation and desensitization [11,19–21]. Moreover, oligomerization of GPCRs is essential because the formation of unique multi-protein complexes may lead to an alteration in signaling (interactions between different classes of GPCRs point to a new level of molecular cross-talk) and drug specificity [22–25]. Thus, understanding the structural mechanisms involved in receptor association and identification of the dimer interface is of fundamental importance.

Currently, data regarding when and how GPCRs dimerization occurs remains limited. Several studies suggest that ligand binding regulates dimer formation, either by promoting [26,27] or inhibiting [28] dimerization. On the other hand, many other studies have shown that homo- and hetero-dimerization are a constitutive phenomenon and may be initiated during protein synthesis in the endoplasmic reticulum [12,13,29,30]. The outcomes are equivocal probably because different methods are often applied to understanding protein–protein interactions.

The mechanism of intermolecular interactions involved in the formation of dimers between receptors is still unknown. The precise role of specific regions of receptor molecule(s) in the dimerization process has not yet been elucidated. However, several models have been described. Data indicate that covalent bonds (i.e., disulfide bonds

Abbreviations: CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; FRET, fluorescence resonance energy transfer; GPCRs, G protein coupled receptors; HEK 293, human embryonic kidney 293 cell line; ic3, third intracellular loop; TCSPC, time-correlated single photon counting measurements; TM, transmembrane domains of a receptor; 5-HT_{2A}R, 5-HT_{2A} receptors; D_2R, D_2 receptors; D_1R, D_1 receptors

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in the case of the m3 muscarinic receptor) are formed between the extracellular domains of the receptor [26,31,32]. Alternatively, interactions between the intracellular domain (i.e., a coiled-coil interaction within the C-tail of the gamma-aminobutyric acid $(GABA)_B$ receptor) are engaged in the process [33]. Interactions between the transmembrane domains (i.e., hydrophobic interactions detected for the dopamine D_2 receptors (D_2R)) are also suggested as a potential mechanism by which GPCRs associate with one another [17,34–39]. However, the interaction between transmembrane domains is not the only point of contact and a combination of these mechanisms likely plays a role in GPCRs oligomerization.

Moreover, electrostatic interactions are also considered as effectively engaged in the formation of complexes between proteins. Electrostatic interactions may occur between characteristic epitopes; one epitope containing mainly two or more adjacent arginine residues and the other epitope consisting of acidic (two or more adjacent aspartate or glutamate) residues or/and a phosphorylated residue [40–42]. In a previous study, we showed that an electrostatic interactions between an arginine-rich epitope from the third intracellular loop (ic3) of D_2R (217RRRRKR222) and two adjacent glutamate residues from the C-terminus of the dopamine D_1 receptor (D_1R, 404EE405) participate in hetero-dimerization between D_1R and D_2R [43]. Moreover, the role of arginine residues within the ic3 of D_2R in the formation of noncovalent complexes between other GPCRs (D_2R and the adenosine A_{2A} receptor and D_2R and the cannabinoid CB1 receptor) has been highlighted in previous studies [44,45]. A similar interaction has also been shown for hetero-dimers of D_1R and the ionotropic N-methyl-D-aspartic acid (NMDA) receptor [46,47].

The widespread biological action of the serotonin (5-hydroxytryptamine or 5-HT) and dopamine are mediated by two classes of membrane receptors, including the serotonin 5-HT_{2A} receptor (5-$HT_{2A}R$) and dopamine receptor (D_2R). Both 5-$HT_{2A}R$ and D_2R belong to the GPCRs superfamily and play essential roles in neurotransmission [48,49]. Although these proteins are representative of distinct classes of GPCRs, their interaction may be significant. Alterations in either serotonin or dopamine neurotransmission have been implicated in many human neurological and psychiatric disorders, including depression, anxiety and schizophrenia [50]. Both D_2R and 5-$HT_{2A}R$ are well-established targets of various pharmacological compounds used to treat some of these pathologies (in addition, second generation anti-psychotic agents have affinity for both of these receptors) [51]. Therefore, a detailed understanding of the dimerization processes is very important in terms of drug discovery, because it provides an opportunity to steer this process towards new and potentially more effective therapeutics.

Identifying whether a hetero-interaction between 5-$HT_{2A}R$ and D_2R exists is very important. A hetero-dimerization may alter the potency of ligands as well as therapeutic compounds. The existence of a hetero-interaction is especially promising since hetero-dimers can be formed only on neurons expressing both 5-$HT_{2A}R$ and D_2R. Therefore, a higher selectivity of the appropriate compounds may be achieved. Importantly, we determine the influence of 5-$HT_{2A}R$ and D_2R specific ligands (agonists and antagonists) on the dimerization process.

Measurement of fluorescence resonance energy transfer (FRET) phenomenon which occurs between fluorescently tagged receptors is considered a very useful tool [52–54], since it allows to study the physical interactions between receptor proteins either in a single cell or in population of living cells. FRET is observed between a fluorescent donor and an acceptor chromophore when they are located within 100 Å of each other and are arranged properly in terms of their transition dipole moments [55]. Here, we use FRET to monitor protein dimer formation between 5-$HT_{2A}R$ and D_2R. We labeled 5-$HT_{2A}R$ and D_2R with cyan fluorescent protein (CFP, the fluorescence donor) and yellow fluorescent protein (YFP, the fluorescence acceptor) and expressed the receptors in human embryonic kidney (HEK) 293 cells to address the question of whether D_2R and 5-$HT_{2A}R$ physically interact. Furthermore, in the present study we investigate the potential role of amino acid residues located in characteristic epitopes in the ic3 (217RRRRKR222) of D_2R and in the C-terminus (454EE455) of 5-$HT_{2A}R$ in the interaction and dimer formation of these receptors.

2. Materials and methods

2.1. Materials

All molecular biology reagents were obtained from Fermentas (Vilnius, Lithuania). Oligonucleotides were synthesized by IBB PAN (Warsaw, Poland). The pECFP-N1 and pEYFP-N1 vectors were purchased from BD Biosciences, Clontech (Palo Alto, CA, USA). The pcDNA3.1(+) plasmids encoding human 5-$HT_{2A}R$ and the human D_2R proteins were obtained from the UMR cDNA Resource Center (University of Missouri-Rolla, MO, USA). *Escherichia coli* DH5α (Dam+) was purchased from Novagen. HEK 293 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell culture materials were purchased from GIBCO and Sigma. The 5-$HT_{2A}R$ ligands were supplied by Hartmann Analitic (Braunschweig, Germany) — [^{3}H]ketanserin, Research Biochemicals International (Natick, MA. USA) — methysergide, Sigma — DOI ((±)-2,5-Dimethoxy-4-iodoamphetamine hydrochloride and butaclamol, and Tocris — quinpirole and ketanserin. Antibodies: monoclonal mouse anti-serotonin 5-HT_{2A} receptors (Pharmingen), rabbit polyclonal anti-dopamine D_2 receptors (Santa Cruz), Alexa 488-conjugated anti-mouse IgM (Molecular Probes), and Cy3-conjugated anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch).

2.2. Construction of fusion proteins

The human 5-$HT_{2A}R$ and dopamine D_2R genes cloned into the pcDNA3.1(+) plasmid were labeled with cDNA encoding enhanced CFP (ECFP) or enhanced YFP (EYFP) by cloning into the pECFP-N1 and pEYFP-N1 plasmids, respectively.

Briefly, the full-length cDNAs encoding 5-$HT_{2A}R$ and D_2R were PCR-amplified. The forward primer was a universal primer for pcDNA3.1(+) and the reverse primer removed the STOP codon and introduced a unique restriction site, *Xho*I, in both receptors. The resulting fragments were inserted, in-frame, into the *Nhe*I/*Xho*I sites of the pECFP-N1 and pEYFP-N1 vectors. The obtained fusion protein constructs were used after expression as the fluorescence donor (receptor-ECFP) or acceptor (receptor-EYFP). Henceforth, the ECFP and EYFP variants are called CFP and YFP, respectively.

