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Improved donor/acceptor BRET couples for monitoring *B*-arrestin recruitment to G protein-coupled receptors

((Supporting information online))

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Abbreviations: BRET, Bioluminescence Resonance Energy Transfer; mBu, milli-BRET unit; Rluc, *Renilla* luciferase; YFP, Yellow Fluorescent Protein; RGFP, Renilla green fluorescent protein; GPCR, G protein-coupled receptors; BARR, Barrestin; V2R, vasopressin 2 receptor; MT₁, melatonin 1 receptor; AVP, Argininine Vasopressine.

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Abstract

We report highly sensitive Bioluminescence Resonance Energy Transfer (BRET) assays with optimized donor/acceptor couples. We combined the energy donors *Renilla* luciferase (Rluc) and the Rluc8 variant with the energy acceptors yellow fluorescent protein (YFP), the YPet variant and the *Renilla* green fluorescent protein (RGFP). Different donor/acceptor couples were tested in well-established assays measuring ligand-induced *B*-arrestin (BARR) intramolecular rearrangements and recruitment to G protein-coupled receptors (GPCRs). We show increased sensitivity with Rluc8/YPet and Rluc8/RGFP couples and measured previously undetectable BRET signals. These tools improve existing BARR assays and offer new options for the development of future BRET assays.

1. Introduction

BRET is a natural phenomenon occurring in several marine organisms. In 1999, Xu *et al.* first applied BRET to the detection of protein-protein interactions in intact cells [1]. This initial version, nowadays called BRET¹, relies on the Rluc energy donor and its substrate, coelenterazine h, and the YFP energy acceptor. The overlap between the donor and acceptor energy spectra is substantial, resulting in a relatively high background signal that needs to be carefully controlled [2]. In contrast, the BRET² version uses Rluc in the presence of the blue-shifted substrate coelenterazine 400A and the GFP10 variant that shows a blue-shifted absorption spectrum [3, 4]. Despite improved spectral resolution in the BRET² version, BRET¹ is still the most widely used method due to its higher sensitivity (>100X higher luminescence signals) [5] allowing BRET measurements at physiological protein expression levels [6].

The primary goal of this study was to further improve the BRET¹ version by testing new donor/acceptor couples. We compared the original energy donor Rluc with the recently described Rluc8 variant, which differs by eight mutations from Rluc. Rluc8 was selected for its higher stability and quantum yield [7] and was successfully applied to BRET imaging in a BRET² setting [8]. Furthermore, we compared the original YFP with the YPet variant and the recently cloned RGFP. YPet was optimized for enhanced fluorescence resonance energy transfer (FRET) in a tandem CFP-YFP fusion protein [9], an experimental setting closely resembling the Rluc/YFP BRET couple. For RGFP, an improved energy transfer

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efficiency was reported in BRET² assays but only if the N-terminus of RGFP was free, considerably restricting the use of RGFP in BRET assays [10].

GPCRs play a central role in the signal transduction of an enormous array of biological stimuli primarily through their coupling to heterotrimeric G proteins. GPCR activation initiates their feedback desensitization, which is mostly mediated by GPCR kinases (GRKs) and ßARR proteins. ßARRs can also act as scaffolds, linking GPCRs to mitogen-activated protein kinase signaling pathways [11]. Because ßARRs bind only the activated, phosphorylated form of the receptor, BRET assays using ßARRs as biosensors for GPCR activation were widely developed in the last decade [12, 13].

In order to test the new BRET couples described above, we chose two wellestablished BRET assays used to monitor agonist-promoted interactions upon GPCR activation: the ligand-induced ßARR intramolecular rearrangement and the ligand-induced ßARR recruitment to GPCRs.

2. Materials and Methods

2.1. Compounds

Arginine vasopressin (AVP) was purchased from Calbiochem/Merck (Notingham, UK), melatonin from VWR (Fontenay sous Bois, France). The d(CH2)5[D-Tyr(Et)2, Ile4, Eda9]AVP compound was provided by Dr. M. Manning (Toledo, Ohio, USA).

