Chapter 9

Combining Conformational Profiling of GPCRs with CRISPR/Cas9 Gene Editing Approaches

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Abstract

Ligand-biased signaling could have a significant impact on drug discovery programs. As such, many approaches to screening now target a larger section of the signaling responses downstream of an individual G protein-coupled receptor (GPCR). Biosensor-based platforms have been developed to capture signaling signatures. Despite the ability to use such signaling signatures, they may still be particular to an individual cell type and thus such platforms may not be portable from cell to cell, necessitating further cell-specific biosensor development. We have developed a complementary strategy based on capturing receptor-proximal conformational profiles using intra-molecular BRET-based sensors composed of a *Renilla* luciferase donor engineered into the carboxy-terminus and CCPGCC motifs which bind fluorescent hairpin biarsenical dyes engineered into different positions into the receptor primary structure. Here, we discuss how these experiments can be conducted and combined with CRISPR/Cas9 genome editing to assess specific G protein-dependent and -independent events.

Key words GPCRs, BRET, Genome-editing, Conformation-sensitive biosensors, CRISPR, Drug discovery

1 Introduction

Despite the broad physiological and clinical significance of G protein-coupled receptors (GPCRs), their signaling properties in their native cellular contexts remains incompletely understood, which presents a critical impediment to drug discovery. Individual cells express numerous GPCRs which can couple to diverse signaling pathways with convergent effects on several downstream processes such as cell division, cell growth, and other phenotypic parameters. While profiling of signaling events has been shown to be of significant utility in designing and performing drug screens [1–4], it is likely that a "one size fits all" approach will not work as we move from one cell type to another. The ability to measure GPCR activity directly at the level of the receptor allows us to

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complement such screens and may help in decisions about whether or not to expand panels of signaling biosensors [5–8].

We have engineered FlAsH-BRET (bioluminescence resonance energy transfer) conformational biosensors into several GPCRs [5–8] and have used them to characterize specific conformational changes that occur in response to ligand or protein interactions. Specific conformational states are likely associated with the activation of different downstream signaling pathways and these state transitions can be detected in real-time in live cells using the FlAsH-BRET method described below. Such profiles do not require knowledge of signaling pathways a priori. Briefly, this technique relies on BRET between a bioluminescent enzyme, *Renilla* luciferase II (RlucII) and a small molecule dye called FlAsH which can bind a short peptide sequence CCPGCC [9, 10]. As the efficiency of energy transfer from the donor (RlucII) to the acceptor (FlAsH) is a function of distance and orientation of the donor and acceptor moieties, it is sensitive to conformational rearrangements within the receptor. FlAsH only fluoresces when bound selectively to the tetracysteine tag above, which can be introduced at multiple locations within the receptor by insertional mutagenesis. By fusing RlucII to the receptor C-tail and inserting FlAsH binding motifs throughout different intracellular loops using insertional mutagenesis, panels of biosensors can be generated to capture different conformational "perspectives" of a receptor of interest in response to ligand or allosteric modulators.

Biosensors designed in our lab for the prostaglandin F (FP), angiotensin II type 1 (AT_1R), and β_2-adrenergic receptors show this approach to be robust and reproducible [5, 6, 8]. Such biosensors could differentiate between balanced or biased ligands with different profiles demonstrating that these assays can be used to guide drug discovery efforts [6]. We also used the technique to study allosteric crosstalk between receptor dimers and with intracellular protein partners [7]. We feel that the FlAsH-BRET approach offers simple and robust insights into the conformational dynamics of GPCRs in a live cell context. Such insights can help understand the complex mechanisms linking receptor conformations and functional outputs as measured using signaling outputs.

1.1 Generation of FlAsH-BRET-Based Conformation-Sensitive Biosensors

The precise strategies to generate FlAsH-BRET-based, conformation-sensitive biosensors within the angiotensin II type I receptor (AT_1R) and β_2-adrenergic receptor (β_2AR) have previously been published [5–8]. To summarize here, the wild-type AT_1R sequence was used as template for overlap PCR in order to introduce a signal peptide and a FLAG-tag at the 5′ end of the receptor and to insert the FlAsH-binding tetracysteine tag, CCPGCC at various different positions within the coding sequence of the receptor. The resulting PCR product was ligated in a restriction enzyme compatible pIRESH plasmid which contained a *Renilla* luciferase