2.3. Construction of genetic variants of the serotonin and dopamine receptors

Three genetic variants of D_2R-YFP in which six amino acid residues (two each) within the arginine-rich epitope (217RRRRKR222) of the ic3 were mutated (D_2R1-YFP: 217AARRKR222, D_2R2-YFP: 217AAAAKR222, and D_2R3-YFP: 217AAAAAA222) were obtained as described in our previous study [43]. We also constructed a genetic variant of 5-HT_{2A}-CFP in which the acidic epitope (454EE455) in the C-tail was mutated (5-HT_{2A}MUT-CFP: 454AA455). The appropriate point mutations were generated using a QuikChange II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol.

Briefly, the 5-$HT_{2A}R$ gene was inserted into the pECFP-N1 vector and was used as the template for the PCR-Quick reaction. Incorporating the oligonucleotide primers, each complementary to the opposite strand of the vector and containing the desired mutation, generated a mutated plasmid. The resulting product was treated with endonuclease *Dpn*I to digest methylated and hemimethylated DNA and select for the synthesized DNA containing the introduced mutations. *E.coli* DH5α cells were then transformed with the mutated plasmid. All mutated sequences were verified by DNA sequencing (IBB PAN Warsaw, Poland).

2.4. Cell culture and transfection

HEK 293 cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 1% L-glutamine and 10% heat-inactivated fetal bovine serum (FBS). The cells were cultured at 37 °C in an atmosphere of 5% CO_2. Transient transfections of HEK 293 cells were performed using the calcium phosphate precipitation method, as described by Sambrook et al. [56]. HEK 293 cells were transfected with plasmids encoding either CFP-tagged 5-HT_{2A}R, and 5-HT_{2A}RMUT or YFP-tagged 5-HT_{2A}R, D_2R, D_2R1, D_2R2, and D_2R3 fusion proteins separately or co-transfected with different combinations of both CFP- and YFP-tagged plasmids. One day before transfection, the cells were seeded into 100 mm dishes at a density of 3×10^6 cells/dish for fluorescence spectra measurements and binding assays. Cells were plated on glass coverslips in 30-mm dishes at a density of 1×10^6 cells/dish for fluorescence lifetime measurements and confocal imaging. The cells were transfected with 12 μg of DNA per 100 mm dish or 2 μg of DNA per 30-mm dish. The ratio of DNA encoding the donor to DNA encoding the acceptor was 1:1 to study homo-dimerization or 1:2 to study hetero-dimerization which was dependent on the expression level of the investigated receptors. Berney and Danseur [57] have shown that the most optimal to FRET measurements is usage donor/acceptor ratio 1:1 or 1:2.

2.5. Membrane preparation and radioligand binding assay

Ligand binding experiments for 5-HT_{2A}R were performed with and without fluorescently tagged proteins, as well as for the 5-HT_{2A} mutant (5-HT_{2A}MUT-CFP). Forty-eight hours after transfection, HEK 293 cells separately expressing the receptors were washed with phosphate-buffered saline (PBS), scraped from the dish in PBS, and centrifuged at $200\times g$ for 5 min. The obtained pellets were frozen at −20 °C until use.

Frozen pellets were resuspended in a 6 ml binding buffer (50 mM Tris-HCl, pH 7.7) using an Ultra Turrax homogenizer. The homogenates were centrifuged twice at $25{,}000\times g$ for 15 min at 4 °C. The obtained suspension was preincubated at 37 °C for 15 min and subsequently centrifuged at $25{,}000\times g$ for 10 min at 4 °C. Then, the pellet was resuspended in a 2 ml of the binding buffer and homogenized. [^{3}H]ketanserin (specific activity of 67 Ci/mmol) was used as the 5-HT_{2A}R-specific radioligand.

Binding assays were performed in a total volume of 400 μl. Saturation studies were carried out on a fresh membrane preparation (final protein concentration of 100 μg/tube) using concentrations of [^{3}H]ketanserin ranging from 0.03 to 4 nM. Nonspecific binding was assessed by the addition 50 μM methysergide. The tubes were incubated for 30 min at room temperature and binding was terminated by rapid filtration through glass fiber filters (GF/B, Whatman). The filters were washed three times with a 5 ml of ice-cold binding buffer and the amount of radioactivity bound to the filter was determined by liquid scintillation counting (WinSpectral-Wallac). Radioligand binding parameters, including the dissociation constant (K_d) and the maximum binding capacity (B_{max}), were estimated using the SigmaPlot 2001 version 7.0 program. Statistical analyses were performed using Student's *t*-test.

Binding experiments for D_2R and the genetic variants of D_2R were performed as previously described [43].

2.6. FRET measurements

Qualitative as well as quantitative methods were used to monitor whether FRET occurs between the CFP-tagged protein (donor) and the YFP-tagged protein (acceptor of fluorescence). We used both spectrofluorimetric measurements of cell suspensions (recorded by a Fluorolog 3-Horiba, Jobin Yvon S.A.S., Longjumeau, France) and measurements of FRET by fluorescence lifetime microscopy and time correlated single photon counting method (TCSPC) in a single living cell expressing the investigated receptors in different combinations (performed on a Nikon Eclipse TE-2000 inverted fluorescence microscope). Each measurement was conducted 48 h after transfection at 37 °C. For each receptor combination, at least four independent experiments were performed. During each TCSPC experiment, fluorescence decay from at least 15 cells on the coverslip was recorded. In order to investigate the influence of specific ligands, the cells were incubated in the presence of agonists and antagonists for 15 min at 37 °C before the measurement. Fluorescence emission profiles were obtained by subtracting signals derived from both mock transfected cells and incubated medium. This analysis was done according to Stanasilla et al. [58]. Fluorescence intensity decays were analyzed both as mono- and multi-exponentials. Analysis of the reduced χ^2 value and residual distribution led to the conclusion that the best fit parameters were obtained with two exponentials. Adding a third exponential did not significantly influence the parameters and the fractional contribution of the additional lifetime was close to zero. Statistical analyses were performed using Student's *t*-test. Detailed information of the methodology used, along with the various control experiments indicating the specificity of the observed effects have been described previously [43,52,59,60]. The use of three independent methods of FRET efficiency measurements (by fluorescence lifetime microscopy and confocal microscopy as well as spectrofluorimetric measurements) used in our laboratory warrants the reliability of the results [52,59]. Moreover studies of interactions of various GPCRs (serotonin 5-HT_{1A}, adenosine A_{2A}, dopamine D_1 and D_2) with alpha subunits of the G protein in the same transfection conditions convincingly showed that FRET can be measured specifically only when the given receptor is co-expressed with the appropriate (interacting) G protein (as for example dopamine D_2 and alpha-i, but not alpha-s subunit) [43,52].

Although energy-transfer based methods are very useful tools for studying protein–protein interactions, they have several limitations. The interpretation of FRET efficiency alterations as an indication of receptor oligomerization has been criticized. We are aware that the effects observed in these kinds of studies may be the result of conformational changes within the receptor molecule tagged with the fluorescent protein. We discussed this issue in detail in our previous paper [60]. In order to avoid misinterpretation of the data, control experiments were conducted. The donor–acceptor distance was calculated using the following equation:

$$\bar{r} = R_0\left[\left(E^{-1}-1\right)^{1/6}\right]. \tag{1}$$

The anticipated possible alteration in the energy transfer induced by the conformational change was estimated according to the following equation (Table 3) [60–62]:

$$\Delta E = \frac{6r^5}{\left[1+(r/R_0)^6\right]^2 R_0^6}\Delta r. \tag{2}$$

2.7. Confocal microscopy

Confocal microscopy was used to analyze the localization of the fluorescently tagged receptors in HEK 293 cells. Images were acquired using a BioRad MRC 1024 confocal system (BioRad) interfaced with a Nikon Diapoth 300 (Nikon) inverted microscope. The microscope was equipped with a 60x PlanApo oil-immersion 1.4 NA objective lens and a 100 mW argon ion air-cold laser (ITL). Detailed information has previously been provided [43].