2.2. Construction of fusion proteins

The coding sequences for humanized Rluc, YFP, and *Renilla mullerie* GFP (RGFP) were obtained by PCR amplification from previously described pcDNA3-OBR-Rluc, pcDNA3-OBR-YFP [14] and pCMV-h-RM RGFP vectors (Prolume, Arizona USA). The Rluc8 and YPet variants were generated by multi-site mutagenesis (Promega, Charbonnières-les-Bains, France) from the Rluc and YFP sequences. Tandem Acceptor-Rluc-Flag and Acceptor-Rluc8-Flag fusions were generated by PCR by inserting the AENLYFQGA linker sequence between acceptors and donors and adding a Flag epitope (DYKDDDDK) before the Stop codon. Tandem Flag-Rluc-RGFP and Flag-Rluc8-RGFP fusion proteins contain the same linker between donors and RGFP and the Flag epitope was inserted after the Start codon of the donors.

The human V2R coding region was cloned in frame with the Rluc coding region to obtain the V2R-Rluc construct. The V2R-Rluc8 construct was obtained by replacing Rluc by Rluc8. Both constructs contain a PVAT linker sequence.

Rluc and YFP of the previously described Rluc-BARR2-YFP vector [13] were replaced by Rluc8, YPet and RGFP, respectively. The linker between donors and BARR2 is composed of the following amino acids GDLRRALENSHASAGYQACGTGS and the linker between BARR2 and the acceptors is CLEDPRVPVAT.

Acceptor-BARR2 fusion proteins were obtained by fusing the coding region of YFP, YPet or RGFP in frame with that of rat BARR2 without linker.

MT₁-Rluc8 construct was obtained by replacing Rluc by Rluc8 from a previously described MT₁-Rluc vector [15]. MT₁-Rluc and MT₁-Rluc8 contained the same linker sequence (VWDPPVAT) between human MT₁ and the donor.

2.3. Cell culture and transfections

HEK 293T cells were grown in complete medium (Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 4.5g/L glucose, 100U/mL penicillin, 0.1mg/mL streptomycin and 1mM glutamine) (Invitrogen, CA). Transient transfections were performed using JetPEI (Polyplus Transfection, France), or Fugene6 HD (Roche, Basel, Switzerland), according to manufacturer's instructions.

For Donor/Acceptor fusion protein BRET assays and corresponding separate coexpressed soluble proteins, HEK 293T cells, which do not express endogenous V2 and MT₁ receptors, were transfected with 2 to 10µg of the different plasmids. For the double-brilliance βARR BRET assay, wild-type HEK 293T cell transiently expressing myc-V2R or HEK 293 cells stably expressing Flag-MT₁ [16] were seeded in 6-well plates and transiently transfected with 500ng of RlucβARR-YFP, Rluc-βARR-YPet, Rluc-βARR-RGFP, Rluc8-βARR-YFP, Rluc8βARR-YPet or Rluc8-βARR-RGFP expression vectors. 24h post-transfection, cells were transferred into 96-well plates and left another 24h before BRET measurements.

For the ßARR recruitment BRET assay, HEK293T cells were seeded in 10 cm dishes and transiently transfected with 1500ng and 750ng of myc-V2R-Rluc and

<u>myc-V2R-Rluc8, respectively. 4h post-transfection cells were transferred into 12-</u> <u>well plates, and transfected 24h later with increasing amounts of YFP-βARR,</u> <u>YPet-βARR and RGFP-βARR expression plasmids. Cells were then transferred</u> <u>into 96-well plates and left another 24h before BRET measurements.</u> <u>Concentration-response curves were performed in presence of saturating amounts</u> <u>of YFP-βARR, YPet-βARR and RGFP-βARR expression plasmids.</u> <u>Measurements were performed 15 min after agonist addition.</u>

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2.4. BRET experiments

For Donor-Acceptor fusion proteins and the corresponding separate soluble forms, cytoplasmic extracts were prepared 48h post-transfection as described previously [10], diluted and submitted to BRET measurements in the presence of 5μ M coelenterazine h (Interchim, Montluçon, France).