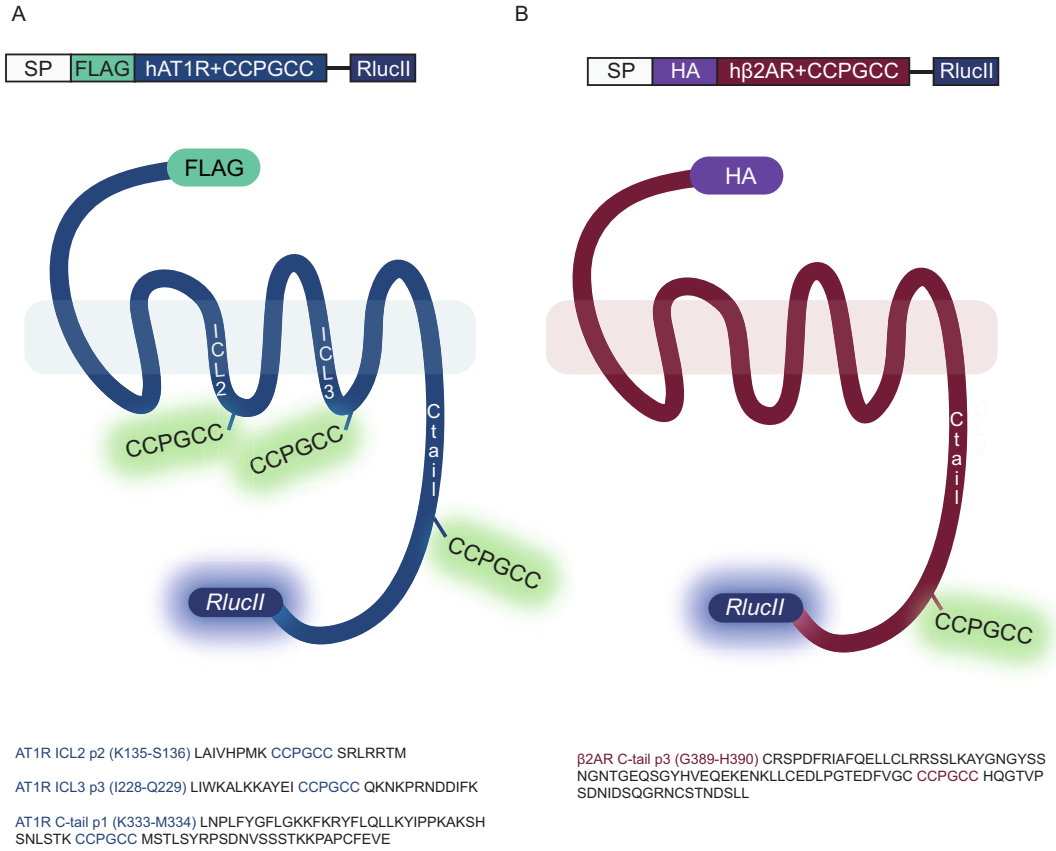

Fig. 1 Cartoon depiction of FlAsH BRET-based conformation sensitive biosensors. Both (**a**) AT_1R and (**b**) β_2AR constructs contain an upstream signal peptide for cell surface targeting as well as a FLAG- or HA-tag for confirming membrane localization. The *Renilla* luciferase donor was introduced onto the C-termini of both the AT_1R and the β_2AR. Within the coding sequence of the AT_1R there is one FlAsH acceptor insertion site located in the second intracellular loop (ICL2 p2), one within the third loop (ICL3 p3) as well as one in the C-tail (C-tail p1). For the β_2AR, the tetracysteine motif was introduced in the distal portion of the C-tail (C-tail p3). This BRET-based biosensor configuration allows to detect relative changes in receptor conformation upon agonist stimulation

construct fused at the 3′ end. Sanger sequencing was performed to confirm the sequence of the resulting plasmids containing SP-Flag-hAT_1R-CCPGCC-RlucII in pIRESH backbone vector (Genome Québec). A similar strategy was used to construct FlAsH-BRET sensors in the β_2AR. The sensors used in this paper are shown schematically in Fig. 1.

This biosensor configuration is ideal to capture the conformational flexibility within a receptor. Introducing the tetracysteine tag at positions distributed within different loops of the receptor is favorable in order to have multiple conformational perspectives to sample from. The small size of the FlAsH motif enables us to

capture conformational dynamics within a receptor without perturbing the native function of the receptor. However, it is wise to generate multiple FlAsH insertion sites within the distinct intracellular loops and carboxyl terminus since certain insertions may disturb wild-type function and will be rendered useless for subsequent analysis. Combining such biosensor-based approaches with genome editing techniques such as CRISPR/Cas9 can be used to interrogate the ability of different G proteins to effect basal and ligand- or allosteric-partner-mediated effects on receptor conformation [5–7]. Here, we compare parental HEK 293 cells, with CL4-4 cells deleted for most Gα subunits.