Image ProPlus 4.5 software was used for the co-localization analysis. For a quantitative estimation of the degree of co-localization between two different proteins of interest, Pearson's correlation coefficients and coefficients of determination were estimated. Pearson's correlation coefficients were calculated from randomly

selected parts of the image (membrane signal) from individual cells co-transfected with different combinations of the constructs (wild-type or fluorescently tagged 5-HT_{2A}R and D_2R mutants). The average intensity of the fluorescence signal was measured for every image in a determined individual area of interest free of cells and subtracted as background. For analysis, these regions were used for which fluorescence intensities were correlated. For each combination of proteins, a minimum of 20 individual regions from different, independently transfected cells were counted. Statistical analyses were performed using Student's *t*-test. To make interpretations of the Pearson's correlation coefficients easier, coefficients of determination (which are squared values of the correlation coefficient) were estimated.

2.8. Immunohistochemical study

2.8.1. Tissue preparation

The rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and were transcardially perfused with a 0.9% NaCl, followed by a 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). After the post-fixation period of 24 h, 50 μm thick sections were cut at the level of the medial prefrontal cortex and susbstanti nigra using a Leica VT-1000 S vibratome.

2.8.2. Immunofluorescence staining

Free-floating sections from medial prefrontal cortex and substantia nigra were developed using immunofluorescence double-labeling protocols to determine the co-localization of serotonin 5-HT_{2A} and dopamine D_2 receptors. The sections were incubated in a blocking buffer (5% normal donkey serum and 0.3% Triton X-100 in 0.01 M PBS) for 1 h. Next, the sections were incubated (48 h at 4 °C) with primary antibodies: monoclonal mouse anti-serotonin 5-HT_{2A} receptors antibody (1:200) and rabbit polyclonal anti-dopamine D_2 receptors (1:100). The specificity of anti-D_2 receptors antibodies has been demonstrated by Park [63] and Kovoor [64].

Antibodies were diluted in 5% normal donkey serum and 0.3% Triton X-100 in 0.01 M PBS. The sections were then washed and incubated overnight (4 °C) in a mixture of secondary antibodies: Alexa 488-conjugated anti-mouse IgM (1:200) for serotonin 5-HT_{2A} receptors and Cy3-conjugated anti-mouse or anti-rabbit IgG (1:300) for dopamine D_2 receptors. Sections were then washed, mounted, and coverslipped. Slices were analyzed with a confocal laser scanning microscope, DMRXA2 TCS SP2 (Leica), using a 63×/1.4−0.7 oil objective (Leica) driven by a confocal software (Leica) using sequential scan settings. Working with argon and GreNe lasers, two laser lines emitting at 488 and 543 nm were used for exciting the Alexa 488- and Cy3-conjugated antibodies, respectively. The background noise of each confocal image was then reduced by averaging four scans/line and six frames/image. The pinhole value of one airy was used in order to obtain flat images (for further details [65,66]).

3. Results

3.1. Radioligand binding assay

We previously showed that the binding parameters obtained for D_2R and its mutants (D_2R1, D_2R2) displayed similar K_d values [43]. The D_2R3 (217AAAAAA222) variant localized within the cell; therefore, no binding parameters could be established for this mutant protein. Binding experiments (Fig. 1) conducted for wild-type 5-HT_{2A}R, as well as fluorescently labeled 5-HT_{2A}R indicate that the K_d values for these two receptors are similar (Table 1). These data show that the fluorescent tag does not cause changes in the ligand binding properties of these receptors. Moreover, similar binding parameters were obtained for CFP-tagged 5-HT_{2A}MUT (Table 1).

Additional binding assays were performed for cells co-expressing both 5-HT_{2A}R and D_2R3. Saturation analysis was performed with [^{3}H] spiperone for D_2R points to a negligible level of binding (data not shown). In this case, although D_2R3 seems to be present within the plasma membrane, it is not functional.

3.2. Analysis of the localization of 5-HT_{2A}R, D_2R and their genetic variant fusion proteins

Confocal microscopy was used to visualize and analyze the localization of 5-HT_{2A}R and D_2R as well as their genetic variants (5-HT_{2A}MUT, D_2R1, D_2R2, D_2R3) in HEK 293 cells. These experiments demonstrate the influence of the introduced mutations on the subcellular localization of the receptor proteins. Fig. 2A shows HEK 293 cells transiently co-transfected with 5-HT_{2A}R and 5-HT_{2A}MUT, as well as D_2R, D_2R1, D_2R2 and D_2R3, in different combinations. The green signal (CFP channel) results from receptors expressing CFP while the red signal (YFP channel) is from receptors tagged with YFP. Merged images (with apparent yellow signal indicating overlap of the green and the red signal) indicate co-localization. Additionally, Pearson's correlation

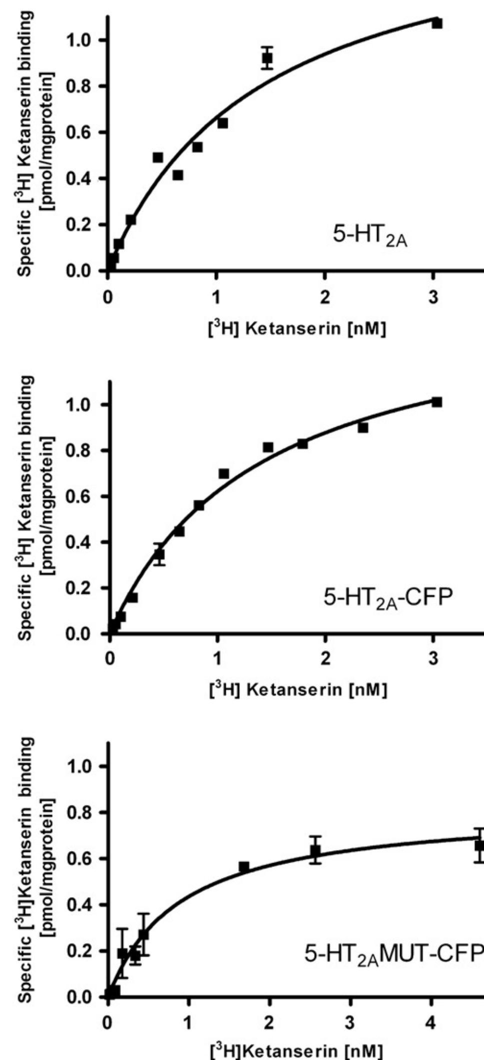


Fig. 1. Saturation binding of [^{3}H]Ketanserin to human 5-HT_{2A} receptors.Data are from a single experiment performed in duplicate and are representative of at least three independent experiments. Linking of the fluorescent tag as well as elimination of the acidic motif in 5-HT_{2A}R do not significantly alter the ligand binding constant.

Table 1
Binding parameters for the serotonin 5-HT_{2A} receptors.

Species	Bmax (pmol/mg protein)	Kd (nM)
5-HT_{2A}	1.61 (±0.23)	1.43 (±0.07)
5-HT_{2A}_CFP	1.10* (±0.16)	1.34 (±0.06)
5-HT_{2A} MUT_CFP	0.82* (±0.18)	0.95 (±0.24)

The standard errors of means are presented in parentheses, $n = 5$. The statistical significance was evaluated using Student t-test.
* $p < 0.05$ vs 5-HT_{2A}.

coefficients and coefficients of determination were estimated to determine the degree of co-localization of the receptors (Fig. 2B).

As seen in Fig. 2, 5-HT_{2A}R localized mainly in the cell membrane and introduction of mutations within the C-tail of the receptor did not change the subcellular distribution of 5-HT_{2A}R. In cells concomitantly expressing 5-HT_{2A}R and D_2R, these receptors not only localized to the plasma membrane, but were also found within the cell. Cells co-transfected with both wild-type and genetic variants of 5-HT_{2A}R (5-HT_{2A}MUT-CFP/5-HT_{2A}-YFP) as well as with 5-HT_{2A}R and one of the variants of D_2R (in the following combinations: 5-HT_{2A}-CFP/D_2-YFP, 5-HT_{2A}-CFP/D_2R1-YFP, 5-HT_{2A}-CFP/D_2R2-YFP, and 5-HT_{2A}-MUTCFP/D_2-YFP), the obtained values of both Pearson's correlation coefficients and coefficients of determination remained approximated. On the other hand, cells co-expressing 5-HT_{2A}R and the D_2R3 mutant displayed a decrease in the degree of co-localization, which correlated with the replacement of all amino acid residues within the ic3 (217–222) of D_2R.

