Chapter 8

BRET Approaches to Characterize Dopamine and TAAR1 Receptor Pharmacology and Signaling

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Abstract

It is evident that G protein-coupled receptors (GPCRs) such as D2 dopamine receptor and functionally related Trace Amine Associated Receptor 1 (TAAR1) can engage both in G protein-dependent (e.g., cAMP-mediated) and -independent β-arrestin-mediated signaling modalities. Both of these signaling events can be monitored in real-time and in live cells by using new biosensors based on a Bioluminescence Resonance Energy Transfer (BRET) approach. Here we discuss the practical applications of BRET to analyze dynamics of cAMP modulation via an EPAC biosensor as well as recruitment of β-arrestin2 to the D2 dopamine receptor. Combination of these approaches allows for a comparison of activity of pharmacological compounds on these signaling modalities as demonstrated for various antipsychotics as regard to D2 dopamine receptor. Furthermore, analysis of cAMP concentrations in cells expressing TAAR1 provides a simple high-throughput screening method to identify new ligands for this receptor. These BRET approaches could be applied for the characterization of pharmacology and signaling of variety of other GPCRs.

Key words: D2R, TAAR1, cAMP, β-arrestin2, BRET, EPAC, 3-Methoxytyramine, β-Phenylethylamine

1. Introduction

Dopamine (DA) is a major monoaminergic neurotransmitter in the mammalian brain and controls many physiological functions, such as voluntary movement, emotion, reward, cognition and neuroendocrine secretion (1). DA is also involved in critical processes in the periphery, including gastrointestinal activity, regulation of kidney function and catecholamine secretion. A variety of human pathologies have been linked to a dysregulation of dopaminergic neurotransmission ranging from schizophrenia and addiction to Parkinson's disease, Tourette's syndrome, Attention Deficit Hyperactivity Disorder (ADHD), and hyperprolactinemia (1). DA exerts its physiological functions via action on five members of a

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family of G protein-coupled receptors (GPCR) that are subdivided into D1-like (D1 and D5) and D2-like (D2, D3, D4) dopamine receptor subtypes. D1-like dopamine receptors are positively coupled to adenylate cyclase and their stimulation increase cAMP level inside the cells, while D2-like dopamine receptors negatively regulate the production of cAMP in the cell. In addition, recent studies have demonstrated a novel G protein-independent pathway for D2 dopamine receptors (D2Rs) that involves the multifunctional scaffolding protein β-arrestin2 (2). While best known for their role in GPCR desensitization, β-arrestins are emerging as key signaling proteins in a variety of physiological processes (3). Regarding D2R, β-arrestin2-orchestrated formation of a protein complex including D2R, and other proteins (PP2A and Akt) has been demonstrated to be important for the full manifestation of certain D2R-related behaviors (4). Intriguingly, a recent comparison of the ability of several clinically active antipsychotics to affect G protein-dependent (cAMP accumulation) versus G protein-independent processes (β-arrestin2 recruitment), has shown that antipsychotics are generally more effective in preventing β-arrestin2 recruitment to the D2R than regulating cAMP concentration within the cell (5).

Another GPCR that is emerging as a functionally important modulator of dopaminergic activity in the brain is Trace Amine Associated Receptor 1 (TAAR1) (6–8). TAAR1 belongs to the family of Trace Amine Associated Receptors (TAARs) that is expressed in several mammalian brain areas including limbic regions and sites containing monoaminergic cell bodies such as the VTA (8). It has been shown that TAAR1 can modulate dopaminergic activity both by altering VTA neurons firing and D2 receptor signaling (9). TAAR1 is a G_s coupled receptor and it is activated by a class of compounds called trace amines (TAs) that include β-phenylethylamine (β-PEA), tyramine, octopamine, and tryptamine (6, 7, 10). Other interesting molecules that bind TAAR1 are amphetamines and the major extracellular dopamine metabolite 3-methoxytyramine (3-MT) (6, 10). This compound has been traditionally considered as an inactive o-methylated (by COMT) metabolite of extracellular DA but recently its role in neuromodulation and DA-independent movement control that is in part mediated by TAAR1 has been described (11).

