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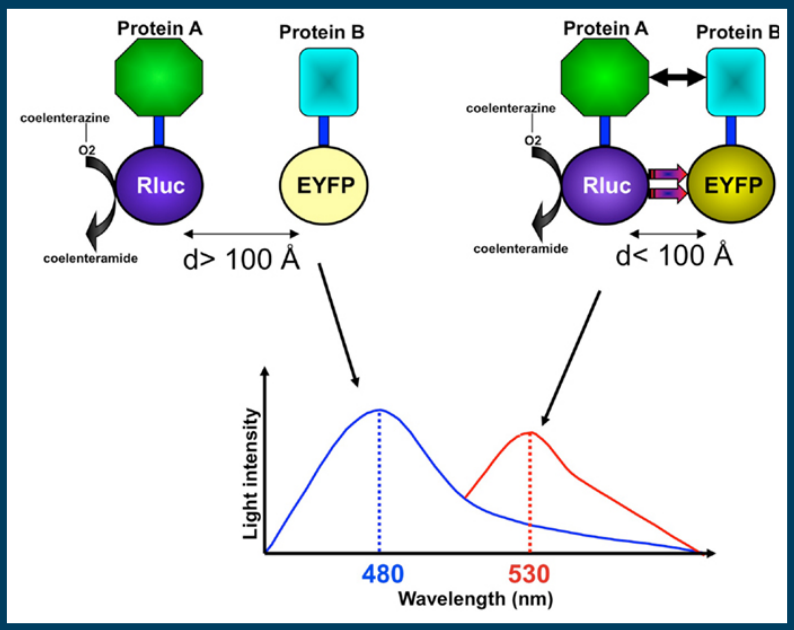
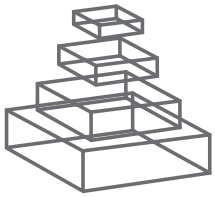


THE USE OF BRET TO STUDY RECEPTOR-PROTEIN INTERACTIONS

Topic Editor
Milka Vrecl

frontiers in ENDOCRINOLOGY



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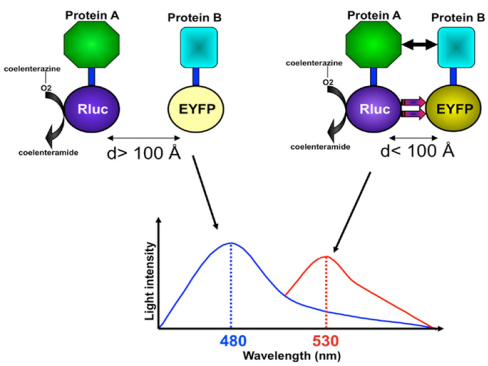
THE USE OF BRET TO STUDY RECEPTOR-PROTEIN INTERACTIONS

Topic Editor:
Milka Vrecl, Veterinary Faculty, University of Ljubljana, Slovenia



Reproduced from Siddiqui S, Cong WN, Daimon CM, Martin B and Maudsley S (2013) BRET biosensor analysis of receptor tyrosine kinase functionality. *Front. Endocrinol.* 4:46. doi: 10.3389/fendo.2013.00046

This Research Topic is intended to provide an up-to date overview of biophysical methods based on bioluminescence resonance energy transfer (BRET) and their utilization in the field of receptor research and the drug discovery process.

Since its first application more than a decade ago, BRET has become a method of choice to monitor protein-protein interactions in live cells. In the seven transmembrane receptors (7TMRs) and receptor tyrosine kinases (RTK) field, it has been widely used to monitor i) receptor homo- / hetero-dimerization, ii) ligand-induced conformational changes in receptors, and iii) activation-promoted receptor complex formation with intracellular protein partners or structural rearrangements within the preassembled receptor signaling complexes. BRET technology has also been successfully utilized in the development of technological platforms for compound medium/high-throughput screening and biosensor technology.

We invited submissions (reviews, research articles, methods, perspectives, etc.) that address the most recent advances in BRET technology and its utilization in the following areas of receptor-protein interactions investigation:

– Receptor homo- and hetero-oligomerization (specificity of BRET results, functional importance, negative cooperativity, allosteric modulation, dimer asymmetry ...)
– Conformational rearrangements within the receptor (e.g. movement of the third intracellular loop) and/or in the preassembled multiprotein signaling complexes
– Ligand-biased signaling (intramolecular BRET)
– Receptor screening challenges (BRET-based screening platforms for the TMRs and RTK)
– Biosensor development (tissue-based biosensors and their use in living subjects)

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EDITORIAL
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Comment on "The use of BRET to study receptor-protein interactions"

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Edited by:
Pierre De Meyts, Hagedorn Research Institute, Denmark

Keywords: BRET, high throughput screening assays, GPCR heteromers, PPI inhibitors, protein–protein interactions

This Research Topic assembles for the first time a comprehensive selection of articles (mini review, review articles, original research, and opinion articles) on the bioluminescence resonance energy transfer (BRET) technology. BRET is a natural phenomenon known in several marine organisms. It relies on the non-radiative transfer of energy from an appropriate energy donor to an energy acceptor, provided that both are located at a distance lower than 10 nm [see Ref. (1) for a basic and theoretical introduction]. BRET has been first applied to the detection of protein–protein interactions (PPIs) in 1999 (2). Since then, the technology constantly evolved by combining new donor and acceptor couples and developing various BRET assay formats as discussed in the Mini Review article of De et al. (3).

Many BRET assays have been developed to study the oligomerization of seven-transmembrane-spanning G protein-coupled receptors (GPCRs). The BRET technology provides an attractive way to study this phenomenon in intact cells without the need of solubilization of receptors from their natural membrane environment. The overwhelming majority of BRET-based studies conclude that GPCRs do indeed exist as dimers or higher-order oligomers when transfected into cells at physiological levels. Three of the articles of this Research Topic discuss BRET assays that have been developed to properly address the specificity of BRET signals obtained upon expression of different GPCRs (1, 4, 5). The current consensus confirms that several different BRET assays are needed to evaluate the specificity of BRET signals. The precise role of each of these assays remains a source of controversy in the field (4). Further refinement of BRET control experiments and application of new techniques like single-molecule measurements and functional *in vivo* studies are likely to provide new insights to the existence and physiological relevance of GPCR oligomerization. Not surprisingly, GPCR oligomerization is the main issue of four articles of this Research Topic (4–7) ranging from studies on class A and B GPCR homo- and heteromers using BRET or alternative approaches like time-resolved FRET measurements.

Apart from performing the proper control experiments, another important issue in the BRET field concerns the proper interpretation of stimulus-induced BRET signals. In the context of GPCR oligomerization, agonist-induced BRET signals can be generated by an agonist-driven oligomerization or agonist-induced conformational changes within preassembled oligomers. Discrimination between these two possibilities is not trivial but has become possible due to the development of BRET donor saturation experiments in the absence and presence of receptor stimulation (8, 9). This issue, to which BRET has made a significant contribution, is obviously of general importance for the field of PPIs. This is illustrated by the articles describing the interaction of the protease-activated receptor 1 and 2 with its cognate G proteins as described by Ayoub and Pin (10) and Ayoub et al. (11).

The BRET technology has been extended toward other receptor families like tyrosine kinase receptors and cytokine receptors. This diversification demonstrates the general feature of this technique. These studies did not only address the question of receptor oligomerization but also monitored the real-time interaction of receptors with various effector molecules such as Grb2, PTP1-B, PLC-γ1, etc. This important aspect is discussed in the review article of Siddiqui et al. (12). Receptor–effector interactions have been also monitored by BRET for two privileged GPCR interacting partners, heterotrimeric G proteins and β-arrestins, as documented in articles of this Research Topic (6, 10, 11, 13).

A more recent application of BRET concerns the development of biosensors to monitor downstream events of cellular signaling like the generation of second messengers and activation of intracellular kinases. These sensors are typically composed of the energy donor and acceptor separated by an assay-specific domain that changes its conformation upon second messenger binding or phosphorylation, thus modifying the position of the donor and acceptor and consequently the BRET signal. Similar sensors have been developed for FRET applications, which served as source of inspiration for the development of the BRET sensors. The articles from Salahpour et al. (13) and Xu et al. (2) describe the design and validation of cAMP and ERK sensors.

The high reproducibility of BRET and the robustness of the measurements make BRET an interesting option for the design of high throughput screening assays. Two applications are discussed in this Research Topic. The first concerns the design of an assay for the identification of compounds that specifically activate GPCR heteromers (6) based on the recruitment of β-arrestin to GPCR heteromers. The second case concerns the identification of PPI inhibitors (14).

Taken together, this Research Topic provides an illustrative overview of the principles and applications of the BRET technology that should be of interest for any scientist interested in monitoring PPI in intact cells.

ACKNOWLEDGMENTS

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ORIGINAL RESEARCH ARTICLE
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Interaction of protease-activated receptor 2 with G proteins and β-arrestin 1 studied by bioluminescence resonance energy transfer

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G protein-coupled receptors are well recognized as being able to activate several signaling pathways through the activation of different G proteins as well as other signaling proteins such as β-arrestins. Therefore, understanding how such multiple GPCR-mediated signaling can be integrated constitute an important aspect. Here, we applied bioluminescence resonance energy transfer (BRET) to shed more light on the G protein coupling profile of trypsin receptor, or protease-activated receptor 2 (PAR2), and its interaction with β-arrestin1. Using YFP and Rluc fusion constructs expressed in COS-7 cells, BRET data revealed a pre-assembly of PAR2 with both Gαi1 and Gαo and a rapid and transient activation of these G proteins upon receptor activation. In contrast, no pre-assembly of PAR2 with Gα12 could be detected and their physical association can be measured with a very slow and sustained kinetics similar to that of β-arrestin1 recruitment. These data demonstrate the coupling of PAR2 with Gαi1, Gαo, and Gα12 in COS-7 cells with differences in the kinetics of GPCR-G protein coupling, a parameter that very likely influences the cellular response. Moreover, this further illustrates that pre-assembly or agonist-induced G protein interaction depends on receptor-G protein pairs indicating another level of complexity and regulation of the signaling of GPCR-G protein complexes and its multiplicity.

Keywords: BRET, PAR2, trypsin, G proteins, β-arrestin, pre-assembly

INTRODUCTION

Recently the study of the interaction of GPCRs with their specific signaling and regulatory proteins has been widely studied using energy transfer-based approaches BRET and FRET (bioluminescence/fluorescence resonance energy transfer) (1–5). These methods allow the measurements, in real-time and live cells, of either the recruitment of specific proteins (i.e., G protein subunits, RGS, arrestins, GRKs ...) to the activated receptor or conformational changes within their preassembled or newly formed complexes (6–10). Consequently, interesting conclusions have been reported regarding GPCR-G protein coupling, interaction between G protein subunits, and GPCR-arrestin association in terms of structure, pharmacology, and kinetic of activation/deactivation. Indeed, many studies have shown the possibility of GPCRs to form preassembled complexes even in the absence of receptor activation [for review (1, 3)]. However, others clearly demonstrate that receptor-G protein association is exclusively mediated by agonist activation with expected or unexpected kinetics [for review (1, 3)].

One of the important GPCR families is protease-activated receptors (PARs) which is composed by four subtypes, PAR1, protease-activated receptor 2 (PAR2), PAR3, and PAR4, which play crucial roles in a number of physiological processes such as thrombosis, vascular development, cell proliferation, and tumorigenesis (11). Therefore they are considered as interesting targets for the treatment of various pathologies like inflammation, cancer, and stroke (12). PARs are known to be activated by various serine proteases such as thrombin, trypsin, plasmin, and the factor Xa (13, 14). PARs activation occurs through a highly specific protease-mediated cleavage of the N-terminal extremity of the receptor unmasking a new N terminus that acts as a tethered ligand which directly activates the transmembrane core of the cleaved receptor (13–15). PARs are characterized by the diversity and overlapping of their signaling pathways involving various G protein classes: Gαi/o, Gαq/11, Gα12/13 proteins as well as arrestins promoting multiple downstream signaling responses in various cellular models (15–19). Furthermore, PARs undergo a rapid desensitization, internalization, and degradation involving the phosphorylation of the receptor by G protein-coupled receptor kinases and the recruitment of arrestins (18, 19). However, except for the prototype member, PAR1, the G protein coupling profile of the different members of PARs is not really a consensus matter and little information is available regarding their coupling to G proteins. This is true for PAR2 which is typically Gαq/11-coupled receptor leading to an increase in intracellular calcium via PLC/IP3 pathway (16, 18, 20, 21). However, a study in the Xenopus oocyte system has reported that PAR2-mediated intracellular signaling events were a pertussis toxin (PTX)-sensitive indicating a role of PAR-Gαi/Gαo coupling (22). Also, PAR2 activation in the epithelial cells elicited a calcium response in both PTX-sensitive and PTX-insensitive depending on the cell model used (23). Recently PAR2 activating

peptide SLIGRL has been shown to induce smooth muscle contraction by triggering the activation of Gαq, Gαi1, and Gα13 (24). However, it has been shown that PAR2, in contrast to PAR1, does not couple to Gαi and Gαo families in COS-7 cells (25). Together, these studies indicate that the pattern of G protein coupling of PAR2 strongly depends on the cellular model considered since the differences can be due to factors such receptor density, the availability of G proteins and other interacting proteins ... etc. [For review (1)].

Many recent studies have used BRET to investigate GPCR-G protein coupling (1, 3) including PARs (6, 7, 26). Indeed, our recent data using both BRET and time-resolved FRET (TR-FRET) technologies revealed the existence of preassembled complexes between PAR1 and Gαi1 protein (6, 7), as well as Gαo (27) in COS-7 cells. In contrast, the physical association of PAR1 with Gα12, but not Gα13, was exclusively observed upon receptor activation with a very slow and stable kinetic indicating the recruitment of Gα12 to the activated PAR1 in parallel to β-arrestin1 recruitment (7). In this study, we aimed to investigate the physical interaction of PAR2 with Gαi1, Gαo, Gα12, and β-arrestin1 before and upon receptor activation by BRET, in real-time and live cells, using Rluc-tagged Gα proteins and YFP-tagged PAR2.

MATERIALS AND METHODS

MATERIALS AND PLASMID CONSTRUCTIONS

Human cDNA for PAR2 were cloned into pcDNA3.1+ (Guthrie Research Institute, Sayre, PA, USA). PAR2-YFP fusion protein and Rluc-tagged G proteins were generated as previously described (6). PAR2-ΔC-YFP mutant corresponds to the δ Tail mutant reported by Seatter et al. removing the entire C-terminus from Serine 348 (28). Such truncation was generated using the similar strategy for PAR1-ΔC-YFP previously reported (6). Rluc-β-arrestin1 were generously provided by M. G. Scott (Institut Cochin, Paris, France). Bovine trypsin pancreas was from Calbiochem Merck KgaA (Darmstadt, Germany) and Ser-Leu-Iso-Gly-Arg-Leu-NH2 (SLIGRL) peptide was from Tocris Cookson Inc., Ellisville, MO, USA. Ninety-six-well white microplates were from Greiner Bio-One SAS (Courtaboeuf, France). Coelenterazine h substrate was from Promega (Charbonnières, France).

CELL CULTURE AND TRANSFECTION

COS-7 cells were grown in complete medium [DMEM supplemented with 10% (v/v) fetal bovine serum, 4.5 g/l glucose, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1 mM glutamine] (all from Invitrogen, Carlsbad, CA, USA). Transient transfections were performed by reverse transfection in 96-well plate using Lipofectamine 2000 following the manufacturer's protocol. Briefly, for each well the different combinations of coding plasmids were used as follow: 25 ng of PAR2-YFP (WT and ΔC mutant), 50 ng of Gαi1/o-Rluc or Rluc-β-arrestin 1, and 150 ng of Gα12-Rluc. The plasmid mixes and Lipofectamine 2000 (0.5 μl/well) were first preincubated 5 min at room temperature in serum-free DMEM (2 × 25 μl/well). Then the two solutions of serum-free DMEM containing plasmids and Lipofectamine were mixed and incubated 20 min at room temperature. Cells (10^5 in 150 μl/well) in DMEM supplemented with 10% FCS were then incubated with the final plasmid-Lipofectamine mix (50 μl/well).

BRET MEASUREMENTS

Forty-eight hours after transfection cells were washed with PBS and preincubated in the absence or presence of trypsin or SLIGRL as indicated in PBS at 37°C. Cells were then washed and resuspended in PBS for BRET measurements. The kinetic and dose-response analysis of BRET signals was performed as described previously (6). The BRET Ratio was defined as the subtraction of the ratio of the emission at 530 ± 25 nm over the emission at 485 ± 20 nm of cells expressing the Rluc fusion protein alone from the same ratio of cells co-expressing Rluc and YFP fusion proteins. Then the resulted values were multiplied by 1000. However, the ligand-induced BRET was calculated by subtracting the BRET Ratio for a PBS-treated cell sample from the same ratio for the aliquot of the same cells treated with agonist. In this calculation only ligand-promoted BRET changes are represented and the PBS-treated cell sample represents the background eliminating the requirement for measuring an Rluc-only control sample especially when fast kinetics and dose-response analysis are performed.

DATA ANALYSIS

All data were represented using Prism GraphPad software (San Diego, CA, USA). Kinetic and dose-response curves were fitted with non-linear regression equations using the different equations as indicated.

RESULTS

BASAL AND LIGAND-INDUCED BRET BETWEEN PAR2 AND Gα PROTEINS

The pattern of G protein coupling for PAR2 is still not completely clarified when compared to PAR1 which is known to activate Gαo, Gαi1/2, Gαq as well as Gα12/13 pathways in various *in vitro* and *in vivo* models (14, 29). Therefore, we wanted to investigate the putative coupling of PAR2 with Gαi1, Gαo, and Gα12, as this has been previously demonstrated for PAR1 (6, 7, 27). For this, we used BRET approach allowing real-time assessment of the receptor-G protein complexes in live cells and BRET measurements were performed in COS-7 cells transiently co-expressing Gα-Rluc and PAR2-YFP fusion proteins and stimulated or not with its specific agonist, trypsin. As shown in **Figure 1A**, significant constitutive BRET signal was measured between PAR2-YFP and either Gαi1-Rluc or Gαo-Rluc compared to Gα12-Rluc. This was observed at similar relative expression levels of PAR2-YFP as well as Rluc-tagged G proteins measured by fluorescence and luminescence, respectively (**Figure 1B**). Interestingly, the stimulation with 100 nM of trypsin for 2 min (for Gαi and Gαo) or 30 min for (Gα12) specifically increased the BRET signal between all the Gα-Rluc and PAR2-YFP indicating functional coupling of PAR2 with Gαi1, Gαo, and Gα12 (**Figure 1A**). Together, these data suggest a possible pre-assembly between PAR2 and Gαi1 and Gαo, but not Gα12. The agonist-induced BRET increase clearly demonstrates a functional coupling of PAR2 with these G proteins which is characterized by conformational changes within the preassembled PAR2-Gαi1 and PAR2-Gαo complexes and probably Gα12 recruitment as previously shown for PAR1 (6, 7, 27).

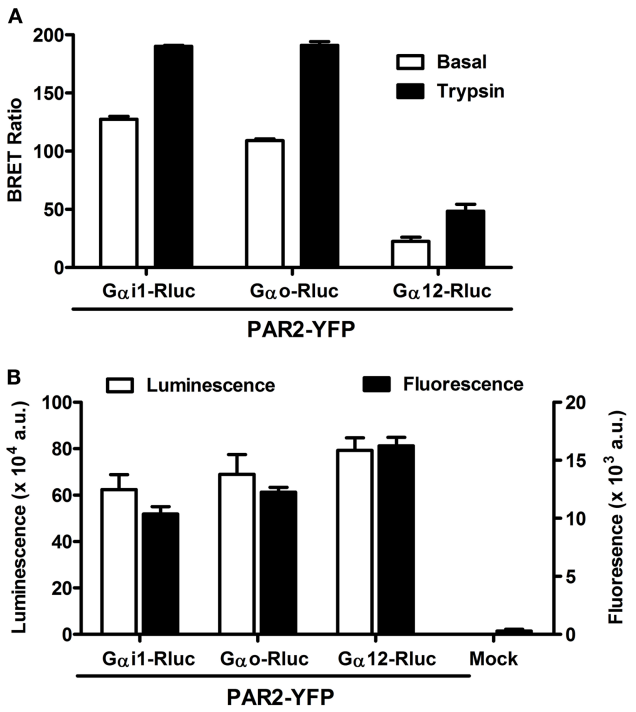

FIGURE 1 | Bioluminescence resonance energy transfer between PAR2 and Gα proteins in live COS-7 cells. **(A)** BRET measurements in COS-7 cells co-expressing PAR2-YFP and either Gαi1-Rluc, Gαo-Rluc, or Gα12-Rluc in the absence (□) and presence of stimulation with 100 nM of trypsin (■) for 2 min (for Gαi and Gαo) or 30 min for (Gα12). **(B)** Quantification of the luciferase (Rluc) activity (□) and YFP fluorescence (■) of BRET partners measured in BRET assay. Data are means ± SEM of three independent experiments performed in triplicate.

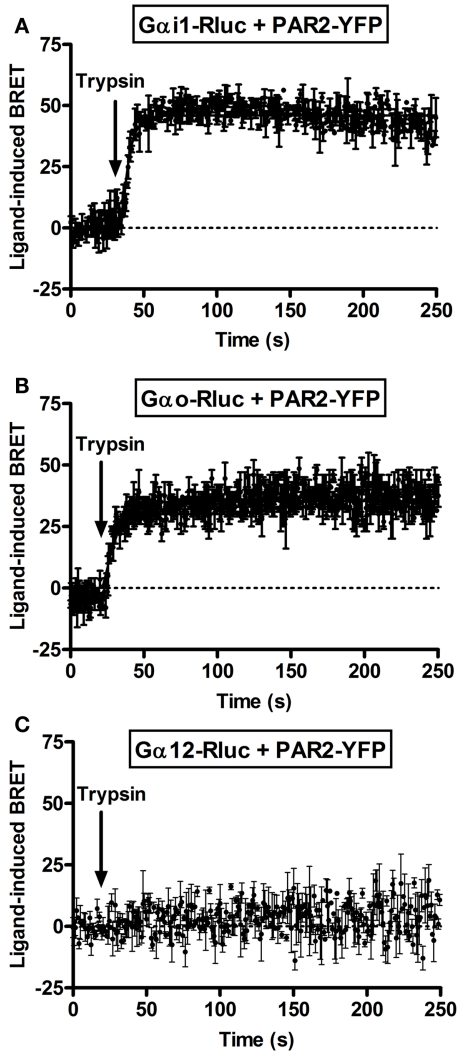

FIGURE 2 | Kinetic analysis of ligand-induced BRET increase between PAR2 and Gα proteins. COS-7 cells transiently co-expressing PAR2-YFP and either Gαi1-Rluc **(A)**, Gαo-Rluc **(B)**, or Gα12-Rluc **(C)** were used for BRET experiments and repetitive signals were recorded before and immediately after the injection of 100 nM of trypsin. The curves were fitted using "Plateau then increase to top" equation of Prism GraphPad software and $Y = \text{IF}[X < X0, \text{Plateau}, \text{Plateau} + (\text{Top} - \text{Plateau})*(1 - \exp(-K*(X - X0)))]$ constraining the plateau to a theoretical value of 0. Data are mean ± SEM of three independent experiments performed in single points.

KINETIC ANALYSIS OF LIGAND-INDUCED BRET BETWEEN PAR2 AND Gα PROTEINS

Next, we performed real-time kinetics before and after agonist addition using the injection system available on the Mithras LB-490. As result, the injection of 100 nM of trypsin rapidly increased the BRET signal between PAR2-YFP and Gαi1-Rluc (**Figure 2A**) as well as Gαo-Rluc (**Figure 2B**) and the increased signal remained stable ~5 min after ligand injection. The $t_{1/2}$ values are in second interval as indicated in **Table 1**. However, no ligand-induced BRET increase was observed between PAR2-YFP and Gα12-Rluc within the first 4 min post-stimulation (**Figure 2C**). These observations are comparable to what we previously reported on PAR1-Gαi1 coupling (6, 7) indicating similar pre-assembly properties and activation kinetics.

Next, we performed long-term kinetics (up to 15–20 min) in the absence or presence of trypsin stimulation. As shown above, for both Gαi1-Rluc (**Figure 3A**) and Gαo-Rluc (**Figure 3C**) we observed a basal BRET signal and trypsin promoted a rapid BRET increase in the first seconds of stimulation and the signal was stable for ~4 min before its slow decline in a time-dependent manner. The kinetic analysis using "Plateau followed by one phase decay" equation of Prism GraphPad software resulted in decay $t_{1/2}$ values close to 10 min (**Table 1**) for both Gαi1-Rluc (**Figure 3B**) and

Table 1 | $t_{1/2}$ Values of trypsin-induced BRET increase signals and its decline.

BRET combinations	BRET increase	BRET decline
Gαi1-Rluc + PAR2-YFP	3.31 ± 0.81 s	9.82 ± 0.38 min
Gαo-Rluc + PAR2-YFP	1.80 ± 0.40 s	9.96 ± 0.57 min
Gα12-Rluc + PAR2-YFP	4.94 ± 0.53 min	ND
Rluc-β-arrestin 1 + PAR2-YFP	1.72 ± 0.29 min	ND
	3.29 ± 0.04 min[a]	

[a] *$t_{1/2}$ Value for SLIGRL. Data are mean ± SEM (n = 3).*

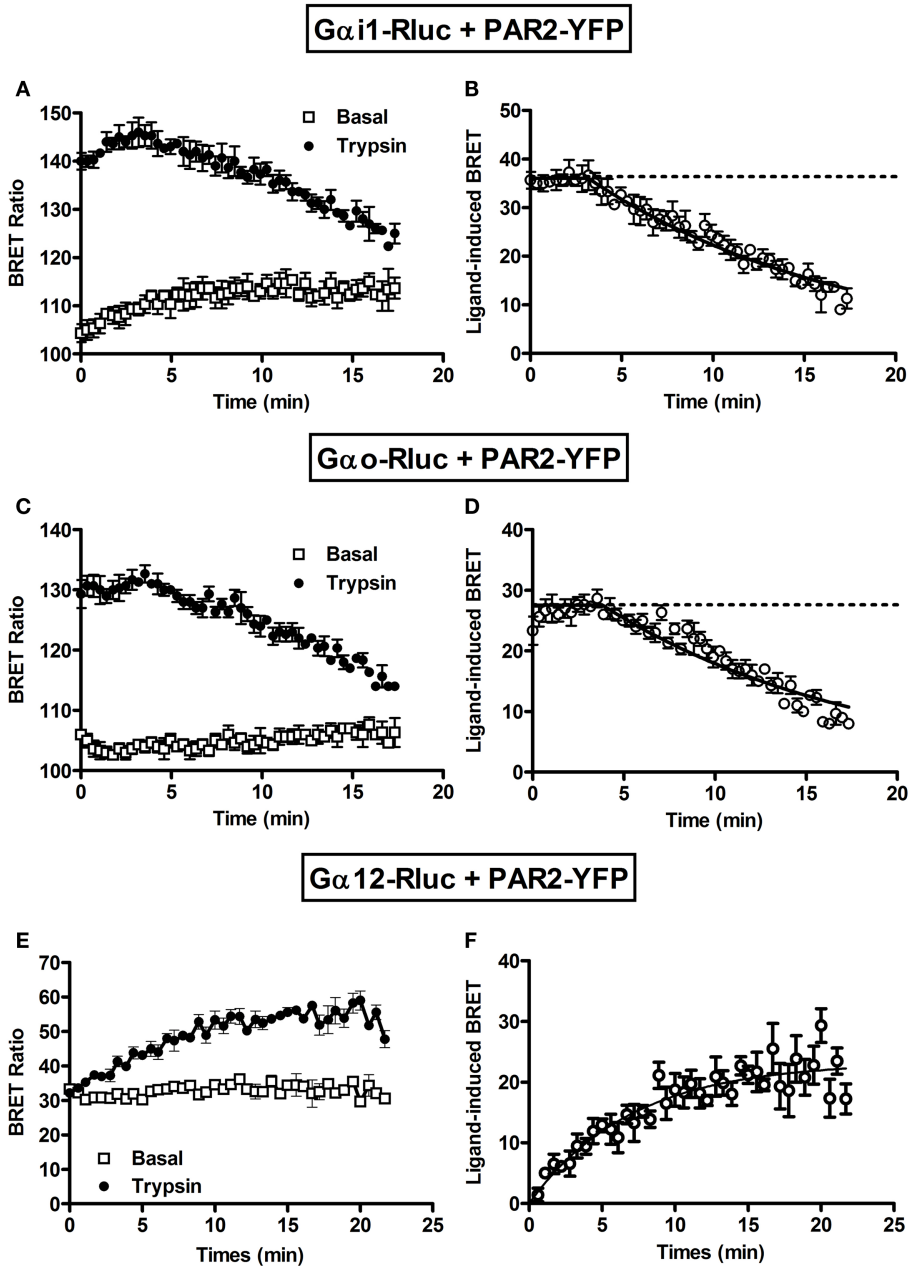

FIGURE 3 | Long-term kinetic analysis of trypsin-induced BRET increase between PAR2 and Gα proteins. COS-7 cells transiently co-expressing PAR2-YFP and either Gαi1-Rluc **(A,B)** or Gαo-Rluc **(C,D)** or Gα12-Rluc **(E,F)** were used for BRET experiments in the absence (□) or presence of 100 nM of trypsin (●). For this, repetitive signals were recorded every ~35 s immediately after trypsin addition during 18–20 min. The panels **(B,D,F)** represent the Prism GraphPad fits of trypsin-induced BRET increase using either "Plateau followed by one phase decay" equation ($Y = \text{IF}[X < X0, Y0, \text{Plateau} + (Y0 - \text{Plateau})*\exp(-K*(X-X0))]$) constraining the plateau to a theoretical value of 0 for Gαi1 and Gαo or "One phase exponential association" equation ($Y = Y_{max}*(1-\exp(-K*X))$) for Gα12. Data are mean ± SEM of three independent experiments performed in single points.

Gαo-Rluc (**Figure 3D**). This analysis demonstrates a reversible trypsin-induced BRET increase reflecting a rapid activation of PAR2-Gαi1 and PAR2-Gαo complexes which is then likely followed by their desensitization. In contrast, a very low BRET signal was measured between Gα12-Rluc and PAR2-YFP as expected (**Figure 3E**) consistent with the data in **Figure 1A**. Interestingly, in the presence of trypsin we observed a gradual increase in the BRET signal between Gα12-Rluc and PAR2-YFP (**Figure 3E**) which reached a plateau after 15 min of stimulation (**Figure 3F**) with a $t_{1/2}$ value close to 5 min (**Table 1**).

Together, our data indicate a pre-assembly of PAR2 with Gαi1 and Gαo but not Gα12 and nicely demonstrate the rapid agonist-promoted activation of the preassembled PAR2-G protein complexes. For Gαi1 and Gαo BRET increase likely reflects

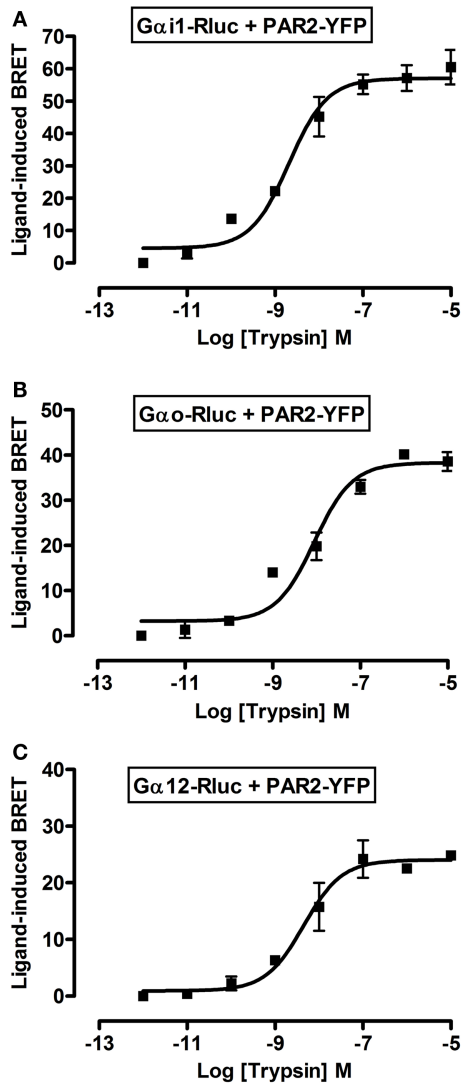

FIGURE 4 | Dose-response analysis of trypsin-induced BRET increase between PAR2 and Gα proteins. COS-7 cells transiently co-expressing PAR2-YFP and either Gαi1-Rluc **(A)**, Gαo-Rluc **(B)**, or Gα12-Rluc **(C)** were used for BRET experiments in the presence of increasing concentrations of trypsin as indicated. Data are means ± SEM of three independent experiments performed in duplicate.

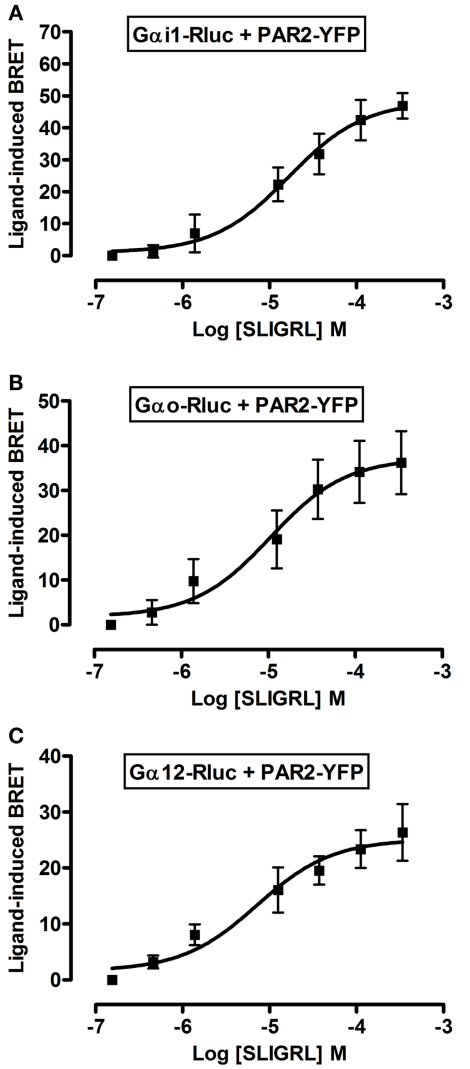

FIGURE 5 | Dose-response analysis of SLIGRL-induced BRET increase between PAR2 and Gα proteins. COS-7 cells transiently co-expressing PAR2-YFP and either Gαi1-Rluc **(A)**, Gαo-Rluc **(B)**, or Gα12-Rluc **(C)** were used for BRET experiments in the presence of increasing concentrations of SLIGRL as indicated. Data are means ± SEM of three to four independent experiments performed in duplicate.

conformational changes within the preassembled complexes leading to their activation followed by their time-dependent desensitization. In contrast, the kinetic data with Gα12 suggest a delayed recruitment in time-dependent manner of the G protein to the activated PAR2. All these observations are in fact consistent with our previous data on PAR1-Gαi1 coupling (6, 7) suggesting similar profile and properties with regard to G protein coupling.