3.3. Fluorescence spectroscopy measurements of 5-HT_{2A}R and D_2R dimerization

Steady state fluorescence spectroscopy measurements in cell suspension allow for a quick estimation of whether there is any energy transfer in the examined sample. However, this is only a qualitative approach, but at the same time is very demonstrative. Therefore, we used this type of measurement to investigate the interaction between 5-HT_{2A}R and D_2R and their genetic variants. Fig. 3 shows the fluorescence emission spectra for HEK 293 cell suspensions co-expressing the fusion proteins in different combinations (5-HT_{2A}-CFP/D_2-YFP, 5-HT_{2A}-CFP/D_2R1-YFP, 5-HT_{2A}-CFP/D_2R2-YFP, 5-HT_{2A}-CFP/D_2R3-YFP, 5-HT_{2A}MUT-CFP/D_2-YFP, 5-HT_{2A}-CFP/5-HT_{2A}-YFP and 5-HT_{2A}MUT-CFP/5-HT_{2A}-YFP) after excitation at 434 nm (donor absorption).

Results presented in Fig. 3A show representative emission spectra of the HEK 293 cell populations co-expressing 5-HT_{2A}R and D_2R fusion proteins (5-HT_{2A}-CFP and D_2-YFP) in comparison to the emission spectra of cell populations co-expressing the 5-HT_{2A}R fusion protein (5-HT_{2A}-CFP) and one of the genetic variants of the D_2R fusion protein (D_2R1, D_2R2 or D_2R3-YFP). Fig. 3B shows the fluorescence emission

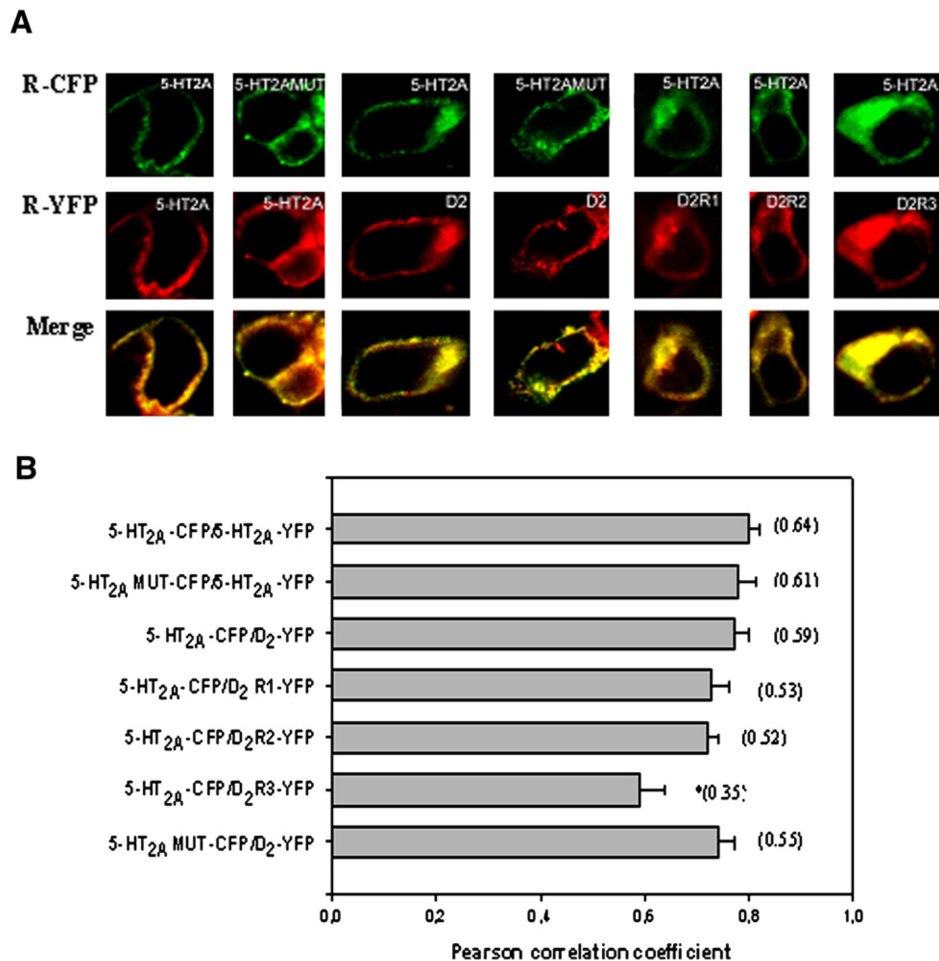
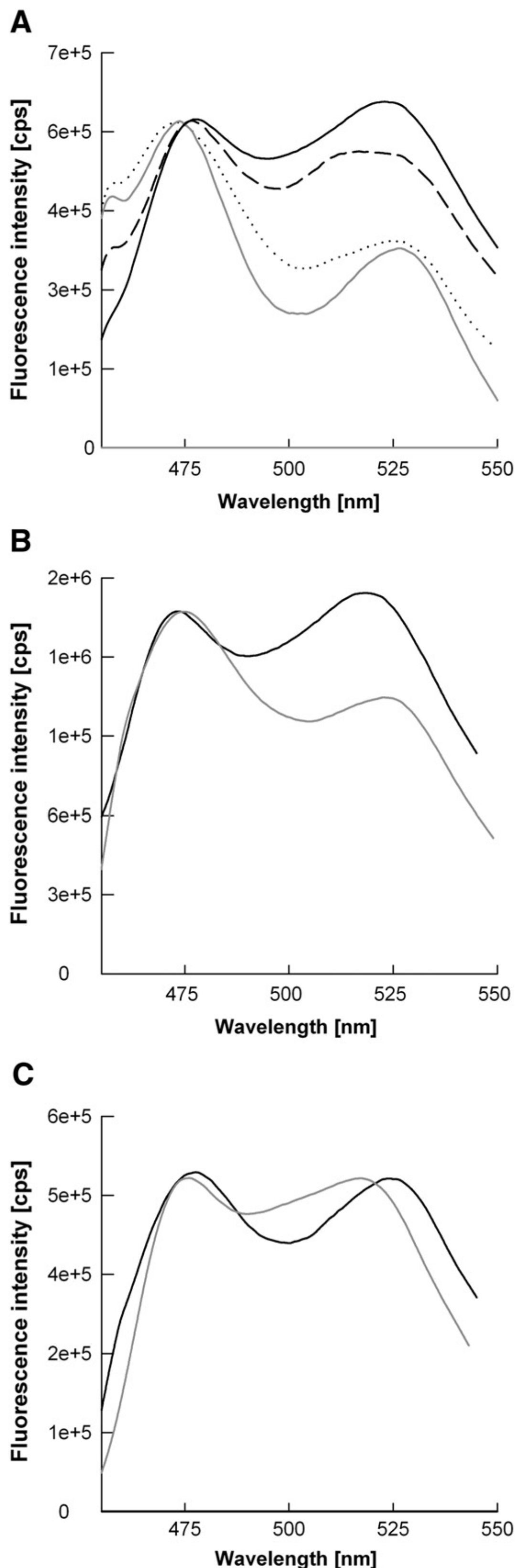


Fig. 2. Expression of 5-HT_{2A}R and D_2R and their mutants in HEK 293 cells.(A) HEK 293 cells were co-transfected with either 5-HT_{2A}-CFP or 5-HT_{2A}MUT-CFP and either D_2-YFP, D_2R1-YFP, D_2R2-YFP, D_2R3-YFP or 5-HT_{2A}-YFP (green and red). Image overlays show extensive co-localization in 5-HT_{2A}/5-HT_{2A}, 5-HT_{2A}MUT/5-HT_{2A}, 5-HT_{2A}/D_2, 5-HT_{2A}MUT/D_2 and 5-HT_{2A}/D_2R1, 5-HT_{2A}/D_2R2 assays and partial co-localization in 5-HT_{2A}/D_2R3 assays. (B) Bar graph of Pearson's correlation coefficient calculated for HEK 293 cells co-transfected with different serotonin 5-HT_{2A} and dopamine D_2 receptor protein construct combination. Data are mean ± SE, and statistical significance was evaluated using Student's t-test. *$p < 0.05$ $n = 20$ for combinations 5-HT_{2A} with D_2R3 variant vs 5-HT_{2A}/D_2. Either 5-HT_{2A}MUT/D_2 and 5-HT_{2A}/D_2R1, 5-HT_{2A}/D_2R2 vs 5-HT_{2A}/D_2 or 5-HT_{2A}MUT/5-HT_{2A} vs 5-HT_{2A}/5-HT_{2A} combinations are not statistically significant. Values of corresponding coefficients of determination (r^2) are reported in brackets.

profiles of cells co-transfected with the genetic variant of 5-HT_{2A}R (5-HT_{2A}MUT-CFP) and D_2R (D_2-YFP) in relation to the spectra obtained for cells co-expressing the wild-type forms of these receptors.