Classical assays that evaluate cAMP concentration consist in detection of the accumulation of the second messenger inside the cell, or in the evaluation of its signaling as measured by a reporter gene. The main drawback of these techniques is their low versatility in terms of temporal monitoring of the sample. In the last few years, new biosensors based on Fluorescence Resonance Energy Transfer (FRET) and Bioluminescence Resonance Energy Transfer (BRET) have been described to measure in real-time physiological events within living cells (12–14). To monitor cAMP variations, a genetically encoded BRET biosensor has been developed (10). The exchange protein activated by cAMP (EPAC) biosensor binds

cAMP in the cell resulting in a conformational change and in an energy transfer between Rluc and YFP in a concentration dependent manner. With a similar BRET approach, it is also possible to monitor, in real time, β-arrestin2 recruitment to the D2 receptor (5). These two systems are increasingly useful in the study of two important processes in vitro that involve GPCRs signaling: cAMP levels modulation and β-arrestin recruitment to the receptor. The major advantages of these approaches are (1) the possibility to monitor the kinetics of signaling events (second by second) within live cells, (2) a direct comparison of activity for a given pharmacological compound on G protein-dependent and independent signaling events within the same cell.

2. Materials

2.1. Cell Culture and Compounds

1. Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) of fetal bovine serum (FBS, Sigma, St. Louis, MO), 10 mM of HEPES (store at 4°C) (Invitrogen), and 0.01 mg/mL of gentamicin (store at room temperature (RT)) (Invitrogen) is used for normal cell culture purpose and it is stored at 4°C. FBS is aliquoted in 50 mL tube and stored at −20°C.
2. Phenol red free Minimum Essential Medium (MEM) containing 2% of FBS, 10 mM HEPES, and 2 mM L-glutamine (Invitrogen) is used for plating the cells before the experiments and it is stored at 4°C. L-glutamine is a 100× solution stocked at −20°C in 5 mL aliquots.
3. Poly-D-lysine (Sigma) is a powder dissolved in tissue-culture water at 0.1 mg/mL and stored at 4°C. Do not discard after the use since it could be reused several times.
4. Phosphate Buffer Saline (PBS) (Invitrogen) is used to wash the cells. PBS containing calcium and magnesium (Sigma) and 0.003% (wt/vol) of ascorbic acid (Sigma) is the medium for the BRET experiments and used to dissolve the compounds (store at 4°C).
5. Trypsin at 0.25% (wt/vol) with ethylenediamine tetraacetic acid (EDTA) (1 mM) (Invitrogen) is used to detach the cells and is kept at 4°C.
6. Burker chamber (Blaubrand, Germany) to count cells and trypan blue solution at 0.4% (Sigma) to stain live ones.
7. Human embryonic kidney 293 cells (HEK293T) (Invitrogen).
8. Coelenterazine *h* (Promega, Madison, WI) is dissolved in anhydrous ethanol to a final concentration of 1 mM and it must be

stored at –20°C with some desiccant compound such as silica gel to keep the humidity at low levels. Coelenterazine *h* is light sensitive and the working solutions must be prepared immediately before the addition to the plate.

9. 3-Isobutyl-1-methylxanthine (IBMX), β-phenylethylamine (β-PEA), 3-methoxy-tyramine (3-MT), dopamine (DA), forskolin, haloperidol, quinpirole, and isoproterenol are from Sigma. IBMX is prepared at 200 mM in DMSO (1,000×) and stored at –20°C. Forskolin is dissolved at 10 mM in DMSO as stock solution and it is stable at RT at least for 6 months. All the other compounds are made ready at 10 mM in PBS (plus ascorbic acid—see Subheading 4) and then diluted to the desired concentration 1:10 each time in PBS except haloperidol that has to be dissolved initially in 10 mM in DMSO.
10. Cells are plated in a culture-treated 96-well plate (Corning, Corning, NY), white and clear-bottom (catalogue number 3610). Before the experiments a white backing tape (Perkin Elmer-catalogue number 6005199) has to be placed to cover the bottom of the plate.

2.2. Plasmids and Transfection

1. Plasmids containing the cDNA for the human Trace Amine Associated Receptor 1 (hTAAR1) were obtained from the cDNA Resource Center at the University of Missouri-Rolla and the American Type Culture Collection (Manassas, VA). A variant of the wild-type form is used to improve the expression at the plasma membrane (10).
2. HA-tagged D2 Long dopamine receptor is fused to *Renilla* Luciferase at the C-terminus (5).
3. Mouse β-arrestin2 is fused to YFP at the C-terminus (5).
4. The BRET biosensor EPAC is a modification from an existing FRET biosensor (10).
5. pRluc-N or pRluc-C vectors are from Perkin Elmer (Downers Grove, IL).
6. pEYFP-N or pEYFP-C vectors are from BD Biosciences (San Jose, CA).
7. pcDNA 3 is from Invitrogen.
8. Regarding calcium-phosphate transfection, calcium chloride (Sigma) is dissolved at 2.5 M in Milli-Q water. HEPES buffered saline (HBS) 2× is composed of sodium chloride (Sigma) (2.5 M), HEPES (0.5 M), and sodium phosphate dibasic (1 M) (Sigma). The pH of the solution is adjusted to 7.1. Both solutions should be stored at 4°C and kept sterile by opening only under the cell culture hood.

