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Detection of Receptor Heteromers Involving Dopamine Receptors by the Sequential BRET-FRET Technology

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Abstract

Until very recently, dopamine receptors, like other G-protein-coupled receptors, were believed to function as individual units on the cell surface. Now it has been described by several groups including ours that dopamine receptors not only function as homomers but also form heteromers with other receptors at the membrane level. Bioluminescence and fluorescence resonance energy transfer (BRET and FRET) based techniques have been very useful to determine the interaction between two receptors, but to demonstrate the existence of higher-order complexes involving more than two molecules requires more sophisticated techniques. Combining BRET and FRET in the Sequential BRET-FRET (SRET) technique permits heteromers formed by three different proteins to be identi fi ed. In SRET experiments, the oxidation of a Renilla Luciferase substrate triggers acceptor excitation by BRET and subsequent energy transfer to a FRET acceptor. Using this methodology here we describe the heteromerization between adenosine A_{2A}, dopamine D_2, and cannabinoid CB_1 receptors in living cells.

Keywords

Dopamine receptors; Dopamine receptors interacting proteins; BRET; FRET; Sequential resonance energy transfer; GPCR; Receptor oligomerization; Heteromer; Protein–protein interaction

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1. If cells are dying after transfection refer to cytotoxicity of transfection Reagent.

2. The amount of fusion proteins transfected must be near the physiological range.

3. If the donor protein expression is high, then the fluorescence background will be high and poor SRET signal will be detected. If there is low donor protein expression, the acceptor protein will not be excited.

5. Add DeepBlueC immediately before SRET detection.

1. Introduction

Dopamine exerts many of its physiological functions by interacting with dopamine receptors. Dopamine receptors are classified in D_1-like, with the D_1 and D_5 receptor subtypes (D_1R and D_5R), which usually couple to Gs/olf proteins, and D_2-like, with the D_2, D_3, and D_4 receptor subtypes (D_2R, D_3R, and D_4R), which couple to Gi/o proteins (1). Like many other G-protein coupled receptors (GPCRs), dopamine receptors function as oligomers forming homomers (2–6) and heteromers with other dopamine receptors (7–10) or other GPCRs (11–13). A receptor heteromer has been recently defined as a macromolecular complex composed of at least two functional receptor units with biochemical or functional properties that are demonstrably different from those of its individual components (14). For this reason, receptor heteromers provide many implications for pharmacology, since they constitute new targets for drug development (15–17). The use of biophysical techniques, such as bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET) techniques has been fundamental in taking the issue of GPCR oligomerization to the front of GPCR research, providing evidence for an increasing number of receptor heteromers in living cells (18–20). Nevertheless, to detect higher order receptor oligomers a significant development in biophysical energy transfer techniques has been needed. To this end, two techniques have been developed in our laboratory to study oligomers formed by three different proteins and applied to determine heterotrimers involving dopamine receptors. One is BRET with bimolecular fluorescence complementation (BiFC), that we used to demonstrate heteromultimerization between adenosine A_{2A} ($A_{2A}R$), D_2R, and cannabinoid CB_1 (CB_1R) receptors and between $A_{2A}R$, D_2R, and glutamate $mGlu_5$ receptors (21, 22). The other technique is Sequential-BRET-FRET (SRET) (23). In SRET, the oxidation of an RLuc substrate by an RLuc-fusion protein triggers the excitation of the BRET acceptor (i.e., protein fused to GFP^2) and subsequent energy transfer to the FRET acceptor (i.e., protein fused to YFP). SRET will only occur with these fusion proteins if the two acceptor–donor pairs, Rluc/GFP^2 and GFP^2/YFP, are at a distance of less than 10 nm. Here the technique is described to detect heterotrimers formed by $A_{2A}R$, D_2R, and CB1R in living cells. In general we conclude that SRET is an invaluable technique to identify oligomeric complexes of more than two proteins localized at the plasma membrane, including more than two GPCRs, which will enable us to better understand how signals are integrated at the plasma membrane level.

2. Materials

2.1. Fusion Proteins and Expression Vectors

1. The cDNA for functionally validated fusion proteins in suitable mammalian expression vectors are used. The human cDNAs for $A_{2A}R$, D_2R, CB_1R, and the negative control human dopamine $D_{4.4}$ receptor, cloned in pcDNA3.1.
2. pRluc-N1 vector (Rluc expressing vector, PerkinElmer, Wellesley, MA).
3. pGFP2-N3(h) vector (humanized pGFP2-N3(h) from PerkinElmer (Waltham, MA)).