Energy transfer (judged by the localization of the appropriate peaks of the spectra) occurred in each combination tested. However, when either the genetic variant of 5-HT_{2A}R (5-HT_{2A}MUT) or the D_2R3 genetic variant of D_2R was present in the sample, the level of FRET was lower.

Efficient energy transfer was observed in spectra obtained from HEK 293 cells expressing 5-HT_{2A}R tagged with either CFP or YFP (Fig. 3C). Efficient energy transfer was also obtained from cells expressing the 5-HT_{2A}R mutant (5-HT_{2A}MUT-CFP) instead of the wild-type 5-HT_{2A}R.

Fluorescence emission profiles derived from a mixture of two HEK 293 cell populations (one transfected with only the plasmid encoding the receptor-CFP fusion protein and the second transfected with the plasmid encoding the receptor-YFP fusion protein) were obtained as a negative control for this experiment. Although both fluorophores were present in the sample, no visible energy transfer after excitation at 434 nm was observed. The shape of the recorded spectra was the same as in HEK 293 cells expressing the receptors separately (data not shown).

3.4. Fluorescence lifetime microscopy studies of serotonin 5-HT_{2A} and dopamine D_2 receptor dimerization

Fluorescence lifetime microscopy measurements exploiting the time-correlated single photon counting (TCSPC) technique provide highly quantifiable data because it is independent of any change in the fluorophore concentration or excitation intensity. Moreover, FRET can be observed in a single living cell. In the present study, the cell is transiently transfected with 5-HT_{2A}R and D_2R, as well as their genetic variants, tagged with fluorescent proteins in different combinations.

To estimate FRET efficiency, precise measurement of the donor fluorescence (CFP) lifetime in the presence and absence of the acceptor (YFP) is required. The typical time-dependent donor intensity decays for 5-HT_{2A}R-CFP bearing donor alone and with both the donor and the acceptor, 5-HT_{2A}R-CFP/D_2R-YFP, are presented in Fig. 4. The value of the average donor fluorescence lifetime changed when the acceptor was additionally present in the cell. The greatest average fluorescence lifetime decrease, regarded as the highest FRET efficiency (approximately 36%), was detected by this approach in our previous studies using the CFP–YFP hybrid (CFP connected by a short 15 amino acid linker with YFP) [52,59]. Additionally, some control experiments were conducted. We measured the fluorescence lifetime of CFP expressed in the cells alone or together with YFP — when cells were loaded with both fluorescent proteins, not linked to any receptors, no FRET was observed, despite their high expression and localization in the cytoplasm (Table 2).

The average CFP fluorescence lifetime estimated in cells expressing 5-HT_{2A}R-CFP or its genetic variant 5-HT_{2A}MUT-CFP was 2.31 ns and 2.33 ns, respectively. When both fluorophores (receptor-CFP, receptor-YFP) were present in the cell, a shortening of the average donor lifetime was observed. The value of the energy transfer efficiency was about 9.0% (average donor fluorescence lifetime of 2.10 ns) when measurements were performed in cells co-expressing 5-HT_{2A}R-CFP and D_2R-YFP fusion proteins. Replacing D_2R with genetic variants (D_2R1, D_2R2 or D_2R3-YFP, respectively) resulted in a change in the value of the energy transfer efficiency. For the combination of 5-HT_{2A}R and D_2R1, the energy transfer efficiency was equal to 4.3% (2.21 ns)

Fig. 3. Fluorescence emission spectra of HEK 293 cells expressing the CFP- and YFP-tagged proteins coupled to 5-HT_{2A}R and D_2R and their mutants.(A) Co-transfection of HEK 293 with 5-HT_{2A}-CFP and either D_2-YFP (*black solid line*), D_2R1-YFP (*black dashed line*), or D_2R2-YFP (*black dotted line*) or D_2R3-YFP (*dark grey solid line*). (B) Co-transfection of HEK 293 with 5-HT_{2A}MUT-CFP and D_2-YFP (*dark grey solid line*) in comparison with 5-HT_{2A}-CFP and D_2-YFP (*black solid line*). (C) Co-transfection of HEK 293 with 5-HT_{2A}MUT-CFP and 5-HT_{2A}-YFP (*dark grey solid line*) in comparison with 5-HT_{2A}-CFP and 5-HT_{2A}-YFP (*black solid line*). CFP was excited at 434 nm, and fluorescence was detected at 450–550 nm through a double monochromator. The spectral contributions arising from light scattering and non-specific fluorescence of cells and buffer were eliminated.

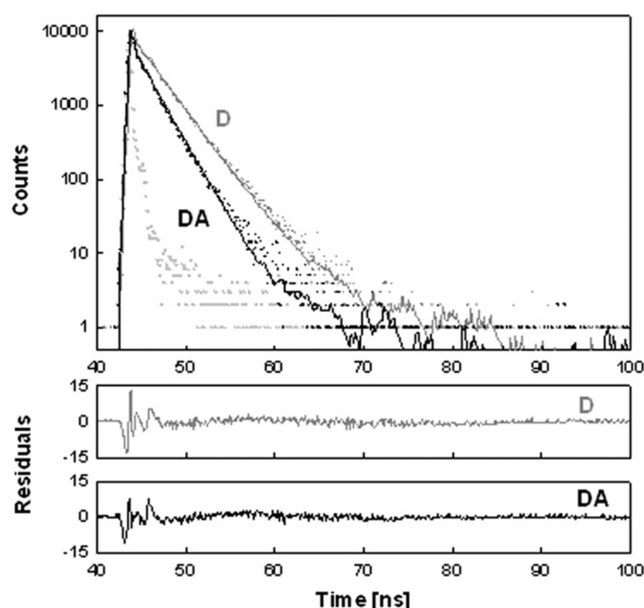


Fig. 4. Time-dependent fluorescence intensity decays of CFP attached to the 5-HT_{2A} receptor with and without YFP attached to the D_2 receptor.The dark grey dotted curve shows the intensity decay of the donor alone (D), and black dotted curve shows the intensity decay of the donor in the presence of acceptor (DA). The dark grey (D) and black (DA) solid lines and weighted residuals (lower panels) are for the best double exponential fits. The grey crosshair curve represents the excitation pulse diode laser profile, set up at 434 nm.

and a further decreased to 3.5% (2.23 ns) was observed for the 5-HT_{2A}R and D_2R2 combination. The energy transfer efficiency was 2.2% (2.26 ns) for the combination of 5-HT_{2A}R and D_2R3. Similarly, when the 5-HT_{2A}R genetic variant (5-HT_{2A}MUT) was used instead of the wild-type 5-HT_{2A}R (specifically, the combination of 5-HT_{2A}MUT and D_2R), a 2.6% (2.27 ns) decrease in the energy transfer efficiency was observed.

Measurement of the cells co-expressing the same type of receptor (5-HT_{2A}R) tagged with both fluorescent proteins (CFP and YFP) indicates a more than 10% energy transfer efficiency with an average lifetime 2.07 ns. Moreover, the replacement of 5-HT_{2A}R with 5-HT_{2A}MUT did not significantly change the value of the energy transfer (10.3%, 2.09 ns).

A summary of the TCSPC results is presented in Table 2. The error of the average fluorescence lifetime is the standard error of the mean obtained from different cells and independent transfections (standard deviations derived from fitting the individual fluorescence decay were ignored because they were very small).