2.3. Experimental Machine and Software

1. To read the plate use the Mithras LB940 (Berthold Technologies, Germany) or the Infinite F500 (Tecan, Switzerland) with a MicroWin 2000 or an i-control software respectively.
2. Data are analyzed using GraphPad Prism 5.

3. Methods

EPAC cAMP biosensor, as well as β-arrestin2 recruitment to D2R, relies on the same principle of function that is BRET. BRET is a form of non-radiative energy transfer between a donor (*Renilla* Luciferase, *R*luc) and an acceptor (YFP) (15). Different couples of donor and acceptor exists, please see ref. (16) for review. Upon oxidation of its substrate coelenterazine *h*, *R*luc emits light with a peak at 475 nm. If the donor is in the proximity of the acceptor (around 0.1 nm), the energy emitted from Rluc can excite the YFP that in turn emits light with a peak at 530 nm. The change in the ratio between emissions by *R*luc and the YFP reflects a change in the distance between the two molecules. In the EPAC biosensor, at basal or low cAMP levels, *R*luc and YFP are in close proximity resulting in a constitutive elevated BRET signal. When cAMP binds EPAC, a conformational change occurs increasing the distance between the donor and the acceptor resulting in a decreased change of BRET ratio. In β-arrestin2-D2R experiments, when DA or another agonist stimulates the receptor (D2-*R*luc), β-arrestin2-YFP is recruited from the cytosol to the plasma membrane (to bind D2R) and this translocation results in an increase of BRET ratio. In this way, it is possible to study in real time these physiological events by integrating the light emission by the two tags with appropriate filters (17). Regarding TAAR1, EPAC biosensor has been shown to be a useful tool in studying trace amines pharmacology and in finding new agonists of the receptors (10, 18). It has demonstrated a good sensitivity and very similar results compared to other techniques (β-PEA and 3-MT potencies to TAAR1). Similarly, β-arrestin2 recruitment to the D2R as measured by BRET assay has been validated as an effective method to study this signaling pathway in antipsychotics pharmacology (5).

3.1. Cells Seeding and Transfection

1. The wild type HEK-293T cells are cultured in 150-mm dishes with DMEM for the maintenance of a certain number of cells according to the experiments planned. The medium should be warmed in a 37°C bath for at least 20 min before using it. Cells are passaged and seeded in 100-mm dishes the day before the transfection using trypsin-EDTA. All the media and solutions must be opened only under the hood (see Note 1).

2. Wash with 10 mL of PBS the 150-mm dishes, add 2 mL of trypsin-EDTA to each dish and let the trypsin to act for 1–5 min at RT.
3. Add about 5 mL of DMEM in order to inactivate trypsin and to help cell harvesting. Gently pipette up and down to detach all the cells from the dish and to break cells lumps.
4. Pour the cells in a 15 mL Falcon tube and count them. Mix 20 μL of cells and 20 μL of trypan blue solution on a piece of parafilm and then pipette 20 μL in the bunker chamber and count the cells under the microscope. Make sure to make a homogeneous cell suspension in the medium with the pipette before collecting the 20 μL in order to have a representative concentration of the solution.
5. After counting the number of cells/mL seed about 3 million of cells for each 100-mm dish in 10 mL of DMEM (see Note 2). Each dish will be used for a single transfection, including control mock transfection with an empty vector (see Note 3).
6. The morning after the seeding, cells will be transfected. The solutions required are the $CaCl_2$ (2.5 M), the HBS 2× and water. All the solutions must be sterilized by filtration with a 0.22 μm filter and then opened only under the hood. All the working aliquots of these solutions (10–50 mL) should be kept in a refrigerator and for more reproducible results equilibrated to room temperature (RT) for 30 min before using them. The remaining aliquots of HBS 2× should be stored at −20°C (see Note 4).
7. Take the DNA tubes out of the freezer (see Note 5) and let them thaw at RT.
8. Place 15 mL Falcon tubes under the hood in a rack, one for each transfection.
9. Put the DNA in each tube. It is important to have the same amount of DNA for each transfection, so equilibrate with empty vector (pcDNA 3). Usually, the amounts for a 1 mL of solution, that is enough for a 100-mm dish, are as follows: EPAC (3 μg), TAAR1 (5 μg), D2R (2 μg), and β-arrestin2 (2 μg). Regarding D1R, they have sufficient endogenous expression in HEK-293T cells to elicit a response detectable by BRET upon their stimulation.
10. Add 50 μL of $CaCl_2$ 2.5 M and enough water to have a total volume of 0.5 mL containing the DNA as well.
11. Pipette up and down for few times to mix the solution.
12. Add 0.5 mL of the HBS 2× drop by drop to each tube and mix by bubbling air inside the solution with the P1000 pipette for 10–15 times.