4. pEYFP-N1 vector (enhanced yellow variant of GFP; Clontech, Heidelberg, Germany).
5. DH5α competent bacterial cells (Invitrogen, Carlsbad CA).
6. DH5α growing medium (LB): 10 g/L NaCl, 10 g/L Tryptone, and 5 g/L Yeast extract in mQ water.
7. Xtra Maxi kit (Nucleobond®, Düren, Germany).

2.2. Cell Culture

1. Human Embrionic Kidney (HEK) 293T cells are grown in 6-well cell culture plates (Techno Plastic Products, Lausanne,Switzerland).
2. As suitable growth medium, Complete Medium (Dulbecco's modified Eagle's medium (DMEM; Gibco (Carlsbad, CA)) supplemented with 2 mM L -glutamine, 100 U/ml penicillin–streptomycin, 5% (v/v) heat inactivated Fetal Bovine Serum (FBS), and 5% (v/v) nonessential amino acids (all supplements are from Invitrogen, Paisley, Scotland, UK) are used.
3. Protein quanti fi cation reagent; Bradford solution (Bio-Rad, Hercules, CA) diluted 1/5 (v/v) in milliQ (mQ water).

2.3. Transfection

1. Branched PEI (PolyEthylenImine, Sigma, Steinheim, Germany). Prepare a 40 μM solution in mQ water.
2. NaCl solution: NaCl 150 mM prepared in mQ water.
3. 0.05% trypsin (Gibco).
4. HBSS buffer: 0.185 g of $CaCl_2 \cdot 12H_2O$, 0.370 g of KCl, 0.060 g of KH_2PO_4, 0.100 g of $MgCl_2 \cdot 2H_2O$, 0.100 g of $MgSO_4 \cdot 7H_2O$, 8.000 g of NaCl, 0.121 g of $Na_2HPO4 \cdot 12H_2O$, and 2.385 g of HEPES in 1 L of mQ water. Use 1 M NaOH to adjust the pH to 7.4.

2.4. SRET

1. Assay buffer: HBSS buffer containing 1 g/L D -glucose. Add glucose to the buffer 10 min before using.
2. 500 μM DeepBlueC (Perkin Elmer) in anhydrous ethanol as luciferase substrate stock solution. Store at −20°C protected from light.
3. 500 μM coelenterazine h (Perkin Elmer) in anhydrous ethanol as luciferase substrate stock solution (Panreac, Barcelona, Spain). Store at −20°C protected from light.

2.5. Equipment

1. Multiskan Ascent Photometer (Thermo Labsystems, San Diego CA).

2. Fluostar Optima Fluorimeter equipped with a high-energy xenon flash lamp and appropriate filters (excitation filter at 485 nm and 410 nm and emission filter corresponding to 530 nm and 510 nm) (BMG Labtechnologies, Offenburg, Germany).
3. Mithras LB 940 equipped with detection filters for shortwavelength (400 nm) and long-wavelength (530 nm) (Berthold Technologies, DLReady, Germany).
4. 96-well white microplates for BRET (Porvair, Norfolk, UK).
5. 96-well black microplates with transparent bottom for fluorescence detection (Porvair).

3. Materials

3.1. Preparation of Fusion Proteins

Generate fusion constructs in Rluc, GFP^2, or YFP expression vectors consisting of the cDNA for the protein of interest, inserted in-frame with the cDNA for the bioluminescent or fluorescent donor or acceptor molecule.