3.5. The influence of specific ligands on dimerization processes — fluorescence lifetime microscopy studies

Next, we examined the effect of typical agonists (DOI and quinpirol) and antagonists (ketanserin and butaclamol) on dimerization processes of 5-HT_{2A}R and D_2R. FRET efficiency values estimated for each receptor combination changed after the addition of the selective ligands to the incubation medium. In the case of homo-complexes of 5-HT_{2A}R, DOI caused an increase in the energy transfer efficiency to the level of 12% (2.03 ± 0.02 ns). When the antagonist ketanserin was added, a decrease in the basal transfer efficiency value to 4.3% (2.21 ± 0.03 ns) was observed. In the 5-HT_{2A}R and D_2R combinations, both the selective 5-HT_{2A}R and the selective D_2R agonists (DOI and quinpirol, respectively) caused a decrease in the FRET value (6.5%, (2.16 ± 0.01 ns) and 6% (2.17 ± 0.02 ns), respectively). Meanwhile, the selective antagonists (ketanserin and butaclamol) increased the transfer efficiency value (11.6% (2.04 ± 0.02 ns) and 11.3% (2.05 ± 0.02 ns), respectively; Fig. 5).

Additional control experiments were conducted to prove that the observed alterations in the FRET efficiency were not the result of conformational changes (within the receptors) as a consequence of ligand binding. The distance of the donor–acceptor pair (r) for the investigated receptor combinations, as well as possible alterations in energy transfer (ΔE) due to the above-mentioned effect, was estimated (Table 3). Moreover, the average donor fluorescence lifetime for cells expressing only CFP-tagged receptors in the presence of the specific ligands used in this study were recorded. Estimated values were from 2.29 ± 0.03 to 2.31 ± 0.01 ns. The obtained data indicate that the observed alterations in the FRET efficiency are not due to the reorientation of the fluorescence donor or differences of its quantum yield.

3.6. Immunohistochemical study

In the medial prefrontal cortex serotonin 5-HT_{2A} receptors have been observed in neurons which by size and shape should be defined as a pyramidal one. 5-HT_{2A} serotonin receptors were also observed in dendritic shafts (Fig. 6A). Dopamine D_2 receptor proteins have been observed mainly in the cell bodies, however rare dopamine positive dendritic processes were observed (Fig. 6A). Different pattern of labeling has been observed in pars reticulate of the substantia nigra (Fig. 6B). Both antigens stain the periphery of the neuronal cell bodies, dendrites emanating or not related to the cell bodies of neurons, which by size and shape might be considered dopaminergic ones.

Table 2
Summary of energy transfer measurements by fluorescence lifetime microscopy in HEK 293 cells.

Species	Average lifetime [ns]		Transfer efficiency
	$\langle\tau_D\rangle$	$\langle\tau_{DA}\rangle$	$\langle E\rangle$ (%)
[a]CFP	2.36 (±0.02)		
[b]CFP + YFP	2.35 (±0.02)		
[c]CFP-YFP		1.52 (±0.03)	35.8 (±1.38)
[d]5-HT_{2A}_CFP	2.31 (±0.01)		
[e]5-HT_{2A}_CFP/D_2_YFP		**2.10 (±0.01)**	**9.09 (±0.59)**
[f]5-HT_{2A}_CFP/D_2R1_YFP		2.21 (±0.02)*	4.33 (±0.96)
[g]5-HT_{2A}_CFP/D_2R2_YFP		2.23 (±0.01)*	3.46 (±0.60)
[h]5-HT_{2A}_CFP/D_2R3_YFP		2.26 (±0.01)*	2.16 (±0.61)
[i]5-HT_{2A} MUT_CFP	2.33 (±0.02)		
[j]5-HT_{2A} MUT_CFP/D_2_YFP		2.27 (±0.02)*	2.58 (±1.20)
[k]5-HT_{2A}_CFP/5-HT_{2A}_YFP		**2.07 (±0.01)**	**10.39 (±0.88)**
[l]5-HT_{2A} MUT_CFP/5-HT_{2A}_YFP		2.09 (±0.01)	10.30 (±0.88)

Excitation was set up at 434 nm, and emission was observed through the appropriate interference filters, as described in the Experimental Procedures. The standard errors of means are presented in parentheses. Statistical significance was evaluated using Student t-test; $n = 53$–62.

[a] Measured in cell expressing CFP not linked to any receptors.
[b] Measured in cell co-expressing CFP and YFP not linked to any receptors.
[c] Measured in cell expressing CFP-YFP hybrid.
[d] Measured in cell expressing CFP coupled to the serotonin 5-HT_{2A} receptor.
[e] Measured in cell co-expressing the serotonin 5-HT_{2A} and dopamine D_2 fusion proteins (5-HT_{2A}-CFP and D_2-YFP).
[f] Measured in cell co-expressing the serotonin 5-HT_{2A} and genetic variant of dopamine D_2 fusion proteins (5-HT_{2A}-CFP and D_2R1-YFP).
[g] Measured in cell co-expressing the serotonin 5-HT_{2A} and genetic variant of dopamine D_2 fusion protein (5-HT_{2A}-CFP and D_2R2-YFP).
[h] Measured in cell co-expressing the serotonin 5-HT_{2A} and genetic variant of dopamine D_2 fusion proteins (5-HT_{2A}-CFP and D_2R3-YFP).
[i] Measured in cell expressing CFP coupled to the genetic variant of serotonin 5-HT_{2A}MUT receptor.
[j] Measured in cell co-expressing genetic variant of the serotonin 5-HT_{2A} and dopamine D_2 fusion proteins (5-HT_{2A}MUT-CFP and D_2-YFP).
[k] Measured in cell co-expressing two serotonin 5-HT_{2A} receptor fusion proteins (5-HT_{2A}-CFP and 5-HT_{2A}-YFP).
[l] Measured in cell co-expressing the serotonin 5-HT_{2A} receptor and genetic variant of the serotonin 5-HT_{2A} receptor fusion protein (5-HT_{2A}MUT-CFP and 5-HT_{2A}-YFP).
* $p < 0.05$ vs. 5-HT_{2A}-CFP/D_2-YFP.

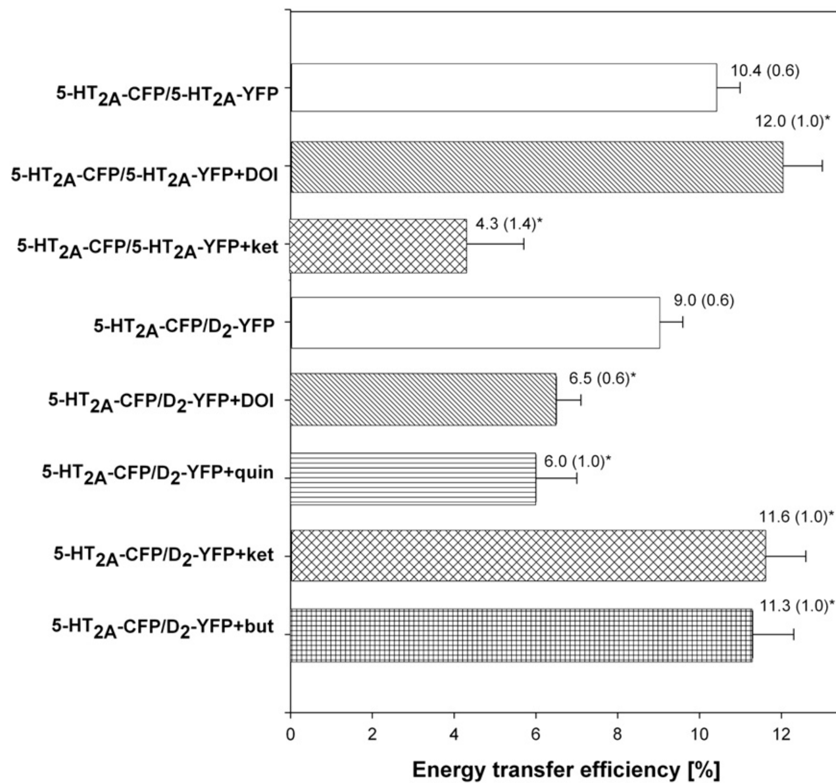


Fig. 5. Bar graph of FRET efficiency values estimated for combination of homo-dimers 5-HT_{2A}/5-HT_{2A} and hetero-dimers 5-HT_{2A}/D_2 after stimulation by DOI, ketanserin, quinpirol and butaclamol, respectively.Values in bracket reflect the standard errors of means. The statistical significance was evaluated using Student *t*-test $^*p<0.05$ vs 5-HT_{2A}/5-HT_{2A} or 5-HT_{2A}/D_2, respectively; $n=57$–65.