DOSE-RESPONSE ANALYSIS OF LIGAND-INDUCED BRET INCREASE BETWEEN PAR2 AND Gα PROTEINS

To further profile PAR2-G protein interactions and demonstrate the specificity of ligand-induced BRET increase between Rluc-tagged Gα and PAR2-YFP being associated to the activation of receptor-G protein complex we carried out dose-response analysis. After stimulation of cells with increasing doses of trypsin according to the kinetic profile of Gαi1-Rluc, Gαo-Rluc, and Gα12-Rluc, shown in **Figure 3**, a significant BRET increase was measured in a dose-dependent manner for Gαi1-Rluc/PAR2-YFP (**Figure 4A**), Gαo-Rluc/PAR2-YFP (**Figure 4B**), or Gα12-Rluc/PAR2-YFP (**Figure 4C**) complexes. To further demonstrate the specificity of trypsin effects, we also performed dose-response experiments using PAR2-selective peptide agonist, SLIGRL, which does not require receptor cleavage to activate PAR2 (20). As shown in **Figure 5**, SLIRGL also induced a significant BRET increase was measured in a

Table 2 | pEC_{50} values of trypsin and SLIGRL on BRET signals.

BRET combinations	Trypsin	SLIGRL
Gαi1-Rluc + PAR2-YFP	8.61 ± 0.08 ($n = 3$)	4.86 ± 0.31 ($n = 4$)
Gαo-Rluc + PAR2-YFP	8.03 ± 0.18 ($n = 3$)	5.18 ± 0.26 ($n = 4$)
Gα12-Rluc + PAR2-YFP	8.31 ± 0.29 ($n = 3$)	5.14 ± 0.37 ($n = 3$)
Rluc-β-arrestin 1 + PAR2-YFP	7.86 ± 0.11 ($n = 3$)	4.91 ± 0.07 ($n = 3$)

Data are mean ± SEM (n = 3–4).

dose-dependent manner between Gαi1-Rluc (**Figure 5A**), Gαo-Rluc (**Figure 5B**), or Gα12-Rluc (**Figure 5C**) and PAR2-YFP. Both trypsin and SLIGRL increased BRET signals with their expected and respective potencies (20) consistent with ligand-induced BRET increase being reflecting PAR2-G protein complex activation (**Table 2**).

LIGAND-INDUCED RECRUITMENT OF β-ARRESTIN 1 TO PAR2

Finally, we examined the interaction of PAR2 with β-arrestin 1 using BRET. Indeed, the activation of PAR2 is known to be followed by its desensitization and phosphorylation at multiple serine/threonine residues in the C-terminal tail (18, 28, 30). Such phosphorylation constitutes a key step for β-arrestin recruitment to PAR2 promoting receptor internalization through clathrin-coated pits (30). As expected no significant basal BRET can be measured Rluc-β-arrestin 1 and PAR2-YFP and both 100 nM of trypsin (**Figure 6A**) and 10 μM SLIGRL (**Figure 6B**) nicely increased BRET signals. The BRET increase was time-dependent before it reached a plateau corresponding to a saturation of all the phosphorylated PAR2 with the recruited β-arrestin 1. After normalization of the data in **Figures 6A** and **6B** to the percentage of maximal BRET in each case we noticed a slight shift in the kinetics between trypsin and SLIGRL curves (**Figure 6C**) with the $t_{1/2}$ values indicated in **Table 1**. This difference in the kinetics may be due to differences in the binding and activation properties of trypsin and SLIGRL. To demonstrate the specificity of the ligand-induced BRET increase as well as the requirement of PAR2 phosphorylation for β-arrestin 1 recruitment, we used a mutant of PAR2 (PAR2-ΔC-YFP) lacking a large part of its C-terminus (from serine 348) containing multiple serine/threonine residues (28). As shown in **Figure 6D**, the deletion of PAR2 C-terminus completely abolished the ligand-promoted BRET increase demonstrating its implication in PAR2-β-arrestin 1 association. Moreover, both trypsin (**Figure 6E**) and SLIGRL (**Figure 6F**) induced β-arrestin 1 recruitment to PAR2 in a dose-dependent manner with similar potencies (**Table 2**). These BRET observations clearly show a recruitment of β-arrestin 1 to PAR2 involving the C-terminus of the receptor as previously shown (28).

DISCUSSION

In this study we investigated the interaction of PAR2 with three different G protein subunits, Gαi1, Gαo, and Gα12 as well as β-arrestin1 in live COS-7 cells and in real-time using BRET. We demonstrated the existence of preassembled PAR2-Gαi1 and PAR2-Gαo complexes which are nicely activated by trypsin and SLIGRL (PAR2-selective peptide agonist) indicating the coupling of PAR2 to Gαi1 and Gαo proteins in our model. However, the association of PAR2 with Gα12 protein was exclusively observed upon receptor activation similarly to β-arrestin1 recruitment suggesting different coupling mode of PAR2 with Gα12. The dose-response analysis indicated the activation of PAR2-G protein complexes with the known potencies of both trypsin and SLIGRL (20). Together, these findings are similar to what we previously reported on thrombin receptor (PAR1) (6, 7, 27) as well as other studies with other GPCR-G protein pairs (8, 31, 32).

The kinetic analysis showed that PAR2 activation led to a rapid and transient BRET increase between the receptor and either Gαi1 or Gαo proteins with $t_{1/2}$ values fluctuating from 1 to 4 s. Such BRET increase likely reflects conformational changes within the activated preassembled complexes as shown for PAR1 (6, 7). Also, the rapid activation of the preassembled complexes is rather slower but still consistent with the activation kinetics observed with other GPCRs (33–35). This is in agreement with the fast kinetic for the activation of these classes of G proteins leading to rapid modulation of intracellular cAMP levels. In fact, such GPCR-G protein pre-assembly has been reported to be important to favor a certain GPCR-G protein stoichiometry required for rapid and targeted downstream cellular responses (36).

Moreover, long-term kinetic analysis revealed that the rapid agonist-induced activation of PAR2-Gαi/Gαo complexes is followed by the desensitization in time-dependent manner of the preassembled complexes. These observations are supported by the assessment of β-arrestin 1 recruitment to the activated PAR2 which showed a time-dependent association between PAR2 and β-arrestin 1 upon receptor activation with either trypsin or SLIGRL. Moreover, we further demonstrated the importance of PAR2 C-terminus for such interaction as previously reported (18, 30).

For the interaction with Gα12 protein, the data with PAR2 support our previous data with PAR1 (7). This G protein seems to be recruited to PAR2 according to an agonist-dependent process with recruitment kinetics similar to that of β-arrestin 1 (**Table 1**). This kinetics may be reconciled with the kinetics the activation of the small G protein RhoA and p115RhoGEF, two major protein effectors of G12/13 family (37, 38), as well as the involvement of G12/13 in slow and long-term cellular responses such as proliferation, differentiation, and migration (39, 40). However, our previous study clearly indicated that slow and sustained Gα12 recruitment cannot be considered general to all GPCRs since its pre-assembly has been demonstrated with other GPCRs (7). Therefore, whether such pre-assembly with Gαi1/o versus agonist-dependent Gα12 recruitment constitute a general feature of protease-activated receptor family or rather reflect similarities in G protein coupling between PAR1 and PAR2 this needs further investigations. Our study demonstrating the functional interaction of PAR2 with Gαi1, Gαo, and Gα12 in COS-7 cells, in a similar way to PAR1 (6, 7) shed more light on the G protein coupling of PAR2. Our observations are in agreement with the

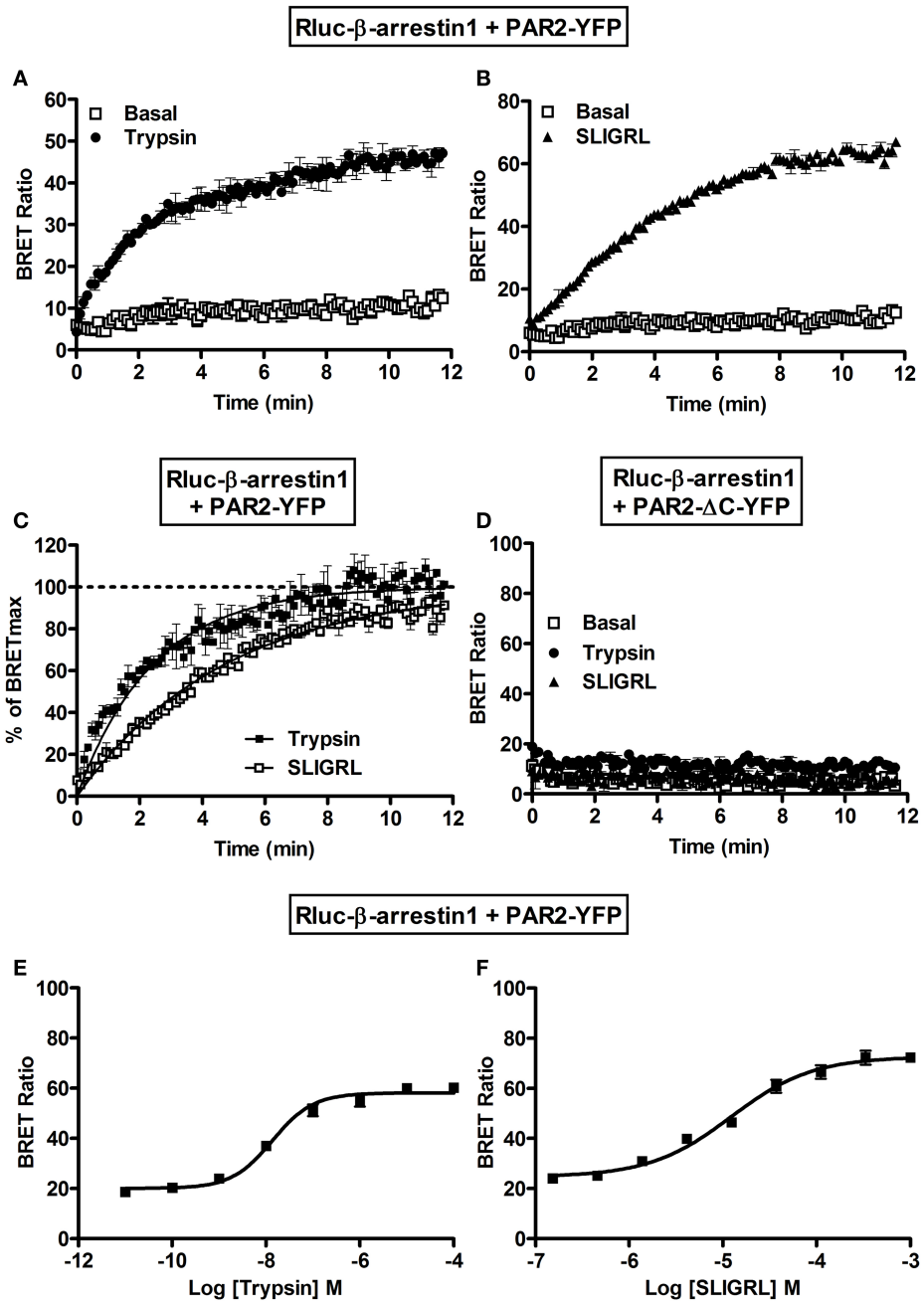

FIGURE 6 | Recruitment of β-arrestin 1 to the activated PAR2 studied by BRET. Time-course analysis on BRET signals measured in COS-7 cells transiently co-expressing Rluc-β-arrestin 1 and either PAR2-YFP **(A,B,C)** or PAR2-ΔC-YFP **(D)** in the absence (□) or presence of 100 nM of trypsin (●) or 10 μM of SLIGRL (Δ). **(C)** Represents the normalization of the curves in **(A,B)** together on the percentage of the maximal ligand-induced BRET signals and the curves were fitted by Prism GraphPad fits of trypsin-induced BRET increase using "One phase exponential association" equation ($Y = Y_{max}*(1-\exp(-K*X))$). Dose-response analysis on the increase of BRET signals between Rluc-β-arrestin 1 and PAR2-YFP upon stimulation with increasing concentrations of trypsin **(E)** or SLIGRL **(F)** as indicated. Data are means ± SEM of three independent experiments performed in duplicate.

previous studies showing the coupling of PAR2 to Gαi1 in the rabbit gastric muscle cells (24) as well as PAR2 forming a stable complex with Gα12 in COS-7 cells (25). However, the latter also reported that PAR2 did not activate Gαi1- and Gαo-dependent signaling pathways (25) illustrating the complexity of PAR2-G protein coupling which appears to be strongly dependent on the cellular model considered.

Finally, our study further illustrates that the GPCR-G protein pre-assembly and agonist-dependent G protein recruitment depend on the receptor-G protein pair and the cellular background

of the model used. This may constitute an important level of integration and regulation of the multiple coupling of GPCRs (1, 41–43), especially when considering the new concepts of GPCR biased signaling and heteromerization.

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Evolution of BRET biosensors from live cell to tissue-scale *in vivo* imaging

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Development of bioluminescence resonance energy transfer (BRET) based genetic sensors for sensing biological functions such as protein–protein interactions (PPIs) *in vivo* has a special value in measuring such dynamic events at their native environment. Since its inception in the late nineties, BRET related research has gained significant momentum in terms of adding versatility to the assay format and wider applicability where it has been suitably used. Beyond the scope of quantitative measurement of PPIs and protein dimerization, molecular imaging applications based on BRET assays have broadened its scope for screening pharmacologically important compounds by *in vivo* imaging as well. In this mini-review we focus on an in-depth analysis of engineered BRET systems developed and their successful application to cell-based assays as well as *in vivo* non-invasive imaging in live subjects.

Keywords: bioluminescence resonance energy transfer, luciferase, fluorescent proteins, optical imaging, protein–protein interactions, cell-based assay

INTRODUCTION

In the post-genomic era, rapid functional evaluation of protein–protein interactions (PPIs), protein phosphorylation, or protease function, which play a key role in various cellular processes such as signal transduction, cell division, transport, etc., in live cell condition is essential. Moreover, the study of such PPIs in normal and diseased cells can help shed light in the understanding of the diseases and to develop suitable therapies. For a long time, conventional biochemical assays like co-immunoprecipitation (1, 2), gel-filtration chromatography (3), sandwich enzyme-linked immunosorbent assay (ELISA) (4), etc., have been used in the investigation of PPIs. These assays though successful, do not suffice as imaging probes because they: (i) are essentially endpoint measurements, (ii) fail to provide spatio-temporal information on specific PPIs, (iii) require mechanical, chaotropic, or detergent based cell lyses, which may alter native PPIs in some cases (5, 6), (iv) are insensitive to transient interactions that regulate certain cellular processes, and (v) have little or no utility for *in vivo* imaging in live subjects. To overcome these limitations, non-invasive imaging approaches such as bioluminescence resonance energy transfer (BRET) have been developed over the last decade, which allow the study of PPIs in their native environment and are capable of providing a unified platform that can be translated from cell culture-based assays to the imaging of live subjects (6, 7). In this mini-review, we will be exploring some hitherto unexplained factors affecting the spectral pattern of several BRET systems and their successful application to cell-based assays as well as *in vivo* imaging of live subjects.

BIOPHYSICAL BASIS OF BRET

Bioluminescence resonance energy transfer is an intrinsic phenomena occurring in the organisms *Renilla reniformis* and *Aequorea victoria*. Exploiting the underlying principles of BRET from nature, literatures demonstrating BRET biosensor applications started since the year 1999. The BRET phenomenon that follows the Förster resonance energy transfer (RET) principle (8), occurs between two proximally situated chromophores – a bioluminescent donor such as a luciferase protein and a fluorescent protein (FP) acceptor with overlapping emission and excitation spectra respectively. Following donor excitation upon substrate addition, part of the electronic excitation energy of the donor is dissipated due to random collisions with other molecules while the remaining electronic relaxation energy is transferred to the acceptor molecule through non-radiative dipole–dipole coupling. Upon excitation, the acceptor molecule now emits its photonic energy at its characteristic wavelength. This results in a decrease in donor emission paralleled by an increase in acceptor emission. The strict dependence of BRET on the inter-chromophoric distance (1–10 nm) makes it an appropriate "molecular yardstick" for determining PPIs. This is true, since the average protein radius is ∼5 nm, which means that a positive BRET signal will only be detected if the two proteins come within ∼10 nm of each other, a distance that is an indicator of direct interaction between the two proteins (9). However, absence of a BRET signal does not necessarily mean that the two target proteins do not interact with each other. Lack of a signal can be accounted for by an unfavorable orientation between the donor and acceptor dipoles. The BRET ratios can be calculated as per Eqs. 1 and 2 (10).

$$\mathrm{BRET} = \frac{\mathrm{BL}_{\mathrm{emission}}\left(\mathrm{Acceptor}\ \lambda\right) - C_f \times \mathrm{BL}_{\mathrm{emission}}\left(\mathrm{Donor}\ \lambda\right)}{\mathrm{BL}_{\mathrm{emission}}\left(\mathrm{Donor}\lambda\right)} \quad (1)$$

where,

$$C_f = \frac{BL_{emission}(\text{Acceptor } \lambda)_{donor\ only}}{BL_{emission}(\text{Donor } \lambda)_{donor\ only}} \quad (2)$$

In the above equation, $BL_{emission}$ is the average radiance measured at the donor (Donor λ) or acceptor (Acceptor λ) filters in BRET-transfected or only donor transfected cells; the correction factor (C_f) represents the BRET signal detected from cells transfected only with the donor plasmid. Upon subtracting this factor from the overall BRET ratio, one can get an idea of the dynamic range for a particular BRET pair. Moreover, since BRET-based assays are ratiometric, any variability due to assay volume or cell number variation or time point of measurement is nullified.

Until recently, the field of BRET-based biosensors has predominantly utilized two basic BRET systems, viz., $BRET^1$ and $BRET^2$. Developed by Xu et al. the $BRET^1$ system combines *Renilla* luciferase (RLuc) with enhanced yellow fluorescent protein (EYFP) (11). However, the spectral resolution (separation of peak donor and acceptor emission spectra) achieved in $BRET^1$ is ∼50 nm only, which is considered suboptimal for macroscopic imaging (12, 13). Another BRET system, named as $BRET^2$, combining RLuc with a UV-excitable GFP variant viz., GFP^2 (14, 15) was developed, that uses a coelenterazine analog-DeepBlueC™ (also known as coelenterazine 400a or Clz400) substrate, which shifts the emission maximum ($E_{m_{max}}$) of RLuc to 400 nm. GFP^2 excites at a maximum ($E_{x_{max}}$) of 396 nm and emits photons at 510 nm. This yields a much larger spectral resolution of 110 nm and has enabled us to perform tissue-scale imaging using wideband filters for the first time (16, 17). However, successful tissue imaging with higher sensitivity of cells located deep inside the animal body calls for the design and development of BRET systems with more red-shifted emissions. This is because, at wavelengths below 600 nm, particularly in the blue-green regions of light, pigments like myoglobin and hemoglobin absorb a significant fraction of the visible light (18).

EXPANSION OF BRET ASSAY FORMATS

In the past few years, improvisations in various components of BRET such as luciferases, FPs, substrates, and instrumentations have contributed to the remarkable expansion in the range of BRET platforms available. Armed with these BRET vectors, the progress of molecular imaging to live cells, animals, and plants with varied applications has been made possible. With the advent of engineered RLuc variants with an elevated photon output and/or a red-shifted $E_{m_{max}}$, *viz.*, RLuc8 ($E_{m_{max}}$ 480 nm; four fold increase in photon output compared to RLuc) (19) and RLuc8.6 ($E_{m_{max}}$ 535 nm; ∼6-fold increase in photon output compared to RLuc) (20), new BRET systems in combination with FPs in the orange and red regions of emission spectra were developed (**Figures 1A–D**). Theoretically, the amplitude of donor emission should always exceed the acceptor emission (**Figures 1A,C**). However, we noted that in the spectral profiles of some of these newly developed BRET systems, the normalized amplitude at the donor emission was lower in comparison to that at the acceptor emission (**Figure 1B**). For example, in the case of TagRFP-RLuc8, only when Clz-*v* substrate was used (shifting the peak donor emission to 515 nm), the amplitude of TagRFP at 585 nm surpassed RLuc8 emission. To explain this anomaly, a deeper understanding of the RET principle is required. RET efficiency is essentially an interplay between the spectral overlap integral of the donor emission and acceptor excitation spectra, in addition to the quantum yield of the donor. We speculate that a donor bleed through signal coupled with the high degree of spectral overlap between RLuc8 and TagRFP (upon the use of Clz-*v*) that favors maximum energy transfer between the pair is detected at the acceptor filter, giving an unnaturally high peak. On a different note, if one tries to define the ideal BRET pair for tissue-scale imaging, it would be the one that gives a high spectral resolution with minimally compromising the BRET ratio. Based on the data compiled from the BRET systems available with us (**Figure 1E**), TurboFP and RLuc8.6-Clz combination would be the ideal BRET partners for both *in vitro* and *in vivo* imaging as they have a high BRET ratio (∼1.19) with an equally high spectral separation of 100 nm.

BRET OPTIONS FOR STUDYING THE KINETICS OF PROTEIN INTERACTIONS

A landmark development in the recruitment of BRET-based systems to capture the PPI kinetics was the creation of protected Clz analogs. The problem associated with the use of normal Clz substrates in live cells was their auto-oxidation, resulting in the loss of peak signal within 2–3 min, which further drops to ∼50% within 17 min. Consequently the measurement of long-term PPI kinetics is impossible using such substrates. To eliminate this problem, Levi et al. (21) reported chemical modifications to protect the putative oxygenation sites of Clz400 and demonstrated that depending on the protective modifications, long-term $BRET^2$ monitoring was achievable. Similarly, another commercial source also developed EnduRen™ substrate that can be used specifically for live cell imaging (22). This is a protected form of coelenterazine-*h* with their active sites blocked by esters or oxymethyl ethers that are only released upon cleavage by intracellular hydrolytic enzymes. The absence of active Clz-*h* in the media significantly reduces the signal attenuation due to background auto-oxidation and the half-life of Clz-*h* increases. Moreover, a steady-state bioluminescence emission of EnduRen™ till about 24 h potentiates its use for monitoring dynamic changes in PPIs from live cell conditions.

Another commonly used luciferase is the North American Firefly luciferase (FLuc; $E_{m_{max}}$ 562 nm) (23). A codon-optimized version of FLuc has been created by commercial sources for use in mammalian cells. The relatively slower and stable emission kinetics of its substrate, D-luciferin, makes it naturally suitable for kinetic measurements from live environments, obviating the need for any chemical modifications to its structure (24). FLuc has been reported to be used in a BRET system in conjunction with red FPs like DsRed (25) as well as with non-protein fluorophores such as Cy3 and Cy3.5 (26). However, its bulky size of 61 kDa, an obligate dependence on Mg^{2+} and ATP as its cofactors (27, 28) and finally, a low spectral resolution with the BRET partners reported so far, makes it a poor choice for BRET.

MULTIPLEXED BRET OPTIONS FOR CO-LATERAL INTERACTION STUDIES

In addition to the simple PPIs assays, one might be interested to monitor two concurrent dependent/independent PPI events

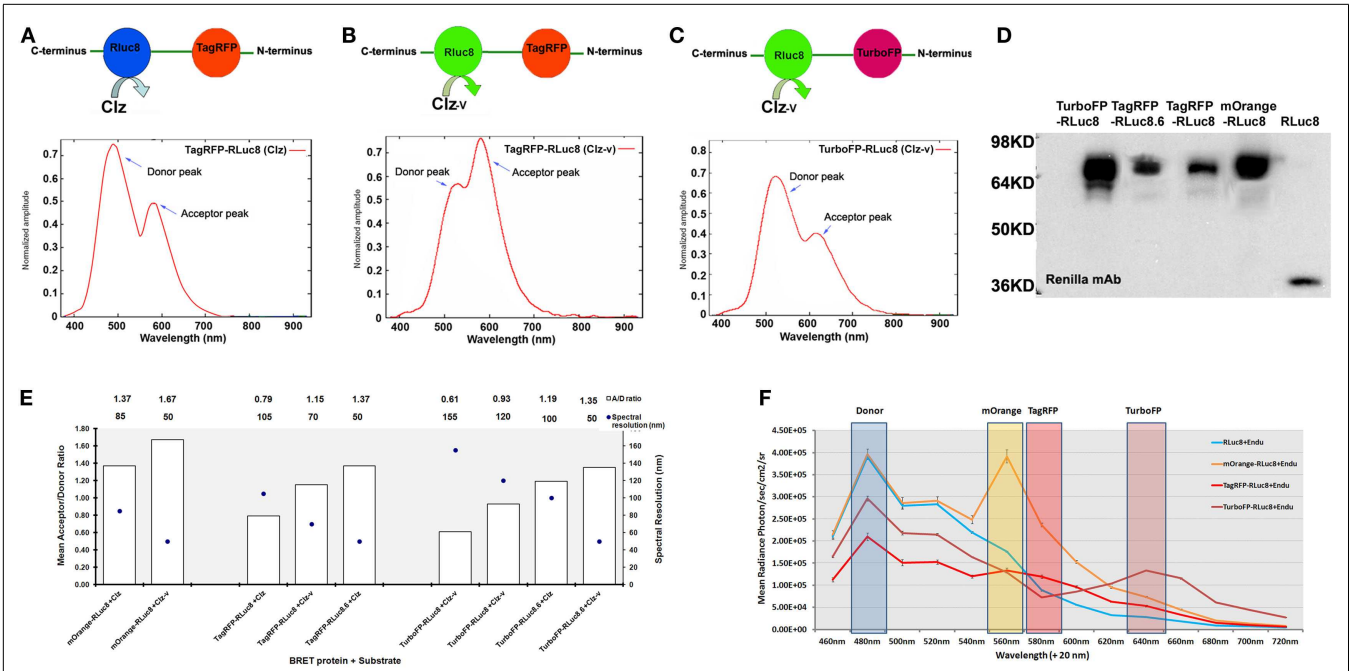

FIGURE 1 | Various new generation of BRET pairs reported using mutant *Renilla* luciferase proteins. **(A)** Represents TagRFP-RLuc8 pair using normal coelenterazine (Clz) substrate. The bottom chart represents normalized amplitude vs. wavelength measured from the purified fusion protein added with Clz substrate. **(B)** Represents the same pair using the coelenterazine-*v* (Clz-*v*) substrate analog. **(C)** Represents TurboFP-RLuc8 pair using the Clz-*v* substrate. The chart represents normalized amplitude vs. wavelength measured from purified protein with Clz-*v* substrate. Note that in majority cases [e.g., **(A)** or **(C)**], typical normalized amplitude emission of the donor is higher than the acceptor emission output, whereas in some cases [e.g., **(B)**], due to efficient transfer of energy, normalized amplitude of acceptor emission is higher than the donor emission. **(D)** Represents western blot analysis of various BRET fusion proteins made by combining mutant *Renilla* luciferases (such as RLuc8 and RLuc8.6) with mOrange, TagRFP, and TurboFP fluorescence acceptors. As control, RLuc8 (RL8) protein of 35 kDa size was also shown on the right most lane. **(E)** BRET ratios (denoted as A/D ratio on *Y*-axis) are calculated by measuring the donor and acceptor $E_{m_{max}}$ from each of these fusions in the presence of indicated Clz (or its analog) substrate from mammalian cells expressing the proteins. Spectral resolutions (difference between the donor and acceptor emission peaks) are also plotted on the *Z*-axis (as dark circles). One can observe an inverse relationship between the BRET ratio measured and the spectral resolution of the BRET partners which is more or less linear in fashion. In some cases, the BRET ratio was seen to be >1 which is theoretically not possible. This is because the values mentioned here are the raw measured values without accounting for the C_f value. Once the C_f value is added into the equation, the BRET ratios drop below 1. Note that either Clz-*h* or Clz-*v* substrate analogs can be used against the same fusion protein to fine tune the spectral resolution. **(F)** Spectral profile measured from live mammalian cells over-expressing RLuc8, mOrange-RLuc8, TagRFP-RLuc8, and TurboFP-RLuc8 fusion proteins. Cells over-expressing these proteins were exposed to live cell substrate EnduRen™ (Endu) and emission profiles were imaged using IVIS spectrum imaging system loaded with 20 nm band-pass filters between 460 and 720 nm. This arrangement provides a multiplexing opportunity, where the same donor protein can be combined with multiple acceptors, whose $E_{m_{max}}$ can be resolved by band-pass filter sets as indicated on the chart.

within the same cell. BRET multiplexing was employed in one of the GPCR studies to monitor the ubiquitination kinetics and its involvement in receptor regulation. Exploiting the distinct spectral emission properties of the RLuc substrates-Clz-*h* ($E_{m_{max}}$ 480 nm) and Clz400 ($E_{m_{max}}$ 400 nm), Perroy et al. co-expressed RLuc-β-arrestin and GFP^2-ubiquitin along with a YFP-labeled vasopressin receptor (V_2R-YFP) (29). In this way, depending on the substrate (Clz-*h* or Clz400) oxidized by RLuc, either BRET^1 or BRET^2 kinetics can be respectively detected. Appropriate negative controls, for instance, the use of Clz-*h* to detect negligible BRET transfer between RLuc and GFP^2 can validate the authenticity of such experiments. Moreover, with the series of BRET systems that are now available to us, one can recruit either a single/dual luciferase system such as RLuc8 and RLuc8.6 with appropriate acceptor FPs (**Figure 1F**), which can facilitate BRET multiplexing of three to four candidate proteins. The ease with which this objective can be achieved and the requirement of only a single substrate, makes it a highly attractive option for co-lateral protein interaction studies.

MULTIPLEXED BRET OPTIONS FOR STUDYING MULTI-PROTEIN COMPLEX

While dual-BRET techniques facilitate the concomitant monitoring of two different PPIs events, elegant approaches such as sequential RET (SRET) (30, 31), bimolecular-fluorescence complementation-BRET (BiFC-BRET) (32, 33), complemented donor-acceptor-RET (CODA-RET) (34), and bimolecular luminescence complementation-BiFC (BiLC-BiFC) (35, 36) have enabled the detection of interactions between higher order protein complexes. In the SRET technique, the three candidate proteins are fused to either RLuc donor or one of the two FP acceptors. In such a situation, a BRET process excites the first fluorescent acceptor, which will now serve as a fluorescence resonance energy transfer

(FRET) donor for the second fluorescent acceptor. Two such systems, SRET[1] (RLuc-YFP-DsRed) and SRET[2] (RLuc-GFP[2]-YFP) utilizing Clz-*h* and Clz400 substrates respectively, were reported in literature, that could detect the heterotrimerization of cannabinoid CB_1 receptor (CB_1R), dopamine D_2 receptor (D_2R), and adenosine A_{2A} receptor ($A_{2A}R$) as well as the assembly of G-protein subunits in living cells. Further, Navarro et al. successfully demonstrated the oligomerization of Calmodulin (CaM), $A_{2A}R$, and D_2R using SRET[2] in live cells, which can open avenues for screening of potential drugs that specifically target these receptor interactions. In another study, the CB_1R-D_2R-$A_{2A}R$ interactions were studied using a BiFC (using N- and C-termini truncated forms of YFP) coupled with a luciferase protein to form a functional BRET system (BiFC-BRET). In yet another recent literature, a split luciferase complementation reconstituting the donor bioluminescence was paired to an acceptor FP to detect the BRET signal. This technique, termed CODA-RET can be used in the study of receptor oligomerization in presence of agonists/antagonists as well as in drug screening. Amalgamating the above two techniques, one can also employ both complemented donor along with bimolecuar fluorescence complementation (BiLC-BiFC) to form a functional BRET system that can explore the interaction of up to four proteins.

BRET FOR TISSUE-SCALE IMAGING

While we have progressed so far in terms of optimizing various BRET platforms with the aim to image PPIs non-invasively in their natural physiological environments *in vivo*, it has not yet been achieved completely. However, scientific endeavors have not been futile. With the introduction of the intensely cooled charge coupled device (CCD) camera-based optical imaging instrumentation, the ability to detect very dim photon signals from live cells in culture or from animal or plant tissues has become possible. To detect signals with detectors placed outside the animal subjects, the cells of interest present at a depth within the subject must produce sufficient signal. Here, primarily the use of red and NIR light signals is favored as they have lesser tissue attenuation and thus, better penetration capacity. Therefore, overall modification of existing assays to adapt them for non-invasive monitoring is a challenging task. Approaching the development of a single format imaging assay that can serve to measure PPIs from isolated single cells as well as physiologically relevant animal/plant models, both BRET[1] and BRET[2] strategies display some form of confinements. Therefore, while attempting live animal BRET assays, we have conducted serial experiments to identify an optimal BRET assay showing satisfactory performance as a single format assay (12, 16, 17). By now, we have introduced an ample variety of the red light emitting BRET vectors, many of which undoubtedly show superior performance over the previous assays used. By withdrawing the traditional method of BRET measurement using a microplate reader, we adapted a method for spectral separation of donor and acceptor signal by using black-box cooled CCD camera macro-imager (16). An important parameter to successfully adapt this imaging method was the use of the BRET formats with relatively large spectral resolution, which allows the selection of wide band-pass emission filters in the device. Thus the CCD camera-based macro-imaging instrument can measure BRET signals from lysed or live cells placed in multi-well plates. The same instrument can then be used for BRET measurement from whole organisms as well. A point worth noting here is that, BRET imaging from animal tissues is further complicated by the consideration of tissue attenuation factor. To address this, a double ratio (DR) which provides a depth-independent measure of the BRET signal in animal experiments was defined (Eq. 3) (7).

$$DR = \frac{BL_{emission}(\text{Acceptor }\lambda)_{BRET} \times \mu_t(\text{Acceptor }\lambda)}{BL_{emission}(\text{Donor }\lambda)_{BRET} \times \mu_t(\text{Donor }\lambda)} \Big/ \frac{BL_{emission}(\text{Acceptor }\lambda)_{donoronly} \times \mu_t(\text{Acceptor }\lambda)}{BL_{emission}(\text{Donor }\lambda)_{donoronly} \times \mu_t(\text{Donor }\lambda)} \quad (3)$$

where, μ_t denotes the total attenuation coefficient.

The main bottleneck of extending FRET strategy in small animal evaluation is associated with the auto-fluorescence correction method. As light travels in and out from animal tissues, the resulting photon attenuation complicates the FRET ratio calculations. In this context, the exclusion of an external photon input makes BRET-based technologies more acquiescent for macro-scale imaging of PPIs. As represented in **Figure 2**, we have also done proof of principle studies by confirming the detection of the rapamycin-dependent interaction of FKBP12 and FRB from living animals (7, 12, 17). Following the successful BRET imaging from small animal model, macro-imaging of plant tissues was also reported (37). Using a modified electron bombardment-CCD camera coupled with a dual-view image splitter, visualization of the constitutive photomorphogenesis 1 protein (COP1) homo-dimerization using RLuc-EYFP BRET assay was demonstrated in the rootlet and cotyledons of tobacco seedlings in order to understand its repressive activity on light regulated development in plants. The same group had previously reported the use of a similar BRET assay in onion epidermal cells as well as in the *Arabidopsis* seedlings to study the effect of COP1 dimerization and its nuclear exclusion on the functional activity of COP1 (38). BRET is better adapted to plant imaging, since it circumvents the issues of photobleaching and auto-fluorescence of photosynthetic pigments as seen in the case of FRET. Considering careful validation of the PPIs in systematic, large-scale models using individual test cases, the molecular imaging assays like BRET appear promising in the current proteomic developments. So far, the major hurdle with BRET strategy was our inability to visualize the interactions of endogenous proteins. However, this is no longer an impediment, as Audet et al. have successfully reported the measurement of BRET signals in cell lines obtained from transgenic mice that are made to express β2-adrenergic receptor fused to RLuc (β2AR-RLuc) and βarrestin-2 fused to a GFP (GFP2-βarr2) (39). Even though this development does not count for an actual detection of endogenous proteins, it is definitely a leap in that direction. With the inception of BRET-based quantum dots (QDs) conjugates (40, 41), *in vivo* imaging in small animal models has been simplified. These new generations of BRET probes follow a similar approach as the conventional BRET systems and act as BRET acceptors for RLuc donor. Some of these QDs can emit at wavelengths as high as 800 nm, enabling the visualization of dynamic PPIs from deep tissues of small, live animals with better resolution.