1.2 CRISPR/Cas9 Genome Editing for Generation of the Δ G Total Cell Line

CL4-4 cells lacking three G protein families (Gαs, Gαq/11 and Gα12/13, and a part of the Gαi family, namely Gαi2) were generated and characterized by sequencing of sgRNA-targeted loci as described [11]. The phenotypes for the loss of Gαs [12, 13] and Gαq/11/G12/13 [6] were characterized as previously described (reviewed in [14]). In addition, Gαi-mediated signaling, as measured by an in-house-modified Glo-sensor assay was reduced as compared with the triple-family-KO cells from which CL4-4 cells were derived. Full loss of Gαi signaling was achieved by treating these cells with PTX [11], and as described below.

2 Materials

2.1 Cell Culture

1. Tissue culture incubator.
2. Biosafety cabinet.
3. T75 flasks.
4. 6-Well dishes.
5. White flat bottom 96-well plates.
6. Dulbecco's Modified Eagle Medium, DMEM.
7. Fetal bovine serum, FBS (Wisent, QC).
8. 0.25% Trypsin-EDTA.
9. Penicillin-streptomycin.
10. Lipofectamine 2000 (Invitrogen).
11. Poly-L-ornithine.
12. Pertussis toxin (Sigma-Aldrich).

2.2 FlAsH Labeling Procedure

1. Chemical Fume Hood.
2. 37 °C incubator.
3. 1.5 mL Eppendorf tubes.
4. 15 mL conical tubes.

5. 50 mL conical tubes.
6. Multi-channel or repeater pipette.
7. Pipette wash basin.
8. Aluminum foil.
9. 1, 2-Ethanedithiol (EDT, Sigma-Aldrich) (*see* **Note 1**).
10. Dimethylsulfoxide (DMSO, Sigma-Aldrich).
11. FlAsH reagent (Invitrogen). Store at −20 °C protected from light.
12. Hank's balanced salt solution 1× (HBSS) without phenyl red, with sodium bicarbonate, calcium, and magnesium. Store at 4 °C and keep sterile (Wisent, 311-513-CL).
13. 25 mM 2,3-dimercapto-1-propanol (BAL wash buffer, Invitrogen). Store at 4 °C (*see* **Note 2**).
14. Kreb's buffer: 146 mM NaCl, 4.2 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES pH 7.4, 0.1% glucose. Store at room temperature, in an environment protected from light.
15. 1× Phosphate Buffered Saline: 137 mM NaCl, 2.7 mM Potassium Chloride, 8 mM Sodium Phosphate dibasic, 2 mM Potassium Phosphate monobasic, pH 7.4.

2.3 BRET Assay

1. A filter-based detection plate-reader that can detect light sequentially at two wavelengths equipped with an injector unit.
2. Timer.
3. Coelenterazine h (powder form, NanoLight Technologies) Reconstitute in 100% ethanol at a 1 mM stock concentration. Aliquot the solution and keep aliquots at −80 °C for long-term storage. It is preferable to store aliquots in dark screw cap tubes to protect the substrate from light (*see* **Note 3**).
4. Ligands of interest.

3 Methods

3.1 Cell Culture

1. *Day 0*—Grow stock flask of parental HEK 293 cells [6] and CL4-4 cells to ~80% confluency in DMEM with 5% fetal bovine serum (vol./vol.) at 37 °C in a humidified atmosphere with 95% air and 5% CO_2 (*see* **Note 4**).
2. *Day 1*—Plate 200,000 parental cells and 800,000 CL4-4 cells in 2 mL DMEM + 5% FBS in a 6-well plate (*see* **Note 5**).
3. *Day 2 AM*—Transfect cells with 1.5 μg total plasmid DNA per well using Lipofectamine 2000 according to the manufacturer's instructions. Briefly, for one well, combine 1 μg of the FlAsH BRET-based conformation-sensitive biosensor plasmid

DNA, in a tube, with 0.5 μg empty vector pcDNA3.1 and complete to 100 μL with DMEM. The Lipofectamine to plasmid DNA ratio should be maintained at 2:1. In a separate tube, add 3 μL Lipofectamine 2000 with 97 μL of DMEM. Wait 5 min and then mix the DNA tube with the Lipofectamine tube for a total volume of 200 μL. Wait 30 min for the lipid–DNA complexes to form. Add this transfection solution drop-wise onto the cells (*see* **Note 6**).