Substantial degree of colocalization of serotonin 5-HT_{2A} and dopamine D_2 receptors has been observed in both the medial prefrontal cortex and substantia nigra.

4. Discussion

Membrane-bound proteins, such as tyrosine kinases, cytokines or transforming growth factor receptors, have been known for many years to exist and been active in an oligomeric form. In contrast, the possibility of GPCRs associations has only recently been widely accepted. Therefore, the molecular details of GPCRs oligomerization processes are still poorly understood. Although many GPCRs have been shown to undergo homo- or hetero-dimerization [1,3,4] either in response to ligand or constitutive [27,52,59,67,68], little is known about the mechanisms involved in the association of these proteins.

In the present study, constitutive dimerization (either homo- or hetero-) between 5-HT_{2A}R and D_2R was investigated in living HEK 293 cells using biophysical approaches (spectrofluorimetric measurements and fluorescence lifetime measurements) based on FRET. FRET is a technique widely used to monitor protein–protein interactions [53,54] and allows for the observation of the physical proximity between molecules by assessing the level of energy transfer occurring between the energy donor and the acceptor (in this study receptor-CFP and receptor-YFP, respectively). In order to monitor energy transfer between investigated proteins, we co-expressed the fusion proteins in HEK 293 cells in different combinations. Generally, GPCRs oligomerization is difficult to analyze in native cells. The HEK cell line has been widely used in resonance energy transfer studies of membrane receptors, because these cells provide an accepted model in which fluorescently tagged receptor proteins can be efficiently expressed. The often discussed problem connected with experiments performed in heterologous expression systems is the issue concerning “overcrowding” of the protein of interest [69]. Meaning, GPCRs dimerization might simply be promoted at relatively high expression levels and may partially be an artifact of over-expression. However, studies indicate that the extent of dimerization of the receptors (shown by bioluminescence resonance energy transfer (BRET)) was unchanged over a 20-fold range of expression level of the β_2-adrenergic receptor (from 1.4 to 26.3 pmol/mg protein). [70]. Additionally, while studying the homo-dimerization of neuropeptide Y receptors, the FRET effect was demonstrated to be independent of the level of receptor expression [71]. Similarly, β_2/β_3-adrenergic hetero-dimers possessed a stable BRET signal for expression levels ranging from 0.58 to 6.5 pmol/mg protein [22]. In the present study, the experiments were conducted with reasonable expression levels of the investigated receptors (ranging from 0.82 to 1.61 pmol/mg protein). These findings imply that examples of the observed GPCRs dimerization are not merely an artifact derived from the high level of expression achieved in a heterologous system. Moreover, data obtained in our previous studies [43,52] concerning the interactions between the GPCRs (D_1R, D_2R and 5-HT_{1A}R) and the appropriate α subunit of G protein confirm the specificity of the methodology (FRET measurements by fluorescence spectroscopy and fluorescence lifetimes) used herein. Using the same expression system and the same amount of DNA for transient transfections, FRET did not occur when two non-interacting fusion proteins (bearing CFP and YFP, respectively) were co-expressed in the same cell. Moreover, using this

Table 3
The anticipated possible alteration in energy transfer induced by the conformational change.

	5-HT_{2A}/D_2	5-HT_{2A}/5-HT_{2A}
r [nm]	7.20	7.02
Δ*E* [%]	1.37	1.59

The values were estimated according to the Eqs. (1), and (2).

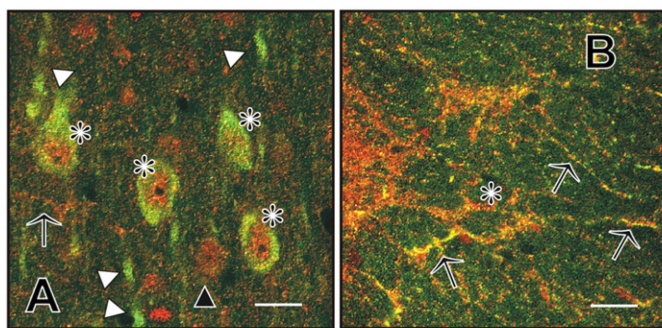


Fig. 6. Representative images (confocal laser scanning microscopy) illustrating colocalization of serotonin 5-HT_{2A} (green), dopamine D_2 receptors (red) in rat medial prefrontal cortex (A) and pars reticulate of substantia nigra (B). Asterisk-points to the neuronal cell bodies positive for both antigens, arrows-dendritic processes. White arrow head in A — dendritic shafts of pyramidal neurons; black arrow head in A — cell body of neuron positive for dopamine D_2 receptors and negative for serotonin 5-HT_{2A} receptors. Scale bars in A and B 20 μm.

methodology we were able to demonstrate a lower FRET efficiency when the expression of one of the studied receptors was decreased [72]. Therefore, we conclude that the observed effects reflect a specific interaction and are not the result of over-expression of the studied receptors.

In the present study, we demonstrate for the first time (using both fluorescence spectroscopy and fluorescence lifetime microscopy to determine FRET efficiency) that 5-HT_{2A}R and D_2R form dimers. In addition, the data obtained in this work indicate that 5-HT_{2A}R forms homo-dimers. These data provide the first evidence that 5-HT_{2A}R/D_2R and 5-HT_{2A}R/5-HT_{2A}R complexes may exist in living cells.

D_2R and 5-HT_{2A}R display complex interactions during neurotransmission [48,49]. The affinity of second-generation antipsychotic drugs for both D_2R and 5-HT_{2A}R is strongly postulated as the mechanism of action of these drugs [51]. Therefore, a detailed understanding of the dimerization process of these receptors is essential. Various studies suggest that GPCRs hetero-dimers form functional complexes with distinct drug specificities and signaling properties, in which each individual receptor subunit may be responsible for a specific function [73–77]. Recently, the importance of a 5-HT_2R and metabotropic glutamate subtype 5 (mGluR5) receptor complex has been implicated in psychosis [78].

The physical interaction between two receptors can take place only if they are concomitantly expressed in the same cell. In the brain, co-expression of 5-HT_{2A}R and D_2R has been shown at least in dopaminergic cells in the ventral tegmental area [79,80]. Another potential site for a direct 5-HT_{2A}R/D_2R interaction is layer V of the prefrontal cortex [50,81].

In the present work – using the immunofluorescence double labeling protocols – we show the data indicating the colocalization of serotonin 5-HT_{2A} and dopamine D_2 receptors in the medial prefrontal cortex as well as in the pars reticulate of substantia nigra of the rat brain.

Both of these brain receptors have been implicated as important sites of action of antipsychotics due to a well-documented serotonin–dopamine interaction and its relevance to schizophrenia [49]. The 5-HT_{2A}R/D_2R complex may be a potential target for more novel effective therapeutics. In this context, it seems very important to estimate the influence of 5-HT_{2A}R- and D_2R-specific ligands on the dimerization process. Typical 5-HT_{2A}R (DOI and ketanserin) and D_2R (quinpirol and butaclamol) ligands display distinct actions depending on the receptor combination. In the case of 5-HT_{2A}R homo-dimers, the agonist DOI increased the level of FRET efficiency, while the antagonist ketanserin decreased the basal energy transfer value. A similar effect was recorded in our previous study on homo-dimers of 5-HT_{1A}R and the adenosine A_{2A} receptors [52]. Opposite effects were observed when we investigated the influence of ligands on hetero-complexes of 5-HT_{2A}R and D_2R. Agonists (DOI and quinpirol) decreased the FRET value while antagonists (ketanserin and butaclamol) increased the FRET value. As a result of analyzing these data, the proportion of the monomeric and oligomeric forms of the receptors on the cell membrane still requires resolution. Homo- and hetero-dimers of the investigated receptors are likely to be formed simultaneously. In the present study, we could not distinguish homo-dimers because the homo-complexes would be built with receptors tagged with the same fluorescent protein. The presence of 5-HT_{2A}R and D_2R homo-dimers most likely influenced the effect of the ligands. Therefore, the observed effects might be the result of the promotion of homo-dimerization over hetero-dimerization after treatment with specific agonists. Hence, a lower level of FRET efficiency was detected. Similarly, the addition of antagonists weakened homo-dimerization, in turn causing an increase in energy transfer from the hetero-dimer combination. Promotion of homo-dimerization over hetero-dimerization has been previously described for D_2R and the adenosine A_{2A} receptors [82].