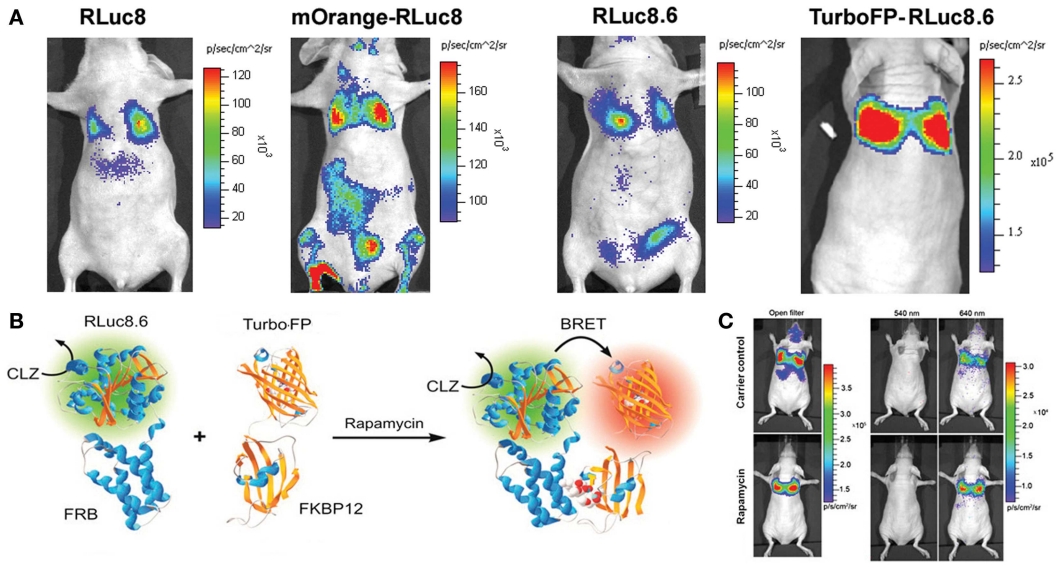

FIGURE 2 | Bioluminescence resonance energy transfer performance in deep tissue imaging experiments. **(A)** Upper panel represents mouse images comparing improvements in the signal output from lungs. Mammalian cells engineered for equivalent over-expression of donor alone (RLuc8 or RLuc8.6) or BRET proteins (mOrange-RLuc8 or TurboFP-RLuc8.6) as marked, were compared. Note the photon output values in the reference color scale bars. Highest signal output from same number of cells placed within lungs was noted with TurboFP-RLuc8.6 BRET protein imaged with Clz substrate. **(B)** Schematic illustration of the most successful BRET format tested for monitoring the rapamycin induced FRB-FKBP12 association. **(C)** Representative bioluminescence images of nude mice with accumulated mammalian cells in the lungs which stably over-express FRB and FKBP12 interacting partners fused to RLuc8.6 and TurboFP respectively. Cells (3×10^6 in 150 μL PBS) were injected through the tail vein, resulting in significant trapping in the lungs. One group of mice ($n = 8$) was injected 2 h before cell injection with 40 μg rapamycin dissolved in 20 μL DMSO and further diluted in 130 μL PBS administered through the tail vein. A second group of mice ($n = 8$) was injected with DMSO (20 in 130 μL PBS). Two hours after cell injection, the mice were injected i.v. with Clz substrate and sequentially imaged using open/donor/acceptor filters. Substrate-only control mice ($n = 4$) were used for background subtraction. The figure is partially represented with permission from PNAS (7).

CONCLUSION

Bioluminescence-based live cell assays are becoming increasingly attractive in biological applications as they are rapid, fairly sensitive, cost effective and easy to perform, some are even acquiescent to high-throughput systems and offer several advantages in comparison to other *in vitro* systems. BRET has been utilized for developing diverse live cell-based assays, many of which have now been adapted in small animal research for tracking specific protein functions, phosphorylation, and protease activation events as well as screening genetic and chemical modulators. By making this technology versatile, their scope for BRET-based molecular imaging of biological events from living cells and subjects will continue to expand.

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REV, a BRET-based sensor of ERK activity

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Networks of signaling molecules are activated in response to environmental changes. How are these signaling networks dynamically integrated in space and time to process particular information? To tackle this issue, biosensors of single signaling pathways have been engineered. Bioluminescence resonance energy transfer (BRET)-based biosensors have proven to be particularly efficient in that matter due to the high sensitivity of this technology to monitor protein–protein interactions or conformational changes in living cells. Extracellular signal-regulated kinases (ERK) are ubiquitously expressed and involved in many diverse cellular functions that might be encoded by the strength and spatio-temporal pattern of ERK activation. We developed a BRET-based sensor of ERK activity, called Rluc8-ERKsubstrate-Venus (REV). As expected, BRET changes of REV were correlated with ERK phosphorylation, which is required for its kinase activity. In neurons, the nature of the stimuli determines the strength, the location, or the moment of ERK activation, thus highlighting how acute modulation of ERK may encode the nature of initial stimulus to specify the consequences of this activation. This study provides evidence for suitability of REV as a new biosensor to address biological questions.

Keywords: biosensor, bioluminescence resonance energy transfer, BRET imaging, fluorescence lifetime imaging microscopy, extracellular signal-regulated kinases, spatio-temporal signaling, Rluc8-ERKsubstrate-Venus

INTRODUCTION

The specificity of cellular responses to receptor stimulation is encoded by the spatial and temporal dynamics of downstream signaling networks. Cells indeed respond to multiple external stimuli thanks to a surprisingly limited number of signaling pathways activated by plasma membrane receptors. To encode and make distinct various external signals these commune pathways have to be precisely regulated in space and time. Thus distinct spatio-temporal activation profiles of a shared repertoire of signaling proteins result in different gene activation patterns and diverse physiological responses (1–3).

An emerging picture of interrelated networks has therefore superseded the former preconceived scheme of discrete linear pathways to convey extracellular signals to specific targets. In fact, various receptor pathways share a common protein catalog that mediates signal transduction. For any individual receptor pathway, there is no single protein or gene responsible for signaling specificity. Rather, specificity is determined by the temporal and spatial dynamics activation of downstream signaling components. To address and answer the questions surrounding the specificity of signal to response events, signaling reporters for individual downstream signaling components activation in intact cellular environment will be required.

In recent years, efforts have been made to study the dynamic cellular processes by the engineering of biosensors specific to various signaling pathways. Resonance Energy Transfer technologies (Fluorescent, FRET or Bioluminescence resonance energy transfer, BRET) have proven to be efficient in this area by enabling the monitoring of protein–protein interactions or protein-conformational changes in living cells. FRET and BRET technologies are both based on the non-radiative transfer of energy between the donor and acceptor molecules via the Förster mechanism and primarily depend on: (1) an overlap between the emission and excitation spectra of the donor and acceptor molecules, respectively; and (2) the close proximity of the donor and acceptor entities (<100 Å) (4, 5). In the case of FRET, both the donor and acceptor are fluorescent molecules, whereas in BRET, the energy donor is a bioluminescent molecule. FRET necessarily requires fluorescence excitation, resulting in problems of photobleaching, autofluorescence, simultaneous excitation of both donor and acceptor fluorophores, phototoxicity, and undesirable stimulation of photobiological processes. Most of these FRET drawbacks tend to be corrected with the emergence of technologies like TR-FRET or Fluorescence Lifetime Imaging Microscopy (FLIM) (6, 7). Nevertheless, the use of BRET allows these practical problems to be bypassed, as it is initiated by an enzymatic reaction (instead of fluorescence excitation). Accordingly, bioluminescence-initiated resonance energy transfer results in greater sensitivity in living subjects because of a higher signal to background ratio, and therefore makes BRET a

technology of choice for measurements from cell lysates or intact cells (8).

The Mitogen-Activated Protein Kinases (MAPK) family is a class of serine/threonine kinases [including the Extracellular signal-regulated kinases (ERK), p38, and JNK sub-families] that are ubiquitously expressed, activated by various stimuli and therefore involved in numerous cellular functions. The ERK signaling cascade is a central MAPK pathway that plays a role in the regulation of various cellular processes such as proliferation and differentiation, development, neuronal plasticity and learning, survival, and apoptosis (9, 10). The ability of this cascade to regulate so many distinct and even opposing cellular processes raises the question of signaling specificity determination by this cascade. Duration and strength of the signals, interaction with specific scaffolds, changes in sub-cellular localization, crosstalk with other signaling pathways, and presence of multiple components with distinct functions in each tier of the cascade seems to determine the ultimate function of ERK activation (11–14). The reliability of signaling and the spatio-temporal activation of ERK are therefore key context-dependent determinants that need to be deciphered in order to resolve the nature of precise biological responses.

To better understand the final outcome of intricate factors controlling the kinetics of ERK activity, signaling reporters in living cells have been engineered (15–18). In the present work we used the most efficient biosensor for ERK activity so far tested (18). We reproduced published data, however the sensitivity of this FRET biosensor was not sufficient to detect subtle variations of ERK activity in neurons. We therefore improved this biosensor by switching the FRET tags to BRET compatible entities. We then defined the proper experimental conditions to record accurate BRET signals with this ERK-biosensor, described the potential and limitation of this upgraded reporter, and discussed possible further improvements. This work also addresses general technical concerns about the use of biosensors.

MATERIALS AND METHODS

REAGENTS

Glycine (200 μM), strychnine (1 μM), GABA (100 μM), U0126 (10 μM), PMA (1 μM), and KCl (50 mM) were all purchased from Sigma-Aldrich, St Quentin Fallavier, France. NMDA (50 μM) was purchased from Tocris (Fisher-Bioblock, Illkirch, France) and epidermal growth factor (EGF) (50 ng/ml) from Calbiochem (Merck-Millipore, Darmstadt, Allemagne). We used the following primary antibodies: p44-42 MAP Kinase Antibody (Cell Signaling Technology, #9102), phospho-p44p42 MAP Kinase (Thr202/Tyr204) antibody (Cell Signaling Technology, #9101), p-Thr-48-Cdc25C antibody (Cell Signaling Technology, #9527), and rabbit GFP antibody (Invitrogen, A11122).

REV CONSTRUCTION

Plasmids coding for nuclear and cytoplasmic Extracellular signal-regulated Kinase Activity Reporter (EKAR), pRK5-Cerulean-EKAR$_{Nucl}$-Venus, and pRK5-Cerulean-EKAR$_{cyto}$-Venus (18) (Addgene, Cambridge, MA, USA) were digested by *Cla*I and *Bam*HI restriction enzymes to remove the Cerulean-coding sequence and replace it by the Rluc8-coding sequence amplified by PCR between *Cla*I and *Bam*HI restriction sites. We thus obtained two plasmids: pRK5-Rluc8-EKAR$_{Nucl}$-Venus and pRK5-Rluc8-EKAR$_{cyto}$-Venus. Inactive mutants were made by mutation in EKAR Cdc25C peptide "PDVPRTPVGK" (Thr-to-Ala substitution). The control pRK5-Rluc8-EKAR (cyto) plasmid was designed from the pRK5-Rluc8-EKARcyto-Venus construct: the original Rluc8-ERKsubstrate-Venus (REV) sequence was removed by a *Cla*I/BrsGI enzymatic digestion and replaced with a Rluc8-EKAR sequence obtained by PCR on pRK5-Rluc8-EKARcyto-Venus with insertion of *Cla*I/BrsGI appropriate restriction sites.

CELL CULTURES AND TRANSFECTION

HEK293T cell culture and calcium phosphate transfection were performed as previously described (19). To determine the optimal level of REV expression we performed many transfections with different amounts of pRK5-Rluc8-EKAR$_{Nucl}$-Venus and pRK5-Rluc8-EKAR$_{cyto}$-Venus plasmids, ranging from 0.1 ng to 4 μg of each plasmid per 100 mm diameter cell dish (3,000,000 cells). The total amount of DNA per plate dish was complemented with the non-coding plasmid pcDNA3 to reach 5 μg of DNA in each transfection. We chose the transfection condition containing 20 ng of REV-coding plasmids for the other experiments. Hippocampal neuronal primary cultures were prepared from 17.5 days embryonic mice (E17.5) and grown in neurobasal medium (Gibco, Invitrogen, Cergy Pontoise, France) supplemented with 2% B-27 (Gibco), glutamax (4 mM, Gibco), glutamic acid (25 μM, Gibco), antibiotics (Penicillin 100 U/ml and Streptomycin 100 μg/ml), and 10% Fetal Bovine Serum (FBS), in 35 mm diameter glass bottom culture dishes (MatTek Corporation, Ashland, MA, USA). After 3 days in culture (DIV3), the culture medium was supplemented with Cytosine β-D-arabinofuranoside hydrochloride 5 μM (Sigma-Aldrich, St Quentin Fallavier, France) for 12 h. Then, 75% of the medium was replaced by neurobasal medium supplemented with B-27, glutamax, and antibiotics. Neurons were then transfected with 100 ng of pRK5-Rluc8-EKAR$_{Nucl}$-Venus and pRK5-Rluc8-EKAR$_{cyto}$-Venus plasmids and 1.8 μg of the non-coding plasmid pcDNA3 using Lipofectamine 2000 (Invitrogen, Cergy Pontoise, France) according to the manufacturer's standard protocol at DIV10 and studied between DIV11 and DIV12.

IMMUNOFLUORESCENCE STAINING AND IMAGING

Immunofluorescence staining was performed on HEK cells, transfected or not with REV, were fixed with 4% PFA for 10 min at room temperature, permeabilized with Triton X-100 0.15% for 10 min, washed and incubated with blocking buffer (FBS 10%, BSA 1% in PBS) for 2 h at RT. Polyclonal antibodies raised against p44-42 MAP kinase were incubated overnight at 4°C in PBS containing 1% BSA. After three rinses with PBS, the anti-mouse Cy3-conjugated antibodies (1:500, Jackson) were added for 30 min at RT. Three rinses with PBS were carried out before mounting cells directly in the wells under cover slips.

Images were obtained with LSCM (OLYMPUS, FV-1000, 60× objective), equipped with appropriate epifluorescence and filters (Green: 475_40 and 530_50 nm for excitation and emission respectively, Red: 545_25 and 605_70 nm for excitation and emission respectively). Images were digitized and saved in TIFF format using the Andor software and further analyzed using the ImageJ software (NIH).

WESTERN BLOTS

Cells were lysed in 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA, anti-protease mixture (Roche Applied Science), phosphatase inhibitors (Na_3VO_4, NaP_2O_3, NaF), and 20 mM Tris-HCl, pH 7.4 (lysis buffer), and the mixture was centrifuged. The supernatant was incubated in a Laemmli buffer at 90°C. Proteins were transferred to nitrocellulose (NC) membranes (Millipore, Bedford, MA, USA) and blocked in blocking buffer (5% non-fat dry milk in TBS and 0.1% Tween 20) for 1 h. The blots were then incubated with primary antibodies at the relevant dilution (Cell Signaling Technology, Beverly, MA, USA) for 1 h at room temperature, and with horseradish peroxidase-linked secondary antibodies (1:20,000; Pierce, USA) for 2 h. Immunoblots were revealed using the enhanced chemiluminescence reagents (Thermo, USA) and visualized using the X-ray film. The density of immunoreactive bands was measured using NIH image software, and all bands were normalized to percentages of control values.

FLUORESCENCE LIFETIME IMAGING MICROSCOPY

Time-domain FLIM was performed with a multiphoton microscopy system, based on a Zeiss Axiovert 200M LSM 510 Meta NLO equipped with a Ti:Sapphire Chameleon-XR pulsed laser (Coherent). Time-resolved detection was afforded by the addition at a non-descanned output of a fast photomultiplier and SPC-830 time-correlated single-photon counting (TCSPC) electronics (7). For EGFP excitation, laser power at 900 nm was adjusted to give average photon counting rates of the order 10^4–10^5 photons s^{-1} (0.0001–0.001 photons/excitation event) and with peak rates approaching 10^6 photons s^{-1}, below the maximum counting rate afforded by the TCSPC electronics to avoid pulse pile-up. Acquisition times of 120 s were used. Analysis of the fluorescent transients was performed with the SPCImage software package (7). Images were taken with a Zeiss 63×/1.0 W Plan-Apochromat objective.

BRET MEASUREMENTS

Bioluminescence resonance energy transfer measurements in cell populations were performed as previously described (19). Single cell BRET imaging in cultured hippocampal neurons to study the sub-cellular localization of REV-conformational changes were performed according to previous protocols (20, 21). Briefly, images were obtained using a Plan-Apochromat 63×/1.40 Oil M27 objective, at room temperature. Hippocampal neurons were transfected at DIV10 and recorded at DIV11 or DIV12 in the following external medium (in millimolar): 140 NaCl, 0.5 $CaCl_2$, 3 KCl, 10 HEPES, 10 D-Glucose, 0.0003 tetrodotoxin, pH 7.4 and osmolarity of 330 mOsm. Transfected cells were first identified using a monochromatic light and appropriate filter to excite Venus (exciter HQ480/40 #44001 – emitter HQ600/50 #42017, Chroma). The light source was then switched off until the end of the experiment. Coelenterazine H (CoelH, 20 μM) was applied for 5 min before acquisition with Metamorph software (Molecular Devices). BRET images were collected every 30 s by sequential acquisitions from the 535 and 480 nm channels of 7 s each, using the evolve camera from Photometrics. Drugs were added 2 min (or four images) after the first acquisition. Sequential acquisitions were performed at 5 MHz (Gain 3950, binning 1) with emission filters D480/60 nm (#61274, Chroma) and HQ535/50 nm (#63944, Chroma) to select em480 and em535 wavelengths respectively. We applied an exclusive threshold on the em480 image, from 0 to 4,000 counts, in order to exclude these non-reliable weak values (see **Figure 3A**). The pixel-by-pixel 535/480 nm ratios were calculated by dividing the absolute blue or yellow intensities per pixel of images obtained at 535 nm over 480 nm. These numerical ratios (comprised between 0 and 1.5) were translated and visualized with a continuous 256 pseudo-color look-up table (LUT) as displayed in the figures. We determined the average intensity ("mean") and distribution ("Standard Deviation") of the 535/480 nm fluorescence ratios, in a square region of pixels drawn on the sub-cellular compartment of interest using Image J software (NIH). The "mean" measured the global BRET intensity in that area, while the "Standard deviation" of BRET from pixel to pixel gave information about the distribution of the BRET signals in that area. These are two complementary pieces of information. We then averaged the mean ± SEM and Standard Deviation ± SEM obtained from three to six cells and seven square regions per cell in the same sub-cellular compartment and in identical stimulating conditions. Please note that a high standard deviation indicates a spatial clusterization of the BRET signals and not a variation of mean between similar areas, this latter being detected by the "SEM" of the mean.

STATISTICAL ANALYSES

Analyses were performed using Prism software. Statistical analyses were performed with the non-parametric Kruskal and Wallis test for more than two independent samples or with Friedman test for paired samples with a "p" risk threshold of 5%.

RESULTS

CONSTRUCTION OF "REV," A BRET-BASED SENSOR OF ERK ACTIVITY

Extracellular signal-regulated Kinase Activity Reporter is a FRET-based sensor of ERK activity optimized for signal-to-noise ratio and FLIM (18). Briefly, the ERK activity sensor includes a substrate phosphorylation peptide from Cdc25C containing the consensus MAPK target sequence (PRTP) (22), and the proline-directed WW phospho-binding domain (23) boxed between FRET compatible entities (EGFP and mRFP1). Phosphorylation of the substrate sequence by ERK activation induces the binding of the phospho-binding domain and subsequent conformational rearrangement, thus triggering a change in FRET between the donor and acceptor entities. Because specificity in MAPK signaling depends on docking domains (24), EKAR contains an ERK specific docking site (FQFP) next to the phosphorylation sequence (25). Finally, a central flexible linker consisting of 72 glycine residues spaces out the phospho-binding domain from the substrate peptide to allow conformational changes (18).

Extracellular signal-regulated Kinase Activity Reporter selectively and reversibly reported ERK activation in HEK293T cells after EGF stimulation (18). Using FLIM, we corroborated these data (**Figure A1** in Appendix). However in our hands, this FRET-based sensor failed to report weaker biological stimuli also thought to modulate ERK activity. In order to detect subtle modulations of ERK activity with this biosensor, we hypothesized that the sensitivity of the assay would be improved by replacing the FRET donor and acceptor pair by BRET compatible entities. We therefore engineered a construct in which the *Renilla Luciferase*

(Rluc8) and acceptor Yellow Fluorescent protein Venus are boxing the ERK substrate (**Figure 1A**), so-called "REV." Because of the nuclear localization of the WW domain, REV expression was restricted to the nucleus when expressed in HEK293T cell (REV_{nucl}, **Figure 1B**). We engineered a second plasmid containing an additional DNA sequence coding for a C-terminal nuclear export sequence, which resulted in cytoplasmic expression (REV_{cyto}, **Figure 1B**). We controlled that REV transfection in HEK cells did not impair endogenous ERK expression. Both of them were broadly expressed in the cell (**Figure A1B** in Appendix). To characterize REV as a new ERK-biosensor, HEK293T cells were transfected with both nuclear and cytosolic plasmids, except when specified.

DEFINING THE EXPERIMENTAL CONDITIONS TO DEPICT RELIABLE BRET SIGNALS TO REPORT ERK ACTIVITY

Rluc8-ERKsubstrate-Venus being an intra-molecular BRET biosensor, the stoichiometry of donor and acceptor entities is constant: one Rluc8 for one Venus per molecule. In its non-phosphorylated form, the reporter adopts an "open" conformation. Upon phosphorylation, conformational bending of the biosensor increases the proximity between the donor and acceptor in a "closed" conformation and induces a BRET-increase to report its phosphorylation by ERK. In an attempt to properly define the experimental conditions allowing reliable BRET signals, we first assessed the optimal expression level of REV. Three main criteria have to be fulfilled. This optimal expression must indeed be sufficient to be within the linear range of detection of the luminescence and fluorescence signals of the donor and acceptor entities respectively. However, too high a level of REV expression could induce non-specific inter-molecular BRET due to random collisions between proteins, which would bias the analysis. Finally, a minimal expression of REV would maximize its phosphorylation by endogenous kinases activation, while an excess of REV expression would preclude the phosphorylation of all molecules by ERK and thus prevent adequate detection of ERK activity. We thus performed several transfections of HEK293T cells with increasing quantity of REV expression plasmids (see Materials and Methods). The BRET signal expressed as a function of the fluorescence (which is proportional to the REV expression level) was constant, except for weak expression of REV revealing the limit of detection of the luminescent and fluorescent signals

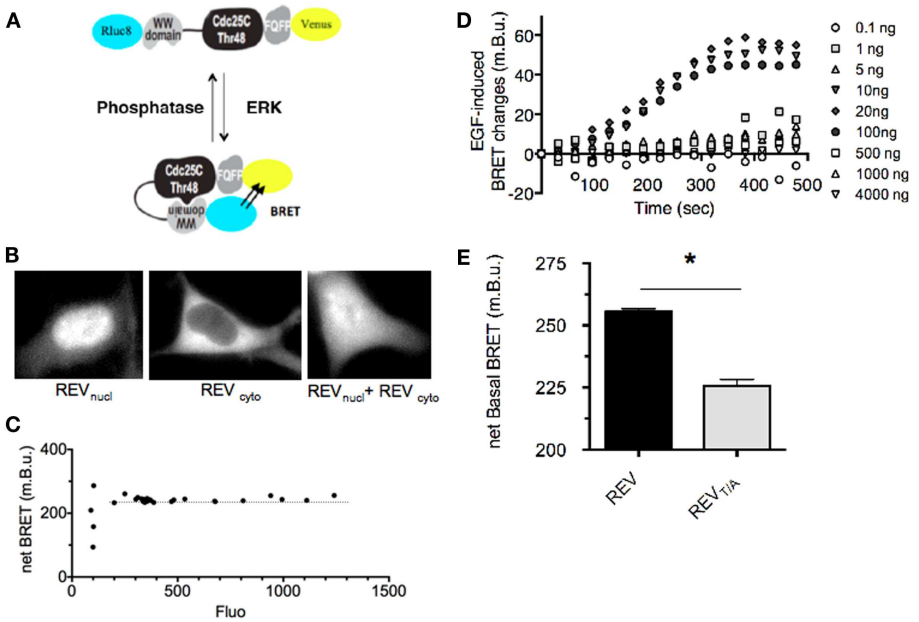

FIGURE 1 | Design and characterization of REV, a BRET-based sensor of ERK activity. **(A)** Schematic representation of ERK sensor-conformational changes induced upon ERK activation, adapted from Harvey et al. (18). The conformational change induced by REV-phosphorylation increases the proximity between Rluc8 and Venus, promoting BRET-increase. **(B)** Fluorescence of REV_{nucl} and REV_{cyto} transfected alone or together in HEK293T cells. **(C)** Determination of the expression level of REV required for a reliable BRET measurement. HEK293T cells were transfected with increasing amounts of pRK5-Rluc8-$EKAR_{Nucl}$-Venus and pRK5-Rluc8-$EKAR_{cyto}$-Venus plasmids. BRET in cell population was expressed as a function of REV-fluorescence reporting the expression level of REV. Note that the BRET remained constant for a fluorescence of REV comprised between 200 and 1,300 photon counts which corresponds to cells transfected with 10–4,000 ng of plasmid per 100 mm diameter culture dish. **(D)** Determination of the expression level of REV required for efficient report of ERK activation. HEK293T cells transfected with increasing amounts of pRK5-Rluc8-$EKAR_{Nucl}$-Venus and pRK5-Rluc8-$EKAR_{cyto}$-Venus plasmids were stimulated with EGF and recorded over time. Note that EGF-induced ERK activity could be reported only in cells transfected with 10–100 ng of plasmid per 3,000,000 cells. **(E)** Basal BRET measured in HEK293T cells transfected with REV or $REV_{T/A}$ inactive mutants. Each bar of the histogram represents the mean ± SEM of five independent experiments performed in triplicate. Note that the unphosphorylated form of REV, $REV_{T/A}$, displays a lower BRET signal than the wild-type REV, highlighting a basal activity of ERK in our experimental conditions. The non-null net BRET of $REV_{T/A}$ also emphasizes a basal BRET in the unphosphorylated (open) conformation of REV. **(C–E)** BRET in cell population were measured in the Mithras luminescence-fluorescence plate reader. Net BRET values were calculated by subtraction of the BRET obtained in cells transfected with Rluc8 alone, and multiplied by 1,000 to be expressed as milli BRET unit (mBu).

(**Figure 1C**). No inter-molecular BRET interference was detected for this range of protein expression. To assess the optimal quantity of DNA coding for REV to efficiently report ERK activation, we applied EGF on cells transfected with 0.1–4,000 ng of plasmid DNA coding for REV (**Figure 1D**). EGF-induced ERK activation was detected only in transfection conditions ranging from 10 to 100 ng of REV plasmid per 100 mm diameter cell dish (3,000,000 cells). In cases of ERK substrate (REV) stronger expression, only a small amount of REV might be phosphorylated by ERK activation, which prevents the optimal detection of ERK activity. In the following experiments, to favor REV-phosphorylation by endogenous kinases, we thus selected the transfection condition giving rise to the weakest expression level of REV within the linear range of BRET (corresponding to 20 ng of DNA per 100 mm cell dish, **Figure 1C**).

HEK293T cells expressing REV displayed a mean basal net BRET signal of 255.55 ± 1.15 milli BRET units (mBu, **Figure 1E**). This basal BRET signal might report either a sufficient proximity between the donor and acceptor entities in the non-phosphorylated conformation of REV and/or a non-null phosphorylation of REV due to a basal activity of ERK. To discriminate between these two possibilities, we mutated the MAPK phosphorylation site in the Cdc25C peptide (Thr-to-Ala substitution, $REV_{T/A}$) (18). The basal BRET displayed by the $REV_{T/A}$ mutant was significantly weaker (225.64 ± 2.6 mBu) than the wild-type biosensor (**Figure 1E**), highlighting a basal ERK activity. This was further confirmed by perfusion of the ERK pathway inhibitor U0126 (10 μM), which induced a BRET decrease of -32.79 ± 0.68 mBu in REV-transfected cells (**Figure 2A**). The remaining basal BRET with REV in presence of U0126 and the non-null BRET displayed by the $REV_{T/A}$ mutant both evidenced a proximity between Rluc8 and Venus in the non-phosphorylated form of REV compatible with intra-molecular BRET.

REV EFFICIENTLY AND SPECIFICALLY REPORTS ERK ACTIVITY IN LIVING CELLS

To assess the efficiency of REV to report ERK activation, REV-expressing cells were stimulated with EGF (50 ng/ml) or phorbol myristate acetate (PMA, 1 μM) to strongly activate ERK signaling. EGF application induced a transient BRET-increase that was maximal 5 min after stimulation (**Figure 2A**). Conversely, the PMA-induced BRET-increase was slower (maximal 15 min after stimulation), but stable for more than 1 h (**Figure 2A**). Both EGF- and PMA-induced BRET-increases could be reversed by application of the ERK inhibitor U0126 (10 μM, dotted lines, **Figure 2A**). Furthermore, no BRET changes were recorded upon PMA application in HEK293T cells transfected with the mutant $REV_{T/A}$, confirming the specificity of the biosensor to report ERK phosphorylation specifically (**Figure 2B**). Under EGF- or PMA-induced ERK stimulations, no difference was found between nuclear and cytosolic REV reporters when expressed separately (**Figure 2C**). These drug-specific profiles of ERK activation reported by REV were in accordance with western blot (WB) analyses to reveal the phosphorylated form of endogenous ERK and transfected REV (using Anti-ERK and Anti-phospho-Thr-48-cdc25c antibodies, respectively) (**Figure 2E**). However, we noticed a slight shift in time of the maximal ERK activity using WB compared to real-time BRET experiments. This temporal shift might be the result of the WB experiment's lack of precision due to the use of detergent to lyse cells and solubilize proteins. The BRET sensor thus appeared to be an accurate reporter of ERK activity, with the main advantage of reporting the modulation of ERK activity in real time in the same living cells.

To characterize the sensitivity of this BRET-based sensor, we measured the ratio of EGF-induced increase in REV BRET (57.32 ± 1.03 mBu, mean ± SEM obtained from three individual experiments) over the standard deviation of BRET signals in basal condition (2.53 ± 0.14 mBu, mean of Standard Deviation ± SEM obtained from three individual experiments) and found that the EGF-induced REV BRET-increase was 22.6 times higher than the standard deviation of the BRET. Similar quantification was performed with EKAR, the most sensitive FRET-based sensor so far published. FLIM experiments showed an EGF-dependent decrease in EGFP fluorescence lifetime of 0.0560 ± 0.0082 ns for a 0.0147 ± 0.0017 ns standard deviation of the signal in basal condition. The EGF-induced FRET increase was therefore 3.8 times higher than the standard deviation. To place emphasis on the higher sensitivity of REV, we normalized EGF-induced EKAR and REV signals modulation to the standard deviation of FRET and BRET signals respectively (**Figure 2D**).

The high sensitivity of REV suggested the possibility of reporting subtle modulations of ERK activity with this BRET-based sensor, which we failed to do with EKAR by FRET/FLIM. For example, GABA-B receptor stimulation in HEK293T cells transfected with GB1 and GB2 subunits induced a shorter and weaker increase in ERK activity as shown by WB (**Figure 2E**). This transient weak activation of ERK activity could successfully be reported in real time with REV in living cells (**Figure 2A**). Taken together, these results identify REV as a sensitive biosensor to report ERK activity in living cells, providing that its expression level was sufficient to reliably read the BRET signals but weak enough to allow its phosphorylation by endogenous ERK.

REV REPORTS STIMULUS-SPECIFIC RESPONSES OF ERK SIGNALING IN NEURONAL SUB-CELLULAR COMPARTMENTS

In light of the good sensitivity of REV, we performed BRET imaging experiments in hippocampal neurons to depict the spatio-temporal dynamics of ERK activation. First, as mentioned previously for BRET in cell population, a sufficient level of expression is needed in order to be in the linear range of detection of luminescence and fluorescence. This consideration also applies to BRET imaging to obtain an accurate evaluation of BRET signals. To determine a cut-off for the minimal level of luminescence emission required for an accurate BRET ratio measurement, we measured the evolution of Em480, Em535, and BRET intensity over time (**Figure 3A**). The BRET signal over time was constant until the luminescence decreased fewer than 4,000 counts where the fluctuation between successive BRET readings strongly increased. Accordingly, in order to exclude these non-reliable pixels of weak emission, we applied an exclusive threshold on the Em480 image before carrying out the pixel-by-pixel division of Em535/Em480 to obtain the BRET image.

One of the major difficulties in establishing BRET imaging is to distinguish the signal originating from the transfer of

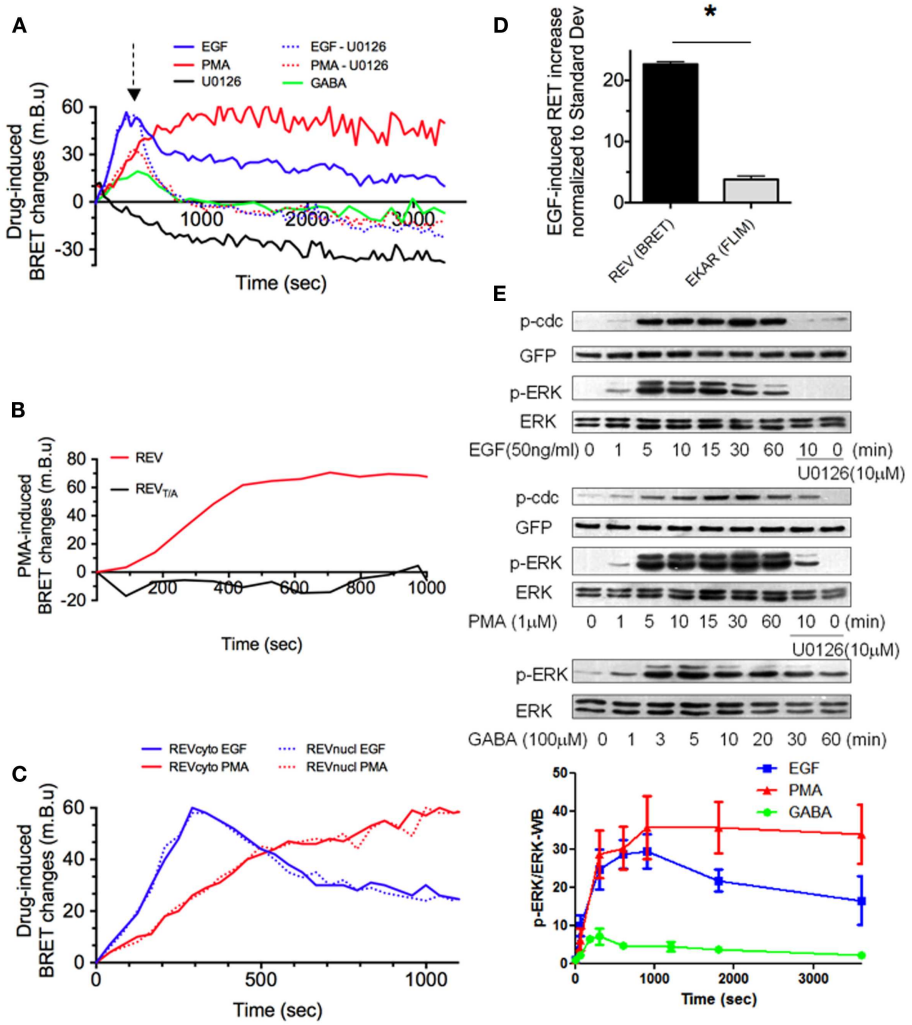

FIGURE 2 | REV, a sensitive BRET sensor of ERK activity. **(A–D)** Drug-induced BRET changes of REV were measured in HEK293T cells population, in the Mithras luminescence-fluorescence plate reader. Cells were transfected with both cytosolic and nuclear constructs of REV **(A,B,D)** or only one of the two constructs **(C)**. In A, green line, GB1 and GB2 subunits of GABA-B receptor were co-transfected with REV. **(A)** EGF, PMA, and GABA application induced a BRET signal increase with different intensity and temporal profile, while U0126 decreased the BRET. The PMA or EGF-induced increase of BRET could be reversed by U0126 application (dotted lines, U0126 application is symbolized by the arrow, 300 s after PMA or EGF perfusion). **(B)** PMA-induced BRET changes was measured in HEK293T cells transfected with REV or REV$_{T/A}$. The absence of effect of PMA in REV$_{T/A}$-transfected cells validated the specificity of REV to report ERK activation. **(C)** BRET changes induced by PMA (red) or EGF (blue) were measured in the cytosol (full lines) or nucleus (dotted lines). **(D)** Sensitivities of EKAR (FRET/FLIM-based reporter) and REV (BRET-based reporter) were compared by normalizing the EGF-effect to the standard deviation in their respective technology. **(E)** ERK, phospho-ERK (p-ERK), REV (GFP), and phospho-REV (p-cdc) staining quantified by western blot in HEK293T cells transfected (GABA – green line) or not (EGF and PMA conditions, blue and red line) with GB1 and GB2 subunits of GABA-B receptor before and up to 60 min after drug application. When specified, following 10 min of EGF or PMA incubation, U0126 was added for 50 min. p-ERK/ERK ratio were calculated in each column and normalized to the p-ERK/ERK ratio measured before stimulation ($t = 0$). Each point of the graph below represents the mean ± SEM obtained from three individual experiments, for each time condition.

energy from that resulting from an overflow of the energy donor output into the energy acceptor detection channel. To control for this basal signal, we engineered a REV construct without Venus (RE). Neither the mean BRET signal nor the standard deviation, were found to be significantly different in the soma and dendritic areas (**Figure 3B**). As expected, RE displayed a homogenous and weak basal BRET, independent on the luminescence level. This validated the accuracy of our experimental conditions.