4. *Day 2 PM*—Change media to 2 mL DMEM + 5% FBS 5 h post transfection.
5. *Day 3 AM*—Replate 30,000 parental and CL4-4 cells per well in a poly-L-ornithine coated white 96-well plate (*see* **Note 7**).
6. *Day 3 PM*—Wait approximately 5–6 h for the cells to adhere to the 96-well plate. For conditions where cells must be pre-treated with PTX, prepare a solution of 100 ng/mL PTX in DMEM + 5% FBS. Aspirate the media and add 100 μL of this 100 ng/mL PTX solution per well. Incubate cells overnight with PTX for at least 16 h in a 37 °C incubator (*see* **Note 8**).
7. *Day 4 AM*—Follow the FlAsH labeling procedure (*see* below), which takes roughly 1.5–2 h to complete.

3.2 FlAsH Labeling Procedure

1. In a water bath, heat the HBSS to reach a working temperature of 37 °C.
2. Make a 25 mM solution of 1,2-ethanedithiol (EDT). To avoid pipetting small volumes, first prepare a 1 M solution of EDT by diluting it in DMSO as follows; in an Eppendorf containing 55 μL DMSO, add 5 μL of 12 M EDT.
3. Mix by vortex.
4. Further dilute the 1 M EDT solution in DMSO to make a 25 mM solution of EDT. As such, pipette 12.5 μL of 1 M EDT into 487.5 μL DMSO.
5. Mix by vortex (*see* **Note 9**).
6. Make the FlAsH-EDT_2 solution. Add one volume of FlAsH reagent (2 mM) to two volumes of 25 mM EDT to make a 667 μM FlAsH-EDT_2 solution (*see* **Note 10**).
7. Incubate the FlAsH-EDT_2 solution for 10 min at room temperature.
8. Then, add 100 μL of HBSS to the 667 μM FlAsH-EDT_2 solution.
9. Continue the incubation for 5 min at room temperature.
10. Complete the volume with HBSS to make a solution with final concentration of 750 nM FlAsH-EDT_2.
11. Mix by vortex.

12. Prior to the FlAsH labeling, wash cells with 150 μL of HBSS (*see* **Note 11**).
13. Aspirate the HBSS from each well.
14. Add 50–60 μL of the 750 nM FlAsH-EDT_2 solution and incubate for 60 min at 37 °C, protected from any source of direct light. Due to the unpleasant FlAsH-EDT odor, prior to incubation at 37 °C, wrap the 96-well plate with parafilm. This will also prevent accidental spills.
15. Wash cells with 2,3-dimercapto-1-propanol (BAL). Make 100 μM BAL wash buffer by pipetting 40 μL of 25 mM BAL solution in 10 mL HBSS. You may adjust the final volume of BAL wash buffer depending on the number of wells labeled on your assay plate.
16. Mix by vortex.
17. Aspirate the FlAsH-EDT_2 labeling solution.
18. Wash cells twice with 100 μL of a 100 μM BAL wash buffer diluted in HBSS buffer. Incubate the first wash for 10 min at 37 °C. Perform the second wash without incubation.
19. Aspirate the BAL-wash solution.
20. Wash cells once with 150 μL of Kreb's assay buffer.
21. Let cells sit in 80 μL of Kreb's buffer for 2 h at room temperature, in an environment protected from light, prior t o the BRET assay (*see* **Notes 12** and **13**).
22. Proper and safe disposal procedures must be taken when discarding the FlAsH and EDT particularly since FlAsH is complexed with arsenic. All solutions containing FlAsH-EDT_2 must be re-collected and disposed of according to the regulations in place by waste management facilities at your institution.

3.3 Setting Up the Assay Protocol

3.3.1 Preparation of the Stock Ligand Concentrations

For angiotensin II (Sigma-Aldrich), dissolve it in ddH_2O to make a stock concentration at 10 mM. For stability purposes, aliquot and store at −20 °C. For isoproterenol (Sigma-Aldrich), dissolve it in 100 mM ascorbic acid to make a stock concentration at 100 mM. For stability purposes, aliquot and store at −20 °C. We recommend using fresh aliquots of isoproterenol, as it is not always stable even when frozen.

3.3.2 Preparation of the Working Ligand Concentrations

Dilute the concentrated ligand stock to make a saturating concentration of the agonist (*see* **Note 14**). For angiotensin II, use at 1 μM final concentration. For isoproterenol, use at 10 μM final concentration.