For the double-brilliance β ARR2 and β ARR2 recruitment assays, cells were washed once with PBS 48h post-transfection, and coelenterazine h substrate was added at a final concentration of 5µM and left for 10min at RT. Cells were then stimulated by a saturating concentration of ligands for 15 min (double-brilliance β ARR2) or 5 min (β ARR2 recruitment). Arginine vasopressin (AVP) and melatonin were used as agonist for V2R and MT₁ experiments, respectively.

BRET readings were performed using a lumino/fluorometer (Mithras; Berthold technologies, France) allowing sequential integration of luminescence with three filter settings (Rluc filter, 485 ± 10 nm; YFP (and YPet) filter, 530 ± 12.5 nm,

RGFP filter, 515 ± 10 nm). Emission signals at 530 or 515 nm were divided by emission signals at 485 nm. The difference between this emission ratio obtained with donor and acceptor fused or co-expressed and that obtained with the donor protein expressed alone was defined as the BRET ratio. Results are expressed in milliBRET units (mBU) corresponding to the BRET ratio multiplied by 1000. Donor saturation curves and determination of maximal BRET values were described previously [17].

2.5. Western Blot

Proteins were separated by 10% SDS-PAGE and immunoblot analysis was carried out with the following antibodies: Mouse-monoclonal anti-Flag M2 (SIGMA, MO), mouse monoclonal anti-myc (Santa Cruz, CA), mouse monoclonal anti-Renilla Luciferase (Chemicon International, CA), rabbit polyclonal anti- β ARR (SIGMA, MO) and rabbit polyclonal anti- α -tubulin (Sigma, MO). Immunoreactivity was revealed using IRDye infrared secondary antibodies using the LI-COR Odyssey infrared imaging system (Courtaboeuf, France).

3. Results

3.1 BRET efficiency of tandem fusion proteins

We first generated a series of N-Acceptor-Donor-C fusion proteins composed of a N-terminal acceptor, a common linker sequence and a C-terminal donor.

Expression of equivalent amounts of fusion proteins in HEK 293T cells revealed the expected strong BRET for direct fusion proteins (**Fig. 1A**). Dilutions of these samples up to 64 fold showed the expected stability of the BRET ratio (data not shown). On the other hand, co-expression of the new donor/acceptor couples as separate proteins resulted in negligible BRET signals (0-30 mBU) demonstrating their suitability for the study of protein-protein interactions (**Fig. 1A**). BRET values were highest for YPet fusion proteins followed by YFP and RGFP fusion proteins and were always higher for Rluc8 fusion proteins as compared to the corresponding Rluc fusion proteins. The use of RGFP in BRET has been reported to be sensitive to its position in the fusion protein (N-terminal *vs.* C-terminal fusion) [10]. We therefore tested N-Donor-RGFP-C fusion proteins with RGFP located at the C-terminus. A robust BRET signal that was higher than that of N-RGFP-Donor-C fusion proteins was observed (**Fig. 1B**). Taken together YPet fusion proteins showed highest BRET signals, whereas BRET signals for RGFP and YFP fusion proteins were comparable.