In neurons, ERK activity is modulated by the neuronal activity and has been involved in opposing cellular processes such as long term potentiation (LTP) or depression (LTD) of the synaptic transmission (26). Several studies have shown that ERK activity is indeed regulated by different stimuli, among which we chose three examples. (1) KCl depolarization induces sustained ERK activation in hippocampal neurons (27, 28). (2) Selective extrasynaptic NMDA receptor activation (as well as NMDA bath application) does not activate ERK pathways, whereas (3) synaptic NMDAR

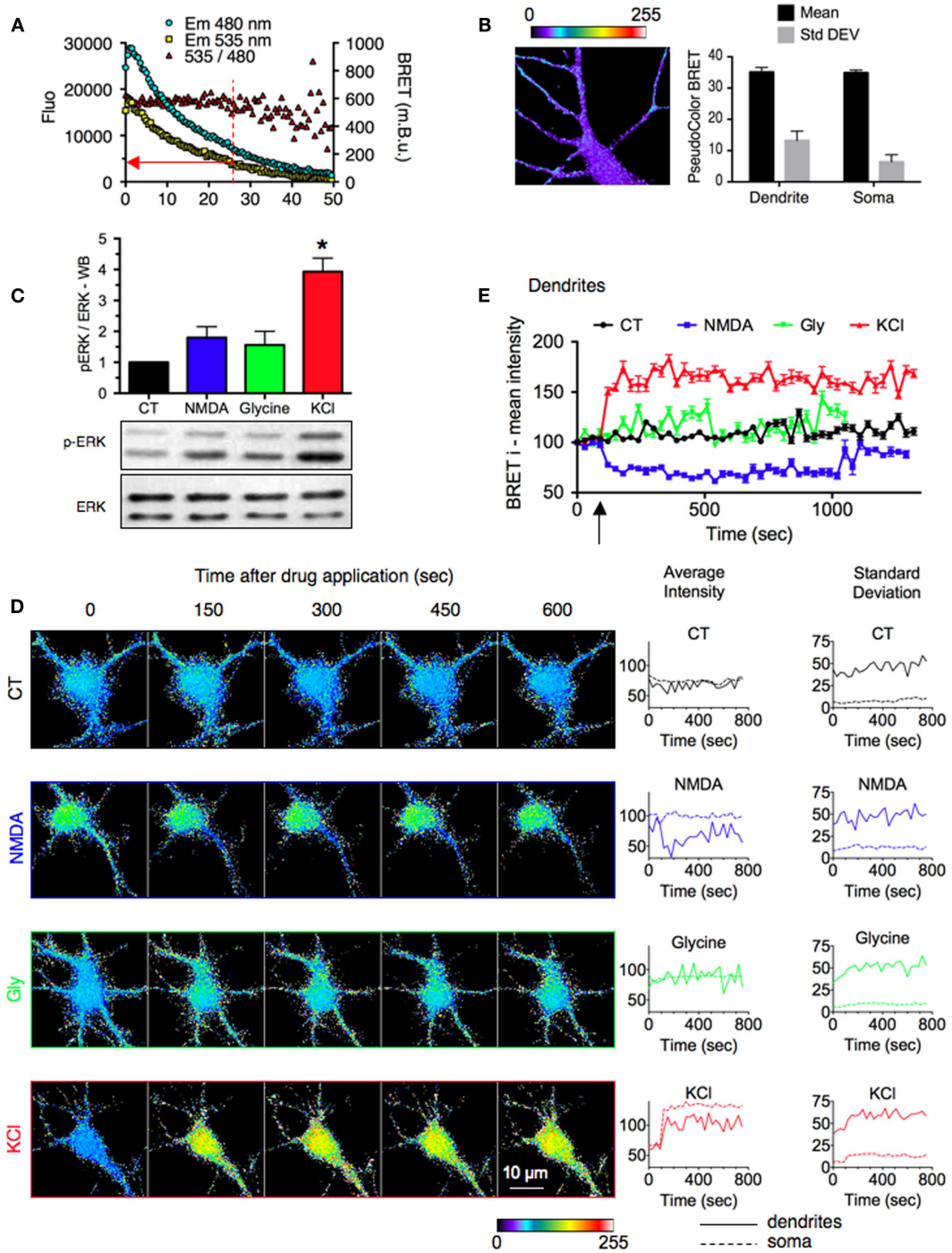

FIGURE 3 | Stimulus-specific responses of ERK signaling reported by REV in neuronal sub-cellular compartments. **(A)** Evolution of Em480, Em535, and 535/480 ratio signals over time. Sequential BRET images on neurons expressing REV were acquired for 50 min. The red arrow indicates the cut-off value for luminescence intensity below which BRET fluctuations prevented adequate measurements. **(B)** Basal signal resulting from the overflow of the energy donor output into the energy acceptor detection channel. Neurons were transfected with RE (REV construct without Venus). Left, representative BRET image. Right, BRET intensity (mean) and distribution (Standard Deviation) in soma and dendrites. Each bar of the histogram represents the mean ± SEM obtained from three to six neurons and seven regions per neuron. **(C)** ERK and phospho-ERK (p-ERK) staining quantified by western blot in hippocampal neurons stimulated or not (black) with NMDA (50 µM, blue), Glycine and strychnine (200 and 1 µM, respectively, green), or KCl (50 mM, red) for 10 min. **(D)** Real-time BRET imaging of four representative hippocampal neurons transfected with REV, in the stimulated conditions described in **(A)**. Changes of average intensity and standard deviation over time were measured on the soma and dendritic shaft of each image. **(E)** Intensities of BRET signals recorded on dendrites of neurons in BRET images over time. Each point of the graph represents the mean ± SEM obtained from three to six neurons and seven regions per neuron, for each time condition.

activation – to induce a chemical LTP – induces ERK activation (29). WB experiments indeed confirmed these findings but only KCl application was found to be significantly different from the non-stimulated condition (**Figure 3C**).

To further decipher stimulus-specific responses of ERK signaling we used REV to report in living neurons the location, duration, and strength of ERK activity in sub-cellular compartments. Compared to control condition (buffer perfusion), KCl application (50 mM, 10 min) induced a sustained increase in BRET intensity both in soma and dendrites, reporting ERK activation upon depolarization (**Figures 3D,E**). The standard deviation increased in dendrites, suggesting an important clusterization of ERK activity induced by KCl application (**Figure 3D**). Conversely, NMDA bath application (50 μM, 10 min) decreased the BRET signal intensity and this inhibition of ERK activity could be seen in dendrites only (**Figure 3D**). Thus, while a global approach such as WB experiment failed to report significant modulation of ERK activity by NMDA (**Figure 3C**), the possible sub-cellular analysis with the BRET-based sensor highlighted a dendritic significant inhibition of ERK activity (**Figure 3E**). In presence of glycine and strychnine (200 and 1 μM, respectively, for 3 min) the BRET intensity slightly increased in soma and dendrites as well as standard deviation in dendrites, compared to control condition (**Figure 3D**). However, in contrast to the KCl-induced increase and NMDA-induced decrease, this Glycine-induced modulation of BRET signals was not significantly different from the control condition when several experiments were pooled (**Figure 3E**).

DISCUSSION

In the present work we have engineered and tested the first BRET reporter of ERK activity, REV. REV selectively and reversibly reported ERK activity after EGF or PMA stimulation in HEK293T cells and following changes in neuronal activity in hippocampal neurons. REV therefore allows the analysis of ERK signaling in time and space in living cells. We here defined the experimental conditions required to use this BRET-based sensor and achieved a proof of principal study to highlight several advantages of REV to improve the detection of ERK activity. Finally REV was used to point out spatio-temporal profiles of ERK activity induced by different stimuli, so far unrevealed by other technologies.

Our results highlight the need to carefully control the expression level of the biosensor to reach optimal conditions and report subtle modulations of endogenous kinases activity. The expression level of the reporter has to be sufficient to allow a reliable detection of the light emitted by the BRET donor and acceptor. However the reporter expression level must not be excessive to efficiently report the activity of endogenous kinases. We precisely defined the limits of REV expression to report ERK activation by EGF application in HEK cells. Similarly, in neurons, we determined a cut-off for the minimal level of luminescence emission required for an accurate BRET ratio measurement. In order to exclude these non-reliable pixels displaying low luminescence value, we applied an exclusive threshold on the Em480 image before dividing Em535/Em480 pixel-by-pixel to obtain the BRET image. The mean BRET values as well as the distribution (Standard Deviation) of BRET obtained from a sensor expressing only the BRET donor entity was found to be homogenous both in dendrites and soma. This result confirmed the accuracy of our experimental conditions. Once again, in order to optimize the number of REV molecules that would be phosphorylated to report endogenous ERK activation, we chose neurons with the weakest fluorescence, i.e., those in which the expression level of REV was low. We further corroborate our BRET experiments by WB experiments to reveal the phosphorylation of the reporter as well as ERK auto-phosphorylation induced by ERK stimulation in different conditions. The consistence of the results obtained with BRET and WB, further validated our experimental conditions. Nevertheless, it is important to mention that the expression level of REV defined herein may not satisfy every kind of ERK activation. In low ERK activation conditions, only a small amount of REV would be phosphorylated by ERK activation, which would prevent the optimal detection of ERK activity. This might be the reason why REV failed to reliably report one of the weakest modulations of ERK activity we found by WB, the glycine-induced modulation.

Rluc8-ERKsubstrate-Venus selectively reported ERK activity. This was assessed by point mutation of the REV-phosphorylation site, and by the use of ERK inhibitor, U0126. U0126 indeed put emphasis on a basal ERK activity and was also effective in reversing EGF- and PMA-induced BRET-increase. Intriguingly, this reversion did not reach the BRET levels obtained with U1026 alone. WB experiments confirmed that a pre-stimulation of ERK by PMA led to ERK auto-phosphorylation and REV-phosphorylation which were not totally abolished by a subsequent 50 min incubation of U0126, **Figure 2E**. Thus U0126 has a higher potency to decrease ERK substrate phosphorylation when applied alone. The residual phosphorylation of ERK or REV following sequential application of PMA and U0126 might come from the fact that once phosphorylated the REV sensor may be a poor substrate for phosphatases and only a part of phosphorylated REV would return to an inactivated state following U0126 incubation. Moreover the dephosphorylation rate of REV may be different in cells incubated only in U0126 compared to cells with high activation of ERK (EGF/PMA) since phosphatases activation could be affected differently. Nor can one exclude a non-specific action of U0126 and some phosphorylation of the ERK sensor, due not only to ERK but to other kinases not inhibited by U0126. However, no BRET changes were recorded upon PMA application in HEK293T cells transfected with the mutant $REV_{T/A}$, arguing in favor of the specificity of the biosensor to report ERK phosphorylation.

This proof of principal study highlights several advantages of REV to improve the detection of ERK activity. One obvious benefit coming from the BRET-based sensor is the possibility to work on living cells and report the kinetics of ERK activation in real time on the same cell. FRET-reporters present the same advantage, but this BRET biosensor displayed a higher sensitivity. Consequently, compared to classical WB experiments, REV avoids the variability of ERK activity measurements from different pools of cells over time. Moreover, BRET imaging reported a variability of ERK activation

status in a cell population. This cellular precision indeed extend the need for biosensors such as REV, that could be used in real time on the same living cell rather than pools of cells in which the mean signal would not change over time. For example, the basal ERK activation status could indeed have been a limiting factor to see the NMDA effect (with a lower basal ERK activity, we may have missed the NMDA-induced decrease of BRET). This comment also applies to the sub-cellular location of the activation of a signaling pathway. With BRET imaging we were able to highlight subtleties in sub-cellular activation of ERK that we missed with a more general approach such as WB experiments (see again the NMDA-induced modulation of ERK reported by WB compared to BRET, **Figure 3**). Even within sub-cellular compartments, BRET imaging enables the characterization of the distribution of BRET signals by measuring the standard deviation between pixels of the same area. For example, the high standard deviation seen in dendrites of neurons expressing REV, even in basal condition, indicates a spatial clusterization of the ERK activity along dendrites. The cellular and sub-cellular precision thus increases the sensitivity of the assay compared to global methods. Finally, one fundamental property of REV is its high reproducibility. We averaged the mean BRET intensity as well as the BRET distribution (Standard Deviation) on several areas drawn in identical compartment and stimulating conditions. Both averages displayed small SEM, highlighting the reproducibility between similar areas, and the consistence and accuracy of the results obtained with the BRET sensor.

In this first study using REV we pointed out spatio-temporal profiles of ERK activity induced by different stimuli, some of them so far unrevealed by other technologies. Firstly, REV displayed a basal BRET signal, which could be decreased by mutation of the REV-phosphorylation site. This highlighted a basal ERK activity. In neurons, this basal ERK activation was spatially clustered along dendrites. One direct consequence of REV reporting basal activity of ERK is to allow the detection of decreases in ERK activity. Secondly, in HEK cells, we found similar kinetics for cytoplasmic and nuclear ERK activation, which confirms the data obtained with FRET sensors by Harvey et al. (18). The exact molecular mechanisms underlying ERK translocation to the nucleus are still unknown and are the subject of interesting debate. This further highlights the need for real-time sensors for ERK activity. Our results suggest that regulation of ERK activity is similar in the somatic cytoplasm and nucleus, possibly because of a rapid diffusional exchange between the two compartments (30). Thirdly, in neurons, we demonstrated that the strength, the location, and the moment of ERK activation depends on the nature of the stimuli, highlighting that ERK is not simply switched on and off, but rather that acute modulation of ERK will encode the nature of the initial stimulus and specify the consequence of its activation. REV is therefore well suited to address such an important biological issue, not only in neurons but also in any other cell type.

Several experimental adaptations may improve the sensitivity of this BRET-based biosensor. First we will increase the length of the central flexible linker to increase the distance between the donor and acceptor entities in the basal (non-phosphorylated) condition. This should decrease the basal BRET signal still detected with REV in presence of U0126 (ERK inhibitor), or with the mutant of $REV_{T/A}$ that cannot be phosphorylated ($REV_{T/A}$). Accordingly, by decreasing basal BRET we expect a larger window for BRET modulations induced by ERK activation, which could help in reporting subtle variations. Second, the use of BRET compatible entities for the third generation of BRET [BRET3, with Rluc8 as donor and mOrange as acceptor (31)] should also increase the sensitivity of this assay. Indeed, the improved spectral resolution between Rluc8 and mOrange compared to Rluc8 and Venus will minimize the bleed-through of the donor fluorescence into the acceptor detection channel, decreasing the background signal and therefore increasing the sensitivity of BRET measurements. Accordingly, we replaced Venus by mOrange in the BRET-based sensor to obtain REO (Rlu8-ERKsubstrat-mOrange). Ongoing experiments are testing this reporter, which will further present the advantage to be compatible with BRET1-based reporters of other signaling pathways. Another way to improve this sensor could be provided with a higher luminescence signal by pixel without increasing the concentration of biosensor per cell, using a new smaller and brighter luciferase called Nanoluc (Promega). Finally, because the level of expression of the BRET-based sensor is a limiting factor to accurately report ERK activity, we wish to control the expression of the biosensor by endogenous promoter in order to be in harmony with the expression levels of ERK targets.

To conclude, this efficient ERK sensor has to be added to the library of BRET-based sensors so far generated (32) and will help understanding integrated signaling dynamics in time and space (33).

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APPENDIX

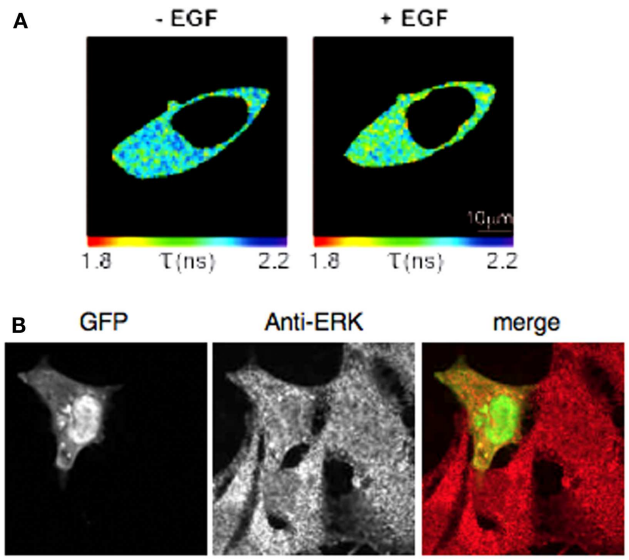

FIGURE A1 | (A) Fluorescence lifetime images of a HEK293 cell expressing cytosolic EKAR before and after (8 min) addition of EGF. **(B)** REV-fluorescence (GFP) and ERK immunostaining (Anti-ERK) in HEK293 cells transfected with plasmids coding for cytosolic and nuclear REV constructs.

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BRET biosensor analysis of receptor tyrosine kinase functionality

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Bioluminescence resonance energy transfer (BRET) is an improved version of earlier resonance energy transfer technologies used for the analysis of biomolecular protein interaction. BRET analysis can be applied to many transmembrane receptor classes, however the majority of the early published literature on BRET has focused on G protein-coupled receptor (GPCR) research. In contrast, there is limited scientific literature using BRET to investigate receptor tyrosine kinase (RTK) activity. This limited investigation is surprising as RTKs often employ dimerization as a key factor in their activation, as well as being important therapeutic targets in medicine, especially in the cases of cancer, diabetes, neurodegenerative, and respiratory conditions. In this review, we consider an array of studies pertinent to RTKs and other non-GPCR receptor protein–protein signaling interactions; more specifically we discuss receptor-protein interactions involved in the transmission of signaling communication. We have provided an overview of functional BRET studies associated with the RTK superfamily involving: neurotrophic receptors [e.g., tropomyosin-related kinase (Trk) and p75 neurotrophin receptor (p75NTR)]; insulinotropic receptors [e.g., insulin receptor (IR) and insulin-like growth factor receptor (IGFR)] and growth factor receptors [e.g., ErbB receptors including the EGFR, the fibroblast growth factor receptor (FGFR), the vascular endothelial growth factor receptor (VEGFR) and the c-kit and platelet-derived growth factor receptor (PDGFR)]. In addition, we review BRET-mediated studies of other tyrosine kinase-associated receptors including cytokine receptors, i.e., leptin receptor (OB-R) and the growth hormone receptor (GHR). It is clear even from the relatively sparse experimental RTK BRET evidence that there is tremendous potential for this technological application for the functional investigation of RTK biology.

Keywords: receptor tyrosine kinase, RTK, protein–protein interaction, neurotrophic, insulin receptor, insulin-like growth factor receptor, epidermal growth factor receptor, cytokine receptors

INTRODUCTION

As a natural phenomenon, bioluminescence is found in marine animals such as the sea pansy *Renilla reniformis* and the jellyfish *Aequorea victoria*. Research has demonstrated that the oxidation of the intrinsically produced substrate coelenterazine to coelenteramide initializes the bioluminescence in those organisms (**Figure 1**) (Hart et al., 1978; Pfleger and Eidne, 2003). Bioluminescence resonance energy transfer (BRET) simply represents an energy transfer from a luminescent donor to a fluorescent acceptor, which re-emits light at another wavelength. BRET requires a sufficient overlap between the emission spectrum of a donor molecule and the absorption spectrum of an acceptor molecule (**Figure 1**) (Issad et al., 2002). BRET also

Abbreviations: Å, Angstrom; AD, Alzheimer's disease; APP, amyloid precursor protein; α(v)β(3), alpha(v)beta(3) (integrins); β_2AR, β_2-adrenergic receptor; BDNF, brain-derived neurotrophic factor; BRET, bioluminescence resonance energy transfer; cAMP, cyclic adenosine monophosphate; COPD, chronic obstructive pulmonary disorder; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ErbB4, erythroblastic leukemia viral (v-erb-b) oncogene homolog 4; EYFP, enhanced yellow fluorescent protein; FAK, focal adhesion kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GFP/YFP, green fluorescent protein/yellow fluorescent protein; GHR, growth hormone receptor; GPCR, G protein-coupled receptor; Grb, growth factor receptor-bound protein; HCS, high-content screening; HD, Huntington's disease; HRG-β1, heregulin beta1; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor-1 receptor; IGFR, insulin-like growth factor; IL-1β, Interleukin-1 beta; IR, insulin receptor; IRA, insulin receptor alpha subunit; IRB, insulin receptor beta subunit; IRS, insulin receptor substrate; Jak, Janus kinase; Jak/STAT, Janus kinases/signal transducers and activators of transcription; Kit, kit receptor; NGF, nerve growth factor; NT-3, neurotrophin-3; OB-R, leptin receptor; p75NTR, p75 neurotrophin receptor; p85, PI3Kinase 85kd subunit; PDGF, platelet-derived growth factor; PDGF-BB, platelet-derived growth factor beta polypeptide; PDGFR, platelet-derived growth factor receptor; PDGFRA, platelet-derived growth factor receptor, alpha polypeptide; PDGFRB, platelet-derived growth factor receptor, beta polypeptide; PIP3, phosphatidylinositol-3 phosphate; PKA, protein kinase A; PLCγ1, phospholipase C gamma 1; PTB, phosphotyrosine binding; PTP1B, protein tyrosine phosphatase 1B; Pyk2, proline-rich tyrosine kinase 2; Rluc, Renilla luciferase; RTK, receptor tyrosine kinase; SCF, stem cell factor; SH2, Src-homology 2; Shc, adaptor protein 46; Socs, silencers of cytokine signaling; STAT, signal transducer and activator of transcription; Stat5a, signal transducer and activator of transcription 5a; Trk, tropomyosin-related kinase; VEGF, vascular endothelial growth factor; VEGF-C, vascular endothelial growth factor-C; VEGFR, vascular endothelial growth factor receptor.

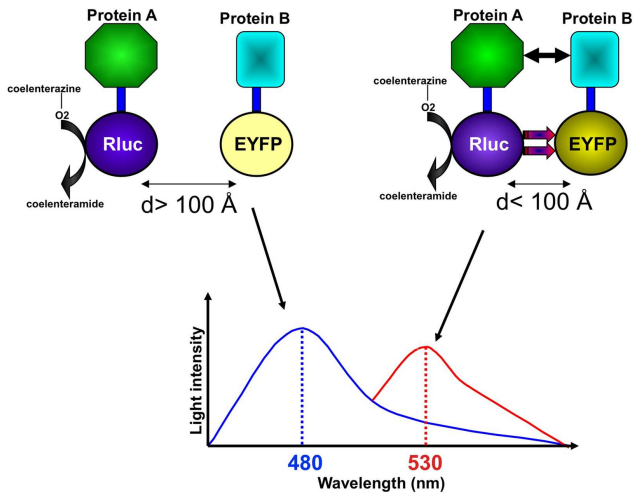

FIGURE 1 | The BRET assay has been developed to study protein–protein interactions. In an example of studying the interaction between protein A and protein B using the BRET assay, fusion proteins with Rluc and YFP are coexpressed, and luminescent signals are measured at 480 nm (Rluc light emission) and 530 nm (YFP light emission) upon addition of the Rluc substrate coelenterazine. If protein A does not interact with protein B and if Rluc and YFP are not at a BRET-permissive distance (>100 Å) and orientation, non-radioactive light emission is mainly measured at 480 nm. If protein A is in close proximity, or interacts with, protein B, placing Rluc and YFP at a BRET-permissive distance (<100 Å) and orientation, non-radioactive energy transfer can be measured at an increased light emission at 530 nm.

depends on the distance between the donor and the acceptor, which should be in the range of 10–100 Å, and on their interacting orientation (**Figure 1**) (Wu and Brand, 1994). Based on this principle, the BRET assay has been developed and applied to study protein–protein interactions as a facile methodological tool.

An important advantage of the BRET assay is that it allows researchers to study dynamic protein–protein interactions in living cells (Hamdan et al., 2006). In general, BRET assays involve proteins of interest fused with either a donor molecule (Renilla luciferase, or Rluc) or an acceptor molecule [usually a variant of green fluorescent protein (GFP)/enhanced yellow fluorescent protein (EYFP)]. BRET fusion proteins are created by expressing specifically engineered cDNAs from both the protein of interest and the donor or acceptor molecule. Subsequently, both donor-tagged and acceptor-tagged constructs are co-transfected into host cells. The presence of energy transfer between the donor and acceptor molecules can then be measured. The amount of energy transference correlates with the extent to which the specific tagged molecules exist within proximity of each other. The wavelengths for detection differ according to the use of BRET (480 nm for Rluc and 530 nm for EYFP) (**Figure 1**). The original BRET technology generally used EYFP as an acceptor, a red-shifted variant of YFP that has an emission maximum at 530 nm. In contrast, the recently introduced BRET-2 uses a codon of humanized wild-type GFP form, termed GFP2. GFP2 has a maximal emission at 510 nm. The BRET-2 system is designed to increase the spectral resolution compared to the original BRET technology. The improved resolution is attributed to the application of DeepBlue C coelenterazine with Rluc and GFP2, resulting in better separation of the luciferase/DeepBlue and GFP2 emission peaks. Whereas, in the original BRET technology, the h form of coelenterazine with Rluc and EYFP is used (Pfleger and Eidne, 2003). However, one of the limitations of BRET-2 is its lower efficiency of light emission that implies overexpression of the partners at supraphysiological levels.

Unlike fluorescence resonance energy transfer (FRET), BRET-based systems do not require the excitation of the donor with an external light source thus, minimizing the unnecessary autofluorescence, light scattering, photobleaching, and the possible photoisomerization of the donor, or even photodamage to the cells. BRET also allows detection of smaller variations in BRET signals as there is low background in the BRET assays due to the absence of any contamination of the light output. Ratiometric measurements of BRET minimize any variations that may occur due to a wide variety of possibilities including: differences in assay volumes, cell types, and numbers, as well as a decay of a signal in a given plate. As with other bioluminescence-based assays, BRET performance can be significantly affected by several factors, including the spectral properties of donor and acceptor molecules (Xu et al., 1999), the ratio of donor to acceptor molecules (Gomes et al., 2002), the distance and orientation of the molecules of interest (Wu and Brand, 1994; Kenworthy, 2001) and the strength and stability of the interactions (Pfleger and Eidne, 2003). Therefore, while presenting multiple advances over previous technologies such as FRET, BRET-based approaches can have their functional limitations. Using BRET to study protein–protein interactions may be critiqued for providing a potentially skewed view of biomolecular interactions. Biomolecular complexes are likely to contain tens or even hundreds of proteins at times and due to the relatively limited number of BRET probes, the number of simultaneous interactions that can be monitored is worryingly limited. As BRET employs ectopically expressed factors there is also an issue of both the lack of endogenous regulation of expression, cellular disposition, and compartmentalization of the factor. Expressing a novel factor in a cell line is highly likely to disrupt the stoichiometry of multiple signaling systems with potentially unknown consequences (Martin et al., 2009a). In addition to this, the variable nature of the host-cell environment, e.g., passage number, differentiation methodologies or viral transformation, will also likely affect signaling systems investigated using ectopically expressed BRET probes. Ideally, molecular interactions should be studied with native-state proteins as the addition of BRET labels may also affect the physico-chemical properties of the protein which may change its transport between different cell compartments, its post-translational modification status, its protein–protein interactions, and even its degradative processing. Changes to any of these properties of the target protein will likely have a significant impact on its perceived functionality using the BRET technique.

The BRET assay was first described in a study on the dimerization of the bacterial Kai B clock protein (Xu et al., 1999). Prior to this first BRET demonstration, non-BRET bioluminescent technologies were employed by Barak et al. (1997) to investigate the functional signaling activity of G protein-coupled receptors via β-arrestin-GFP translocation to the plasma membrane. Following this, a considerable body of BRET-based G protein-coupled

receptor (GPCR) functional analysis has now been generated (Angers et al., 2000; Galés et al., 2005; Ayoub et al., 2007). In addition, the activation or inactivation of second messengers such as cyclic adenosine monophosphate (cAMP) generated by GPCR activation, has also been well-studied using BRET. These techniques include the fusion of the regulatory and catalytic subunits of protein kinase A (PKA) to GFP and Rluc biosensors in order to monitor cAMP activity (Prinz et al., 2006), or the fusion of biosensors to the guanine nucleotide exchange protein activated by cAMP (Jiang et al., 2007; Barak et al., 2008). While BRET has been exhaustively employed for GPCR-based studies, in this present review, we instead focus on the applications of the BRET assays in the functional investigations of the receptor tyrosine kinase (RTK) superfamily. This superfamily contains a variety of distinct receptors associated with diverse functional activities. Hence, the RTK superfamily includes neurotrophic receptors such as tropomyosin-related kinase (Trk) and p75 neurotrophin receptor (p75NTR), insulinotropic receptors including the insulin receptor (IR) and insulin-like growth factor receptor (IGFR), as well as growth factor receptors such as the ErbB receptors including the epidermal growth factor receptor (EGFR), the fibroblast growth factor receptor (FGFR), the vascular endothelial growth factor receptor (VEGFR), and the c-kit and platelet-derived growth factor receptor (PDGFR). Cytokine receptors, e.g., leptin and growth hormone receptors (GHR), while not being traditional RTKs, possess multiple functional similarities with RTKs, e.g., receptor dimerization tyrosine kinase usage, and as such have also been investigated with BRET-based approaches.

INVESTIGATING GPCR SIGNALING WITH BRET

Bioluminescence resonance energy transfer approaches have been extensively applied to the investigation of the dimerization or other protein–protein interactions of multiple types of GPCRs, e.g., melatonin receptors (Ayoub et al., 2002), chemokine receptors (CXCR1, 2, and 4 and CCR2 and 5) (Milligan et al., 2005), α/β-adrenergic receptors (Angers et al., 2000; Small et al., 2006), cholecystokinin receptors (Harikumar et al., 2006), yeast α-factor receptors (Gehret et al., 2006), opsin receptors (Vrecl et al., 2006), protease-activated receptor 1 (Ayoub et al., 2012), and secretin receptors (Lisenbee and Miller, 2006). BRET has also been used to study the ability of muscarinic acetylcholine receptors, M3 and M5, to form homo- and hetero-dimers in living cells in a manner independent of receptor activation (Borroto-Escuela et al., 2010). As mentioned previously, one of the earliest BRET studies was used to assess whether the human β_2-adrenergic receptor (β_2AR), existed as a homodimer in living cells (Angers et al., 2000). This study found that GPCRs exist as functional dimers in the *in vivo* setting and therefore, BRET-based assays could be applied for the study of both constitutive and hormone-promoted selective protein–protein interactions (Angers et al., 2000). In addition to GPCR–GPCR interactions, both membrane and cytosolic protein interaction with GPCRs have been studied with BRET (Milligan, 2004; Pfleger and Eidne, 2005; Pfleger et al., 2006). For example, BRET1-based β-arrestin 2 translocation assays have been used to quantify receptor activation/inhibition (Hamdan et al., 2005). The BRET1 experimental approach is commonly used when it is important to maintain a systemic physiological protein expression level (Bacart et al., 2008). One pertinent study describes a BRET1-β-arrestin recruitment assay in stable mammalian cells and its successful application in high-throughput screening for GPCR antagonists (Hamdan et al., 2005).

INVESTIGATING TYROSINE KINASE-BASED RECEPTOR SYSTEMS WITH BRET

While GPCRs form perhaps the most important pharmacotherapeutic target for drug research (Maudsley et al., 2005) it is still crucial to generate a diversity of therapeutic strategies to contend with disease pathophysiologies. Therefore, the development of RTK-based drug discovery is vital to support the already mature field of GPCR-based drug design. In addition to the important use of BRET-based techniques for GPCR research, BRET has also proven to be useful in monitoring RTK receptor functionality and assisting in drug discovery efforts for identifying novel RTK modulators (Tan et al., 2007). BRET has also been used to study the nature of the ligand-induced conformational changes that accompany signal transduction pathway activation in RTKs (Boute et al., 2001).

Receptor tyrosine kinases are a varied group of transmembrane proteins acting as receptors for cytokines, growth factors, hormones, and other signaling molecules. RTKs are expressed in many cell types and play important roles in a wide variety of cellular processes, including growth, differentiation, and angiogenesis. Many RTKs, characterized by the archetypical EGFR, are composed of a single transmembrane helical region, a large extracellular immunoglobulin-like N-terminal domain and an intracellular C-terminal domain possessing an intrinsic tyrosine kinase activity. Cytokine receptors, while not possessing an intrinsic tyrosine kinase activity in their C-terminal domain, do actively recruit Janus kinase (Jak) family tyrosine kinase molecules to their intracellular domain to effect downstream signal transduction. Receptor dimerization, either ligand-driven or constitutive, forms an important component of the activation process of RTKs. These phenomena, therefore, make the investigation of their functionality with BRET highly analogous to the use of BRET in GPCR studies. Ligand-mediated RTK dimerization, e.g., for EGFR or PDGFR, or constitutive dimerization, e.g., for insulin/insulin-like growth factor-1 receptor, results in the stimulation of either tyrosine kinase recruitment (Jak2) or activation of intrinsic tyrosine kinase activity (EGFR). These active tyrosine kinases can then phosphorylate downstream signaling molecules as well as the opposing dimer unit of the RTK (auto-tyrosine phosphorylation). These auto-tyrosine phosphorylation sites conform to the C-terminal domain of the RTK into a series of high-affinity binding sites for downstream signaling proteins which possess canonical Src-homology 2 (SH2) or protein phosphotyrosine binding (PTB) motifs. The assembly of multiple proteins with the C-terminal domain of the RTKs then serves to propagate and "*condition*" the downstream signaling of the receptor (Maudsley et al., 2000b; Martin et al., 2009a). A significant advancement in the appreciation of functional transmembrane receptor systems was made by Maudsley et al. (2000a,b) through their demonstration of the creation of "higher-order" multi-protein signaling entities between active GPCRs and RTKs. The discovery that GPCR-based signals can then merge and also condition RTK-mediated signaling

has since been developed into an important field of research into the nature of receptor signaling transfer for many receptor systems (Gschwind et al., 2001; Sabri et al., 2002; Piiper et al., 2003; Sales et al., 2004; Flajolet et al., 2008; Chadwick et al., 2011a). This productive interaction therefore opens up the potentially important application of BRET-based techniques for the investigation of this emerging paradigm in receptor biology. Eventually it is likely that with BRET-mediated high-content screening (HCS) techniques, receptor ligands possessing a predilection for activating this RTK-associated GPCR "ensemble" may be rationally discovered and therefore constitute a novel and unique pharmacological resource (Maudsley et al., 2005). In the following sections of this review, we will discuss the most recently developed experimental evidence and concepts derived from RTK-associated BRET research. Each of the target receptor systems is likely to represent some of the most important future therapeutic targets, given the need for increased diversity in therapeutic mechanisms for the future pharmacopeia.