3.3.3 Preparing the Plate Reader

A filter-based detection plate-reader that can detect light sequentially at two wavelengths (F485, F530) with a relatively rapid read speed is optimal for kinetic-based measurements, however single point assays also work. For ideal performance, the plate reader should be equipped with injector units as they are essential for capturing kinetic readings pre- and post-ligand injection. A temperature control unit is also necessary to maintain the temperature constant for the duration of the assay. In BRET-based assays, we use white bottom microplates to maximize the amount of light collected by the detector considering that white plates reflect the light to increase the signal. In our lab, we use the TriStar2 multimode plate reader from Berthold Technologies and the Victor X Light from Perkin Elmer.

1. Set the temperature of the plate reader between 25 and 28 °C (*see* **Note 15**).
2. Wash the injectors once with Kreb's assay buffer.
3. Prime the injectors with the appropriate assay vehicle and agonist.
4. Set up the kinetic protocol.

3.3.4 Preparing the BRET Substrate

The donor moiety of the FlAsH BRET conformation-sensitive biosensors is a mutated version of the bioluminescent enzyme *Renilla* luciferase (RlucII). The production of light thus requires the oxidation of the substrate, coelenterazine h.

1. Prepare the working solution of the substrate. Dilute coelenterazine h in Kreb's assay buffer to attain a 20 μM working concentration (*see* **Note 16**).
2. Add 10 μL of the 20 μM coelenterazine h solution in 6–18 wells maximum (*see* **Note 17**).
3. Incubate 5 min at room temperature (*see* **Note 18**).
4. Start the BRET protocol on the plate reader (*see* **Note 19**).

Our basic experimental protocol is depicted in Fig. 2. Using this kinetic protocol, basal BRET readings are first collected followed by vehicle/agonist injection onto the cells where BRET is continuously monitored thereafter. The BRET ratio can subsequently be calculated by computing the F530/F485 ratio. The change in BRET as a response to the addition of the vehicle/agonist can also be computed by subtracting the pre-injection BRET from the post-injection BRET. The pre-injection or basal BRET is defined by the average of the first ten measurements and the post-injection BRET can be calculated by averaging the last ten measurements. Such changes in BRET can then give us insight on relative conformational changes within the structure of the receptor as a response to agonist. An increase or decrease in BRET after