1. Select the donor and acceptor combination to perform SRET (i.e., A_{2A}R-Rluc, D^2R-GFP^2, CB_1R-YFP, and a negative control $D_{4.4}$R-Rluc). Since in SRET experiments, the oxidation of an Rluc substrate triggers acceptor excitation by BRET and subsequent energy transfer to a FRET acceptor (Fig. 1) it is important to select the optimal combination (see Note 4).
2. The human cDNAs for A_{2A}R, D_2R, CB_1R, or $D_{4.4}$R cloned in pcDNA3.1 are amplified, without their stop codons, using sense and antisense primers harboring unique Eco RI and Bam HI sites to clone A_{2A}R in the Rluc corresponding vector, Eco RI and Kpn I to clone D_2R in the GFP^2 corresponding vector and Bam HI and Eco RI to clone CB_1R in EYFP corresponding vector or Xho I and Bam HI sites to clone $D_{4.4}$R in pRluc-N1 vector. To avoid transcription mutations, it is recommendable to use a high- fidelity DNA polymerase that offers extreme performance for all PCR applications (i.e., iProof™ from Bio-Rad). It is important to select the annealing temperature for each primer as indicated by the supplier and use 15–30 s/kbase for extension times, since longer times could induce a loss of bases.
3. The amplified fragments are subcloned to be in-frame into restriction sites of a multiple cloning region within pRluc-N1, $pGFP^2$-N3(h), or pEYFP-N1 vectors respectively yielding the plasmids corresponding to A_{2A}R-Rluc, $D_{4.4}$R-Rluc, D_2R-GFP^2, and CB^1RYFP. To do this, the amplified fragments (dephosphorylated or not, depending on the activity of the enzymes) and vectors are cut with the specific enzymes (see step 3). Then 100 ng of vector and 60 ng/Kb of insert are mixed with 1 unit of T4 cDNA ligase (Promega, Fitchburg MA) for 3 h at room temperature. The ligation product is transformed in DH5

4. The relative orientation of donor and acceptor can be unsuitable for SRET.

α competent bacterial cells and the subcloned vectors are selected using the specific selection antibiotics of each vector. The positive colonies are grown in LB medium in the presence of the selection antibiotic concentration indicated by the vector supplier.

4. Sequence cDNA to test the correct sequence of the fusion protein. Obtain enough cDNA to do several transfections using a Xtra-Maxi kit (usually we obtain 500 μL of cDNA from 1–5 μ g/μL concentration).

5. Check that the luminescence or fluorescence is detectable after fusion protein expression (represent the amount of cDNA transfected in HEK 293T cells versus bioluminescence or fluorescence detected). If possible, use confocal microscopy to visualize (by its own fluorescence or using antibodies) correct cellular localization of fusion proteins.

6. Validate the fusion proteins of interest, including suitable control proteins, by comparing fusion and wild-type proteins in functional assays. ERK1/2 phosphorylation and cAMP production can be used for this purpose as described previously (13, 24).

7. Generate positive controls for SRET experiments. Rluc-expressing vector (pRlu-N1) is amplified without its stop codon using sense and antisense primers harboring unique Hind III and Kpn I sites to clone Rluc in-frame into restrictions sites of a multiple cloning site of GFP 2 -YFP vector (pcDNA3.1-GFP^2-YFP; Biosignal Packard) to obtain the fusion protein Rluc-GFP^2-YFP.

8. Reconstitute and store the luciferase substrate stock solution containing DeepBlueC with anhydrous ethanol. Protect the solution from light.

3.2. Cell Transient Transfection

1. HEK 293 T cells are passaged when approaching confluence with trypsin/EDTA to provide new maintenance cultures in 150 cm^2 flasks. One 150 cm^2 flask is required for transfection in order to obtain enough transfected cells to perform a SRET saturation curve (see Subheading 3.4, step 4). Aliquot cells in 6-well cell culture plate in growth medium. They should be 60–80% confluent after 24 h. Maintain at 37°C, 5% CO_2 and 90% of humidity.

2. Transfect the expression vectors corresponding to the desired fusion proteins at the suitable ratios (see legends of Figs. 2 and 3) using the PolyEthylenImine (PEI) method. Other methods of transfection may be used as well. To do this, two Falcon tubes are needed. In the Falcon A each μg of cDNA is mixed with 25 μL of NaCl solution. In Falcon B the same amount of NaClsolution is mixed with 1.25 μ L/μg cDNA of 40 μM branched PEI solution. Both Falcon tubes are vortexed a few seconds. After this, Falcon B solution is added to Falcon A. The final solution is strongly vortexed for 10 s. Ten minutes later, 1 mL of serum starved medium is added. Cells are incubated for 4 h with the cDNA-PEI solution. Then cells are placed in a fresh complete culture medium.

3. Around 48 h after transient transfection, detach the cells and resuspend them in HBSS buffer containing 1 g/L D-glucose. Wash cells twice with the same buffer for 5 min and resuspend them in the same buffer.

4. Using 10 μL of cell suspension, quantify the amount of protein using a Bradford assay and dilute cells to 200 μg/mL. It is important to maintain the same amount of protein in each sample. Aliquot cells into 96-well white and black isoplate (100 μL per black well and 90 μL per white well).