BRET FOR LABELING OF NEUROTROPHIC RECEPTORS

The neurotrophins are a family of closely related signaling proteins that control a number of crucial aspects of neuronal (both central and peripheral) activity, i.e., survival, development, responses to stress, and synaptic reinforcement (Mattson et al., 2004a; Skaper, 2008; Stranahan et al., 2009; Golden et al., 2010; Chadwick et al., 2011b; Driscoll et al., 2012). In mammals, the Trk subfamily of RTKs constitutes one major class of neurotrophic tyrosine kinase receptors. Sharing the typical features of RTKs, the activation of Trk receptors is often triggered by neurotrophin-mediated dimerization and/or transphosphorylation of an activation loop kinase (Huang and Reichardt, 2003). Most mammalian neurotrophins elicit their biological functions by activating one or more of the three members of the Trk family of RTKs (TrkA, TrkB, and TrkC) (Kaplan et al., 1991; Klein et al., 1991; Lamballe et al., 1991; Chadwick et al., 2010; Park et al., 2011). Being able to accurately monitor Trk activities in living cells will likely provide a platform for both drug development and mechanism-based research.

Based on the original BRET technology, Tan et al. (2007) further developed BRET-2 assays specifically for evaluating the interactions between Trk receptors (TrkA, TrkB, TrkC) and three kinds of effectors (p85, Shc46, phospholipase C gamma, PLCγ1) with three different neurotrophic stimulators (nerve growth factor, NGF, brain-derived neurotrophic factor, BDNF, neurotrophin-3, NT-3). To briefly describe the BRET-2 process, the size of the BRET-2 signal is expressed as the ratio of GFP2 and luciferase emissions, which correlates with the extent of recruitment of the effector proteins to the Trks, once Trks are activated. Under the stimulation of agonists including NGF (TrkA), BDNF (TrkB), and NT-3 (TrkC), interactions of TrkA-p85/Shc46/PLCγ1, TrkB-p85/Shc46/PLCγ1, and TrkC-Shc46 were continuously monitored, generating both BRET-2 ratio/log [concentration] curves as well as the EC_{50} for each ligand. Similarly, under the inhibition with the antagonist K252a, the same recruiting interactions were also captured, generating IC_{50} values, as well. All together, using BRET-2, this group successfully demonstrated that multiple forms of Trk activity can be investigated in live cells and may represent a reliable core technology for evaluating Trk activity and responsiveness to novel therapeutics.

The BRET assay-based monitoring system has also been used to answer several conformational and mechanistic questions related to functions of Trk receptors. Overexpression of TrkB has been linked to neuroblastomas (Brodeur, 2003) as well as other types of cancers (Moon et al., 2011; Fujikawa et al., 2012). TrkB kinase activity has also been shown to be responsible for the induction of metastasis by the suppression of anoikis, a form of apoptosis due to incorrect or inadequate cell and extracellular matrix attachment (Douma et al., 2004). Additionally, a growing body of evidence demonstrates that TrkB-mediated BDNF signaling plays a critical role in the pathogenesis of multiple neurodegenerative disorders such Alzheimer's disease (AD) and Huntington's disease (Martin et al., 2009b, 2012; Chadwick et al., 2011b; Cong et al., 2012). With the application of the BRET assay, De Vries et al. (2010) demonstrated a conformational rearrangement of preformed TrkB/Shc complexes initialized by BDNF-dependent activation, revealing a complex level of interaction between TrkB and Shc. It is noteworthy that in the study by De Vries et al. (2010), both TrkB receptor mutants as well as compound blockers were tested with the BRET assay. Therefore again, this further suggests that the TrkB BRET assay could be utilized to investigate Trk signaling and potential therapeutic design and provides a good example for the BRET assay application in labeling neurotrophic receptors. This study highlights the application of the BRET saturation assay which allows the determination of a conformational rearrangement of preformed complexes versus the recruitment of one signaling molecule to another, the latter being indicative of the relative affinity of two interacting molecules. This application has also been highlighted in earlier studies (Lacasa et al., 2005; Nouaille et al., 2006).

The p75NTR, a C-terminally truncated, non-signaling Trk receptor modulator (Segal, 2003; Makkerh et al., 2005) is involved in the regulation of multiple neuronal activities, e.g., development of neurodevelopmental processes (Nykjaer et al., 2005), neuronal migration (Johnston et al., 2007; Snapyan et al., 2009), and also neuronal growth inhibition (Yamashita et al., 1999; von Schack et al., 2001). Physically p75NTR can potentiate Trk signaling by potentiating neurotrophin ligand binding to TrkA receptors (Barker and Shooter, 1994; Hantzopoulos et al., 1994) thus enhancing cellular neurotrophin sensitivity (Yamashita et al., 1999; von Schack et al., 2001; Ito et al., 2003). The BRET assay has also been used for studying the interactions between the amyloid precursor protein, that is strongly implicated in AD pathophysiology, and p75NTR (Fombonne et al., 2009). Based on the BRET results, the connection between amyloid precursor protein and p75NTR is one of the most selective interactions observed in AD.

BRET ASSAY FOR LABELING INSULINOTROPIC RECEPTORS

Insulin, a complex peptide hormone secreted by the beta cells of the Islets of Langerhans in the pancreas, controls energy metabolism in the liver, muscle, and adipose tissue by binding to its cognate transmembrane tyrosine kinase receptor, i.e., the IR. Alterations in insulin signaling and action lead to pathophysiological conditions such as obesity, Type 2 diabetes mellitus (T2DM), and generalized metabolic syndrome (Maudsley et al., 2011). The IR is composed of two extracellular alpha-chains that bind ligands and two transmembrane and intracellular β-subunit chains that

possess the tyrosine kinase activity. The IR can be considered to be a "pre-dimerized" analog of growth factor receptors such as the EGFR. While the IR is effectively dimerized before the interaction with the peptide ligand, binding of insulin induces a conformational change that allows transphosphorylation of one β-subunit of the IR by the ligand-mediated stimulation of the intrinsic tyrosine kinase activity of the other β-subunit. BRET assays are highly sensitive for quantifying ligand-independent (constitutive), agonist-induced or antagonist-inhibited RTK activity levels (Tan et al., 2007). The first use of BRET to quantify constitutive, agonist-induced and antagonist-induced RTK activity was performed by Boute et al. (2001), using hormones, growth factors, as well as monoclonal antibodies (Boute et al., 2001). Blanquart et al. (2008) have utilized BRET to characterize ligand-induced conformational changes that occur within hybrids of IRA/IRB, the two isoforms of IR either containing or not containing exon 11 (Blanquart et al., 2008). IRA/IRB hybrids have been reported to be produced randomly in cells (Blanquart et al., 2008).

The discovery of pharmacological agents that specifically activate the tyrosine kinase activity of the IR will be of great importance for the treatment of insulin-resistant or insulin-deficient patients. As functional homologs to insulin, the insulin-like growth factors (IGF-I and IGF-II) play important roles in regulating growth, development, and differentiation of cells (Dupont and LeRoith, 2001) by binding to their cognate IGF-I receptor (IGF-1R). Similar to the IR, IGF-1R also belongs to the RTK superfamily (De Meyts and Whittaker, 2002). IGFRs are widely expressed throughout the central nervous system (CNS) as well as in the majority of peripheral tissues. BRET has facilitated the detection of the activation state of the IGF-1R, independently of any phosphorylation event by allowing the measurement of structural changes to the receptor in response to its cognate ligand (Blanquart et al., 2005). Activation of IGFR has been strongly implicated in generating a protective mechanism favoring neuronal cell survival and regeneration, which makes IGFR a potential therapeutic target for treating brain ischemic injury and neurodegenerative disorders (Roudabush et al., 2000; Mattson et al., 2004b; Harvie et al., 2011; Zemva and Schubert, 2011).

In order to evaluate the activity of IR and IGFR signaling pathways, both rapidly and in real-time, different BRET assays have been optimized for multiple applications. BRET assays for the real-time monitoring of the IR activity in living cells have been applied to investigate the molecular nature of binding partner interactions [growth factor receptor-bound protein 14, Grb14 (Nouaille et al., 2006)], the identification of novel IR system interactors [e.g., Sam68 (Quintana-Portillo et al., 2012)], as well as the activation mechanism of the IRs themselves (Boute et al., 2001). Furthermore, the BRET assay can also be applied to demonstrate or verify poor interactions between the IR and its substrates. IR substrates (IRS)-5 and -6 are two recently identified members of the IRS family. With the application of the BRET assay, Versteyhe et al. (2010) illustrated the finding that IRS-5 and IRS-6 are poor substrates for the IR compared to IRS1 and Shc (Versteyhe et al., 2010). More recently, using the BRET-2 assay in IR-Rluc8 and IRS(1,4,5)/Shc-GFP2 co-transfected HEK293 cells, Kulahin et al. (2012) examined interactions between IR and the canonical IRS (IRS1, IRS4) as well as the bifunctional SH2-domain-containing adaptor protein Shc. With this experimental paradigm, this group was able to demonstrate that specific insulin analogs may possess a 10-fold more potent capacity for the recruitment of IRS1, IRS4, and Shc, compared to human insulin. These varied studies suggest that the IR-based BRET assay may be a valuable tool to discover molecules with insulin-like properties.

Blanquart et al. (2005, 2006) have also applied BRET assays to pursue mechanistic questions into greater depth concerning the conformational changes of IGFR or IR induced by negative regulators such as PTP1B. Earlier, Boute et al. (2003) described the monitoring of the interactional dynamics of IR with PTP1B upon insulin stimulation. In 2005, using BRET, it was demonstrated that with insulin stimulation, the interaction of IR with receptor-like protein tyrosine phosphatases (PTPalpha and PTPepsilon) was due to conformational changes within preassociated IR/protein tyrosine phosphatase complexes (Lacasa et al., 2005). Later in 2011, Boubekeur et al. (2011) showed the interaction of PTP1B with the IR precursor during its biosynthesis in the endoplasmic reticulum. Similar to the IR-based BRET assay, co-transfection of Rluc or YFP-fused IGFR in HEK293 cells constitutes the ligand-induced conformation monitoring BRET assay. Additionally, by co-transfecting both IGF-1R-Rluc and YFP-PTP1B in HEK293 cells, the researchers were able to further reveal the interactions between IGF-1R and the negative regulator PTP1B in response to IGF1, IGF2, or insulin. Taken together from these varied studies, BRET assays are a useful technique for studying ligand-induced IR/IGFR conformational changes, assessing interactions between IR/IGFR and their negative or positive cellular partners or modulators, and setting up the platform of high-throughput screening for leading compounds relevant to related disorders.

Recently, in 2012, BRET was used to study the effects of insulin analogs on IR/IGF-1R hybrids. The group reported that when using MCF-7 cells (human breast adenocarcinoma cell line), glargine, which possibly acts via IR/IFG1R hybrids, demonstrated higher potency while its metabolites, M1 and M2, display lower potency than insulin for the stimulation of proliferative/anti-apoptotic pathways (Pierre-Eugene et al., 2012). They further developed a highly sensitive BRET-based assay that would allow monitoring of the production of phosphatidylinositol-3 phosphate (PIP3) upon stimulation of endogenous IR and IGF-1R in living cells (Pierre-Eugene et al., 2012).

BRET LABELING OF GROWTH FACTOR RECEPTORS

Bioluminescence resonance energy transfer-based techniques can be used to either measure direct EGFR dimerization or to assess the binding of downstream signaling factors to the activated state of the receptor. BRET assays for EGFR have proven to be a useful tool to study the effective pharmacology of ligand-induced interaction between EGFR and signaling pathway-specifying adaptor proteins (Schiffer et al., 2007). Probing these interactions is crucial as EGFR has been classified to have a central role beyond cancer research in neurometabolic aging (Siddiqui et al., 2012) and conditions such as asthma, where EGFR has been shown to be upregulated in asthmatics (Amishima et al., 1998; Puddicombe et al., 2000), and chronic obstructive lung disease (COPD) where there is abundant mucus production, in which EGFR is known to play a role (Takeyama et al., 1999). *In vivo* rodent models

confirm the importance of EGFR in asthma (Vargaftig and Singer, 2003; Tamaoka et al., 2008; Le Cras et al., 2011). The structural nature of the cognate ligand for EGFRs can also profoundly affect EGFR signaling. EGFR activation by stimulants such as histamine (Hirota et al., 2012), which does not classify with the commonly known axis of EGFR ligands, can also be assessed using BRET. Somatic mutations in epidermal growth factor (EGF) can produce ligand variants that quantitatively differ in their pharmacological and downstream signaling properties. This variability suggests the possibility of differential clinical responsiveness to treatment with EGFR inhibitors (Divgi et al., 1991; Perez-Soler et al., 1994; Modjtahedi et al., 1996; Baselga et al., 2000; Robert et al., 2001; Woodruff et al., 2010). EGFR is amongst other RTKs being probed as potential drug targets for asthma (Siddiqui et al., 2013).

In a profound BRET-facilitated study by Tan et al. (2007), the EGFR was shown to interact with Grb2 (growth factor receptor-binding protein 2) as well as Shc46 (MAP kinase proliferation pathway), PI3K-p85 regulatory subunit (PI3K-Akt survival pathway), PLCγ1 (protein kinase C/calcium signaling pathway), and STAT5a (from the signal transducers and activators of the transcription pathway) upon stimulation with the EGF. The ErbB4 growth factor receptor has also been shown to interact with Grb2 and p85 upon stimulation with one of the various ligands able to stimulate this receptor, i.e., heregulin-beta 1 (HRG-β1) (Tan et al., 2007). PDGFR A and B interacted with Grb2 and PLCγ1 when platelet-derived growth factor-BB (PDFG-BB) was used as a stimulant, while PDGFRA also interacted with p85 (Tan et al., 2007). Employing stem cell factor (SCF)-mediated activation of the c-Kit RTK, c-Kit was shown to dynamically interact with both Grb2 and p85 (Tan et al., 2007). Furthermore, vascular endothelial growth factor-C (VEGF-C) stimulation resulted in VEGFR3 and Grb2 interaction (Tan et al., 2007).

Fibroblast growth factor receptor and Grb14 intercommunication has also been investigated with BRET (Browaeys-Poly et al., 2010). Grb14 was found to bind to the phosphorylated FGFR where it induces a conformational change, and thereby unmasks a PLCγ-binding motif on Grb14, resulting in the inactivation of PLCγ (Browaeys-Poly et al., 2010). Therefore, using BRET analysis the authors of this study demonstrated their ability to measure the dynamic capacity of Grb14 to functionally inhibit FGFR signaling. In 2011, BRET was also used to assess the likelihood of FGFR1 homodimer formation upon stimulation by various FGF agonist ligands in HEK293T cells (Romero-Fernandez et al., 2011). FGFR1 is activated by homodimerization when FGF agonist ligand and heparin sulfate glycosaminoglycan are both present.

BRET LABELING OF CYTOKINE RECEPTORS

Activation of cytokine receptors by their cognate ligands induces a rapid recruitment of the Janus family of tyrosine kinases (Jak1/Jak2) in a Fyn- (Src-family tyrosine kinase) dependent manner. In the case of cytokine receptors (e.g., growth hormone, leptin, prolactin, or interleukin) the recruitment of the Jak kinases substitutes for the lack of an intrinsic tyrosine kinase activity in the C-terminal domain of these receptors. Hence, the ligand-induced association of Jak kinases with cytokine receptors in part recapitulates the functional signaling behavior of EGFR-like growth factor receptors. However, a specific function of the Jak recruitment is their ability to tyrosine phosphorylate downstream activators of transcription from the STAT family of proteins. The Jaks phosphorylate the intracellular tyrosines of the receptor complex, creating docking sites for STATs, which themselves become tyrosine-phosphorylated, thereby forming homo- or hetero-dimeric complexes that translocate to the nucleus. In the nucleus, STATs bind to specific gene promoters to activate the transcription of a range of targeted genes. In addition, autoinhibitory *Socs* (silencers of cytokine signaling) genes are also activated by cytokine receptor signaling via this Jak–STAT pathway (Starr et al., 1997). An assay-based on BRET was developed to detect the dimerization and action of the leptin receptor (OB-R), a type I cytokine receptor (Couturier and Jockers, 2003).

The short form of the prolactin receptor inhibits prolactin-induced activation of gene transcription by the long form of the prolactin receptor. In 2009, it was demonstrated using BRET that there is a higher homodimerization affinity of the mutated form of the short form of the prolactin receptor, reduced heterodimerization associations, long form homodimerization, and subsequent prolactin-induced signaling (Xie et al., 2009). Recently, a new genetically encoded biosensor based on BRET technology has been developed to allow real-time monitoring of inflammasome activity (Compan et al., 2012). The primary functional features of this sensor are similar to the endogenous IL-1β, which makes this probe an ideal tool for the characterization of pro-IL-1β processing and for the high-throughput screening of compounds that may underpin the initiation of inflammation (Compan et al., 2012).

Bioluminescence resonance energy transfer has also been successfully applied for the study of GHR activation (Brown et al., 2005). Along with FRET and co-immunoprecipitation in this particular study, BRET studies have generated important evidence that GHR subunits undergo specific transmembrane interactions independent of hormone binding (Brown et al., 2005).

USE OF BRET FOR THE STUDY OF RTK-INTERACTING PROTEINS

In addition to investigating receptor-specific RTK events, BRET can also be used to monitor RTK accessory protein binding. As briefly discussed earlier, currently 22 BRET assays for 9 RTKs, derived from 4 subfamilies [erythroblastic leukemia viral (v-erb-b) oncogene homolog (ErbB), PDGF, neurotrophic Trk, VEGF] have been reported that allow real-time monitoring of interactions with multiple effectors, i.e., Grb2, p85, Sta5a, Shc46, PLC-γ1 (Tan et al., 2007). Demonstrating BRETs utility in this field, BRET studies helped identify tyrosine residues 1068, 1114, 1148 as the main residues mediating interaction of EGFR with Grb2 (Tan et al., 2007). The use of BRET has also proven to be useful in understanding the often complex relationships between ligand-mediated RTK activation and sensitivity to chemical inhibitors of their function. BRET assays have thus suggested that the conformational rearrangement of preformed TrkB-Shc complexes, following BDNF-dependent activation, may prove extremely useful for the HCS of potential pharmacological blockers of TrkB signaling in a physiologically relevant context (De Vries et al., 2010).

Furthermore, BRET has also been used to study how alpha (v) beta (3) [α(v)β(3)] integrins cooperate with transmembrane

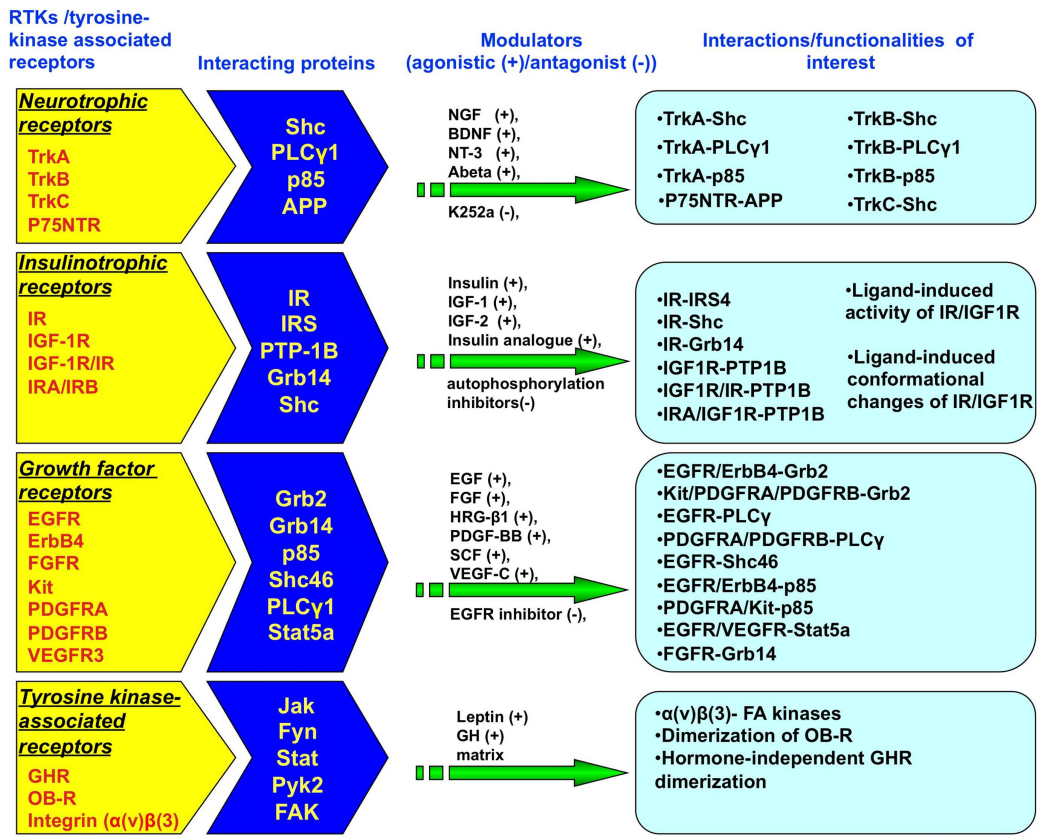

FIGURE 2 | Studies assessing protein–protein interactions for RTK and tyrosine kinase-associated receptors using Bioluminescence Resonance Energy Transfer (BRET). BRET assays have been established to study both the association of multiple RTK/tyrosine kinase-interacting proteins with the receptor superstructure as well as stimulator/inhibitor-mediated conformational changes in receptor structure. The RTK receptors include neurotrophic receptors (TrkA, TrkB, TrkC, p75NTR), insulinotropic receptors (IR, IGF-1R, IR-IGF-1R hybrid receptors), and growth factor receptors (FGFR, EGFR, ErbB4, kit, PDGFRA/B, VEGFR3). Tyrosine kinase-associated receptors include GHR, OB-R, and integrin receptors.

receptor systems, such as tyrosine kinases, to enhance cellular responses (Scaffidi et al., 2004). Integrins are single-pass transmembrane receptors for extracellular matrix proteins such as fibronectin. While integrins themselves do not possess intrinsic tyrosine kinase activity, upon interaction with their extracellular matrix "ligand" molecule, they rapidly associate with tyrosine kinase scaffolding proteins such as focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (Pyk2) (Della Rocca et al., 1999; Davidson et al., 2004; Maudsley et al., 2006, 2007). These scaffolding proteins, in a similar manner to the intrinsic tyrosine kinase domains of growth factor receptors such as the EGFR, upon interaction with integrin molecules activate their tyrosine kinase catalytic function. Once this activity is stimulated, these scaffolding proteins then undergo auto-tyrosine phosphorylation to create signaling protein docking sites. Therefore integrin receptors, as with cytokine receptors, replicate a form of classical RTK activity.

CONCLUSION AND PERSPECTIVES

Bioluminescence resonance energy transfer is an advanced technology that can be applied in live cells and has been successfully applied to the investigation of protein–protein interactions, structure-function analysis, and in the mapping of signal transduction pathways (e.g., RTK-interacting proteins) for RTKs and tyrosine kinase-associated receptors (**Figure 2**). BRET possesses various advantages compared to standard protein investigation procedures that require invasive or cell-destructive processes such as co-immunoprecipitation or even the previously developed FRET technique. The advances made with BRET, i.e., removing the need for external energy stimulation, have also resulted in an overall improved signal-to noise-ratio when compared to earlier versions of the resonance energy transfer technologies. With respect to cell signaling research, its utility has now significantly gone beyond studying GPCRs. The use of BRET for studying RTKs has great benefit especially as researchers continuously strive to maximize the capacity of BRET as a facilitator to probe for novel drugs and related signaling pathways. In the future, we will most likely witness an increasingly successful number of applications and improvements to the technology.

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Setting up a bioluminescence resonance energy transfer high throughput screening assay to search for protein/protein interaction inhibitors in mammalian cells

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Each step of the cell life and its response or adaptation to its environment are mediated by a network of protein/protein interactions termed "interactome." Our knowledge of this network keeps growing due to the development of sensitive techniques devoted to study these interactions. The bioluminescence resonance energy transfer (BRET) technique was primarily developed to allow the dynamic monitoring of protein/protein interactions (PPI) in living cells, and has widely been used to study receptor activation by intra- or extra-molecular conformational changes within receptors and activated complexes in mammal cells. Some interactions are described as crucial in human pathological processes, and a new class of drugs targeting them has recently emerged. The BRET method is well suited to identify inhibitors of PPI and here is described why and how to set up and optimize a high throughput screening assay based on BRET to search for such inhibitory compounds. The different parameters to take into account when developing such BRET assays in mammal cells are reviewed to give general guidelines: considerations on the targeted interaction, choice of BRET version, inducibility of the interaction, kinetic of the monitored interaction, and of the BRET reading, influence of substrate concentration, number of cells and medium composition used on the *Z'* factor, and expected interferences from colored or fluorescent compounds.

Keywords: BRET, PPI, P2I2, RET, HTS, screening assay, inhibitor compound, modulator compound

INTRODUCTION

Protein/protein interactions (PPI) govern all key events in a cell life, from division, to adaption or response to extracellular signals leading to biological effects. However, this view was not so obvious in the past, as convincing examples demonstrating such phenomena were exceptional and hard to achieve. In the last decade, numerous methods with increasing sensitivities and potencies have been developed, allowing the monitoring of those interactions (Xu et al., 1999; Tavernier et al., 2002; Chan, 2004; Brovko and Griffiths, 2007; Michnick et al., 2007; Ventura, 2011; Hamdi and Colas, 2012). Evolution of such methods has allowed the dynamic detection of PPI in living cells (Xu et al., 1999; Coulon et al., 2008; Lee et al., 2010; Quiñones et al., 2012) and nowadays in whole living organisms (Subramanian et al., 2004; Audet et al., 2010). Following this evolution scheme, PPI pathways have been deciphered and furthermore organized in higher protein networks ranging from PPI taking place in molecular complexes, to entire organelles and to whole organisms (Coulon et al., 2008; Chautard et al., 2009; Jaeger and Aloy, 2012). Our current knowledge of these PPI networks has further increased in recent years with the emerging idea that more than PPI networks themselves, the biological context in which they occur is important. System wide analyses of PPI crossing genetic data or pathological states of the cells from which they were generated have been performed and led to new data pointing out the changes in PPI networks in some human pathology (Bader et al., 2008). Deciphering that a fine PPI change can lead to a drastic PPI network modification was the bases of a pathological state, has opened new views for drug discovery. Applying this concept by using the current knowledge of protein interaction network modification in glioblastoma cancer cells, a recent study allowed the successful screening of inhibitory peptide disrupting PIKE-A/Akt and their capacity to inhibit the proliferation of these cells (Qi et al., 2012). Attempts to gain exhaustive interactome taking place in diseases have became common. These growing data demonstrate that most proteins interact with more than one partner (Krause et al., 2004) and lead to better drug target choosing. Indeed the deciphering of deregulated or key interactions in diseases crossed with interactions involved in the less pathways allows to minimize or avoid unexpected side effects (Chen et al., 2012).

To search for inhibitors of PPI, the same methods used to detect the interactions can be used. The need for robust and high throughput screening (HTS) compatible method, when performing screening assays, has lead to the preferential use of techniques

such as yeast two hybrid and derivatives (Hamdi and Colas, 2012), Fluorescence polarization (Smith and Eremin, 2008), MAPPIT (Lievens et al., 2011); and protein complementation assay (Morell et al., 2009; Michelini et al., 2010). Other methods based on resonance energy transfer (RET) to monitor PPI, offers great advantages as they allow full length proteins dynamic interaction monitoring in intact cellular contexts and are applicable to HTS (De, 2011). In this review, the use of RET and more advantageously PPI inhibitors (P2I2) bioluminescence resonance energy transfer (BRET)-based screening assays in mammalian cells will be developed.

THE DIFFERENT RET METHODS

To date, three main RET methods have been developed and used in drug screening assays: FRET (Forster Resonance Energy transfer), BRET and HTRF (Homogeneous Time Resolved fluorescence). All RET methods are based on the use of compatible energy donor and acceptor couples allowing RET to take place when donor and acceptor are in close proximity (<10 nm). To be a compatible couple, the energy donor emission wavelength has to overlap the energy acceptor excitation one in order to gain energy transfer (**Figure 1A**). The energy donor and acceptor are each linked to one of the interacting partners and resonance can occur if the two partners interact and close the donor and acceptor by a distance less than 10 nm. In the FRET method (**Figure 1B**), donor and acceptor are both fluorophores and a proper excitatory light is needed to promote donor emission (Fruhwirth et al., 2011). In FRET cellular screening assays, donor and acceptor are two fluorescent proteins each genetically fused to one of the interacting partners. In the BRET method (**Figure 1C**), the energy donor is a bioluminescent enzyme, converting its substrate into light emission able to promote RET with a compatible fluorescent acceptor (Pfleger and Eidne, 2006; Bacart et al., 2008). For live cell screening purpose, BRET assays involve genetically engineered fusion protein of the studied partners respectively with the donor and acceptor. HTRF is an enhanced FRET derivative method which circumvents the major FRET problem due to simultaneous excitation of acceptor by donor excitatory light. This method is based on energy transfer monitoring in a time resolved manner (Degorce et al., 2009). Indeed the donor used is a fluorescent molecule able to emit light for a short time period after the excitatory light has been turned off (**Figure 1D**). This last property allows the monitoring of energy transfer to a compatible acceptor once the excitatory light is switched off.

All these RET methods have several advantages over the other methods to monitor PPI, that make them the best suited method to detect PPI in mammalian cells. FRET, HTRF, and BRET are homogenous assays as the energy transfer signal is only emitted from the interacting partners, and then, no artifact prone washing steps are required before reading. Each of these methods has its advantages and limits that make them best suited methods in certain fields. In P2I2 live cell screening assays BRET present several advantages over other RET methods.

WHY CHOOSING BRET TO SCREEN FOR PPI INHIBITORS?

Classical FRET and BRET screening assays have a subsequent advantage over HTRF as they mostly rely on genetically fused

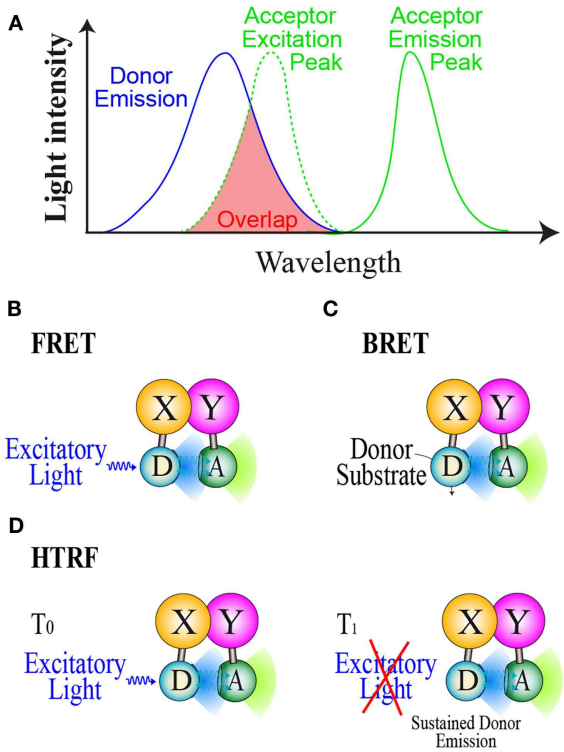

FIGURE 1 | Resonance energy transfer methods. (A) Basic properties of donor/acceptor compatible couple in order to gain RET. Principles of **(B)**: the FRET method, **(C)**: The BRET method, and **(D)**: the HTRF method. D, Donor; A, Acceptor; S, Substrate.

energy donor and acceptor proteins respectively to both partners implicated in the monitored interaction. Using such fusion proteins can however be a disadvantage as fusion can promote steric hindrance hindering wild type interactions. On the other hand, HTRF is able to monitor unmodified protein interactions but involves a latter step of protein labeling with antibodies or chemical linkage (Degorce et al., 2009) which lower its interest in live cell P2I2 HTS assays. BRET shows several advantages over FRET (Boute et al., 2002): first, the excitation of the donor fluorophore by monochromatic light in FRET also lead to the concomitant excitation of the acceptor then hardening the results interpretation; second, this excitatory light promote photobleaching of the donor and cell autofluorescence; and third, BRET signal/noise ratio has been shown to be 10-fold higher than FRET thus allowing the use of 40-fold less amount of protein to reach the same signal level than FRET (Arai et al., 2001). This last parameter is important for screening P2I2 as over-expression of proteins (excess of the monitored complex) might titer a potential active molecule leading to its inability to promote the expected decreased in signal. Indeed, BRET superiority was shown by its ability to monitor PPI using endogenous level of protein expression (Couturier and Jockers, 2003; Pfleger and Eidne, 2003) and its consequent application to various live cell screening assays (Pfleger et al., 2007; Bacart et al., 2008; Kocan and Pfleger, 2011). Finally, using this method to screen for P2I2 is further supported as BRET is prone to disruption

or modulation by co-expression of untagged interacting partner (Bacart et al., 2008; Ayoub and Pfleger, 2010; Kulahin et al., 2011) and by incubation with inhibitory peptides (Granier et al., 2004; Harikumar et al., 2006; Jarry et al., 2010) or inhibitory chemical compounds (Mazars and Fåhraeus, 2010; Corbel et al., 2011).

FOR WHICH KIND OF TARGET INTERACTION CAN THE BRET BE CHOSEN?

The BRET method has already been applied to monitor interaction between various kinds of proteins partners and in various cellular components (Bacart et al., 2008; Alvarez-Curto et al., 2010). This range from two soluble proteins, two transmembraneous ones, one transmembraneous, and one soluble, with interactions taking place in cytoplasm, nucleus, and cytoplasmic or internal membranes (Coulon et al., 2008; Guan et al., 2009; Bacart et al., 2010). Indeed BRET is able to monitor all kinds of interaction, however, certain concerns have to be taken into account when designing P2I2 BRET-based assays. First, the BRET signal is dependent on the donor/acceptor ratio as described by the well-known donor saturation assay (DSA; Mercier et al., 2002; Bacart et al., 2008; Ayoub and Pfleger, 2010; **Figure 2A**). The DSA leaded to further analyze of the BRET signal and demonstrated that the maximal BRET intensity is dependent on the ratio of energy donor interacting with an energy acceptor versus free energy donor present in the cell (Couturier and Jockers, 2003; Ayoub et al., 2004; **Figure 2B**). Indeed at equimolar ratio, if all donors and acceptors molecules interact together, a maximal BRET would be raised. However, this is rarely the case and free donors molecules (or interacting with other but non-acceptor-tagged proteins) lead to decrease this maximal BRET value. Given that, in order to gain the higher BRET signal, the acceptor fusion protein would be highly expressed compared to the energy donor to lower the free donor proportion. However, to ensure the monitoring of active compounds effects, the titration of the compound by excess acceptor has to be avoided. In order to prevent this phenomenon, the level of expression of both partners would result in an ideal window leading to high BRET signal still located in the dynamic range of DSA curves (**Figure 2C**).