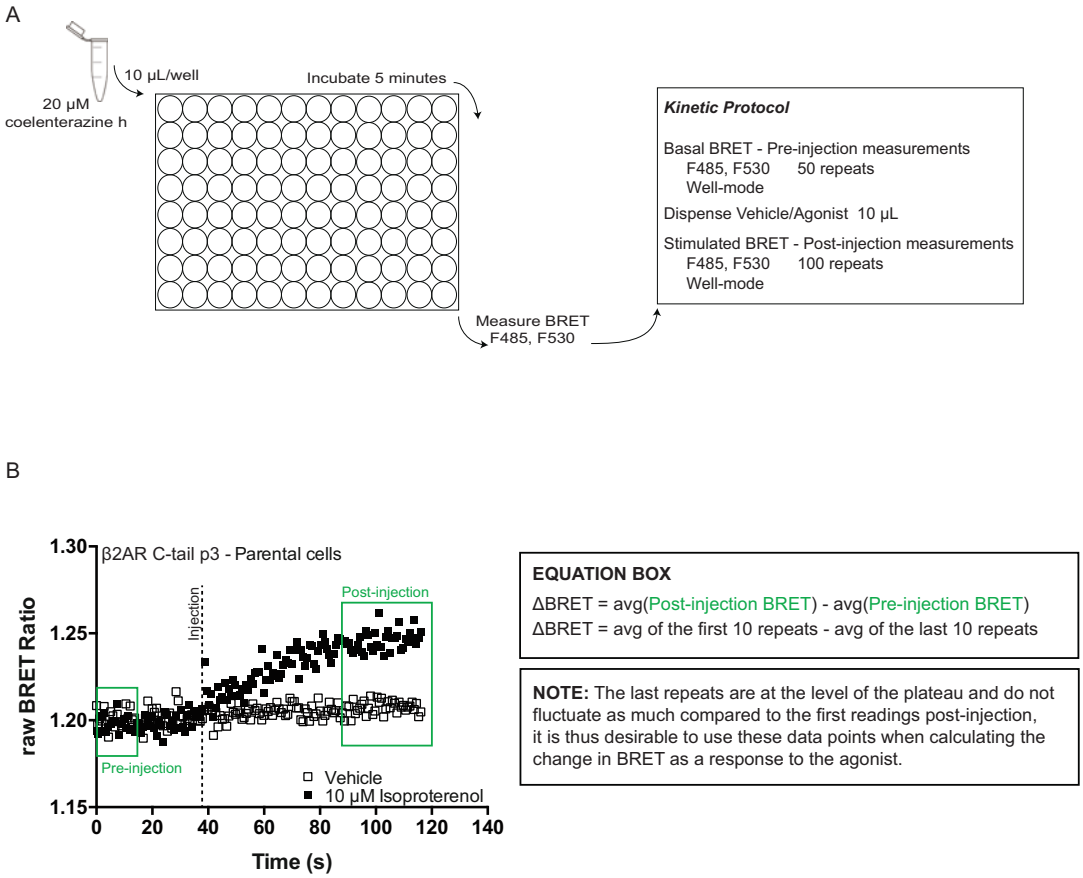

Fig. 2 Overview of the experimental protocol when performing and analyzing FlAsH BRET-based conformation-sensitive biosensor experiments. (**a**) Upon substrate addition (coelenterazine h), the bioluminescent enzyme RlucII emits light at 485 nm. If the acceptor moiety is in close enough proximity (within 10 nm), this leads to the transfer of resonance energy and the subsequent emission of light at 530 nm. (**b**) Kinetic measurements can be taken both pre- and post-stimulation by vehicle or agonist. The BRET ratio can be calculated by dividing the acceptor emission (F530) by the signal from the luminescent donor (F485). The equation box contains details on how to calculate the corresponding change in BRET as a result to vehicle/agonist stimulation as exemplified above

agonist stimulation is indicative of distinct conformational changes. Conformational profiles can then be generated by comparing the conformation at different FlAsH insertion sites giving us insights on the determinants of receptor conformation. The expression of our various biosensors in different CRISPR lines can also give us insight on the requirements of different G proteins or β-arrestins in regard to agonist-induced receptor conformation.

3.4 Data Analysis

1. Compute the BRET ratio. The software driving many plate readers collates the raw data in an excel file (.xls). The BRET ratio can then be computed by dividing the fluorescence

(FlAsH, acceptor channel, F530) by the luminescence (RlucII, donor channel, F485) as follows: $\frac{F530\ \text{FlAsH Acceptor}}{F485\ \text{RlucII Donor.}}$

2. Compute the change in BRET or ΔBRET. The ΔBRET, as referred to in this protocol, refers to the change in BRET in response to the addition of an agonist. To compute this value, we need to first calculate the pre-injection and post-injection BRET; the BRET before and after the addition of vehicle or agonist respectively. Since kinetic readings are taken, according to our assay protocol, our pre-injection BRET is the average of the first 10–50 repeats whereas the post-injection BRET is represented by the last 100 repeats following agonist injection.
3. Then, to obtain the change in BRET, subtract the pre-injection BRET from the post-injection BRET, as follows: $\Delta\text{BRET} = \text{avg.}[\text{BRET}_{\text{Post-injection}}] - \text{avg.}[\text{BRET}_{\text{Pre-injection}}]$ (*see* **Note 20**).

In these experiments, we measured conformational changes in two GPCRs induced by their respective orthosteric agonists in the presence or absence of a number of their known and putative G protein partners. We compared the parental cell line to a cell line in which Gαs, Gαq/11, Gα12/13, and Gαi2 have been knocked out via CRISPR-Cas9 genome editing. To generate a "total" Gαi phenotype, we also used pertussis toxin to inactivate Gαi proteins. As shown in Fig. 3a, b, the changes in BRET in response in the β_2AR biosensor to 10 μM isoproterenol in the parental and in the CL4-4 cells were relatively similar. This result argues that the absence of the various Gα subunits does not seem to influence the