13. Add the transfection solution drop wise to each dish, gently and trying to cover the entire plate. Rock the dishes back and forth for a few times before replacing them in the incubator. After few hours, a small dark precipitate should be observed in the cells under the microscope.

3.2. Plating

1. Cells are normally plated in the 96-well plate after 16–24 h, but for TAAR1 it is better to plate the same day, in the afternoon, so as to do the experiment 1 day after the transfection since TAAR1 is partially degraded in vitro (10).
2. After 4–6 h post transfection for TAAR1 and 16–24 h for dopamine receptors and β-arrestin2 (see Note 6) cells have to be plated.
3. The assay plate is a culture-treated, white, clear-bottom 96-well plate. The plate is sterile so it has to be properly handled under the hood. At this step the phenol red free Minimum Essential Medium (MEM) containing 2% of FBS, 10 mM HEPES, and 2 mM L-glutamine should be used to avoid color in the medium that could disturb the light emission during the experiment ("clear medium").
4. Place the poly-D-lysine and the PBS under the hood.
5. While pre-warming the medium, pipette approximately 100 μL of poly-D-lysine in each well of the 96-well plate and incubate for 10–20 min (see Note 7).
6. After this period re-collect the poly-D-lysine with the pipette and wash once all the wells with PBS (about 150 μL for each well). Then remove the remaining PBS with a vacuum aspirator.
7. Wash twice the transfected cells in the 100-mm dishes with PBS and detach them by pouring 1 mL of trypsin-EDTA for each dish.
8. After 5 min of incubation (see Note 8), add 4 mL of clear medium in each dish. Gently pipette the cells up and down to detach them. Pour them in a labeled 15-mL Falcon tube, one for each transfection.
9. Spin the tubes in a centrifuge for 5 min at 200 × *g* to pellet the cells.
10. Remove the medium from the tube and resuspend the cells with 1 mL of clear medium and count the cells for each tube as described above.
11. Then dilute the cells solutions of each tube with the clear medium so as to have a concentration of 750,000 cells for each mL.
12. Put 100 μL of cells suspension for each well in the assay plate for all the different transfections, according to the experiment

design. This step can be done either using a normal p100 pipette or a repeater pipette (see Note 9).

13. The 96-well plate is stored in the incubator for at least 12 h before the experiment.

3.3. BRET Experiment

24–48 h post-transfection the EPAC sensor and the other proteins should be sufficiently expressed. Since *R*luc produces a signal independently from BRET, an internal control of the level of transfection is represented by the basal *R*luc counts. For Mithras, 100,000 counts are the minimum for a good signal to noise ratio.

1. Pre-warm to RT or to 37°C (depending on the planned experiment) the PBS with calcium and magnesium and add 0.003%(wt/vol) of ascorbic acid. 30–50 mL of PBS solution is usually enough for one 96-well plate and preparation of the test compounds.
2. The compounds should be freshly prepared even if some compounds are stable for some time if stored at –20°C in working aliquots. For example IBMX is dissolved in dimethylsulfoxide (DMSO) at the concentration of 200 mM (1,000× stock) and it could be stored at –20°C in small aliquots (e.g., 200 µL). Prepare all compounds to a concentration of 10 mM in the same PBS with the ascorbic acid and then make the suitable dilutions. β-PEA (TAAR1 agonist) is soluble in water and PBS in the same way as 3-MT (TAAR1 agonist), isoproterenol (β2-adrenergic receptor agonist), quinpirole hydrochloride (D2R agonist). Haloperidol (D2R antagonist) is soluble at 10 mM in DMSO but the subsequent dilutions (1:10, 1:100, 1:1,000, and so on) should be made in PBS (see Note 10).
3. In case of experiments at RT, all the media and solutions should be equilibrated at RT. In case of 37°C assay, switch on the plate reader heating system at least 20 min before the experiments and keep the PBS warm in a 37°C water bath (see Note 11).
4. Take the plate out of the incubator and check the cells under the microscope. Before proceeding with BRET, stick the white backing tape to the bottom of the plate to prevent light dispersion.
5. Remove the clear medium from all the wells and replace it with the PBS with calcium, magnesium and ascorbic acid.
6. Dilute the coelenterazine *h* stock solution 1:20 in PBS (10× solution) and add to each well to yield a final concentration of 5 µM (see Note 12).
7. For the evaluation of cAMP levels using the EPAC sensor with TAAR1, D1R or in general with a G_s-coupled receptor, after adding coelenterazine, if necessary, also add IBMX to a final concentration of 200 µM (see Note 13).