Furthermore, this last parameter will guide the choice for the design of the fusion proteins. As the proportion of free donor will lead to decrease the BRET signal, it has to be the lowest and the choice to fuse it to a *X* or *Y* protein will be the global ratio of *X*/*Y* complexes versus *X* or *Y* that are free or engaged in other complexes than the one studied.

Bioluminescence resonance energy transfer is also well suited to monitor transitory interaction but with the same restriction: when performing the reading, the BRET signal will depend on the percentage of donor/acceptor complexes versus the donor alone and would be hard to monitor if this percentage is low. Some modifications can enhance the monitoring of such interaction like substrate trapping strategy that disables the substrate/enzyme dissociation (Boute et al., 2003; Issad et al., 2005; Boubekeur et al., 2011).

WHICH BRET VERSION TO CHOSE?

To screen for P2I2, compound titration by excess reporter amount has to be avoided. For *in vitro* interaction methods, setting up the protein quantities to use is easily done, however this is harder to achieve for live mammalian cell BRET-based assays. Indeed, choosing the most sensible and most compatible with HTS over the different BRET versions available seems to be the only way to gain the necessary highest readout. This choice became difficult nowadays as several BRET methods based on different substrates and different compatibles donor/acceptor couples have been developed (Bacart et al., 2008; De et al., 2009; Lohse et al., 2012).

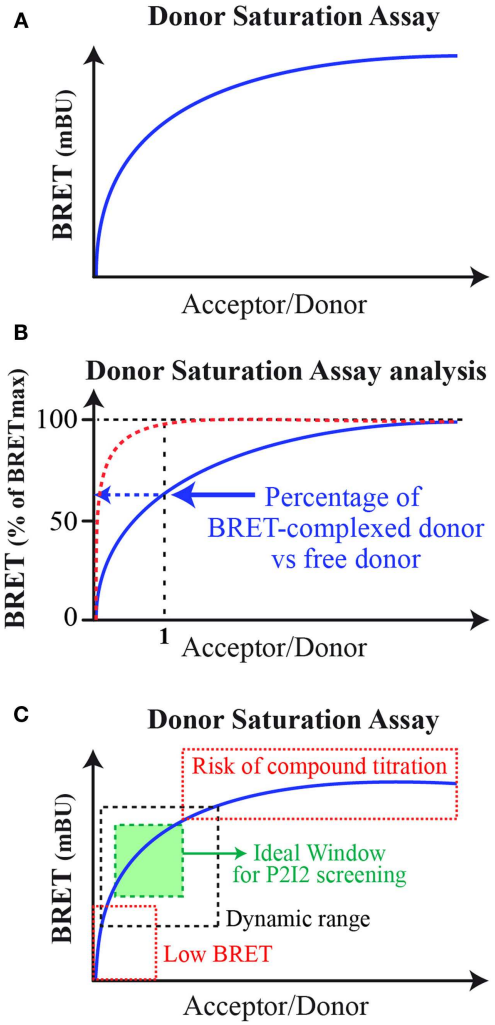

FIGURE 2 | Bioluminescence resonance energy transfer donor saturation assay. (A) Basic donor saturation assay expressed as milliBRET unit (mBU); **(B)** Donor Saturation Analysis for dimeric complexes formation: in red, theoretical curve if 100% donor and acceptor interact with each other at a 1/1 molar ratio. In blue, the common DSA curves obtained showing lower percentage of donor/acceptor complexes in cells. **(C)** Donor saturation assay for analysis to set up a P2I2 BRET-based screening assay. In hatched black, the dynamic windows of BRET monitoring. In hatched red, the two areas of the DSA curve to avoid. In green, the ideal window to be chosen when setting up a P2I2 BRET-based screening assay.

BRET1

Original BRET1-based on the Rluc/YFP couple showed low signal (Xu et al., 1999) hindering its use in HTS. Higher signals were obtained using mutants or new cloned acceptors such as YFP

Topaz, YFP citrine, YFP Venus, YPet, or the Renilla-GFP (R-GFP; Bacart et al., 2008; Molinari et al., 2008; Kamal et al., 2009; Ayoub and Pfleger, 2010). YFP Venus was used to demonstrate the feasibility of a BRET1 HTS assay in CCR5 ligands screening (Hamdan et al., 2005). The BRET1 readout signal was also enhanced by the concomitant use of these acceptors with mutants of Rluc or other luciferases. Rluc2 or Rluc8, mutants of Rluc with higher stability and quantum yield (Loening et al., 2006), greatly increased BRET1 signal (Kocan et al., 2008; Kamal et al., 2009; Schelshorn et al., 2012). Recently, BRET1 was used to develop two P2I2 screening assays (Mazars and Fåhraeus, 2010; Corbel et al., 2011).

BRET1 has also been achieved using Gaussia Luciferase (Gluc). Gluc is a smaller and brighter luciferase known to date and was cloned from a marine copepod (Tannous et al., 2005; Welsh et al., 2009). It shares some spectral properties with Rluc and has been recently used in BRET1 assays (Li et al., 2012).

BRET1 method using quantum dot (Qdot) as energy acceptors has also been reported these past few years. These photostable fluorescent nanoparticles are excitable at 480 nm and have a size dependent emission wavelength tunable to the overall rainbow colors (Weng and Ren, 2006). Qdot BRET-based assay have first shown energy transfer efficiency (So et al., 2006) and *in vitro* protease assays have been later developed (Xia et al., 2008; Kim and Kim, 2012). However, the coupling to proteins (Algar et al., 2010) and the cellular toxicity (Soenen et al., 2012) of Qdot are still an obstacle to their use in live mammalian cell for PPI monitoring.

BRET2

Bioluminescence resonance energy transfer 2 method was developed by Packard Biosciences by increasing the separation of the two emitted wavelength to circumvent the poor signal/noise ratio of BRET1. This enhancement relies on the concomitant use of coelenterazine 400a (or deep blue C), a coelenterazine derivative that forces the Rluc emission to a 397 nm peak, and the compatible energy acceptor GFP2 (a mutant of aequorea GFP; Ramsay et al., 2002). BRET2 has been successfully used for ligands screening (Vrecl et al., 2004; Elster et al., 2007), and virus protease inhibitors screening (Hu et al., 2005; Oka et al., 2011). However, BRET2 has suffered from a weak and short lasting light emission that greatly limited its use to develop P2I2 BRET-based HTS assays. Indeed, high expression of BRET partners is necessary to ensure signal recording. BRET2-based PPI assay using Rluc2 or Rluc8 have shown enhanced BRET dynamic range and kinetic of the reading up to 1 h (De et al., 2007; Kocan et al., 2008; Dacres et al., 2009, 2012; Kulahin et al., 2012). However, BRET2 has not been used in P2I2 screening assays yet and its use in this field would still need to be demonstrated.

BRET3

A BRET3 method using firefly luciferase (Fluc) and dsRed or Cy3 as compatible acceptor has been developed (Arai et al., 2002; Yamakawa et al., 2002). However, the huge overlap of donor/acceptor emission peaks of this method leads to extremely low signal to noise ratio that impaired its application to really study protein/protein interactions. A better proof of concept was gained by the use of mOrange as acceptor that allowed PPI monitoring in live cells and animals (De et al., 2009). More recently, new analogs of luciferin (the firefly substrate), leading to different spectral properties of the emitted light, were synthesized and showed their efficiency in BRET3 experiments (Takakura et al., 2010, 2011). One of these, coumarylaminoluciferin allowed a mutant of Fluc to emit light compatible with the use of YFP as acceptor (Takakura et al., 2010) and may promote advances of the BRET3 version by using the various YFP variants developed for BRET1. At this stage of development, BRET3 has not been yet demonstrated to be a valuable method to screen for P2I2.

FUTURE BRET ENHANCEMENTS

Although major advances have already been made since the 1999s BRET version, further improvements of BRET methods are still expected. As described above, the BRET enhancements were based on the use of variants of luciferases or fluorescent acceptors, coupled to the concomitant use of modified substrates. New improvement of know luciferases are on the way and would probably lead to new BRET advances. A systematic pairing of luciferases with compatible substrates have highlighted best couples: Rluc/enduren and Gluc/native coelenterazine h are 8- to 15-folds brighter than the princeps BRET1 (Kimura et al., 2010). Another study sorted mutants of Gluc with a up to sixfold enhancement in light emission and a 10-fold prolonged bioluminescence than native Gluc which was already the brighter luciferase (Kim et al., 2011). *Vargula* luciferase (Vluc) shares quite the same spectral properties than Rluc and has been applied to BRET1 (Otsuji et al., 2004). *Metridia pacifica* luciferase 1 (MPluc1) and *Metridia longa* luciferase (Mluc) or its mutants emits in the 450–500 nm range and have thus potential to be used in BRET assays in the future (Takenaka et al., 2008; Kim et al., 2011; Markova et al., 2012). Recently, Nanoluc™, a new deep-sea shrimp evolved luciferase has been introduced by Promega (Hall et al., 2012). This 171 amino acids (19 kDa) ATP independent glow-type luciferase using furimazine as substrate is announced to have more than 100-fold higher luciferase activity than Rluc or FLuc. Its maximal emission peak at 465 nm makes it compatible with current BRET acceptors and its efficient application in two BRET-based assays has furthermore been shown. Its high activity allows lowering Donor amount needed to ensure sufficient BRET signal and may thus enhance the sensitivity of the method.

BRET1 OR BRET2?

Due to recent advances, the proper choice between BRET1 and 2 versions became difficult. Due to the lack of studies systematically comparing each BRET enhanced methods with each other, a ranking of the BRET signal and the amount of protein needed to reach it is hard to achieve. Both methods recently reached higher sensitivity, readout, and kinetics parameters that render them fully compatible with HTS. However, BRET1 basic method has been shown to be able to monitor PPI at endogenous expression level of proteins (Couturier and Jockers, 2003; Pfleger and Eidne, 2003) thus allowing the use of lower protein expression level than BRET2 in order to avoid active compound titration. Furthermore, Rluc and Rluc 8 as energy donor were systematically tested in BRET1 and BRET2 identical assays and showed the better sensitivity of BRET1 over BRET2 in living cells (Kocan et al., 2008). However, another study found the opposite (Dacres et al., 2009). To date, only BRET1-based P2I2 screening assays have been

described and showed the feasibility of this approach (Mazars and Fåhraeus, 2010; Corbel et al., 2011). BRET1 seems to be nowadays the best suited BRET method to develop P2I2 screening assays until proven otherwise.

HOW TO SET UP A BRET ASSAY TO SCREEN FOR PPI INHIBITORS?

VALIDATION OF THE SPECIFICITY OF THE INTERACTION

The BRET signal is dependant on the ratio of donor/acceptor as it has been shown for years, using the well-known DSA, to show the specificity of the interaction. The first step to screen for P2I2 using BRET in cells is to verify this point by performing DSA experiments or other characterization such as untagged competitor protein cotransfection or effect of a known ligand promoting change of the BRET signal (Bacart et al., 2008; Ayoub and Pfleger, 2010).

PRODUCTION OF INDUCIBLE BRET CELL LINES

In order to set up a screening assay, the BRET signal has to be high, reproducible and stable, however, as revealed by DSA, fine changes in the donor or acceptor expression in transitory transfections will lead to a change in the BRET signal (**Figure 2A**). To ensure the stability and reproducibility of the signal needed for a screening assay, cell clones stably expressing the donor alone (Control cell line) and the donor/acceptor couple (BRET cell line) would be prepared as this was done for most BRET-based screening assays. Disrupting a PPI might be hard or quite worthy to achieve, this is why P2I2 screening assays developed until now were designed *in vitro* to allow compound tested to inhibit interaction before it takes place. For BRET-based assay, it is easily achieved if the studied interaction is naturally induced such as promoted receptor/effectors interaction upon ligand addition (Kamal et al., 2009; See et al., 2011). However, for constitutive interactions, designing such successful assays in living cell using BRET implies the use of a fast inducible system to add the chemical compound before inducing the target interaction (Corbel et al., 2011). Several mammalian tight inducible systems have been developed to reach this goal (Clackson, 1997). However, for screening protocol conveniences; repressed gene expression systems overcame by inducer molecule represents the best strategy. Several inducible systems are based on this scheme: Tet-on systems, based on a tet repressor (TetR) binding to tet operator elements of a promoter and displaced by addition of tetracycline derivatives thus allowing the target gene expression (Shockett and Schatz, 1996; Sun et al., 2007); Ecdysone systems and derivatives, based on glucocorticoids promoted association of an active steroid hormone nuclear receptor enabling expression of a target promoter (No et al., 1996; Xiao et al., 2003), and Q-mate™ based on a steric hindrance due to cumate repressor protein CymR bound to operator sites on the target promoter and which is released by addition of the inducer molecule cumate (Mullick et al., 2006). Two cell lines have to be developed to allow subtraction of the background BRET signal (from control cell line) from the interaction promoted BRET signal (BRET cell line). In order to gain comparable background luciferase activity in both cell lines, the BRET cell line would be advantageously prepared by introducing the acceptor tagged protein in the genome of the control cell line.

WHICH BRET PARTNER TO INDUCE?

Given the DSA curves, the maximal BRET signal is achieved when the donor is saturated by the acceptor. The resulting strategy would then be to express this one constitutively and the donor-fused partner in an inducible way. This kind of inverse DSA would lead to a high BRET signal tending to its maximal value as soon as the donor expression is induced. To ensure this ideal scenario, several parameters have to be taken into account when selecting the cellular clones. First of all, a low background expression of the donor is needed; otherwise a high BRET signal would be readily present before induction. Second, a sufficient acceptor expression has to be reached to ensure high maximal BRET values but low enough to avoid titration of compounds targeting this moiety. Third, during the induction process, the molecular amount of expressed donor would not exceed the one of the acceptor as the BRET signal would then decrease by free donor accumulation. Another important point to take into account is the location of the monitored interaction. Constitutively expressed acceptor would have reached its proper location whereas, upon induction, the donor will be neo-synthesized and a delay is then expected for it to reach final location and interact with its partners. The BRET signal appearance is then expected to be delayed, however, unless compound modify translation rate or transit through/between cellular compartments, this delay would be the same in presence or absence of screened compound incubation when verifying primary hits.

HOW TO OPTIMIZE A P2I2 BRET-BASED ASSAY?

When setting up a primary screening assay, efforts have to be made to make it easy, fast, highly reproducible, and to lower the associated costs. To this aim, several parameters described below can be optimized when setting up P2I2 BRET-based screening assays to assume these efforts.

FAST AND EASY PROTOCOL

The use of an inducible and stably expressing cell clones seems to be a prerequisite to ensure ease and reproducibility of such P2I2 BRET-based screening assays. An example using transitory transfection has shown that a known inhibitory compound was active in this assay (Mazars and Fåhraeus, 2010), however no hits based on this assay has been further published. On the contrary, a successful P2I2 screening assay using yeast stably expressing donor and acceptor respectively in an inducible and constitutive way has led to identification of chemical hits able to prevent the interaction between human cdk5 and p25 (Corbel et al., 2011). This study showed for the first time a real success for such P2I2 BRET-based screening assays. In order to keep the homogeneity of the test, efforts to set up a protocol avoiding unnecessary washing steps would be done. This can be achieved by some typical protocol as shown on **Figure 3A**: cells are first dispatched in wells, allowed to adhere, and rinsed (or not) to lower background donor expression. After this last step, addition of medium, compounds, inducer of the donor expression, and finally the donor substrate to perform the reading are then chronologically added.

KINETIC OF THE MONITORED INTERACTION

The use of an inducible system to ensure compound inhibitory action before the interaction takes place also lead to the problem of the kinetic of the studied interaction after induction. In

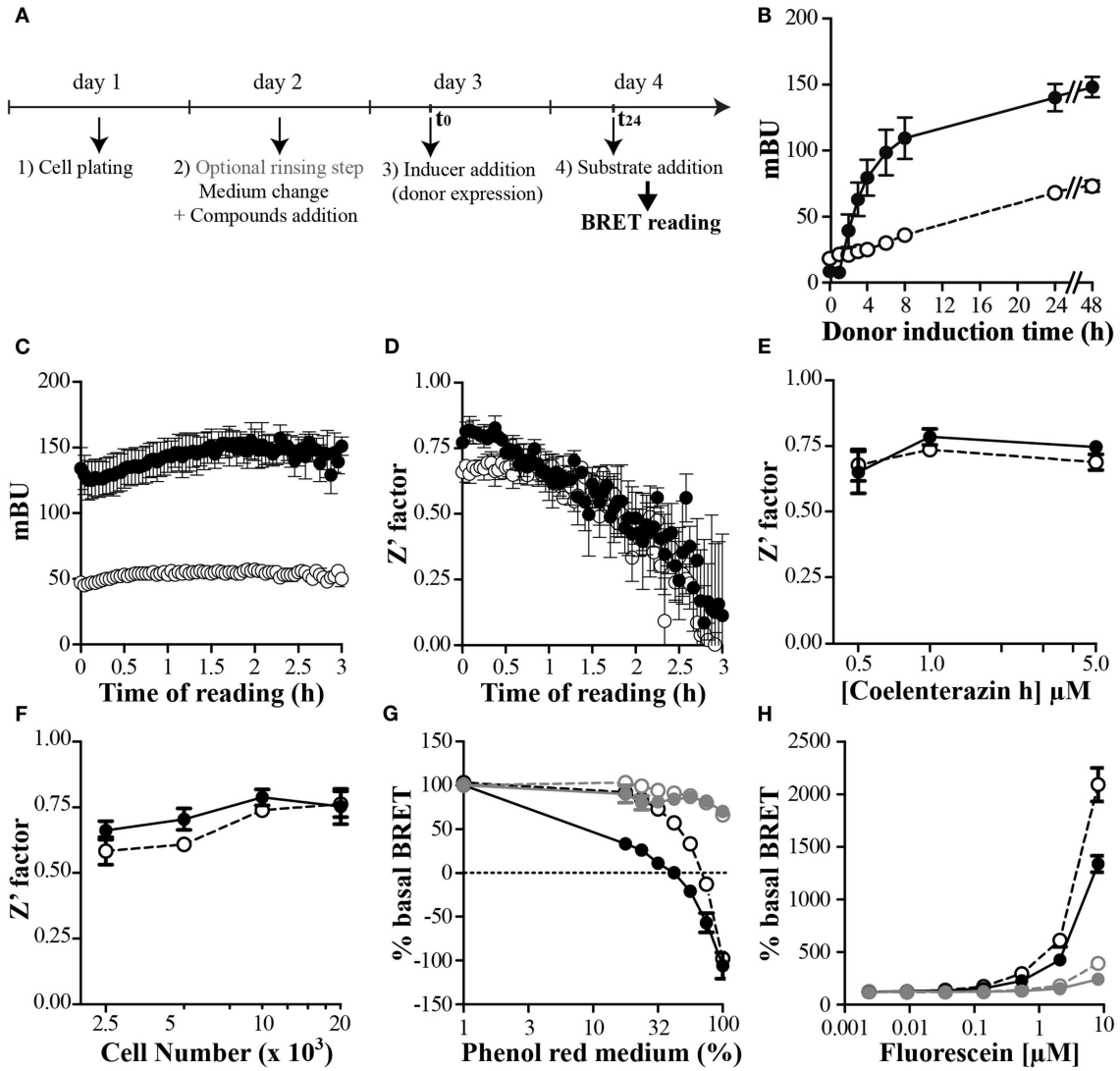

FIGURE 3 | Setting up and optimize a P2I2 BRET-based screening assay. (A) Basic protocol of P2I2 BRET-based screening assay; **(B–H)** parameters analysis of the BRET signal monitored using OBRc/OBRGRP (●) and CD4/PLSCR1 (○) BRET-based screening assays, engineered using CHO-Trex cell lines to allow doxycyclin induction of donor. $n = 3$; **(B)** kinetic of the BRET induction: BRET signal monitored as a function of time after inducer addition (doxycyclin 0.1 µg/ml). **(C)** Kinetic of the BRET reading after coelenterazine h addition and **(D)** effect on the Z′ factor calculated from 8 points. $n = 3$. **(E)** Effect of substrate concentration or cell number used **(F)** on the Z′ factor (from 8 points). $n = 3$; **(G)** dose dependent effect of red phenol or Fluorescein **(H)** interfering compound in the medium when reading BRET. In gray when medium was removed before reading: OBRc/OBRGRP (●) and CD4/PLSCR1 (○).

order to know the maximal BRET value reachable as a function of induction time, a kinetic of the induction would be performed for each inducible P2I2 BRET-based assay developed. To show the feasibility of such an inducible BRET approach in mammalian cells, two cellular (tet-on based) inducible P2I2 BRET-based assays were developed to monitor the kinetic of the induction. The first test was based on a previous BRET demonstration that interaction of the leptin receptor (OBR) with OB-RGRP negatively regulated OBR expression at the cell surface and was implicated in leptin resistance (Couturier et al., 2007). The second, monitoring the interaction between CD4 and phospholipid scramblase 1 (PLSCR1) was developed, based on finding showing that disrupting this interaction may inhibit HIV entry into cells (Py et al., 2009). As seen on **Figure 3B**, a maximal BRET value was reached after 24–48 h of induction by doxycyclin for both assays and BRET signal was very stable. In order to shorten the screening campaign, a 24 h induction time would be chosen in the present cases.

KINETIC OF THE BRET READING

When reading BRET, the kinetic of the *Rluc* emission is a crucial point as the BRET ratio is known to be stable only when it decreases. Depending of the temperature, the level of protein expressed, and of the developed test, the time to reach the decreasing activity step of the *Rluc* may vary from seconds to 5–15 min.

The proper kinetic has then to be determined for each developed BRET assay and a corresponding delay has to be added in the process before reading. Another crucial point when performing a screening assay is the emitted light that have to last long enough to ensure at least the reading of an entire 96 or even 384 wells plate. However, it is well-known that the *Rluc* activity and *per se* the BRET signal cannot be monitored for a long time period. To date some BRET kinetic experiments have shown reliable signal for at least 30–60 min using coelenterazine h (Kocan et al., 2008; Matthiesen and Nielsen, 2011).

To circumvent the short lasting period of the Rluc emission and increase its light output, efforts have been made to develop some new substrates for BRET 2 or BRET 1 (Zhao et al., 2004). Two BRET1 compatible substrates have been produced by Promega to gain better kinetics parameters for *Rluc in vivo* and *in live* cells (ViViren and Enduren respectively). These substrates have protected oxidation sites to lower the autoluminescence due to their degradation and are metabolized to coelenterazine h by cellular esterases. The light output superiority over coelenterazine h has been shown for both these substrates (Otto-Duessel et al., 2006; Kimura et al., 2010), and the interest of using enduren was confirmed by studies showing maintained luciferase activity and BRET1 signal for up to 9 h (Dinh et al., 2005; Pfleger et al., 2006). However, the expensive cost of such substrates may explain their restricted use and hinder their application in BRET-based assay screening campaigns.

Using both our cellular inducible P2I2 BRET-based assays, we tested the kinetic of the BRET reading using common ceolenterazine h. Unexpectedly the BRET ratio remained reliable for as long as 3 h after substrate addition (**Figure 3C**) however, the Z' value was compatible with screening (>0.5) for at least 80 min (**Figure 3D**). BRET monitored using coelenterazine h substrate is then sensitive enough and finally sufficiently long lasting to allow the automated addition of substrate in several plate and their reading over an extended time period using stackers.

INFLUENCE OF SUBSTRATE CONCENTRATION

The cost of a screening assay is a question of matter and regardless the price of compounds collection to be tested, a BRET-based assay includes the cost for the necessary substrate for each well to be read. To lower this cost, the total volume incubated in the wells has to be as low as possible to add the lower amount of substrate to reach the proper final concentration. Since the princeps publication describing BRET1 and until now, most BRET-based screening assays mostly used coelenterazine h at a final concentration of 5 μM (Boute et al., 2001; Charest et al., 2005; Hamdan et al., 2005; Laursen and Oxvig, 2005; Pfleger et al., 2006; Percherancier et al., 2009; Corbel et al., 2011; Kang et al., 2011) or even up to 30 μM (Vizoso Pinto et al., 2011). In order to monitor the effect of lowering the concentration of substrate on the BRET ratio and the Z' parameter, both our P2I2 BRET-based assays were used. As shown on **Figure 3E**, the Z' factor remain higher than 0.5 for a concentration of 1 μM and even 0.5 μM however it comes closer to the limit of 0.5. A final concentration of 1 μM can then be safely used when performing a P2I2 BRET-based screening campaign.

INFLUENCE OF THE NUMBER OF CELLS

When performing P2I2 screening assay, efforts have to be done to lower the amount of targeted complex to avoid or at least lower titration of the tested compound to gain high sensibility. The easy way to do it is to lower the number of plated cells, but the signal has to be still reliable and reproducible. To test this we plated varying number of cell from both our P2I2 assays we developed in 96 wells plate format and calculated the Z' factor from the BRET results. As can be seen on the **Figure 3F**, the Z' factor remains compatible with screening using lower cell number than 5000 but closest the 0.5 limit. 5000–10000 cells might then be used when performing such assay to ensure proper reliability.

INFLUENCE OF THE BRET READING BUFFER ASSAY

To perform a BRET assay in live cell, one would keep the cells in an as physiological context as possible and then perform the full experimental protocol from compound incubation to reading in proper cell culture medium. This is done currently in most studies, except for the final reading step which is mostly performed by replacing the medium with PBS containing the proper BRET substrate (See et al., 2011) or phenol red free medium. Indeed, when performing the BRET reading using medium containing red phenol, a shift in the BRET ratios is expected (**Figure 3G**), probably due to a change of the properties of the donor and/or the acceptor emissions or simply a physical change in the propagation of the light waves in the medium. Until recently, no dedicated study was done to monitor the effect of the reading buffer assay. Using a hGluc-(enterokinase cleavage site:-EYFP fusion, it has been shown that current buffers used to perform BRET reading such as Tris, Tricine, Sodium, HEPES, or MOPS are not the best to choose (Li et al., 2012). This study also showed that pH change of the medium promoted a change in the BRET signal (with a maximal value at ph 9), and furthermore that divalent cations such as Mg2+ and Ca2+ promoted a decrease in the BRET ratios. Most importantly, they have shown that adding imidazole to the reading medium promoted a 10-fold increase in the sensitivity of the assay and a sevenfold increase of the detection limit of the enterokinase activity. Although this was done using hGluc as donor, this study opens the way to monitor these parameters for other donors, as the monitored effects were not due to a drastic change in the luciferase activity but rather a change in the transfer efficiency. Future studies would find enhanced BRET buffers for BRET1 and BRET2 assays, in regards to the donor and acceptor used.

INFLUENCE OF COLORED AND FLUORESCENT COMPOUNDS

As describe in the previous paragraph, the BRET signal can be modulated by the composition of the medium in which the reading is performed. Interfering compounds used in the reading buffer, on both control and BRET cell lines of a P2I2 BRET-based assay, would not be such a problem as the effect would be present in all wells measured leading to an overall increase or decrease of the signal. However, when performing a compound screening assay, if a compound in a particular well lead to such a change, a false positive or negative signal would be expected, as this well is compared to controls incubated with vehicle only. As shown using red phenol versus red phenol free medium (**Figure 3G**) a BRET decrease is monitored. A colored compound having such properties would

be expected to lower the BRET signal due to a change in the medium properties, but not to a decrease in the studied interaction. On the other hand, fluorescent compounds sharing the same spectral properties than the acceptor, would also promote a change in the BRET signal, due to a saturation of the reading medium. A donor saturation effect leading to a BRET change would be expected by the free concentrated fluorescent compound if the donor emitted light overlaps the excitation one of this compound. In the case that the emission wavelength of the fluorescent compound is close to the acceptor emission, an artifactual BRET enhancement would be expected. Indeed, by incubating increasing concentration of Fluorescein on both our BRET screening assays, a huge BRET increase was monitored in a dose response manner. However, by replacing the medium containing Fluorescein by PBS before reading, the same BRET modulation was shown to be decreased by a 2 order dilution (**Figure 3H**) indicating that this effect was mostly mediated by the simple presence of Fluorescein in the medium. Therefore, when performing a P2I2 BRET-based screening assay, the reading of the fluorescence is necessary to exclude or to evidence those artifacts.

WHAT ARE THE EXPECTED RESULTS?

If a molecule inhibits the studied interaction, a decrease in BRET signal is expected (**Figure 4A**). The BRET method is a well suited method for this purpose as the signal relies on the ratio of the two emitted wavelengths (respectively from the donor and the acceptor). The BRET intensity is then dependent of the percentage of interacting partners in the cell (**Figure 2A**). A PPI inhibitory compound is then expected to reduce the amount of the BRET interacting partners as well as increasing the non-interacting donor proportion, leading to an enhanced BRET signal decrease, higher than just decreasing the interacting partner amount. Such P2I2 screening using energy transfer methods, might then lead to lower the IC50 values, and therefore to enhance the detection limit of such active compound when using a given concentration.

As the energy transfer is dependent of the distance between donor and acceptor but also the relative orientation of their dipole moment (Stryer and Haugland, 1967; Hickerson et al., 2005; Majumdar et al., 2005), RET methods allows to monitor the presence of the targeted interaction as well as fast conformational changes in the studied complex (Vilardaga et al., 2003; Milligan, 2004; Lohse et al., 2008; Alvarez-Curto et al., 2010). Such conformational changes, prone to promote a RET signal change (increase or decrease), lead to expect a higher hit rate than other PPI monitoring methods. Hence, such conformational modulators are unable to be detected using classical methods basically monitoring the presence of the interaction, unless they also promote a dissociation of the targeted complex.

Among RET methods, BRET has been shown to allow the monitoring of intramolecular or intermolecular conformational changes with high sensitivity and even only tiny changes due to point mutations (Milligan, 2004; Bacart et al., 2008; Alvarez-Curto et al., 2010; Darbandi-Tehrani et al., 2010). P2I2 BRET-based screening assays might then detect interactions inhibitors but also conformational modulators (**Figure 4A**) that do not promote interaction disruption but might lead to a change in the biological function as well. BRET experiments have been successfully used

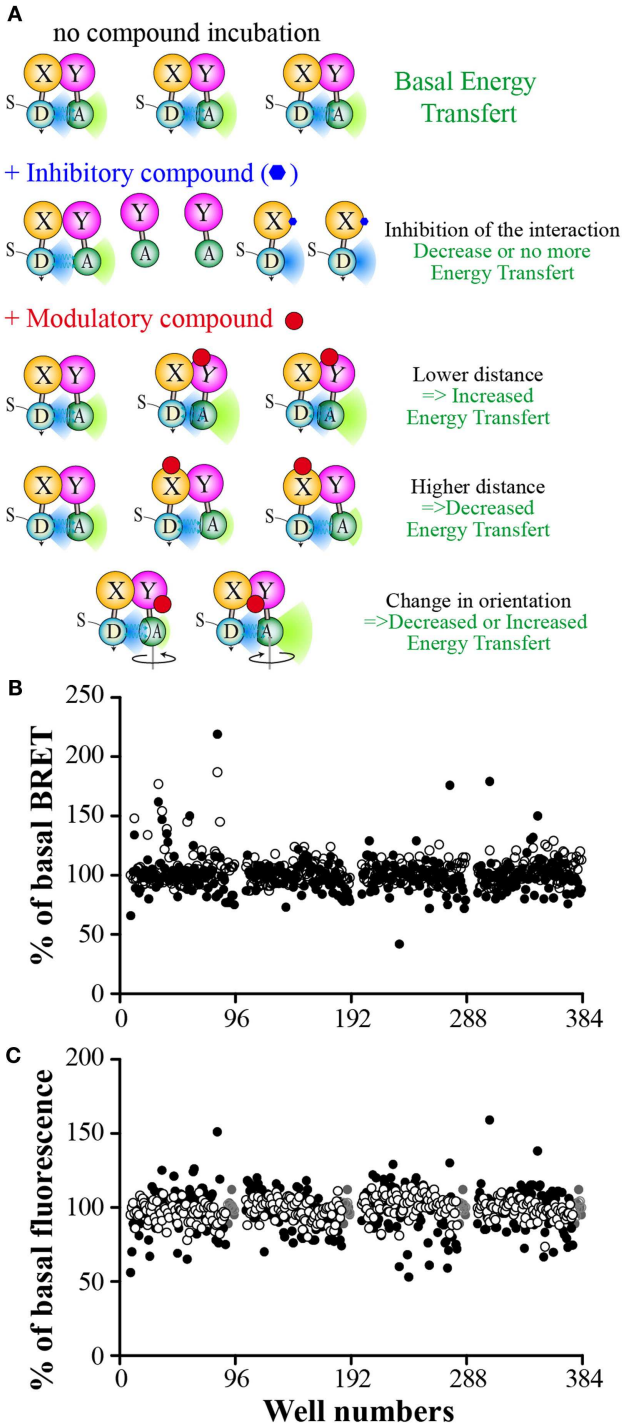

FIGURE 4 | Expected results from a P2I2 BRET-based screening assay. **(A)** Different expected BRET change upon inhibitor or modulator compound action compared to basal BRET signal. *X* and *Y*: Protein *X* and *Y*; D: energy donor; A: energy Acceptor; S: BRET Substrate. **(B)** Results of a 320 compounds miniscreen using OBRc/OBRGRP (●) and CD4/PLSCR1 (○) BRET-based screening assays, expressed as% of basal BRET in absence of compound in each plate. **(C)** Fluorescence measured from the same plates as in **(B)** expressed as % of fluorescence value in absence of compound (represented by ●).

to show ligands promoted conformational changes of receptors upon binding and leading to biological effects (Boute et al., 2001; Ayoub et al., 2002; Couturier and Jockers, 2003; Blanquart et al., 2006; Galés et al., 2006; Audet and Piñeyro, 2011). However, no systematic correlation between BRET increase or decrease and the biological effect is expected as agonists and antagonists were shown to promote a similar BRET change (Ayoub et al., 2002), no change (Terrillon et al., 2003), or even different BRET changes on same BRET assays (Elster et al., 2007), fully disrupting the correlation between the monitored signal and the expected biological effect.

Therefore, in PPI modulators BRET screening assays, if a known biological inducing control molecule is available, efforts would be focused on the design of a BRET assay able to monitor signal changes in presence of this compound. Nevertheless, compound promoting an opposite BRET change than the control used might represent another acting mechanism that could lead to a biological effect also.

To verify the feasibility of such a BRET approach to screen for P2I2 in mammal cells, we performed a miniscreens of 320 compounds using both our two PPI screening assays. As seen on **Figure 4B**, compounds were able to lower the BRET signal but also to increase it. Interestingly, some compounds were active on one assay but not the other. As expected, the total fluorescence reading (**Figure 4C**) showed that some compounds promoted changes in the overall fluorescence properties of the reading buffer in some wells. However, increased fluorescence was mild compared to those gained by Fluorescein but leading to no change in BRET signal (>10 and >2-fold increase respectively for OBRc/OBRGRP and CD4/PLSCR1; not shown). This indicates that these modifications prone to BRET signal increase might be of minor importance when performing P2I2 BRET-based screening assays, depending on the compound concentration used. On the contrary, BRET signal decrease, promoted by colored compounds might be more of concern as the decrease seen in the prescreen reached 50% of basal fluorescence, a change that promoted high BRET decrease when studying red phenol containing medium promoted BRET change (**Figure 3G**).