3.3. SRET Detection

Using aliquots of transfected cells (20 μg of protein), four different determinations are performed in parallel:

1. Quantification of protein-YFP expression by determination of the fluorescence due to protein-YFP. Cells distributed into 96-well microplates (black plates with a transparent bottom), are read in a Fluostar Optima Fluorimeter using an excitation filter at 485 nm, and a 10 nm bandwidth emission filter corresponding to 530 nm (527–536 nm).

2. It is also important to quantify the protein-GFP 2 expression to control for BRET energy transmission and to develop the linear un-mixing arrangement. Cells distributed into 96-well microplates (black plates with a transparent bottom), are read in a Fluostar Optima Fluorimeter using an excitation filter at 410 nm and a 10 nm bandwidth emission filter corresponding to 510 nm (506–515 nm). To determine the exact fluorescence amount of protein-GFP^2 and protein-YFP it is necessary to calculate the linear un-mixing. First, analyze the contribution of GFP^2 and YFP proteins alone using the two detection channels (see Note 6). Fluorescent determinations are measured in parallel in experiments with cells expressing only one of these proteins and normalized to the sum of the signal obtained in the two detection channels. Values obtained in 1 and 2 are corrected considering this linear un-mixing. The sample fluorescence is the emission at 530 nm corrected as described minus the fluorescence ofcells expressing only protein-Rluc and protein-GFP^2. The protein-GFP^2 fluorescence is the emission at 510 nm corrected as described minus the fl uorescence of cells expressing only protein-Rluc.

3. Quanti fi cation of protein-Rluc expression by determination of the luminescence due to protein-Rluc. Cells are distributed in 96-well microplates (white plates) and luminescence is determined 10 min after the addition of 5 μM coelenterazine H in a Mithras LB 940 multimode reader.

4. SRET measurements. Cells are distributed in 96-well microplates (white plates) and 5 μM DeepBlueC is added. SRET signal is collected using a Mithras LB 940 reader 30 s after the substrate addition with detection filters for short-wavelength (400 nm (370–450 nm)) and long-wavelength (530 nm (510–590 nm)). Net SRET is de fined as [(long-wavelength emission)/(short-wavelength

6. Given the spectral emission of the GFP^2/YFP pair, the contribution of GFP^2 or YFP proteins to the two detection channels

emission)] − Cf, where Cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for cells expressing protein-Rluc, protein-GFP^2 and the other protein partner not fused to a fluorescence protein (similar values are obtained measuring Cf in cells expressing protein-Rluc only and protein-GFP^2). Linear un-mixing is done for quantification, taking into account the spectral signature to separate the two fluorescence emission spectra (see Note 6). SRET determined in cells expressing A_{2A}R-Rluc, D_2R-GFP^2 and CB_1RYFP or the corresponding positive or negative controls is shown in Fig. 2.

3.4. SRET Saturation Curves

SRET saturation curve is further proof for the specificity of the interaction observed.

1. Transiently transfect HEK 293T cells with a constant amount of the constructs corresponding to the protein-Rluc and protein-GFP^2 and with increasing amounts of the construct corresponding to protein-YFP indicated in the legend of Fig. 3.
2. After 48 h of transient transfection determine SRET as indicated (see Subheading 3.3) for each transfection condition.
3. Both fluorescence and luminescence for each sample are measured to confirm similar donors expression (approximately 100,000 bioluminescence units and 6,000 GFP^2 fluorescence units) while monitoring the increase in acceptor expression (1,000 to 20,000 fluorescence units) (see Note 7).
4. Represent the net SRET values as a function of the amount of the acceptor. In each saturation curve, the relative amount of acceptor is given as the ratio between the fluorescence of the acceptor (YFP) and the luminescence of the first donor (Rluc). Curves are fitted to a nonlinear regression equation, assuming a single phase (i.e., with Graph-Pad Prism software, San Diego, CA, USA). From these saturation curves, an apparent $SRET_{max}$ and an apparent $SRET_{50}$ can be determined (see Note 8). A SRET saturation curve obtained by increasing CB_1R-YFP expression while maintaining the same A_{2A}R-Rluc/D_2-GFP2 ratio (Fig. 3) allows determination of the following parameters for the trimer A_{2A}R-Rluc/D_2R-GFP2/CB_1R-YFP: apparent SRET max of 0.18 ± 0.05 and apparent SRET 50 of 0.013 ± 0.007.