8. To evaluate agonist activity, such as β-PEA or 3-MT on TAAR1, add the compounds 10 min after coelenterazine *h* (see Note 14) and immediately read the plate. If the experiment is conducted at 37°C, place the plate in the incubator during the lag of time before the agonist addition. β-PEA and 3-MT are active at low micromolar concentration (see Fig. 1a, b).
9. To evaluate antagonistic activity against a known agonist (e.g., putative antagonists of TAAR1), the different compounds are added 5 min before the agonist.
10. In case of unknown activity of the compounds or of the receptor, a positive control could be made with the endogenously expressed β2-adrenergic receptor and the agonist isoproterenol

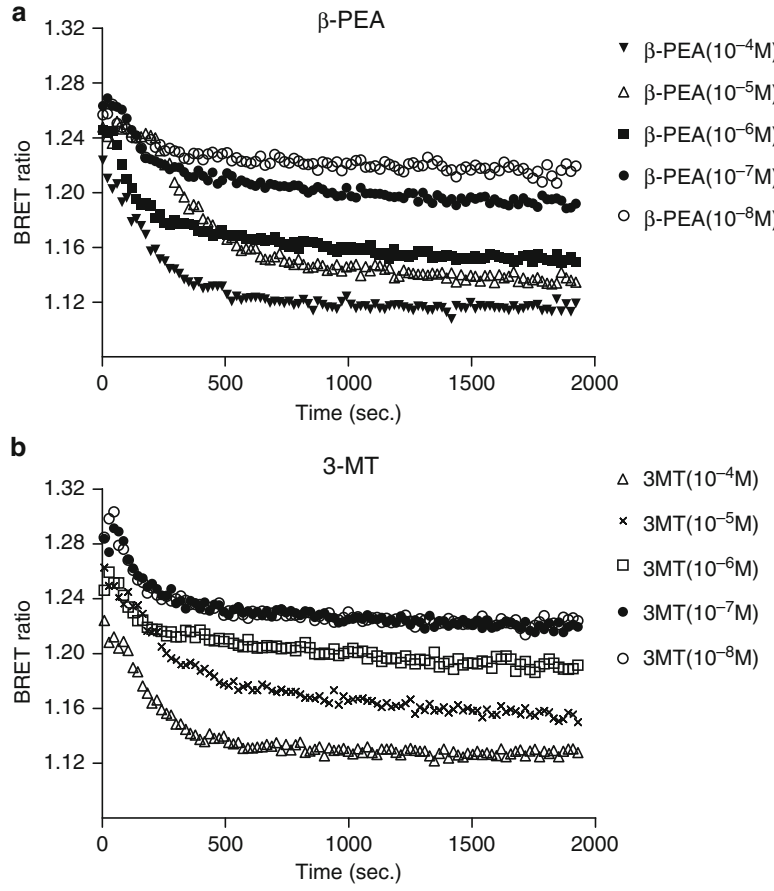

Fig. 1. Evaluation of cAMP levels using cAMP BRET biosensor in HEK-293T cells expressing hTAAR1. (**a**) Time course evaluation of cAMP variations using different concentrations of β-PEA. BRET ratio is calculated as *R*luc/YFP ratio and the reading started after β-PEA addition. The decrease in BRET ratio indicates an increase in cAMP levels. This experiment is performed at RT with the addition of 200 μM of IBMX. (**b**) Similar experiment using 3-MT as TAAR1 agonist.