CONCLUSION

Bioluminescence resonance energy transfer technique is well suited to set up high throughput P2I2 screening assays. It has several advantages over other methods: it is homogenous; it can be performed in live cells like FRET but with higher sensitivity; and allows the monitoring of the studied interactions in a whole intact cellular context. However, general guidelines have to be respected when setting up such assays. As in any BRET interaction monitoring, the specificity of this interaction has to be checked using classical DSA. Stable cells lines would be selected in order to assume ease and reproducibility of the assay and expression of the donor would be inducible to allow compound to inhibit the target interaction before it happens. Kinetic of the induction and interaction have then to be determined. The kinetic of the BRET signal reading and influence of substrate concentration has to be checked in order to choose the parameters leading to the best dynamic BRET output and highest Z' factor value for the developed assay. Despite the fact that only one example of such a successful P2I2 screening assay (perfomed in yeast) has been published so far, this is a promising method to develop such assays in mammalian cells. One huge advantage of P2I2 BRET-based assay compared to classical methods is its ability to detect not only P2I2 but also conformational modulators of PPI, also able to promote the final biological targeted effect. A higher hit rate is then expected when using P2I2 BRET-based assays rather than with classical assays, only able to detect P2I2. Taken into account this huge advantage over other PPI monitoring techniques, its important optimization from the last years, and the still growing data of PPI leading to new potential drug target selection, a booming use of BRET to develop P2I2 assays would be expected in future years.

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APPENDIX

ENGINEERING OF OBRc/OBRGRP AND CD4/PLSCR1 INDUCIBLE BRET-BASED SCREENING ASSAYS

Fusion protein expression vector cloning

Leptin receptor c isoform (OB-Rc) and phospholipide scramblase 1 (PLSCR1) were cloned in phase with Rluc8 coding sequence in pcDNA5 vector to gain $pcDNA_5$-OBR-Rluc8 and $pcDNA_5$-Rluc8-PLSCR1 fusion protein expressing vectors. Transcript Leptin receptor overlapping transcript 1 (OB-RGRP) and CD4 were cloned in $pcDNA_3$ vector in phase with YPet.

Control and BRET cell lines engineering

Rluc8 fusion vectors were transfected in CHO-Trex (invitrogen) cell lines expressing a TetR and blasticidin antibiotic selection allowed to select resistant clones expressing luciferase activity. In both these BRET control cell line expressing the donor alone, the respective YPet-tagged partner was transfected and resistant clones selected using further G418 antibiotic selection to obtain BRET cell lines. OBRc-Rluc8/OBRGRP and CD4/PLSCR1 BRET cell line expressing the highest BRET signal upon stimulation by doxycyclin at 0.1 μg/ml for 24 h were selected. The use of the respective corresponding control cell lines allows to monitor BRET background to calculate mBRET from BRET cell lines.

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BRET biosensors to study GPCR biology, pharmacology, and signal transduction

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Bioluminescence resonance energy transfer (BRET)-based biosensors have been extensively used over the last decade to study protein–protein interactions and intracellular signal transduction in living cells. In this review, we discuss the various BRET biosensors that have been developed to investigate biology, pharmacology, and signaling of G protein-coupled receptors (GPCRs). GPCRs form two distinct types of multiprotein signal transduction complexes based upon their inclusion of G proteins or β-arrestins that can be differentially affected by drugs that exhibit functional selectivity toward G protein or β-arrestin signaling. BRET has been especially adept at illuminating the dynamics of protein–protein interactions between receptors, G proteins, β-arrestins, and their many binding partners in living cells; as well as measuring the formation and accumulation of second messengers following receptor activation. Specifically, we discuss in detail the application of BRET to study dopamine and trace amine receptors signaling, presenting examples of an exchange protein activated by cAMP biosensor to measure cAMP, β-arrestin biosensors to determine β-arrestin recruitment to the receptor, and dopamine D2 receptor and trace amine-associated receptor 1 biosensors to investigate heterodimerization between them. As the biochemical spectrum of BRET biosensors expands, the number of signaling pathways that can be measured will concomitantly increase. This will be particularly useful for the evaluation of functional selectivity in which the real-time BRET capability to measure distinct signaling modalities will dramatically shorten the time to characterize new generation of biased drugs. These emerging approaches will further expand the growing application of BRET in the screening for novel pharmacologically active compounds.

Keywords: arrestins, GRKs, EPAC, cAMP, FRET, screening assay, TAAR1

INTRODUCTION

Bioluminescence resonance energy transfer (BRET) is a process in which a non-radiative transfer of energy occurs between an excited luminescent enzyme/substrate donor complex and a fluorescent molecular acceptor that are separated by less than 100 Å. In many BRET studies published to date, the donors are variants of the enzyme, *Renilla reniformis* luciferase (Rluc), the enzymatically cleaved chemical substrate is coelenterazine, and the light emitting acceptors are variants of green fluorescent proteins (GFPs; Pfleger and Eidne, 2006). Degradation of a luminescent substrate by Rluc excites the GFP which in turn emits fluorescence. Resonance energy transfer (RET) techniques such as fluorescence resonance energy transfer (FRET) and BRET have become experimental techniques of choice for measuring constitutive and dynamic protein–protein interactions and interrogating changes in the activity of many biochemical signaling pathways, with FRET having the advantage of allowing cellular localization of the biological phenomenon that is studied. On the other side, BRET has advantage over FRET since it does not require an external illumination to initiate the energy transfer, which may lead to high background noise resulting from direct excitation of the acceptor or photobleaching. BRET experiments much like FRET can be conducted under conditions that more closely reflect the biochemical environments occurring in living organisms. As such, experimental platforms for BRET measurements have included model systems composed of bacteria, mammalian, and plant cells, and over 400 BRET related studies have been published since the first publication of Kai transcription factor interactions in bacteria (Xu et al., 1999). Over the last decade, the main usage of BRET has resided into investigating various protein–protein interactions (see Ayoub and Pfleger, 2010; Ferre et al., 2010; Lohse et al., 2012 for review) with major usage in the field of G protein-coupled receptors (GPCRs). However, more recently, several studies have applied BRET for the study of dynamic cellular processes, be it the modulation of the interaction of two proteins following a pharmacological treatment or the development of biosensors for various signaling pathways. In this review, we will discuss the development of some of these biosensors for the study of GPCRs. The term biosensor in this review will be applied to pharmacologically responsive interactions of GPCRs with other interacting proteins designed to

study GPCR signaling pathways. We will however only briefly discuss the numerous studies that have reported pharmacologically evoked BRET variations on either homo or heteromeric GPCR complexes as those have in large part been reviewed elsewhere (Ayoub and Pfleger, 2010; Ferre et al., 2010; Lohse et al., 2012). Specifically, we will illustrate why BRET biosensors have become such an enabling technology for studying GPCR biochemistry *in cellulo* by presenting their use in characterizing the pharmacology and signaling of two important GPCRs implicated in monoamine transmission, the trace amine-associated receptor 1 (TAAR1) and dopamine D2 receptor (D2R).

BRET BETWEEN RECEPTORS, G PROTEINS AND EFFECTORS

The first such study to utilize BRET to monitor the interaction between a GPCR and a G protein examined the interaction of β_2-adrenergic receptor (β2AR) with $G_{\alpha s\beta 1\gamma 2}$ (Gales et al., 2005). The β2AR was C-terminally tagged with Rluc, while the αs, β1 and γ2 were tagged with GFP10, a blue shifted variant of the GFP protein. Importantly, while the β1 and γ2 subunits were N-terminally tagged with GFP10, for the αs, the GFP10 was inserted between the helical and the GTPase domain of the protein in order to preserve αs functionality. Interestingly, a basal BRET signal indicative of constitutive interaction between all the tagged subunits and the receptor was observed. This BRET signal could be further enhanced with agonist stimulation when the heterotrimeric G protein was expressed, indicating either an increase in interaction between receptor and G proteins or a conformational change within the heterotrimeric complex. Since this first study, many others have been conducted on various receptor/G protein complexes, describing the kinetics, the orientation and the effects of ligands on the G protein–receptor interaction (Ayoub et al., 2007, 2009; Hasbi et al., 2007; Audet et al., 2008; Harikumar et al., 2008; Lohse et al., 2008; Kuravi et al., 2010; Oner et al., 2010; Busnelli et al., 2012).

Other BRET studies have investigated the interaction of GPCR with downstream effectors such as adenylyl cyclases or ion channels. Using a BRET approach, a constitutive interaction between β2AR–GFP and Kir3.1–Rluc potassium channel or adenylyl cyclase–Rluc was reported (Lavine et al., 2002). Importantly, the Kir3 BRET studies required the expression of a functional channel and therefore a significant BRET signal for Kir3.1–Rluc also required co-expression of the Kir3.2 or Kir3.4 subunits. Interestingly, agonist stimulation did not modulate these interactions, indicating that the complex between the receptor and these effectors did not dissociate during signal transduction.

β-ARRESTIN-BASED BRET SENSORS

The first and most reported BRET-based sensor studies for GPCRs have been investigations into the dynamics of β-arrestin recruitment. Non-visual arrestins, β-arrestin1 (arrestin-2) and β-arrestin2 (arrestin-3), are cytosolic proteins that bind agonist stimulated receptors. In a pioneering study, Barak et al. (1997) showed that stimulation of β2AR with the full agonist isoproterenol resulted in the recruitment of GFP-tagged β-arrestin2 to the plasma membrane from a cytoplasmic localization. The recruitment of β-arrestin to a receptor is a signal event that initiates two important biological effects. First, β-arrestin recruitment leads to the internalization of the receptor into endocytic vesicles. Second, β-arrestin recruitment is associated with the stimulation of additional signal transduction pathways that are G protein independent (Lefkowitz and Shenoy, 2005). Over the years it has become clear that G protein- and β-arrestin-dependent signaling pathways may lead to different physiological effects. This has led to the idea that novel therapeutic approaches can be pursued based upon the selective modulation of either the G protein or the β-arrestin-dependent pathway (Whalen et al., 2011). As such, devising new approaches to interrogate β-arrestin signaling becomes an important avenue for drug development. Considering the many advantages of BRET, the measurement of β-arrestin recruitment to receptors using BRET became a complementary approach to the technique developed by Barak et al. (1997) that measures β-arrestin redistribution by analyzing high content images. The first study investigating β-arrestin recruitment to a GPCR using BRET employed a β-arrestin2–YFP molecule and β2AR–Rluc (Angers et al., 2000; see **Figure 1** for example and principle). Since this seminal study, β-arrestin recruitment has been reported in more than 30–40 manuscripts, where the time course, dose response, ligand dependency, and effects of receptor homo/hetero-oligomerization on recruitment have been assessed (for reviews, see Pfleger and Eidne, 2003; Pfleger et al., 2007). In addition, a β-arrestin2 recruitment approach by BRET was also successfully used in a high-throughput screening (HTS) platform to identify new antagonists of the chemokine CCR5 receptor (Hamdan et al., 2005), demonstrating the versatility of this assay.

In order to better understand the conformational changes incurred by β-arrestin2 upon its recruitment to the receptor, Charest et al. (2005) developed a β-arrestin2 biosensor termed double brilliance. The double brilliance is a single molecule biosensor in which β-arrestin2 is tagged with Rluc and YFP at the N and C terminus, respectively. The double brilliance can be used to monitor stimulation of GPCRs, since agonist stimulation of the receptors leads to an increase of the constitutive basal BRET signal of the double brilliance. Interestingly, GPCR stimulation does not always lead to an increase in the BRET signal of double brilliance. For angiotensin AT1 receptor signaling, it was observed that while stimulation with angiotensin increased the BRET signal, stimulation of the receptor with a β-arrestin-biased agonist (SII), produced a decrease in the BRET signal of double brilliance (Shukla et al., 2008). A similar observation was made for the parathyroid hormone receptor type 1 (PTH1R). Stimulation with PTH-(1–34) led to an increase in the BRET signal and treatment with (PTH-IA), a β-arrestin-biased ligand, resulted in a decrease in the BRET signal (Shukla et al., 2008). These observations indicate that β-arrestin can adopt multiple different conformations that are dependent on the ligand that stimulates the receptor, and that these changes can be probed using the double brilliance biosensor.

β-Arrestin2 is ubiquitinated following GPCRs activation and in the case of many receptors this process has been shown to be required for receptor endocytosis (Shenoy et al., 2001; Ahmed et al., 2011; Shenoy and Lefkowitz, 2011). Two major classes of GPCRs (class A and B) have been described with regard to the stability of their interaction with β-arrestins. Class A receptors form transient complexes, while class B receptors form stable complexes with β-arrestins after receptor stimulation. It has been

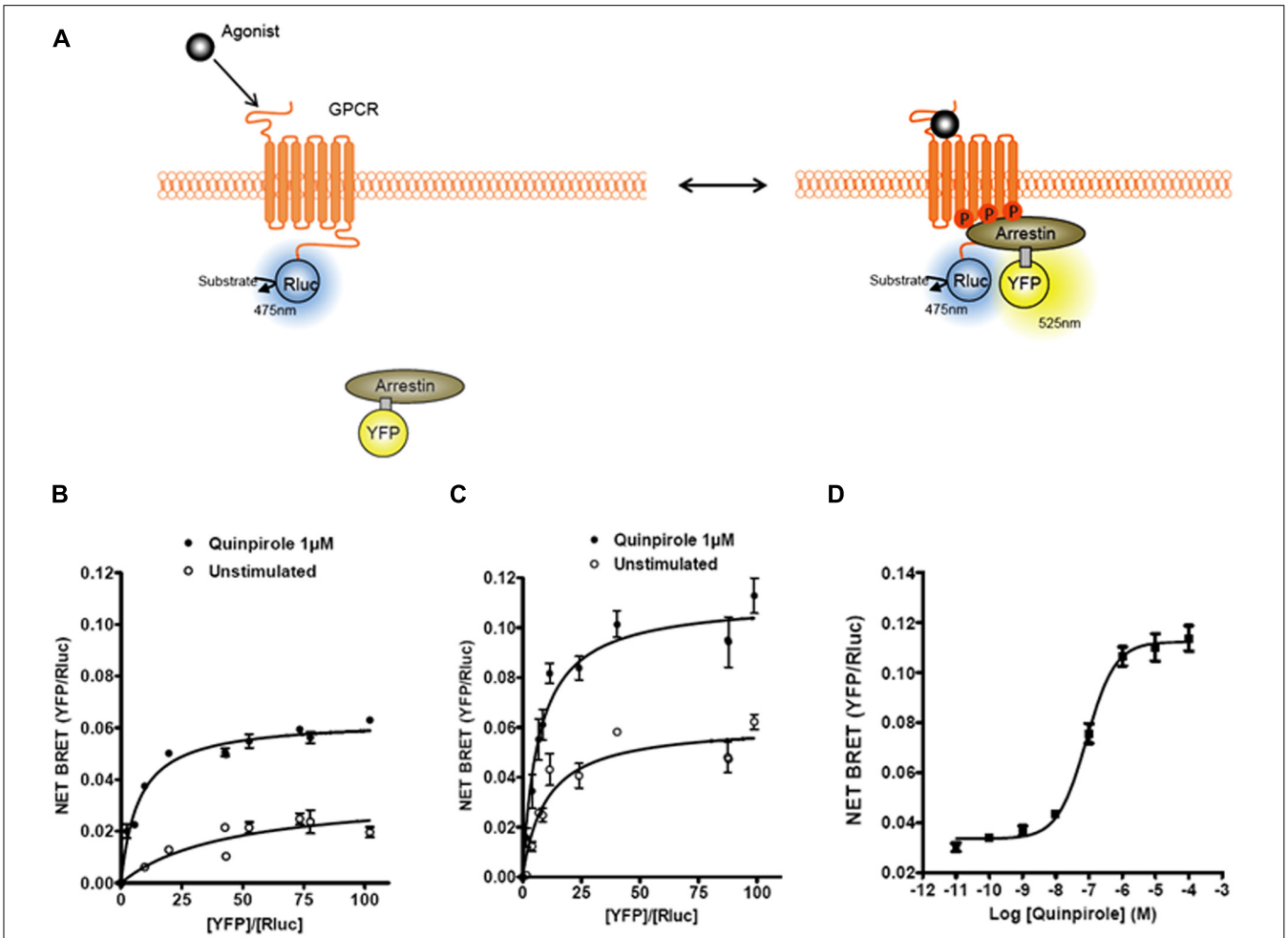

FIGURE 1 | Bioluminescence resonance energy transfer (BRET) monitoring of β-arrestins recruitment to activated GPCRs. (A) Cartoon of postulated molecular events following activation of a GPCR by agonist that lead to recruitment of arrestin to phosphorylated receptor causing related BRET signal. Arrestins can be tagged with different versions of GFP (YFP, Citrine, or Venus; for review, see Pfleger and Eidne (2006)) and GPCRs are tagged with Rluc to monitor intermolecular interaction by BRET. **(B)** Titration curves of β-arrestin1–YFP recruitment to D2R–Rluc with or without D2R agonist quinpirole. **(C)** Titration curves on β-arrestin2–YFP recruitment to D2R–Rluc with or without D2R agonist quinpirole. **(D)** Dose–response curve of quinpirole effect on β-arrestin2 recruitment to D2R.

shown that stable β-arrestin receptor complexes lead to sustained ubiquitination of β-arrestin while transient β-arrestin receptor complexes result in more temporary ubiquitination states (Shenoy and Lefkowitz, 2011). A BRET-based ubiquitination assay has been developed that allows the monitoring of β-arrestin ubiquitination in real time in live cells (Perroy et al., 2004). In these studies, β-arrestin2 was tagged with Rluc while the GFP was fused to ubiquitin. Agonist stimulation of both the β2AR, a class A receptor, and vasopressin V2 receptor (V2R), a class B receptor, results in a dose-dependent increase in BRET signal between Rluc–β-arrestin and GFP–ubiquitin, indicative of receptor-induced β-arrestin ubiquitination. Time course analysis of the BRET signal between β-arrestin and ubiquitin revealed that the signal was transient for β2AR stimulated samples while persistent for the V2R stimulated sample (Perroy et al., 2004). This observation is in agreement with prior experiments describing the dynamics of β-arrestin ubiquitination with class A and B receptors and demonstrates the utility of this sensor for studying the dynamics of β-arrestin ubiquitination in live cells.

Receptor interaction with G protein-coupled receptor kinases (GRKs) has also been investigated using BRET sensors. GRKs are a family of serine/threonine protein kinases that phosphorylate agonist stimulated receptors and for the most part desensitize their G protein signaling activity (Reiter and Lefkowitz, 2006). The first study utilizing BRET investigated the dynamics of an interaction between the Rluc–oxytocin receptor (Rluc–OTR) with GRK2–YFP (Hasbi et al., 2004). The authors showed that GRK2 is rapidly recruited to the receptor after agonist stimulation and that after several minutes of sustained stimulation the BRET signal decreases, consistent with the transient nature of the GRK/receptor interaction. Interestingly, the kinetics of GRK2 recruitment to OTR preceded β-arrestin2 recruitment to the same receptor suggesting that in this case, GRK2-mediated phosphorylation of OTR occurred before β-arrestin2 was recruited to the receptor. Other

studies using BRET to investigate the interaction of GRKs with various GPCRs can be found in the following references (Huttenrauch et al., 2005; Small et al., 2006; Jorgensen et al., 2008, 2011; Namkung et al., 2009).

PKA cAMP BRET BIOSENSORS

By coupling to either stimulatory Gs or inhibitory Gi protein pathways, many GPCRs modulate cAMP production, a second messenger that directly affects the function of many regulatory proteins. To study the dynamics of cAMP production, different BRET biosensors have been developed. The first BRET cAMP biosensor was based on protein kinase A (PKA; Prinz et al., 2006). PKA, a serine/threonine kinase, is composed of two regulatory and two catalytic subunits that dissociate upon binding of cAMP. There are two major PKA isoforms (I and II) that are expressed in mammalian cells with distinct biochemical and cellular functions. Prinz et al. (2006) created PKA fusion constructs by tagging the catalytic subunit with GFP2 (GFP-C) and regulatory subunits RI and RII with Rluc (RI–Rluc and RII–Rluc). Co-transfection of GFP-C subunit with either RI–Rluc or RII–Rluc results in a constitutive BRET signal that decreases in response to cAMP elevations from forskolin/IBMX treatment. However, only the RII–Rluc/GFP-C BRET sensor is sensitive to cAMP production by isoproterenol, a β2AR agonist, indicating that only PKA isoform II is in the correct spatial localization for detecting cAMP increases resulting from plasma membrane GPCR activation. However, when cells were treated with both isoproterenol and IBMX, both PKA isoform (RI and RII) were able to pick up the cAMP production induced by β2AR indicating that IBMX treatment alleviates the necessity for proper spatial localization to detect cAMP changes. Nevertheless, even in the presence of IBMX the magnitude of change in the BRET signal was greater from isoform II compared to isoform I, indicating that RII-based sensors may be more functionally sensitive and suitable for following GPCR-induced cAMP signals.

Although the PKA-based cAMP BRET biosensor is a powerful tool, it has an inherent caveat in that it is an intermolecular biosensor requiring the expression of two different proteins, i.e., regulatory and catalytic subunits. Therefore, a new generation of less complex single molecule cAMP BRET biosensors was developed from the guanine nucleotide exchange protein activated by cAMP (EPAC). To date two independent EPAC BRET biosensors have been described and used to study the modulation of the cAMP pathway by various GPCRs (Jiang et al., 2007; Barak et al., 2008).

APPLICATION OF BRET BIOSENSORS FOR THE STUDY OF D2R AND TAAR1

The recent development of BRET and FRET biosensors has allowed the study of a variety of physiological processes, such as formation of second messengers, protein kinases activity, protein–protein interactions, and protein trafficking (Vilardaga et al., 2003; Ni et al., 2006; Ayoub and Pfleger, 2010; Milligan, 2010; Lohse et al., 2012). With the application of different biosensors to the same receptor it is also possible to monitor the multidimensional complexity of signaling of a given receptor under the same experimental conditions. For example, the D2 dopamine receptor (D2R) is a Gi coupled receptor that is known to decrease cAMP levels upon stimulation by an agonist (Beaulieu and Gainetdinov, 2011). Recently, a new modality of G protein-independent β-arrestin2-mediated signaling has been described for D2R (Beaulieu et al., 2007; Beaulieu and Gainetdinov, 2011). Both G protein-dependent and -independent signaling pathways play important roles in dopamine-related physiological and pathological processes (Beaulieu et al., 2005, 2007; Beaulieu and Gainetdinov, 2011). Using a heterologous expression system and two different BRET biosensors, we assessed cAMP signaling and β-arrestin2 recruitment following activation or blockade of D2R (Masri et al., 2008). In another set of studies aimed at better understanding of monoamine transmission, we used BRET to study the signaling properties and pharmacology of TAAR1. In recent years, the TAAR1 has attracted attention as a potential new target for the modulation of the dopaminergic system (Lindemann et al., 2008; Sotnikova et al., 2009; Revel et al., 2011). Using BRET assays, we have evaluated the ability of TAAR1 and D2R to form a functional heterodimer and alter each other's signaling and functions (Espinoza et al., 2011).

USE OF EPAC cAMP BRET BIOSENSOR TO STUDY D2R AND TAAR1

The cAMP EPAC biosensor used in our studies was originally developed as a FRET biosensor (DiPilato et al., 2004) and later adapted for BRET applications (Barak et al., 2008). In this sensor, both the donor and the acceptor are located within the same protein leading to an intramolecular energy transfer. The sensor is made of EPAC, a protein that changes conformation upon binding cAMP. The premise behind the original FRET biosensor was to tag the full-length protein by a donor (enhanced cyan fluorescent protein, ECFP) and an acceptor (Citrine) on each extremity of the protein (see **Figure 2** for example and principle). The sensor was further improved by using a truncated form of the protein, comprised only of the cAMP binding domain and named ICUE2. ICUE2 was transformed in a BRET sensor by replacing the ECFP with the Rluc (Barak et al., 2008). At resting levels, there is considerable basal energy transfer between Rluc and Citrine resulting in a high BRET ratio, suggesting that in the absence of cAMP the donor and the acceptor are in close proximity. As cAMP increases, BRET ratios decrease, presumably due to a conformational change leading to increased distance between the Rluc and Citrine. As for other BRET sensors, the main advantage of EPAC is the possibility to measure the fluctuations of cAMP in real time and so to evaluate the contribution and the kinetic of different systems that modulate cAMP levels. GPCRs that couple to Gs or Gi are thus suitable targets for investigations with this sensor. Importantly, the BRET response with this sensor is reversible since the removal of the agonist or the addition of an antagonist results in a decrease of the response (DiPilato et al., 2004). Stimulation of both TAAR1 and β2AR leads to increases in cAMP levels readily measurable with the EPAC biosensor (Barak et al., 2008; Violin et al., 2008). But the degree of desensitization of these two receptors is different, being stronger for the β2AR. In comparison to β2AR, over-expressed TAAR1 has relatively minor level of plasma membrane expression, having predominantly an intracellular localization. Due to either poor surface expression or the fact that TAAR1 may be less prone to desensitization, the

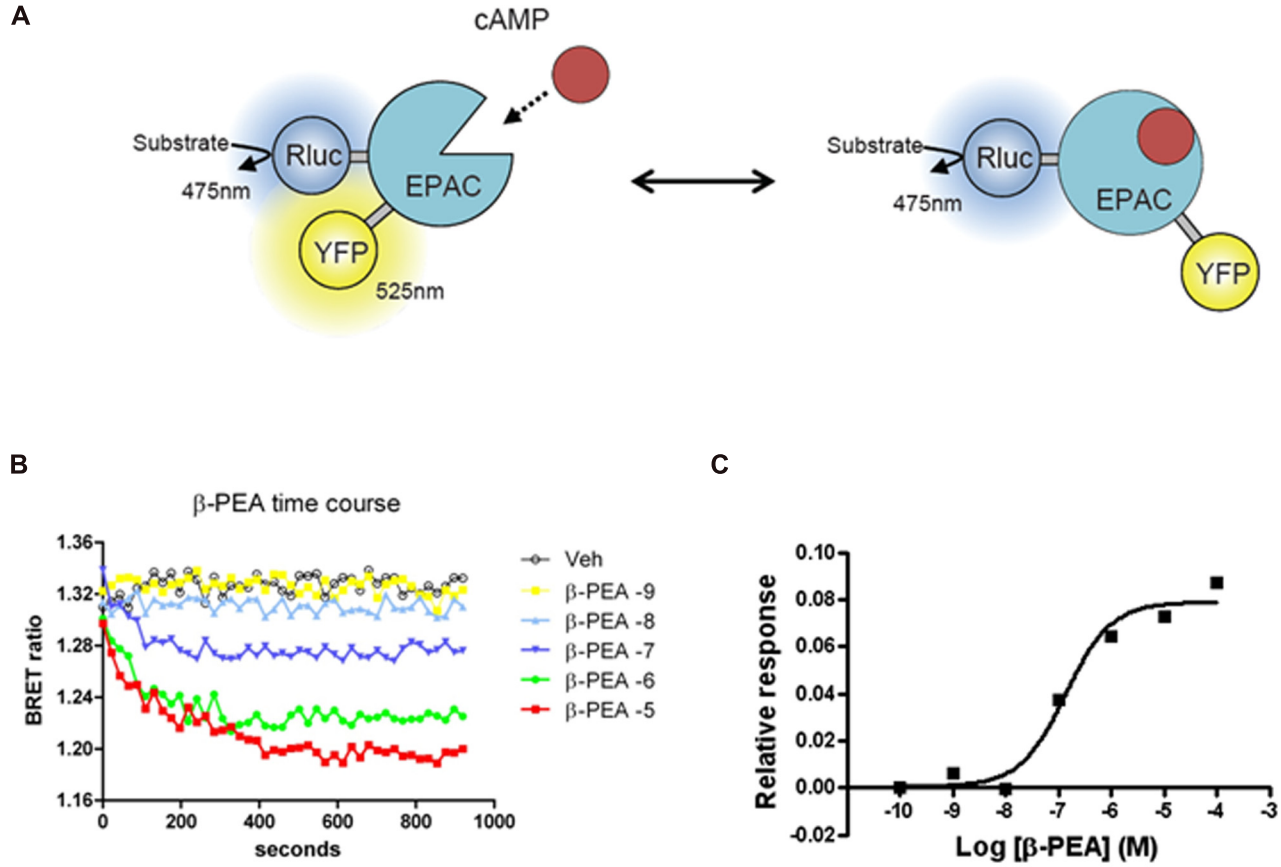

FIGURE 2 | Bioluminescence resonance energy transfer (BRET) EPAC biosensor for cAMP monitoring. (A) Cartoon of the postulated molecular rearrangement of the full-length EPAC protein with and without intracellular cAMP increase causing related BRET signal. EPAC, a protein that changes conformation upon cAMP binding, was tagged with a BRET donor (Rluc) and a BRET acceptor (YFP) on each extremity of the protein to monitor intramolecular BRET signal. **(B)** Time course effects β-PEA in cells transiently transfected with EPAC and TAAR1. BRET ratio is measured as YFP/Rluc ratio and the readings are started right after β-PEA addition. Cells are exposed to different concentration of β-PEA (from 1 nM to 100 μM) or control medium. The decrease in BRET ratio indicates an increase in cAMP concentration. β-PEA induces a robust increase in cAMP level that lasts along the duration of the entire experiment (20 min). **(C)** Dose–response of β-PEA effect on TAAR1-dependent cAMP accumulation after 10 min of stimulation.

over-expression of β-arrestin2 has substantially less of an effect on the kinetic of cAMP response of TAAR1 in comparison to β2AR (Barak et al., 2008).

Considering the simplicity of BRET experimental procedures it is evident that the EPAC sensor is a suitable tool for medium/HTS for identification of ligands of GPCRs that modulate cAMP. For example, we tested many potentially active compounds for their activity at membrane-expressed human TAAR1, confirming activity of some of the already known ligands and discovering interesting new ligands (Barak et al., 2008). In a follow-up study, we used EPAC sensor to screen for novel TAAR1 ligands against a limited library containing 1,000 drug-like compounds (data not published). About 20 potentially active compounds were initially identified but after excluding compounds that showed activity in cells expressing only the EPAC, four were confirmed as putative human TAAR1 agonists. Three of these compounds were known agonists of TAAR1, tyramine, 3-methoxytyramine, and 4-methoxytyramine, thereby validating specificity and sensitivity of this screening approach. Interestingly, the fourth compound was guanabenz that was later identified, by another group, as one of the most potent agonists of TAAR1 (Hu et al., 2009). In general, comparing with a standard radioactive cAMP column chromatography assay, the BRET method was found to be qualitatively similar but slightly more sensitive.

In another study, we used the EPAC biosensor to study the functional interaction between TAAR1 and D2R and observed that blockade of D2R selectively enhances TAAR1 signaling indicating a functional interaction between these two receptors (Espinoza et al., 2011). We have also used the EPAC sensor to evaluate the ability of different antipsychotics to block D2R-mediated signaling (Masri et al., 2008). Almost all antipsychotics showed an intrinsic activity as inverse agonists, with the exception of aripiprazole, a known partial D2R agonist. However, against quinpirole, a selective D2R agonist, the compounds blocked the response with different potencies and affinities, while aripiprazole's maximum efficacy of blockade was only 30%. All these are examples of how the EPAC sensor is a powerful tool for studying cAMP fluctuations in living cells.

β-ARRESTIN2 RECRUITMENT STUDIES FOR D2R

β-Arrestins were originally identified for their role in desensitizing GPCRs while subsequent studies have shown that β-arrestins can also activate signaling cascades independently of G proteins by acting as multifunctional scaffolding proteins (for review: Shenoy and Lefkowitz, 2011). Therefore, to fully characterize the mode of action of GPCR ligands, it is useful to evaluate their effects on both the G protein-mediated pathway and the β-arrestin pathway. A study conducted using β-arrestin2 knockout mice demonstrated that D2R can engage the Akt/glycogen synthase kinase 3 (GSK-3) signaling pathway by a mechanism that involves a signaling complex comprised of β-arrestin2, Akt, and the multimeric protein phosphatase PP2A (Beaulieu et al., 2005). This signaling pathway is essential since it is involved in the expression of some dopamine associated behaviors that are sensitive to antipsychotic drugs (Beaulieu et al., 2004). In fact, the clinical efficacy of almost all antipsychotic drugs (typical and atypical) is directly correlated with their binding affinity to D2R and their capacity to antagonize this receptor (Creese et al., 1976). The activity of these compounds has been extensively studied for cAMP signaling but little is known about ligand selectivity for β-arrestin-mediated signaling pathways. As described above, using BRET, it is possible to monitor real-time translocation of β-arrestin to an activated GPCR as well as to measure the effective dose (EC50) of an agonist or the inhibitory concentration (IC50) of different antagonists and compare their activity on this signaling pathway.

We used the β-arrestin and the EPAC BRET biosensors to define more precisely how antipsychotics affect dopamine D2 receptor signaling. While antipsychotics had antagonistic properties with very variable efficacies and potencies regarding Gi/o-mediated cAMP production, all these molecules were highly effective at antagonizing β-arrestin2 translocation to D2R with potencies between 3- and 150-fold higher than at the G protein-mediated pathway (Masri et al., 2008). These results suggested that clinically effective antipsychotics may act as preferential antagonists for D2R/β-arrestin2-mediated signaling rather than Gi/o-mediated signaling by this receptor. It is tempting to speculate that antipsychotics may exert their therapeutic effects in part by blocking β-arrestin2-mediated D2R signaling while inducing some of their side effects through modulation of other pathways. This idea was recently explored by using analogs of the atypical antipsychotic aripiprazole. These functionally selective β-arrestin2-biased D2R ligands exhibit antipsychotic activity *in vivo* and do not induce catalepsy in wild type mice (Allen et al., 2011). Thus, biased pharmacological approaches aimed at selectively targeting the β-arrestin2 signaling pathway activated by dopamine D2R may provide safer and more effective antipsychotics and protect against some of the motor side effects associated with this class of drugs.

D2R AND TAAR1 HETERODIMERIZATION

Direct protein–protein interaction is a commonly accepted concept in cell biology and in the recent years the possibility of homo- or heterodimerization of GPCRs has been fully appreciated. Using different techniques, it has been shown that many GPCRs exist as homo-, heterodimers or even as oligomers and techniques such as FRET and BRET have strongly contributed to the characterization of these physiological phenomena. The interaction between two or more GPCRs can alter important functions of these receptors such as cell surface delivery, G protein coupling and pharmacology, to name a few (Dalrymple et al., 2008; Milligan, 2010).