3.2. Improved BRET signals with Rluc8/YPet and Rluc8/RGFP couples when monitoring ligand-induced β ARR2 intramolecular conformational changes

We next generated intramolecular BRET biosensors based on the recently described double-brilliance ßARR assay [13]. This assay monitors the conformational rearrangement of ßARR2 bringing its N-terminus and C-terminus into closer proximity upon recruitment to agonist activated GPCRs. Equivalent

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amounts of the indicated N-Donor-BARR2-Acceptor-C fusion proteins, as indicated by Western Blot, were transiently expressed in myc-V2 vasopressin receptor (myc-V2R)-expressing cells, known to recruit BARR2. BRET values were monitored for 15 min in the absence or presence of a saturating concentration of the V2R agonist AVP (10µM). Maximal BRET signals were generally observed as early as 4 min after agonist addition, indicating that the double-brilliance fusion constructs are functional (Supplementary Fig. 1B). Comparison of agonist-induced BRET after 15 min of stimulation showed enhanced induced-BRET for N-Rluc8-BARR2-YPet-C and N-Rluc8-BARR2-RGFP-C (2-3 fold, respectively) when compared with other constructs (Fig. 2A). Agonist concentration-response curves were performed showing BRET EC50 values ranging 75 +/- 45 nM and 79 +/- 29 nM for N-Rluc8-BARR2-YPet-C and N-Rluc8-βARR2-RGFP-C constructs, respectively (Fig. 2B). Similar values were obtained for the other double-brilliance fusion constructs (data not shown). We then expressed the different BARR2 fusion proteins in HEK 293T cells stably expressing Flag-MT₁ melatonin receptors a GPCR whose BARRs recruitment is difficult to monitor using the classical Rluc-BARR2-YFP fusion protein (Fig. 2A). Whereas agonist-induced BRET signals (10 µM melatonin) were barely detectable for all Rluc-containing fusions, BRET signals became clearly detectable as early as 2 min after agonist addition for N-Rluc8-BARR2-YPet-C and N-Rluc8-BARR2-RGFP-C constructs (Fig. 2A) (Supplementary Fig. 1C). Comparable BRET EC₅₀ values of 6.3 +/- 3.7 nM and 8.2 +/- 4.9, were obtained

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for melatonin using N-Rluc8-BARR2-YPet-C and N-Rluc8-BARR2-RGFP-C constructs, respectively (Fig. 2C).

Expression of equivalent amounts of the indicated N-Donor- β ARR2-Acceptor-C fusion proteins in HEK 293T cells in the absence of V2R and MT₁ did not result in agonist-induced BRET signals, demonstrating the specificity of the assay (data not shown). Taken together, these results show that the Rluc8/YPet and particularly the Rluc8/RGFP couple significantly amplify weak and previously undetectable BRET signals of the intramolecular β ARR2 biosensor.

3.3. Improved BRET signals with Rluc8/YPet and Rluc8/RGFP couples when monitoring ligand-induced β ARR2 recruitment to GPCR

We further analyzed the donor/acceptor couples in the ßARR2 recruitment assay [12, 18]. Similar amounts of V2R-Rluc or V2R-Rluc8 C-terminal fusion proteins were co-expressed with increasing amounts of N-Acceptor-BARR2-C fusion constructs and ligand-induced BRET was measured at maximal ßARR2 recruitment (5 min after agonist addition). BRET donor saturation curves were generated in the absence or presence of AVP (10µM) as previously described [14, 19] to determine BRET_{max} values that correspond to the saturation of all donor molecules by acceptor molecules (**Fig. 3**)(**Supplementary Fig. 2**). All couples showed significant agonist-induced BRET signals with highest BRET_{max} values for the V2R-Rluc8/ N-RGFP-ßARR2-C couple (<u>Table 1</u>). No significant ligandinduced BRET signals were observed in the presence of the V2R antagonist

<u>Vd(CH2)5[D-Tyr(Et)2, Ile4, Eda9]AVP (Table 1)(Supplementary Fig. 2).</u> Basal BRET values were highest for the V2R-Rluc8/ N-RGFP-βARR2-C couple indicating the increased sensitivity of this couple to monitor basal βARR2 recruitment. This recruitment is specific as expression of V2R-Rluc8 in the presence of soluble RGFP gave negligible BRET values (Supplementary Fig. 2A). Basal βARR2 recruitment to V2R has been observed recently [10]. Moreover, agonist concentration-response curves, in the presence of saturating amounts of acceptors, were performed showing BRET EC₅₀ ranging from 5 to 15 nM AVP for the different N-Acceptor-βARR2-C constructs (data not shown).