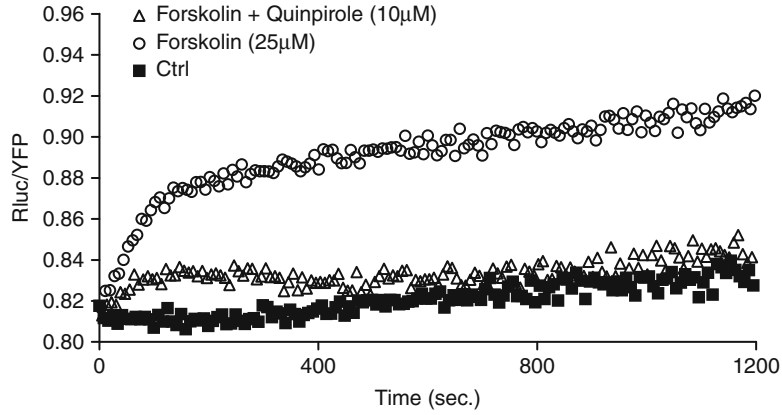

Fig. 2. Evaluation of D2R activation using EPAC BRET biosensor at 37°C. Time course of cAMP variations in HEK-293T cells expressing D2R and EPAC. Forskolin is added at the beginning of the experiment at the time = 0. Quinpirole at 1 μM is added 5 min before forskolin and 5 min after coelenterazine h (see Subheading 3). Forskolin, by the activation of adenylate cyclase, increases cAMP levels and this effect is prevented by D2R activation with quinpirole.

that is active at nanomolar concentrations. Alternatively, the activator of adenylate cyclase forskolin may be used (2–20 μM).

11. For receptors such as D2R, that inhibit adenylate cyclase and the formation of cAMP, forskolin at 2–20 μM is used to increase basal cAMP level (see Note 15).
12. Add quinpirole at 1 μM 5 min after *R*luc substrate, forskolin 5 min after quinpirole and then read the plate immediately (Fig. 2) (see Note 16).
13. In case of evaluation of antagonists on quinpirole effect, these compounds are added simultaneously with coelenterazine *h*. For example, for a haloperidol dose–response, add in each well different concentrations from 10^{-11} to 10^{-5} M (Fig. 3a).
14. For the evaluation of β-arrestin2 recruitment to D2R, the incubations are carried out at RT but the protocol remains the same, add antagonists 5 min and agonist 10 min after *R*luc substrate and read the plate right after agonist addition. Haloperidol is active in preventing β-arrestin2 recruitment at low nanomolar concentrations (Fig. 3b).
15. Usually the plate is read for 20–30 min but, depending on the receptor, the time course could be longer or shorter (see Note 17).

3.4. Plate Reader Settings and Data Analysis

The plate reader used for this assay is the Mithras LB940 that allows the integration of the luminescent signal detected in the 465–505 nm and 515–555 nm windows by using filters with the appropriate band pass and the MicroWin software. The BRET