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7. In SRET saturation curves, be sure that the amount of luminescence due to protein-Rluc and the amount of fluorescence due to protein-GFP^2 are constant while increasing the fluorescence of the acceptor protein-YFP.

8. It should be noted that the SRET max and SRET 50 will differ when different ratios of donor–acceptor for BRET are used. This is reminiscent of what occurs in enzymology, when the V_{max} and K_M for an enzyme using two substrates are calculated by maintaining the concentration of one substrate constant but varying the concentration of the other. For such enzymes, the calculated values are known as “apparent V_{max}” and “apparent K_M”. Accordingly, we propose the denomination of “apparent $SRET_{max}$” and “apparent $SRET_{50}$”.

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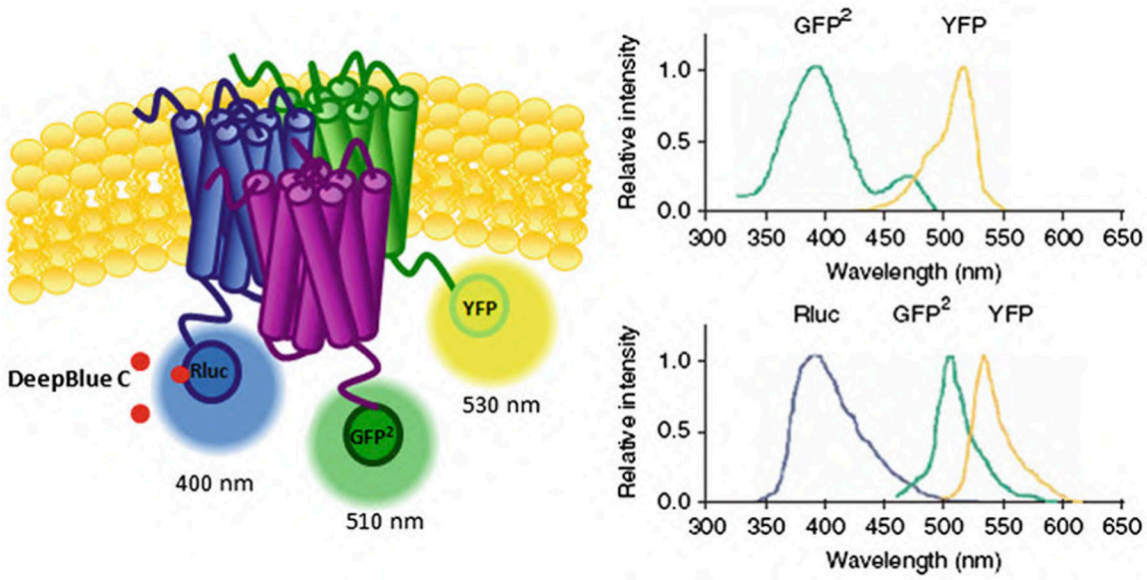

Fig. 1.

Sequential BRET-FRET (SRET). SRET combines BRET and FRET involving two energy donors and two acceptors. BRET and FRET techniques are combined to detect heterotrimers at the membrane level. Signal is initiated by oxidation of DeepBlueC by the Rluc-fused protein (A_{2A}R-Rluc) that generates light emission at the indicated wavelength (blue). The acceptor in BRET is a GFP^2-fused protein (D_2R-GFP^2) that, after excitation, results in emission at the indicated wavelength (green) that excites a YFP-fused protein (CB_1R-YFP) by a FRET process with concomitant light emission peaking at the indicated wavelength (yellow). Emission of YFP after addition of the Rluc substrate is only possible if the three fusion proteins are in close proximity (<10 nm) allowing bioluminescent and fluorescent sequential resonance energy transfer (SRET) to occur. A representation of excitation (top) and emission (bottom) spectra of fused proteins is shown in the right.