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We then measured β ARR2 recruitment to MT₁-Rluc and MT₁-Rluc8. Whereas the recruitment to MT₁-Rluc was undetectable, recruitment to MT₁-Rluc8 became detectable in combination with YPet and RGFP further demonstrating the superiority of these new BRET couples.

4. Discussion

The ßARR recruitment assay has become increasingly popular to monitor GPCR activation. Since the development of the BRET technology in 1999 and the first ßARR assay in 2000, several improvements have been reported to increase signaling amplitude and stability [20] [21]. Here, we focused on improving the most sensitive and widely used BRET¹ version by combining recently developed energy donors (Rluc, Rluc8) and acceptors (YFP, YPet, RGFP) in a systematic manner. By using two well-established BRET assays measuring ßARR2

intramolecular rearrangements and recruitment to GPCRs, we show that Rluc8/YPet(RGFP) couples significantly increase the sensitivity of these assays, allowing the detection of previously undetectable BRET signals.

We observed that Rluc8 and Rluc8 fusion proteins emit 2-4 times more light than the corresponding Rluc proteins expressed at equivalent levels as previously reported (Supplementary Fig. 3) [7, 8, 21]. The higher luminescence intensity of Rluc8 implies a more efficient acceptor excitation and re-emission and the possibility to further decrease protein expression levels to physiologically relevant levels. In agreement with previous FRET experiments using YPet-CFP fusion proteins, we show that YPet is a better BRET acceptor than YFP [9]. Whereas the energy transfer efficiency in BRET assays is typically low, Molinari et al. recently reported the striking observation that RGFP in combination with Rluc can generate close to 100% energy transfer associated with a dramatic increase in acceptor light output [10]. The underlying molecular basis of this phenomenon, which is only observed when the N-terminus of RGFP is free, remains unclear. By using N-terminal RGFP fusion proteins in two independent assays (RGFP-Rluc tandem fusion protein and GPCR-Rluc/RGFP-BARR2 couple), we were unable to confirm these observations. Indeed, no major difference in BRET signals generated by YFP- and RGFP-containing fusion proteins were observed. Accordingly, Rluc light output did not vary significantly with increased BRET (not shown). The reason for this discrepancy remains currently unknown and warrants further studies. We furthermore noted that BRET values obtained with RGFP fusion proteins may be subject to some variation as illustrated by the

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scattering of BRET values obtained in the donor saturation experiment with RGFP-BARR2 and V2R-Rluc8 (Supplementary Fig. 2). These results indicate that RGFP is a highly sensitive energy acceptor. RGFP can be therefore considered as an interesting BRET acceptor, which can result in superior BRET values, particularly when compared to YFP. The data show that the use of RGFP is not restricted to N-terminal fusion proteins, which considerably increases its attractiveness for the BRET assay. More importantly, RGFP shows improved sensitivity compared to YFP when combined with the Rluc8 variant.

Overall, Rluc8/YPet and Rluc8/RGFP couples provide superior results compared to the classical Rluc/YFP couple. According to the data obtained in the ßARR2 recruitment assay, these BRET couples primarily increase the overall sensitivity of the assay whereas the signal-to-noise ratio appears to be comparable for all combinations (3-5 fold increase between basal and AVP-induced BRET signals) (**Table 1**). Despite obvious conceptual advantages of the double brilliance ßARR2 biosensor such as the use of unmodified wild-type GPCR and high assay stability due to the fixed donor/acceptor ratio, the assay is not broadly used in the literature [13, 22]. The low amplitude of ligand-induced BRET signals is one of these reasons. The increased sensitivity observed with the new donor/acceptor combinations clearly increases the attractiveness of the assay. A widespread application to monitor ßARR-dependent signaling events in the context of ligandbiased signaling can be anticipated.