Generally, transmembrane domains and hydrophobic interactions are considered the most effective and probable elements engaged in the GPCR family dimerization process [36,83,84]. However, participation of the C-terminal, the N-terminal and the ic3 regions are also documented [26,28,44]. Previous work suggests that heteromerization involves noncovalent interaction, and in this particular case, the noncovalent bond has been proven to be even more stable than a covalent one [47]. Noncovalent interactions seem to be more effective [67,85–87]. Stable noncovalent complexes between peptides might form through electrostatic interactions between cationic guanidinium groups (located at the terminus of the arginine side chain) and the anionic carboxyl groups of aspartate or glutamate and/or anionic phosphate group on phosphorylated residue [40,42,88,89]. Motifs composed of a minimum of two adjacent arginine residues "RR" or "RKR" on one peptide and two adjacent aspartate "DD" or glutamate "EE" or/and one phosphorylated residue (usually a serine residue) in close proximity to one another have been shown to be sufficient to generate stable noncovalent complexes between these two peptides [46,47,89].

Various reports suggest that the mechanisms involved in hetero-dimerization of some GPCRs are connected to the presence of the motifs described above and the formation of salt bridges [20,40,45,90]. For instance, Ciruela et al. [44] used pull-down assays and mass spectrometry to postulate that hetero-dimerization of the adenosine A_{2A} receptor and D_2R strongly depends on electrostatic interactions between the Arg-rich epitope within the ic3 of D_2R and the acidic epitope in the C-tail of the adenosine A_{2A} receptor. This interaction between the ic3 and the C-tail is also described by Lee et al. [45] for D_2R and the cannabinoid CB1 receptor. In addition, Woods et al. [40,41] described a similar interaction between the glutamate NMDA (NR1 subunit) receptor and D_1R.

In our previous study (using FRET methodology), we showed that D_1R and D_2R dimerize [43,59]. Additionally, experiments conducted with the use of site-directed mutagenesis indicated the role of specific regions in the dopamine receptor in the hetero-dimerization process. Both the Arg-rich epitope from the ic3 of D_2R and the acidic epitope within the C-terminus of D_1R were engaged during hetero-association of these proteins and had no influence on homo-dimerization [43]. Due to the presence of a similar acidic motif (consisting of two glutamate residues, 454EE455) within 5-HT_{2A}R, we wanted to determine its role in homo- or hetero-(with D_2R) dimerization.

The residues located within the ic3 and the C-terminal tail have opposite charges, allowing for the formation of salt bridges between the epitopes. As a result of replacing the Arg residues in the ic3 with Ala, the level of FRET decreased in HEK cells co-expressing 5-HT_{2A}R and one of the genetic variants of D_2R. The degree of 5-HT_{2A}R/D_2R hetero-dimerization depended on the number of exchanged residues within the ic3 (217–222) of D_2R. The efficiency of energy transfer estimated for wild-type 5-HT_{2A}R/D_2R hetero-dimers was reduced by

about 50% upon exchange of the first two Arg residues (combination of 5-HT_{2A}R and D_2R1). Replacement of the two additional Arg residues in that region (combination of 5-HT_{2A}R and D_2R2) caused a further decrease in the FRET efficiency to approximately 20%. When the entire basic region (217–222) of D_2R contained Ala residues, the lowest level of energy transfer was observed (2.2%; combination of 5-HT_{2A}R and D_2R3). A similar value of energy transfer (2.6%) was obtained after replacement of the two acidic glutamate residues in the C-tail of 5-HT_{2A}R. These results suggest that the basic region of the ic3 of D_2R and acidic region of the C-tail of 5-HT_{2A}R are involved in the association between these two receptors.

However, a different conclusion is drawn on the role of the acidic epitope (from the C-tail of 5-HT_{2A}R) in the homo-dimerization of 5-HT_{2A}R. Our data suggest that the acidic epitope does not participate in the formation of homo-complexes between 5-HT_{2A}R. Both qualitative estimations of FRET by fluorescence spectroscopy measurements in a cell suspension as well as quantitative measurements of fluorescence lifetimes in a single living cell co-expressing serotonin receptors (wild-type and the genetic variant 5-HT_{2A}MUT (454AA455)) yield the same results. Keeping in mind the nature of electrostatic interactions, the conclusion that the acidic epitope is not involved in the homo-dimerization of 5-HT_{2A}R appears to be justified, especially since both receptors within the homo-complex possess the same motifs with the same charge. A similar effect was observed in our previous study when the role of the amino acids residues from either the C-tail (404EE405) of D_1R or the ic3 (217RRRRKR222) of D_2R in homo-dimer formation was investigated [43].

Even advanced biophysical techniques cannot solely be used in the studies of receptor oligomerization, especially when specific sequence motifs responsible for membrane protein dimerization are investigated. Therefore, we examined using confocal microscopy the subcellular localization of 5-HT_{2A}R and D_2R as well as their genetic variant fusion proteins. Results obtained from cells co-expressing only the serotonin receptors show the same cell surface localization of both the wild-type and the mutant receptors. Thus, the glutamate residues in the C-tail of 5-HT_{2A}R have no influence on membrane expression of 5-HT_{2A}R. In cells co-expressing different types of these receptors (5-HT_{2A}R and D_2R), the distribution of 5-HT_{2A}R was changed. In addition to being localized on the cell surface, a considerable portion of the receptor was also present inside the cell. We previously observed a similar situation [43] for D_1R and D_2R. Moreover, experiments conducted by So et al. [91] show that when expressed alone, D_1R and D_2R localize to different regions of the cell. D_1R was found predominantly on the cell surface, whereas D_2R localized at the cell surface but was also found intracellularly. Simultaneous synthesis of both dopamine receptors induced alterations in the cellular distribution of these proteins, causing them to be localized either on the cell surface or in intracellular compartments [91]. These observations may explain an increase in the cellular localization of 5-HT_{2A}R in the presence of simultaneous expression of D_2R.

In conclusion, the data presented herein, obtained using methodology based on FRET as an analytical tool, demonstrate that hetero-dimerization of 5-HT_{2A}R and D_2R results from an interaction between specific motifs in the two receptors (454EE455 in 5-HT_{2A}R and 217–222 in D_2R). Although the data obtained for combinations involving the D_2R3 mutant may be considered controversial (in this case, a decrease in FRET efficiency may be connected with a decrease in receptor membrane density or misfolding of the receptor and hence the receptor is unable to interact with 5-HT_{2A}R), the data obtained for the 5-HT_{2A}R/D_2R1 and the 5-HT_{2A}R/D_2R2 combinations clearly indicate the key role for the ic3 region in the dimerization of 5-HT_{2A}R and D_2R. However, this region does not seem to be the only point of contact between these receptors and another type of interactions may also be engaged during this process. On the other hand, the acidic motif located within 5-HT_{2A}R has no significant role in homo-dimerization of 5-HT_{2A}R. Similar to what we have shown previously for D_1R/D_2R interactions, the current study indicates the importance of simultaneous use of both well controlled biophysical methodology together with monitoring of the subcellular localization of 5-HT_{2A}R and D_2R and their genetic variants. Further detailed description of the action of specific ligands on receptor dimer complexes may have an impact on our understanding of the mechanism by which distinct signaling pathways are directly linked and receptor function is integrated. This information may have therapeutic implications for several human diseases. It is especially important in light of the data provided in the present work, showing the presence of serotonin 5-HT_{2A} and dopamine D_2 receptors in the same neuronal cells in the rat brain.

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