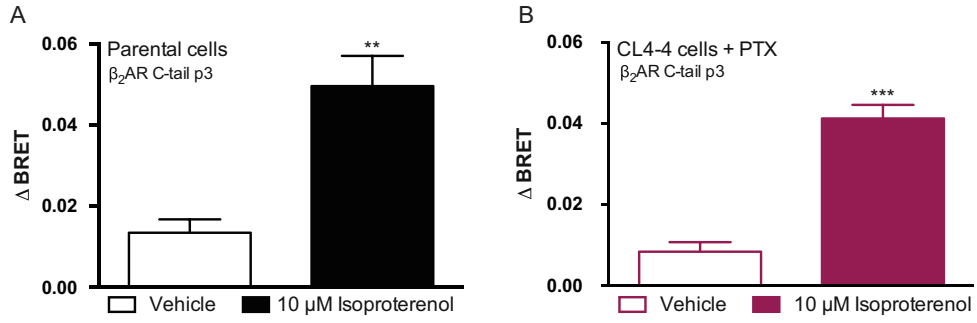

Fig. 3 Isoproterenol-driven conformational rearrangements in the β_2AR expressed in two different genetic backgrounds; (**a**) parental and (**b**) in CRISPR-mediated absence of Gαs, Gαq/11, Gα12/13, and Gαi2 (CL4-4) cells (with additional treatment with PTX) measured by the FlAsH BRET method. Parental cells and CL4-4 cells were plated and transient transfected with HA-hβ_2AR-CCPGCC-Ctail-p3-RlucII biosensor. CL4-4 cells were pre-treated for 16 h with 100 ng/mL PTX. Parental and CL4-4 cells were labeled with the FlAsH dye and stimulated with vehicle or 10 μM isoproterenol followed by kinetic BRET measurements. Data represent four independent experiments; error bars represent mean ± SEM. *T*-tests were performed analyzing the vehicle with the agonist response, asterisks represent $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ Graphs were plotted using GraphPad Prism software

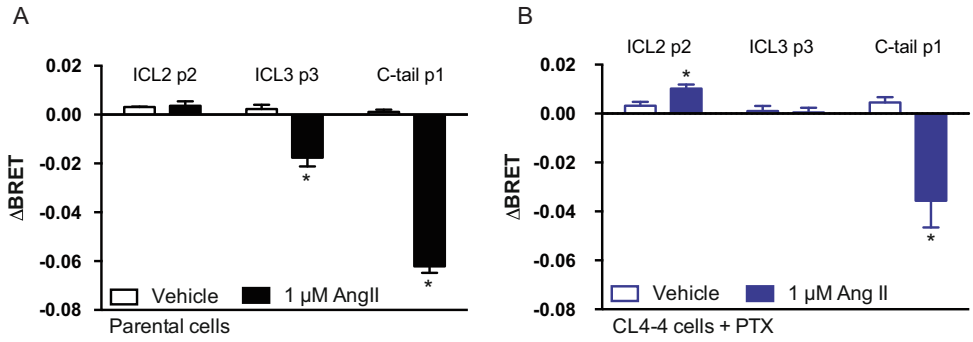

Fig. 4 Angiotensin II-induced BRET changes from the three AT_1R sensors in (**a**) parental and (**b**) CL4-4 cells treated with PTX. Both cell types were transiently transfected with either ICL2 p2, ICL3 p3, and C-tail p1 AT1 FlAsH-based sensors for 48 h followed by FlAsH labeling and BRET assessment; 16 h before the end of the transfection procedure, the CL4-4 cells were treated with 100 ng/mL PTX. Data represent averaged values from 3 (parental) or 4 (CL4-4 cells) independent experiments. Error bars represent mean ± SEM. *T*-test with correction for multiple comparisons were performed between vehicle and Ang II-treated groups using the GraphPad Prism software; $^{*}p < 0.05$

isoproterenol-induced conformational rearrangement of the β_2AR at the C-tail p3 position. Figure 4 shows results from the three conformation-sensitive AT_1R biosensors [6] expressed in both the control parental cell line (Fig. 4a) and in the CL4-4 KO cells treated with PTX (Fig. 4b). In contrast to measurements with the β_2AR, when comparing angiotensin II-induced BRET changes (ΔBRET) from the 3 AT_1R sensors, we noticed a different response profile between the two cell lines. For ICL2 p2, we observed a statistically significant Ang II response (when compared to vehicle treatment) in the CL4-4 KO cell line which was absent in the parental cells while this cell-specific response profile was inverted for ICL3 position 3 sensor. The lack of most of the Gα proteins in the CL4-4 KO cells led to an abrogated Ang II-response from the C-tail p1 sensor when compared to the response measured in the parental cell line. Finally, although all experiments on CL4-4 KO cells were done in presence of PTX to assure complete Gαi inactivation, similar results were observed in absence of the toxin (data not shown). Taken together, our data indicate that these biosensors can capture both G protein-dependent and -independent conformational changes induced by orthosteric agonists.

4 Notes

1. EDT has a distinct unpleasant odor which necessitates its use in a chemical fume hood. The procedure should be performed under dim lighting where possible given that the FlAsH reagent is light-sensitive.