Several BARR activation/recruitment assays have been developed over the years, particularly for high-throughput screening purposes. Each of these assays has its

own advantages and disadvantages. BRET assays measure ligand-induced signals. This is not the case for the fluorescence-based assay that monitors the cellular localization of BARR-EGFP fusion proteins [23, 24]. If the changes in BARR localization following agonist stimulation are small, data analysis may become difficult. The TANGO[™] (Invitrogen corporation) BARR assay that is coupled to a protease-mediated transcriptional reporter gene readout shows excellent assay sensitivity, due to the sensitive reporter gene read-out [25]. However, a high number of false positives can be anticipated in a high-throughput screening assays due to multiple intermediates between receptor activation and gene transcription initiation. BRET assays do not suffer from these potential drawbacks as receptor activation is closely coupled to the final read-out. This is also the case of the recently described enzyme fragment complementation assay for BARR, now commercially available as PathHunter technology from DiscoverX [26]. Conditions for successful enzyme complementation might be more demanding than conditions for successful energy transfer leading potentially to the detection of less compounds compared to BRET assays. Relatively low signals can be considered as one of the major disadvantages of BARR-based BRET assays. The new donor-acceptor couples presented here improve this parameter, in particular in the double-brilliance assay.

In conclusion, the new BRET¹ couples, Rluc8 and YPet/RGFP clearly widen the application of ßARR BRET assays as previously weakly detectable or even undetectable BRET signals are now reliably detected. These new BRET couples are likely to become of general interest for the detection of protein-protein

interactions in intact cells at physiological expression levels. Enhanced ligandpromoted BRET signals might also be beneficial for high-throughput screening procedures.

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Legends to figures:

Figure 1: BRET measured with different donor/acceptor couples. (A) Donor/acceptor proteins were co-transfected (\Box) or transfected as N-Donor-Acceptor-C tandem proteins (\blacksquare) and BRET was measured. (B) Comparison of BRET measured for N-Donor-RGFP-C and N-RGFP-Donor-C fusion proteins. n=5 independent experiments.

Figure 2: Monitoring of <u>ligand-induced βARR2 intramolecular rearrangement.</u> (A) Bar Charts showing the intramolecular rearrangement of the indicated βARR2 double-brilliance constructs upon recruitment to agonist activated V2R (\Box) or MT₁ (\blacksquare) in HEK 293T cells (15 min stimulation with agonist); n=4 independent experiments. (B) AVP concentration-response curves showing for N-Rluc8βARR2-YPet-C (\bigcirc) and N-Rluc8-βARR2-RGFP-C (\Box) (n=2). (C) Melatonin concentration-response curves for N-Rluc8-βARR2-YPet-C (\bigcirc) and N-Rluc8βARR2-RGFP-C (\Box) (n=2). Expression of βARR2 was verified by western blotting using anti-βARR antibodies. Further experimental details and controls are shown in **Supplementary Fig. 1**. A, acceptor; C, C-terminus; D, donor; N, Nterminus; α-tub, α-tubulin.

Figure 3: BRET measured with the β ARR recruitment assay. Agonist-induced recruitment of the indicated β ARR2 fusion constructs to V2R-Rluc or V2R-Rluc8 (\Box) (10 μ M AVP, 5 min), and MT₁-Rluc or MT₁-Rluc8 (\blacksquare) (10 μ M melatonin, 5 min). Expression of V2R-Rluc and V2R-Rluc8 was verified by western blot using

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anti-Rluc antibodies (that recognize Rluc and Rluc8 equally well, data not shown). Ligand-induced BRET was defined as the difference between BRET_{max} values obtained from donor saturation curves +/- ligand. Saturation curves and further controls are shown in Supplementary Fig. 2; α -tub, α -tubulin. <u>n=4</u> independent experiments.