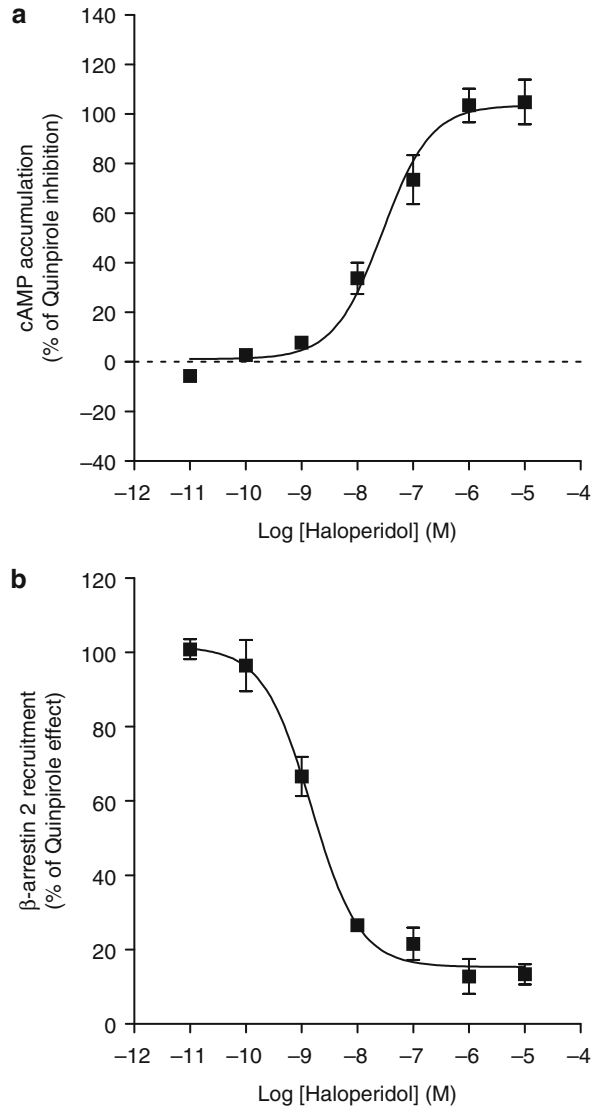

Fig. 3. Evaluation of antagonistic activity of haloperidol on G_i activation or on β-arrestin2 recruitment induced by quinpirole. (**a**) HEK-293 T cells are transfected with EPAC biosensor and D2R and are treated with different concentrations of haloperidol and with 1 μM of quinpirole in the presence of forskolin. (**b**) Dose–response of haloperidol to evaluate the inhibition of β-arrestin2 recruitment induced by 1 μM of quinpirole. Cells are transfected with D2R-*R*luc and β-arrestin2-YFP. Data are plotted as SEM of 3–5 independent experiments.

signal is determined by calculating the ratio between the light emitted at 465–505 nm and the light emitted at 515–555 nm. The data are plotted as BRET ratio over the time in time course experiments. In dose–response graphs, curves are fitted using a nonlinear regression and log vs. response fit using GraphPad Prism 5 (Fig. 4). In the experiment regarding D2R antagonist effect on

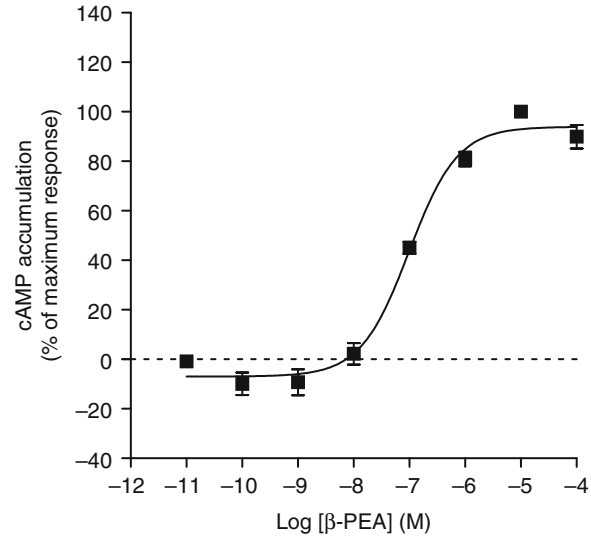

Fig. 4. cAMP variations induced by a β-PEA dose–response in cells co-expressing TAAR1 and EPAC. Cells are treated with different concentrations of β-PEA and plotted as a dose–response experiment. β-PEA is used at concentrations ranging from 10^{-11} to 10^{-4} and the plate is read 15 min after agonist addition. A non-linear regression with one site-specific binding is used to draw the curve using GraphPad Prism5. Data are plotted as SEM of 3–5 independent experiments.

quinpirole activity, in the *y*-axis BRET ratio is transformed in the percent of quinpirole effect (Fig. 3a). In β-arrestin2 recruitment, the D2R antagonists should be plotted with the percentage of quinpirole effect over the log of the concentration of the antagonist (Fig. 3b) (see Note 18).

4. Notes

1. All the media, solutions and instruments used for cell culturing should be maintained sterile. It is important to open the medium only under the hood and clean every object by spraying some ethanol 70°C before bringing them under the hood. Bacteria or yeast contaminations of cell dishes are easily detected by looking at them under the microscope or by looking at the color and clearness of the medium. The water and the solutions for the transfection must be sterilized by filtration under the wood with a 0.22 μm filter. The water used for all procedures is a Milli-Q grade water, with a resistivity of 18.2 MΩ-cm and total organic content of less than 5 parts per billion.
2. Depending on the speed of growth of the clone of HEK-293T cells or the time of the day of seeding it is possible to seed less

or more cells. It is important to have cells at 70–80% confluency the day of transfection.