2. Alternatively, concentrated BAL can be purchased from Sigma-Aldrich and diluted in ddH_2O to 25 mM and kept at 4 °C. As such, from the 10 M BAL stock, pipette 2 μL in 800 μL ddH_2O. Vortex. Bear in mind that BAL also has an unpleasant odor and all work must be carried out in a chemical fume hood.
3. Coelenterazine h is an air-sensitive compound and aliquots should be flushed with argon gas prior to long-term storage to prevent moisture/oxygen infiltration. For added protection, use parafilm to seal the cap.
4. As a consequence of CRISPR-Cas9 genome editing, cell morphology as well as growth rate can occasionally be affected. In this particular case, the removal of the various Gα subunits in CL4-4 cells has significantly slowed down cell growth and the cells are also smaller in size. Therefore, it is wise to observe and monitor the cells for a week or more prior to conducting experiments.
5. Growth rate is an important factor when determining the seeding density prior to transfection. Since the CL4-4 cells grow at a significantly slower rate compared to the parental line, we plated four times more cells. Confluency of the two cell types must be relatively similar as such, optimization is required at this step.
6. If culturing cells in presence of antibiotics, it is important to remove them during transfection, as they will reduce the transfection efficiency.
7. Prior to plating the cells in flat bottom white 96-well plates, it is essential to coat the wells with poly-L-ornithine. Briefly, pipette 50 μL of 1× poly-L-ornithine solution in phosphate-buffered saline at 0.1 mg/mL per well and incubate for 10 min. Aspirate and wash once with sterile water. Keep the plate opened in the cell culture hood for any remaining liquid to dry off. This step is vital otherwise the cells will most likely lift off during the FlAsH labeling protocol due to the multiple washing steps.
8. Caution must be taken when working with pertussis toxin (PTX) as it is a highly dangerous toxin that comes from the bacterium *Bordetella pertussis*. Extreme care must be taken when handling this product. Quick spin tubes prior to opening. You will need to work under Biosafety containment level 2. You should also bleach all surfaces and microtips that have come in contact with PTX. Contact the Environmental Health & Safety Office at your institution for approval of your standard operating procedure prior to working with this toxin.
9. We recommend changing gloves regularly as they will be contaminated with EDT (i.e. the unpleasant odor).

10. Considering that the volumes are small, first pipette the EDT as a small droplet on the side of the 15 mL conical tube and then add the required FlAsH volume to the same droplet.
11. If cells have been pre-treated with pertussis toxin, it is best to remove the media in a biosafety cabinet and subsequently bleach contaminated media and other solutions as required. The same safety precautions apply when removing the HBSS prior to the FlAsH labeling as the HBSS may also contain traces of the toxin.
12. The final assay volume depends on the assay to be performed. Typically, in our assays, 80 μL is standard since we add the BRET substrate (10 μL) and then the ligand (10 μL) for a final volume of 100 μL. If pre-treatments are required, the volume can be altered.
13. The ideal location to incubate the assay microplate is in the plate reader to be used. This will allow time for the cells to equilibrate to the assay temperature. This is possible for the Victor X Light but not for the Tristar2 multimode plate reader. If using the latter, incubate the plate in the same room as the instrument, and protected from any source of direct light.
14. Consider using a tenfold higher concentration because we inject a small volume of agonist.
15. Program the temperature unit approximately 45 min earlier than the time you envision starting the experiment. This will allow sufficient time for the targeted temperature to be reached and stabilize.
16. When thawing a new aliquot of coelenterazine h, keep the tube at room temperature for approximately 20 min prior to opening the tube.
17. Due to the high surface tension in the wells of the 96-well plate, it is important to carefully pipette the substrate solution to the bottom of the well. Gently shaking the 96-well plate will also help evenly distribute the substrate throughout the well.
18. Considering that the luminescence signal degrades over time, the 20 μM coelenterazine h solution should be prepared only when needed. For example, prepare a 100 μL solution to stimulate 6 wells. Likewise, reading 6–18 wells at a time ensures that the luminescence output remains stable during the experiment. For this reason, it is important to prepare the 20 μM coelenterazine h solution right before its addition onto the cells. After the addition of the substrate into the well, wait 5 min prior to measuring the luminescence signal. This lag time will ensure that all the readings will be comparable in intensity considering that after the 5 min the luminescence signal is reasonably stable.

19. If kinetic measurements are not required, single point measurements are also feasible. However, optimization may be required. We also recommend generating stable cell lines for more consistent results when considering collecting data using single point measurements.
20. The equation provided as to how to compute the ΔBRET is only our recommendation. The change in BRET can be calculated by taking the average of the last 10–50 measurements and subtracting them by the first 10–50 measurements. For the post-injection BRET, taking the last ten measurements is recommended, as it is the plateau region which is relatively stable. Plotting the BRET ratio as a function of time is also a way to gain a better comprehension of how the BRET ratio is changing over time.

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