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Table 1: BRET ßARR2 recruitment ass	ay.
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	E	BRET max	Fold stimulation	
	Basal	AVP	Antagonist	AVP/Basal
V2R-Rluc/YFP-bARR2	9±1	35±3	12±1	3.9
V2R-Rluc/Ypet-bARR2	10±2	59±5	10±3	5.5
V2R-Rluc/RGFP-bARR2	9±1	37±3	13±1	4.1
V2R-Rluc8/YFP-bARR2	13±1	52±4	13±1	4.0
V2R-Rluc8/Ypet-bARR2	16±1	67±4	20±2	4.2
V2R-Rluc8/RGFP-bARR2	58±4	192±18	65±5	3.3
V2R-Rluc8/RGFP	6±1	4±1	3±1	0.7

BRET_{max} and fold stimulation values following agonist stimulation.

BRET_{max} values were determined as described in the "Material and Methods" section and ligand-induced BRET (shown in Fig. 2) was defined as the difference between BRET_{max} values in the absence and presence of ligand. BRET was measured in the absence and presence of the V2R agonist AVP and V2R antagonist Vd(CH2)5[D-Tyr(Et)2, Ile4, Eda9]AVP. The fold induction values are expressed as the ratio of the agonist induced Bretmax/Basal Bretmax.

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Figure 1: BRET measured with different donor/acceptor couples. (A) Donor/acceptor proteins were co-transfected (o) or transfected as N-Donor-Acceptor-C tandem proteins (<) and BRET was measured. (B) Comparison of BRET measured for N-Donor-RGFP-C and N-RGFP-Donor-C fusion proteins. n=5 independent experiments. 122x87mm (600 x 600 DPI)

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Figure 2: Monitoring of ligand-induced β ARR2 intramolecular rearrangement. (A) Bar Charts showing the intramolecular rearrangement of the indicated β ARR2 double-brilliance constructs upon recruitment to agonist activated V2R ($_{0}$) or MT1 ($_{0}$) in HEK 293T cells (15 min stimulation with agonist); n=4 independent experiments. (B) AVP dose-response curves showing for N-Rluc8- $_{\beta}$ ARR2-YPet-C ($_{1}$) and N-Rluc8- $_{\beta}$ ARR2-RGFP-C ($_{0}$) (n=2). (C) Melatonin dose-response curves for N-Rluc8- $_{\beta}$ ARR2-YPet-C ($_{1}$) and N-Rluc8- $_{\beta}$ ARR2-RGFP-C ($_{0}$) (n=2). Expression of $_{\beta}$ ARR2 was verified by western blotting using anti- $_{\beta}$ ARR antibodies. Further experimental details and controls are shown in Supplementary Fig. 1. A, acceptor; C, C-terminus; D, donor; N, N-terminus; α -tub, α -tubulin.

138x144mm (600 x 600 DPI)





Figure 3: BRET measured with the BARR recruitment assay. Agonist-induced recruitment of the indicated BARR2 fusion constructs to V2R-Rluc or V2R-Rluc8 (o) (10 μ M AVP, 5 min), and MT1-Rluc or MT1-Rluc8 (<) (10 μ M melatonin, 5 min). Expression of V2R-Rluc and V2R-Rluc8 was verified by western blot using anti-Rluc antibodies (that recognize Rluc and Rluc8 equally well, data not shown). Ligand-induced BRET was defined as the difference between BRETmax values obtained from donor saturation curves +/- ligand. Saturation curves and further controls are shown in Supplementary Fig. 2; α -tub, α -tubulin. n=4 independent experiments. 71x155mm (600 x 600 DPI)

Supplementary Figures:

Supplementary Fig. 1: Supplementary data of donor/acceptor tandem fusion protein BRET assay and double-brilliance β ARR2 biosensors shown in Fig. 1. (A) Expression of Myc-V2R and Flag-MT₁ in Fig. 1B was verified by western blot using anti-Myc and anti-Flag antibodies. (B) Kinetics of AVP- and melatonin-promoted BRET changes of the indicated double-brilliance β ARR2 biosensors in HEK 293T cells. AVP (V2R agonist) and melatonin (MT₁ agonist) were used at 10 μ M. NT, non-transfected.