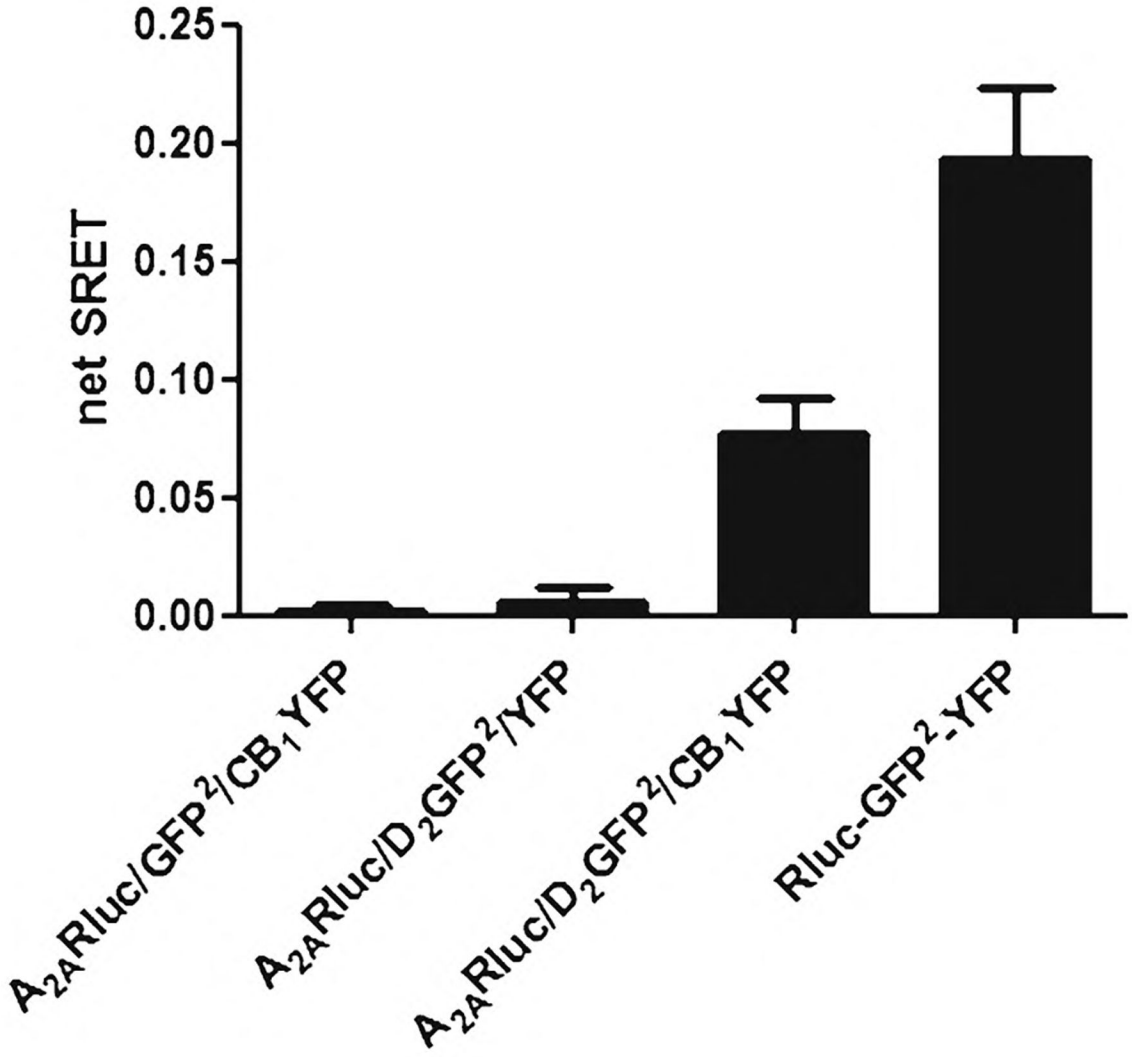

Fig. 2.

SRET for $A_{2A}R$, D_2R, and CB_1R in living cells. SRET assays are performed 48 h post-transfection in cells expressing $A_{2A}R$-Rluc (2 μg of cDNA; approximately 100,000 luminescence units), D_2R-GFP^2 (3 μg of cDNA; approximately 6,000 fluorescence units), and CB_1R-YFP (9 μg of cDNA; approximately 18,000 fluorescence units) or the equivalent amounts of the fluorescence or luminescence proteins or transfected with the positive SRET construct (1 μg of cDNA of Rluc-GFP^2-YFP construct). Net SRET was obtained by monitoring the YFP fluorescence emission after DeepBlueC addition, with subtraction of the value obtained with cells expressing the same amount of $A_{2A}R$-Rluc and the corresponding BRET acceptor. Significant net SRET was detected for $A_{2A}R$-Rluc/D_2R-GFP^2/CB_1R-YFP coupling or for the positive SRET control, while negligible net SRET was obtained in cells expressing equivalent amounts of $A_{2A}R$-Rluc, GFP^2, and CB_1R-YFP, or $A_{2A}R$-Rluc, D_2R-GFP^2, and YFP. Data are expressed as the mean net SRET ± S.E.M. of four independent experiments performed in duplicate. One-way ANOVA followed by Newman-Keuls test showed significant differences with respect to negative controls (***: $P < 0.001$).