3. Since each well (of the 96-well plate) should contain approximately 75,000 cells, usually one 100-mm plate is enough for single transfection to yield a full 96-well plate. In case of multiple transfections and when the experiment design calls for fewer cells, 35-mm dishes can be used to transfect cells. In this case the procedures remain the same by just scaling down all the reagents and solutions used proportionally. In contrast, when many cells with a single transfection are needed, more than one 100-mm dish could be used. In order to have a homogeneous sample, mix all the similar transfections together before seeding them in the assay plate.
4. The critical parameter for the success of the transfection is the pH of the HBS 2× solution. It is suggested to prepare a fairly good amount of solution since it could be tedious to find the right pH each time the solution has to be prepared. After all the reagents are dissolved, the pH should be adjusted to 7.1 ± 0.05. Since there are some variables such as incubator or pH meter dysfunctions or unpredictable ones, it is advisable to prepare different HBS 2× solutions in a range of pH from 7.0 to 7.2 and test them in different transfections in order to find the one with the highest transfection efficacy. Then sterilize by filtration and aliquot into 50 mL Falcon tube and store at –20°C. Working aliquots can be stored at 4°C.
5. Normally, keep the DNA at –20°C but for long-term storage put it at –80°C. Be sure that the solution is clear and there are no precipitate or strange materials that could indicate the presence of molds. In such a case, it is imperative to re-prepare the plasmid.
6. For TAAR1 it is better to do the experiments 24 h after transfection. D2R and β-arrestin2 also have a strong protein expression after 24 h of transfection, so in cases of co-transfecting TAAR1 and D2R, it is possible to do experiments at 24 h.
7. Sometimes the poly-d-lysine treatment is prolonged for hours, for low adherent cells, but usually for HEK cells 10–20 min should be enough. It is possible to reuse the poly-d-lysine up to three times, so re-collect it and keep it sterile in the refrigerator after two uses.
8. Some HEK clones are less or more “attached” to the dish. In some case, while passaging cells, PBS is enough to detach the HEK cells; in other cases trypsin is necessary. The latter is especially the case after a calcium-phosphate transfection.
9. With these dilutions, 75,000 cells/well is seeded and should be enough to have a good signal to noise ratio. The number may change from 25,000 to 100,000 per well depending on

the BRET signal, cells availability (e.g., in case of BRET experiments in primary neurons) and other variables.

10. To minimize the final concentration of DMSO to a maximum of 0.1–0.5% in order to avoid DMSO toxicity, haloperidol and other water-insoluble compounds should be diluted as much as possible in PBS. Appropriate controls should be used to verify the inactivity of the dissolving medium.
11. For β-arrestin2 recruitment to D2R, RT experiment is more convenient since the recruitment is slower and more visible when analyzing the data. cAMP evaluation with EPAC sensor could be done at 37°C or even at RT. The desensitization of the cAMP response of the receptor of interest is more readily observable at 37°C.
12. For BRET experiments, it is convenient, in term of calculations, to have a final volume of 100 µl. In this case, add 10 µL of coelenterazine *h* and prepare all compounds as 10× solutions in Eppendorf tube in order to add 10 µL for each of them that have to be tested. In this case, make the calculations of how many microliters of PBS that have to be added at the beginning of the experiment (e.g., for testing 1 agonist (10 µL) and 1 antagonist (10 µL), plus coelenterazine *h* (10 µL) and IBMX (10 µL), put 60 µL of PBS in each well in order to attain a final volume of 100 µL, $10+10+10+10+60=100$).
13. To increase cAMP levels, IBMX may be used. IBMX blocks the degradation of the cAMP by endogenous phosphodiesterases and may be useful to increase the signal induced by the receptor of interest.
14. The oxidation of coelenterazine *h* by *R*luc takes several minutes to equilibrate, so to have a stable and good signal it is better to wait at least 5 min (ideally 10 min) from the addition of the substrate. If the transfection is efficient, each well of the plate consists of one data point and thus it is not necessary to perform the experiment in duplicates. Duplicate reads however are often recommended.
15. In vitro, without any stimuli, the inhibition of adenylate cyclase does not induce a significant decrease of cAMP that is detectable by our technique. So, to study G_i-coupled receptors such as D2R activity, adenylate cyclase has to be activated by forskolin.
16. Quinpirole has its maximum effect at 1 µM and the EC_{50} is 5.7 nM.
17. The effect of the agonist on cAMP production is quite fast even at RT, so it is necessary to work rapidly in order to measure the beginning of the effect. As an alternative, the injector in the machine could be used to dispense the agonist to different wells, but take into account the number of compounds

that have to be dispensed and the availability as well as the cost of the substances.

18. Another machine tested for this application is the Infinite F500 (Tecan). It has two appropriate filters to select the light emitted by *R*luc and the YFP and as the Mithras two injectors and the heating system. While the Mithras is fairly more sensitive, Infinite's software is more easy-to-use and flexible.

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