Supplementary Fig. 1: Supplementary data of donor/acceptor tandem fusion protein BRET assay and double-brilliance ßARR2 biosensors shown in Fig. 1. (A) Expression of Myc-V2R and Flag-MT1 in Fig. 1B was verified by western blot using anti-Myc and anti-Flag antibodies. (B) Kinetics of AVP- and melatonin-promoted BRET changes of the indicated double-brilliance ßARR2 biosensors in HEK 293T cells. AVP (V2R agonist) and melatonin (MT1 agonist) were used at 10µM. NT, non-transfected.

Supplementary Fig. 2: Supplementary data of the β ARR2 recruitment assay shown in Fig. 2. BRET donor saturation curves for V2R-Rluc and V2R-Rluc8 were generated in HEK 293T cells in the presence of different N-Acceptor- β ARR2-C fusion constructs. BRET was measured 5 min after ligand addition. BRET was measured in the absence and presence of the V2R agonist AVP and V2R antagonist Vd(CH2)5[D-Tyr(Et)2, Ile4, Eda9]AVP. The absence of ligand-induced BRET in the presence of the antagonist demonstrates the specificity of the assay. Basal condition (\bullet); 0.1 μ M Vd(CH2)5[D-Tyr(Et)2, Ile4, Eda9]AVP (\odot); soluble RGFP (\Box). **Supplementary figure 2**



Supplementary Fig. 2: Supplementary data of the ßARR2 recruitment assay shown in Fig. 2. (A) BRET donor saturation curves for V2R-Rluc and V2R-Rluc8 were generated in HEK 293T cells in the presence of different N-Acceptor-ßARR2-C fusion constructs. BRET was measured 5 min after ligand addition. BRET was measured in the absence and presence of the V2R agonist AVP and V2R antagonist Vd(CH2)5[D-Tyr(Et)2, Ile4, Eda9]AVP. The absence of ligand-induced BRET in the presence of the antagonist demonstrates the specificity of the assay. Basal condition (\bullet); 0.1µM Vd(CH2)5[D-Tyr(Et)2, Ile4, Eda9]AVP (\bullet); 0.1µM AVP (\bigcirc); soluble RGFP (\Box).

Supplementary Fig. 3: Comparison of emitted light intensities of Rluc and Rluc8 fusion proteins. Rluc (A) and the indicated β ARR2 double-brilliance (B) and V2R (C) constructs were expressed in HEK 293T cells. Luciferase activity was determined in the presence of 5µM coelenterazine h (middle panels) and another aliquot submitted to western blot analysis (upper panels) using anti-Flag (A), anti- β ARR (B) and anti-Rluc (C) antibodies. Protein bands were quantified and luciferase activity of Rluc8 fusion proteins expressed as % of the corresponding Rluc fusion protein (lower panels).





Supplementary Fig. 3: Comparison of emitted light intensities of Rluc and Rluc8 fusion proteins. Rluc (A) and the indicated ßARR2 double-brilliance (B) and V2R (C) constructs were expressed in HEK 293T cells. Luciferase activity was determined in the presence of 5?M coelenterazine h (middle panels) and another aliquot submitted to western blot analysis (upper panels) using anti-Flag (A), anti-ßARR (B) and anti-Rluc (C) antibodies. Protein bands were quantified and luciferase activity of Rluc8 fusion proteins expressed as % of the corresponding Rluc fusion protein (lower panels).