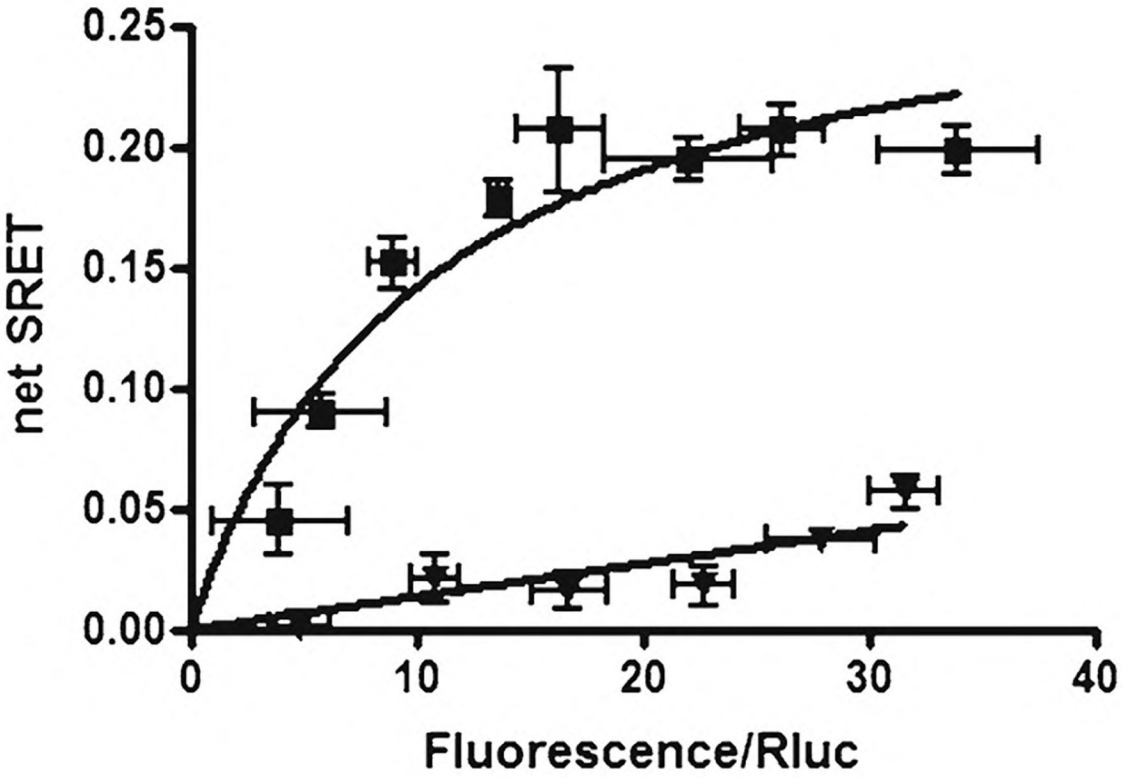

Fig. 3.

SRET assays are performed 48 h post-SRET saturation curve for $A_{2A}R$-D_2R-CB_1R heteromers in living cells. SRET saturation,curves were obtained using HEK-293T cells transfected with 2 μg of the cDNA for $A_{2A}R$-Rluc (approximately 100,000 luminescence units) and 3 μg of the cDNA for D_2R-GFP^2 (approximately 6,000 fluorescence units) and increasing amounts of the cDNA for CB_1R-YFP (8,000 to 18,000 fl uorescence units). Values, expressed as net SRET, represent the mean ± S.E.M. of two independent experiments performed in triplicate. Negative control is constituted by cells expressing the equivalent amounts of $D_{4.4}R$-Rluc/$A_{2A}R$-GFP2/CB_1R-YFP giving linear (nonspecific) SRET with similar amounts of fluorescence and luminescence as those giving saturable SRET.