Intriguingly, when TAAR1 and D2R were co-expressed in the same cells, TAAR1-mediated cAMP signaling was increased when D2R receptors were blocked with the antagonist haloperidol (Espinoza et al., 2011). This signaling enhancement was selective for TAAR1 and did not occur with other Gs-coupled receptors. One possibility that we explored was heterodimerization between TAAR1 and D2R. Using BRET, we observed that TAAR1 and D2R can indeed exist as heterodimers when co-expressed in HEK-293 cells (Espinoza et al., 2011). Since BRET, like other techniques, has certain limitations, it was important to have appropriate controls to verify the reliability of the interaction (Marullo and Bouvier, 2007). We therefore performed BRET titration assays with a constant amount of the donor fusion protein (TAAR1–Rluc) and an increasing amount of the acceptor fusion protein (D2–YFP). The hyperbolic curve obtained for TAAR1–Rluc and D2–YFP, but not for D1–YFP, confirmed the selectivity of the interaction between these two receptors (see **Figure 3**). Furthermore, in a BRET competition assay, where an equivalent quantity of untagged D2R was co-expressed with TAAR1–Rluc and D2–YFP, the BRET signal was significantly decreased, while expression of untagged D1R had no effect on the BRET. Using a whole cell ELISA, we also showed that TAAR1 and D2R can co-internalize upon agonist stimulation of D2R, further confirming the physical interaction between the two receptors. Using cellular fractionation, we further noted that the heterodimer was mainly expressed at the plasma membrane and that treatment with D2R antagonist haloperidol could almost completely abolish the BRET signal from the heterodimer. These results suggest that haloperidol treatment either leads to the disassembly of the dimer or that this treatment induces a conformational change such that the distance between the donor and acceptor fluorophores are increased to the point where there is a decline in energy transfer. Notably, ligand-promoted BRET changes have been reported for other GPCR homo/heterodimers as well (Dalrymple et al., 2008; Milligan, 2009). Finally, we have observed some functional consequences of the putative TAAR1–D2R interaction where in TAAR1-KO animals, haloperidol-induced striatal c-Fos expression and cataleptic responses were significantly reduced (Espinoza et al., 2011).

CONCLUSION AND PERSPECTIVES

Bioluminescence resonance energy transfer biosensors have been instrumental in advancing our understanding of GPCR signal transduction by providing optical tools to study real-time interactions between receptors, the recruitment of binding partners to receptors, and variations in concentrations of second messengers generated downstream of receptors. Most importantly, BRET studies are conducted in live cells and enable the study of a wide variety of signaling systems to be probed under biologically relevant conditions, with minimal perturbation and in a quantitative manner. GPCR-related BRET biosensors have already established utility as screening platforms in drug discovery process.

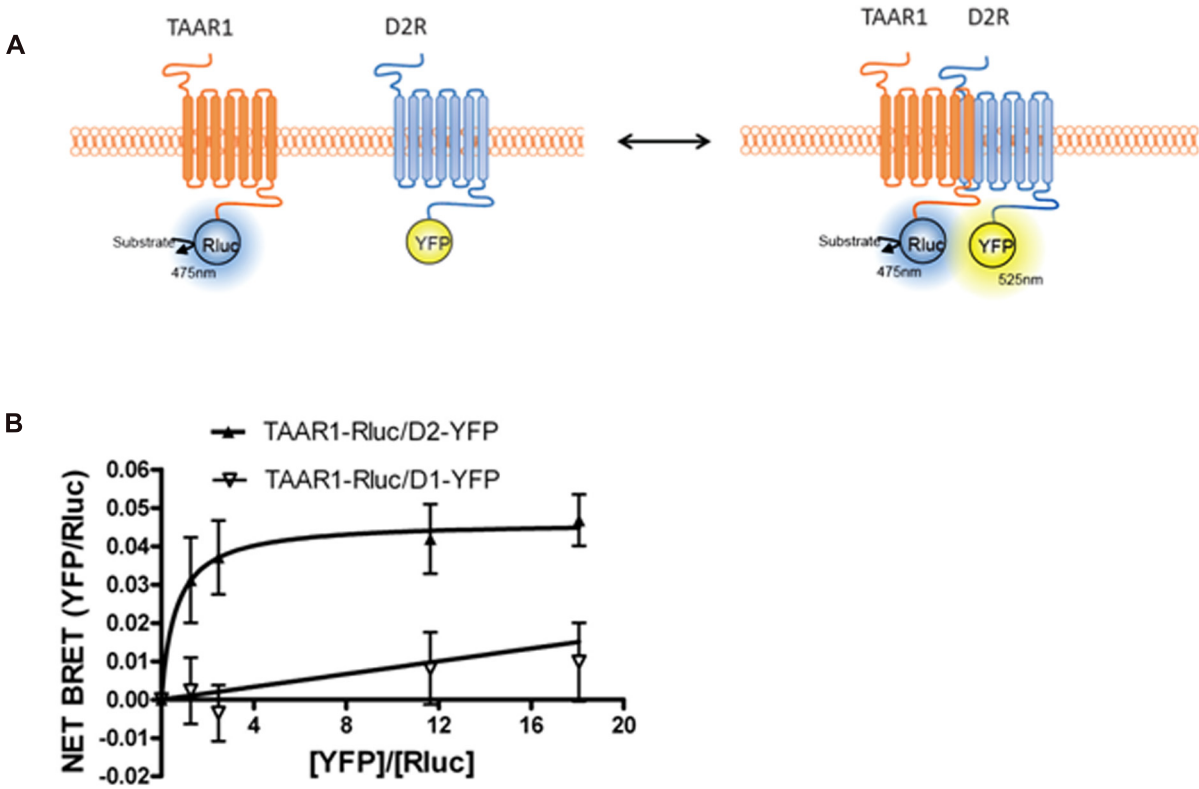

FIGURE 3 | Bioluminescence resonance energy transfer (BRET) monitoring of TAAR1 and D2R heterodimerization. (A) Cartoon of postulated molecular events during heterodimerization of GPCRs causing related BRET signal. TAAR1 is tagged with Rluc and D2R is tagged with YFP to monitor intermolecular interaction between these two receptors. **(B)** BRET titration curve of physical interaction between TAAR1–Rluc and D2R–YFP. A fixed amount of TAAR1–Rluc and increasing amount of D2R–YFP were co-expressed in the same cells. BRET was measured 20 min after the addition of the substrate, coelenterazine h. To test specificity of BRET signal between TAAR1 and D2R, BRET was also measured between TAAR1–Rluc and increasing amount of D1R–YFP. The hyperbolic shape of the curve indicates that TAAR1–Rluc and D2R–YFP form a constitutive heterodimer when co-expressed in the same cells. A linear increase in the BRET signal is observed between TAAR1–Rluc and D1–YFP indicating a non-specific, bystander BRET between these receptors.

At the same time, the number of BRET sensors described to date represents only a small fraction of their potential applications in biology and the list of new sensors to interrogate various signaling pathways and protein–protein interactions is rapidly growing. In particular, we anticipate that a new generation of BRET sensors will enable us to probe GPCR signaling in much greater detail than currently possible; and potentially lead us to new classes of "biased" ligands with unique therapeutic profiles (Beaulieu et al., 2005, 2007; Masri et al., 2008; Beaulieu and Gainetdinov, 2011; Whalen et al., 2011)

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Mathematical models for quantitative assessment of bioluminescence resonance energy transfer: application to seven transmembrane receptors oligomerization

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The idea that seven transmembrane receptors (7TMRs; also designated G-protein coupled receptors, GPCRs) might form dimers or higher order oligomeric complexes was formulated more than 20 years ago and has been intensively studied since then. In the last decade, bioluminescence resonance energy transfer (BRET) has been one of the most frequently used biophysical methods for studying 7TMRs oligomerization. This technique enables monitoring physical interactions between protein partners in living cells fused to donor and acceptor moieties. It relies on non-radiative transfer of energy between donor and acceptor, depending on their intermolecular distance (1–10 nm) and relative orientation. Results derived from BRET-based techniques are very persuasive; however, they need appropriate controls and critical interpretation. To overcome concerns about the specificity of BRET-derived results, a set of experiments has been proposed, including negative control with a non-interacting receptor or protein, BRET dilution, saturation, and competition assays. This article presents the theoretical background behind BRET assays, then outlines mathematical models for quantitative interpretation of BRET saturation and competition assay results, gives examples of their utilization and discusses the possibilities of quantitative analysis of data generated with other RET-based techniques.

Keywords: 7TMRs, BRET, oligomerization, mathematical models, quantitative analysis

INTRODUCTION

Seven transmembrane receptors form the largest and an evolutionarily well conserved family of cell surface receptors, with more than 800 members identified in the human genome. They are the targets both for a plethora of endogenous ligands (e.g., peptides, glycoproteins, lipids, amino acids, nucleotides, neurotransmitters, odorants, ions, and photons) and therapeutic drugs, and they transduce extracellular (ECL) stimuli into intracellular (ICL) responses mainly via coupling to guanine nucleotide binding proteins (G-proteins; McGraw and Liggett, 2006). These receptors are characterized by seven α-helices, which serve as transmembrane spanning domains (TMs) that are connected by three ECL and three ICL loops. The amino (N)-terminal fragment is ECL and the carboxyl (C)-terminal tail is ICL. This common structural topology was resolved by the three-dimensional crystal structure of individual 7TMR members (reviewed by Salon et al., 2011). In addition to their well-established ligands and G-proteins, these receptors can interact with a diverse set of protein partners, including G-protein coupled receptor kinases (GRKs), adaptor proteins such as beta-arrestins, scaffolding proteins that assemble and localize receptor-signaling complexes in specific cell membrane microdomains, as well as with each other/other receptor members, thereby forming homo-/heteromeric complexes (reviewed by Maurice et al., 2011). The specificity of agonist-promoted receptor interactions with protein partners such as GRK2 (Hasbi et al., 2004; Jorgensen et al., 2008) and β-arrestins (Angers et al., 2000) is not in doubt and the 7TMR/β-arrestin interaction has been successfully exploited to develop new bioluminescence resonance energy transfer (BRET)-based screening platforms (Bertrand et al., 2002; Vrecl et al., 2004, 2009; Hamdan et al., 2005; Heding and Vrecl, 2011). In contrast, 7TMR homo-/heteromeric complexes are more difficult to investigate, since these interactions are in general constitutive and ligand-independent.

METHODOLOGICAL APPROACHES TO STUDYING 7TMRs DIMERIZATION

Indirect evidence of receptor self-association already existed in the 1970s, before they were even shown to be seven transmembrane receptors (7TMRs). Following classical radioligand studies on the insulin receptor (De Meyts et al., 1973), negative cooperativity, for which dimerization is a prerequisite, was demonstrated for the β_2-adrenergic receptor (β_2-AR; Limbird et al., 1975) and thyrotrophin-stimulating hormone (TSH) receptor (De Meyts, 1976). In 1982, the receptor mosaic hypothesis was formulated, which proposed the functional importance of clustered receptors organized by receptor–receptor interaction (Agnati et al., 1982). Additional evidence supporting 7TMR dimerization/oligomerization was provided in the 1970s and 1980s by the use of radiation inactivation, photo-affinity labeling, cross-linking, and gel filtration methods (reviewed by Szidonya et al.,

2008). Trans-complementation studies were subsequently introduced (Maggio et al., 1993a,b) in which co-expression of two non-functional mutant/chimeric receptors resulted in a gain of function. Co-immunoprecipitation, which was first utilized to study β_2-AR dimerization (Hebert et al., 1996), was then the most frequently used method for detecting 7TMRs dimerization. Despite apparent receptor dimerization/oligomerization, there were concerns that higher order structures might be non-specific aggregations following detergent extraction of proteins from cells and membranes (reviewed by Milligan and Bouvier, 2005). Another less frequently utilized method for studying 7TMRs dimerization is sandwich ELISA (Biebermann et al., 2003; Rediger et al., 2009). The first widely accepted demonstration of 7TMR heterodimerization came from $GABA_B$ receptors, which exclusively function in a heteromeric form (White et al., 1998). Atomic force microscopy also demonstrated an oligomeric arrangement of rhodopsin and opsin in the form of large paracrystalline arrays, which showed receptors organized into rows of dimers (Liang et al., 2003; Fotiadis et al., 2004). Again, though, it has been suggested that the observed structure is an artifact of the preparation process (Chabre et al., 2003; Chabre and le Maire, 2005). Several functional studies have also reported co-internalization and modulation of the signaling activity of hetero-dimers/-oligomers, supporting the concept of receptor oligomerization (Terrillon and Bouvier, 2004). The introduction of biophysical techniques based on resonance energy transfer (RET), such as FRET and BRET, were then needed for taking the subject of 7TMRs oligomerization to the fore of 7TMRs research, since they enable the detection of protein–protein interactions in live cells and in real-time (reviewed by Pfleger and Eidne, 2005). BRET was first used to demonstrate β_2-adrenergic receptor (β_2-AR) dimerization (Angers et al., 2000) and BRET-based information about 7TMRs homo-/heterodimerization has been rapidly accumulating since then (for recent reviews see Gurevich and Gurevich, 2008a,b; Kubale et al., 2008; Ferre et al., 2009; Ayoub and Pfleger, 2010; Ferre and Franco, 2010; Palczewski, 2010; Achour et al., 2011). Over 20 different biochemical and biophysical methods that have been utilized in 7TMRs oligomerization studies were recently reviewed by Kaczor and Selent (2011). This review gives a short overview of BRET technology development and then discusses the possibilities of quantitative analysis of generated data.

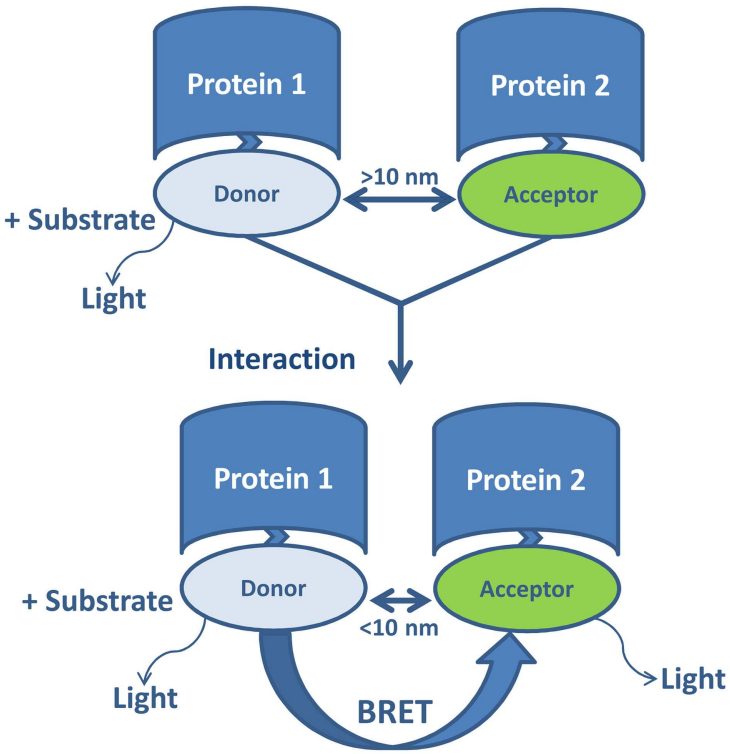
BRET PRINCIPLE AND BRET TECHNOLOGY DEVELOPMENT/OPTIMIZATION

Bioluminescence resonance energy transfer enables the monitoring of physical interactions between two proteins fused to a BRET donor or acceptor moieties, depending on their intermolecular distance (1–10 nm) and relative orientation due to the dipole-dipole nature of the RET mechanism (Förster, 1959). The BRET donor is a bioluminescent enzyme (a version of *Renilla luciferase*, Rluc), which reacts with the substrate to produce excitation. Part of this excitation can be non-radiatively transferred by RET to the acceptor molecule, usually a version of the green fluorescent protein GFP (**Figure 1A**). In addition to the original $BRET^1$ technology (Xu et al., 1999, 2003), which is based on Rluc as a donor and YFP as an acceptor, several versions of BRET assays have been developed that use different substrates and/or energy donor/acceptor pairs (**Figure 1B**). In $BRET^2$, Rluc is used as the donor (emission peak 395 nm for coelenterazine analog DeepBlueC™) and GFP variant two (GFP^2) as the acceptor molecule (excitation/emission peaks at 400/510 nm). $BRET^2$ enables superior separation of donor and acceptor emission peaks (Stokes shift of 50 and 115 nm for $BRET^1$ and $BRET^2$, respectively), as well as efficient filtration of the excitation light, thereby enabling detection of the weak fluorescence signal. The major disadvantage of $BRET^2$ compared to $BRET^1$ is the 100–300 times lower intensity of emitted light and its very fast decay (Heding, 2004). This was improved by the development of suitably sensitive instruments (Heding, 2004) and the use of Rluc mutants with improved quantum efficiency and/or stability (e.g., Rluc8, Rluc8.6, and Rluc-M) as donor (De et al., 2007; Loening et al., 2007). The use of $BRET^1$ and $BRET^2$ is largely limited to *in vitro* cell culture systems because they emit light in the green to yellow region of the visible spectrum (510–570 nm), which is strongly absorbed by biological tissues such as blood and highly vascularized tissue. This was overcome by $BRET^3$, which combines Rluc8 with the mutant red fluorescent protein (DsRed2) variant mOrange and coelenterazine or EnduRen™ as a substrate (De et al., 2009). In $BRET^3$, the donor spectrum is the same as in $BRET^1$, and the red shifted mOrange acceptor signal has excitation/emission peaks at 480/564 nm. Due to tissue attenuation of the light emitted at a wavelength <600 nm, its utilization in live animals is limited to superficial locations (e.g., subcutaneous tumors). Recently developed $BRET^3$ variants ($BRET^4$, $BRET^5$, and $BRET^6$), which have been optimized for deep-tissue imaging, combine Rluc8/Rluc8.6 with two red fluorescent proteins, i.e., TagRFP (emission peaks at 584 nm) or TurboFP635 (emission peak at 635 nm) and coelenterazine or its synthetic derivative (coelenterazine-v) as a substrate (Dragulescu-Andrasi et al., 2011).

BRET RESULTS – INTERPRETATION AND POSSIBLE SHORTCOMINGS

The distance (1–10 nm) at which BRET typically occurs is comparable with the dimensions of most biological macromolecules engaged in complex formation or conformational changes, thus making this technique suitable for monitoring protein–protein interactions in living cells/organisms (Wu and Brand, 1994). The experimentally determined Förster distance R_0, which leads to 50% of energy transfer from the donor to the acceptor, is 4.4 and 7.5 nm for $BRET^1$ and $BRET^2$, respectively (Dacres et al., 2010). Even though the working distance of $BRET^1$ is comparable with FRET (4.4 vs. 4.8 nm; Evers et al., 2006), the selection of RET systems depends on the particular application. FRET, under microscopic observation, allows visualization of protein interactions in living cells at the subcellular level, while BRET might be more suitable for non-imaging applications, such as the dynamic study of protein–protein interactions in a cell population (Boute et al., 2002). Direct comparison of $BRET^2$ with both FRET and $BRET^1$ showed the superiority of $BRET^2$ over FRET and $BRET^1$ in proximity-based assays such as protein–protein interaction assays (Dacres et al., 2009a,b). The working distance of 7.5 nm determined for $BRET^2$ could also make it more suitable for the study of larger proteins and/or multiprotein complexes, including 7TMR complexes (Dacres et al., 2010). For comparison, the 7TMR

A



B

Assay type	Donor	Acceptor	Substrate	Donor emission peak (nm)	Acceptor emission peak (nm)	Stokes shift (nm)
BRET[1]	RLuc/Rluc8	YFP	Coelenterazine h/EnduRen™	475/475	527	~50
BRET[2]	RLuc/Rluc8	GFP[2]	DeepBlueC™/ Coelenterazine 400A	395/395	510	115
BRET[3]	RLuc/Rluc8	mOrange	Coelenterazine h/ EnduRen™	475/475	564	85
BRET[3.1]	Rluc8	mOrange	Coelenterazine-v	515	564	~50
BRET[4.1]	Rluc8	TagRFP	Coelenterazine-v	515	584	~70
BRET[5]	Rluc8.6	TagRFP	Coelenterazine h	535	584	~50
BRET[6]	Rluc8.6	TurboFP635	Coelenterazine h	535	635	100
BRET[6.1]	Rluc8.6	TurboFP635	Coelenterazine-v	570	635	65

FIGURE 1 | Schematic representation of the BRET assay and various BRET variants for studying protein–protein interaction. (A) BRET enables monitoring of physical interactions between two proteins genetically fused to donor and acceptor molecules. The BRET donor is a bioluminescent enzyme (a version of *Renilla luciferase*, Rluc), which reacts with the substrate to produce excitation. The acceptor molecule is usually a version of a green fluorescent protein (GFP). If the distance between donor and acceptor is more than 10 nm, light is emitted with an emission spectra characteristic for Rluc. When the distance is less than 10 nm, part of this energy is non-radiatively transferred by RET from donor (Rluc) to acceptor (version of GFP), resulting in an additional signal emitted by the acceptor. **(B)** A summary of BRET variants and their basic characteristics.

transmembrane core spans ∼4 nm across the ICL surface (Palczewski et al., 2000), the intradimer distance between rhodopsin monomers is 3.5 nm and the distance between rhodopsin dimers is 4.5 nm (Fotiadis et al., 2004). In spite of that, the following limitations of this method need to be considered when interpreting BRET results. Firstly, the size of fluorescent proteins (∼27 kDa) and Rluc (∼34 kDa) is comparable to that of the transmembrane core of 7TMRs (diameter ∼4 nm). These proteins are usually attached to the receptor C-terminus, which varies in length in different 7TMRs from 25 to 150 amino acids. Polypeptides of this length in extended conformation can cover 8-48 nm. A BRET signal thus indicates that the donor and acceptor moieties are at distance less than 10 nm, which may occur when receptors form a structurally defined dimer or when they are >50 nm apart (reviewed by Gurevich and Gurevich, 2008a). The use of acceptor and donor molecules genetically fused to 7TMRs can alter the functionality of the receptor; fusion proteins can also be expressed in ICL compartments, making it difficult to demonstrate that the RET results from a direct interaction of proteins at the cell surface. The use of fusion proteins can therefore be a major limitation for this application. Secondly, quantitative BRET measurements are limited by the quality of the signal and noise level. Fluorescent proteins and luciferase yield background signals arising from incompletely processed proteins inside the cell and high cell autofluorescence in the spectral region used (Gurevich and Gurevich, 2008a). Thirdly, so called bystander BRET results from frequent encounters between over expressed receptors and has no physical meaning (Kenworthy and Edidin, 1998; Mercier et al., 2002). Bystander BRET is also problematic when the studied proteins are confined to a subcellular compartment, such as the plasma membrane. BRET assays should therefore be able to discriminate between true dimerization/oligomerization and random collision due to over-expression. To determine the specificity of a BRET signal, the following experiments have been proposed: negative control with a non-interacting receptor or protein, BRET saturation, and competition assays, as well as experiments that observe ligand-promoted changes in BRET (Ayoub and Pfleger, 2010; Ferre and Franco, 2010; Achour et al., 2011). Correct execution of these experiments requires graded control of protein expression over a broad range of concentration, its accurate measurement, and knowledge about the subcellular location of the expressed proteins. The choice of appropriate control is also crucially important. These requirements are not always easy to fulfill in practice and this further complicates the interpretation of results. A general method (third-party BRET), which does not require graded expression or quantification of acceptors or donors, has also been developed to detect specific constitutive BRET between proteins located in subcellular compartments of living cells; again, though, it has the same limitations as other RET methods (Kuravi et al., 2010). Additionally, proper interpretation of BRET results also requires quantitative analysis of the result, which has so far only been done in a small number of studies (Ayoub et al., 2002; Mercier et al., 2002; Vrecl et al., 2006). The theoretical background of the assays described below could serve as a guiding principle for the quantitative extrapolation of data from BRET experiments performed with 7TMRs and, presumably, also with other cell surface receptors that form dimer/oligomers.

BRET MEASUREMENT

In BRET experiments luminescence is measured at the peaks of donor and acceptor emissions (Xu et al., 1999). Given that a fixed number of quanta are produced by luciferin-luciferase reactions, the majority of light is emitted by the donor molecules. If RET takes place, then part of the energy is transferred to the acceptor molecules and is emitted at a longer wavelength. Since part of the donor emission spectra overlaps with the acceptor emission spectra, this background has to be subtracted; the BRET signal is then calculated as:

$$\mathrm{BRET} = \frac{I_2}{I_1} - \frac{I_{20}}{I_{10}}, \tag{1}$$

where I_2 and I_1 are measured luminescences at the two peak positions in the case of donor-acceptor interactions, and I_{10} and I_{20} represent luminescence intensities at the two peak positions for donor only transfected cells. Samples with different concentrations of donors or variations in light quanta produced by the luciferin-luciferase reaction can in this way be compared.

In order to obtain a correct BRET value that can be compared between different laboratories, the BRET luminometer should be properly calibrated, which means that the same proportion of donor (I_1) and acceptor (I_2) emissions is detected. The sum of the two emissions is then proportional to the concentration of donors, which is again proportional to the total luminescence I_{tot} (luminescence measured in the whole visible spectrum). In general, the magnitude of measured luminescence depends on the selection of emission filters and detector sensitivity. A calibration factor b is thus introduced:

$$I_1 + b * I_2 = k * I_{\mathrm{tot}}. \tag{2}$$

Using the above equation, the value of parameter b can be assessed by completing two experiments with different donor-acceptor ratios while measuring I_1, I_2, and I_{tot}. The calibrated BRET is then:

$$\mathrm{BRET}_{\mathrm{cal}} = b * \left(\frac{I_2}{I_1} - \frac{I_{20}}{I_{10}}\right). \tag{3}$$

For conducting BRET assays, information about relative donor and acceptor concentrations is needed. Donor and acceptor concentrations can be assessed by luminescence and fluorescence measurements, respectively. First, however, the calibration curves of luminescence and fluorescence vs. receptor concentration must be obtained by using an immuno-based method or radioligand binding assay (Ayoub et al., 2002; Mercier et al., 2002; Ramsay et al., 2004).

BASIC BRET THEORY

Bioluminescence resonance energy transfer is defined as the ratio between transferred T and not-transferred energy Q:

$$\mathrm{BRET} = \frac{T}{Q}. \tag{4}$$

The probability that excitation is transferred from donor to acceptor in a single BRET pair is governed by the energy transfer efficiency E:

$$T = E * Q_0, \tag{5}$$

where Q_0 is total energy ($Q_0 = T + Q$). The energy transfer efficiency is inversely proportional to the sixth power of the distance R between the donor and the acceptor, as described by the Förster equation (Förster, 1959), where the Förster radius R_0 depends on the spectral overlap and dipole orientations:

$$E = \frac{R_0^6}{R_0^6 + R^6}. \tag{6}$$

The calculations in a quantitative BRET assay are derived from the Veatch and Stryer model (Veatch and Stryer, 1977) covering FRET experiments with gramicidin dimers. The calculations are usually simplified by assuming that E is small enough for the following approximation to be used:

$$\mathrm{BRET}_{E \ll 1} = \frac{T}{Q_0}. \tag{7}$$

Before using the small energy transfer approximation, the magnitude of the energy transfer efficiency E must be determined. For dimers, E can be calculated from maximum BRET, which is obtained when all donor molecules are accompanied by acceptors (using Eqs 4 and 5):

$$E = \frac{\mathrm{BRET}_{\max}}{\mathrm{BRET}_{\max} + 1}. \tag{8}$$

QUANTITATIVE BRET ASSAYS

Although determination of the BRET signal is quite simple, the interpretation of results obtained from oligomerization studies is not unambiguous. If the receptor expression level is in the physiological range, there is a problem of distinguishing random collisions of donors and acceptors from stable binding. With increasing receptor concentration, there is a higher probability of two receptors being in the range of the Förster radius and producing so called bystander BRET. Several quantitative assays have been developed to distinguish these two processes, including dilution, saturation, and competition assays, which allow an assessment of the receptor oligomerization state and relative affinities for homo- and hetero-dimer formation (Ayoub et al., 2002; Mercier et al., 2002). Interpretations of quantitative BRET assays have been summarized in several review articles (Issad and Jockers, 2006; Pfleger and Eidne, 2006; Ayoub and Pfleger, 2010; Achour et al., 2011; Kubale et al., 2012).

BRET DILUTION ASSAY

A dilution assay is the simplest control experiment to check for oligomerization. RET takes place if the distance between donor and acceptor molecules is in the range of the Förster radius R_0. Molecules can also get close enough for BRET by random collisions (bystander BRET) if their density is high enough (Kenworthy and Edidin, 1998; Mercier et al., 2002). Excluding random collisions, there should be no concentration dependence for coupled donor and acceptor molecules. In practice, the BRET signal can be approximated by:

$$\mathrm{BRET} = \mathrm{BRET}_0 + k\,([D] + [A])\,, \tag{9}$$

where $[D]$ and $[A]$ are donor and acceptor concentrations. By simultaneously lowering the concentration of both receptors (dilution), the BRET signal decreases toward BRET_0, which is the real oligomerization signal (**Figure 2**). When performing this experiment, care should be taken to keep the receptor ratio $[A]/[D]$ constant (Mercier et al., 2002).

There is a low limit of receptor concentrations that can be used in BRET assays because of the increasing noise in calculated BRET at low luminescence intensities. The upper limit of the receptor concentration used in saturation and competition assays should be set at the point at which the BRET value does not significantly differ from BRET_0. The dilution assay has been used to set the concentration range for saturation and competition assays and to distinguish monomers from dimers (Mercier et al., 2002; Terrillon et al., 2003; Breit et al., 2004; Ramsay et al., 2004).

BRET SATURATION ASSAY

The saturation assay involves expressing a constant amount of donor-tagged receptor with an increasing amount of acceptor tagged receptor. Theoretically, the BRET signal should increase with increasing amounts of acceptor until all donor molecules are interacting with acceptor molecules. A saturation level $\mathrm{BRET}_{\max}$ is therefore achieved, beyond which a further increase in the amount of acceptor does not increase the BRET signal (Mercier et al., 2002; Hamdan et al., 2006; Ayoub and Pfleger, 2010; Achour et al., 2011). The BRET saturation curve derived from the Veatch and Stryer model in an approximation of small energy transfer efficiency

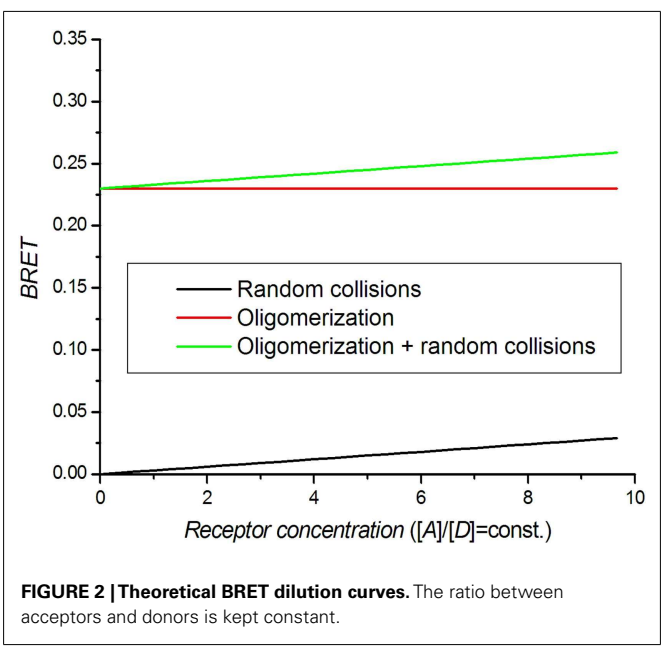


FIGURE 2 | Theoretical BRET dilution curves. The ratio between acceptors and donors is kept constant.

(Eq. 7) is commonly used:

$$\frac{\text{BRET}}{\text{BRET}_{\max}} = 1 - \frac{1}{\left(1 + \frac{[A]}{[D]}\right)^N}, \tag{10}$$

$N = 1$ for dimer, $N = 2$ for trimer and $N = 3$ for tetramer. The detailed derivation can be found in articles by James et al. (2006), Vrecl et al. (2006). Theoretical BRET saturation curves are presented in **Figure 3**. BRET for higher oligomers shows faster saturation. For comparison, the monomer BRET signal that corresponds to random collisions is presented. If the receptor concentration is very high, then random collisions can generate a quasi-linear saturation curve similar to that of the dimers. A dilution experiment should thus be done first to distinguish random collisions from true oligomerization. Mercier et al. (2002) provided an equation that differs from that above for $N > 1$:

$$\frac{\text{BRET}}{\text{BRET}_{\max}} = \frac{([A] + [D])^{N+1} - [A]^{N+1} - [D]^{N+1}}{([A] + [D])^{N+1} - [A]^{N+1} - [D]^{N+1} + (N+1)[D]^{N+1}}. \tag{11}$$

For dimers, the two equations simplify to a saturation binding curve that is usually used in saturation assays:

$$\frac{\text{BRET}}{\text{BRET}_{\max}} = \frac{\frac{[A]}{[D]}}{1 + \frac{[A]}{[D]}}. \tag{12}$$

In **Figure 3**, a comparison can be made between the theoretical BRET curve for dimers and quasi-linear curve from non-specific interactions for which high $\text{BRET}_{\max}$ values can be obtained in the case of high receptor concentrations. On the other hand, the dimer curve remains insensitive to total receptor concentration. For higher oligomers, the saturation curve is shifted to the left and allows a determination of the oligomerization state (Mercier et al., 2002; Vrecl et al., 2006). The ability to determine the oligomerization state from the saturation assay is hampered by systematic errors in the determination of receptor concentrations and variations in sample treatments, resulting in a large spread of data.

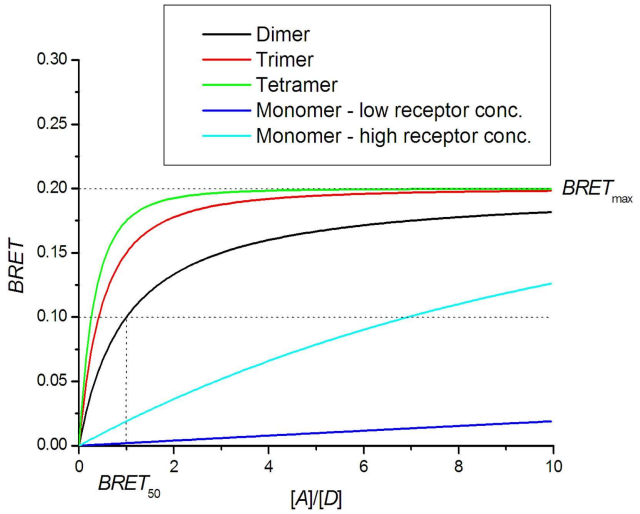


FIGURE 3 | BRET saturation assay. Theoretical curves for oligomer formation are plotted as a function of the ratio of receptors tagged with acceptor [*A*] and donor [*D*] molecules. In the case of monomers, the BRET signal is created by random collisions.

BRET_{50} represents the receptor concentration ratio at which the saturation curve reaches half-maximum value. The theoretical BRET_{50} value for homo-dimers is 1. BRET_{50} values obtained for hetero-dimers can be compared with that of homo-dimers as a measure of the relative affinity for their formation. If the affinity for hetero-dimer formation is lower, the saturation curve is shifted to the right, yielding a higher BRET_{50} value (Mercier et al., 2002; Terrillon et al., 2003; Breit et al., 2004; Goin and Nathanson, 2006). In a few cases, the affinity for hetero-dimers is higher than that for homo-dimers, as shown for melatonin receptors MT_2-MT_1 and MT_2-MT_2 receptor pairs (Ayoub et al., 2004).

The $\text{BRET}_{\max}$ value can be used to detect conformational changes of the receptors forming a certain dimer (Eidne et al., 2002; Issad and Jockers, 2006). Percherancier et al. (2005) showed that ligands can cause modulation in the $\text{BRET}_{\max}$ without affecting the receptor BRET_{50} concentration ratio, revealing the change in energy transfer efficiency E (Eq. 5).

When using a low energy transfer approximation, it should be checked that E is small ($E < 0.2$) for all receptor pairs. A general formula should otherwise be used (see below).

GENERAL BRET SATURATION CURVE

The BRET saturation curve for a general case is derived from the Veatch and Stryer model using Eq. 4 instead of Eq. 7 (Vrecl et al., 2006):

$$\frac{\text{BRET}}{\text{BRET}_{\max}} = 1 - \frac{1}{E + (1-E)\left(1 + \frac{[A]}{[D]}\right)^N}; \tag{13}$$

N represents the oligomerization state: $N = 1$ for dimer, $N = 2$ for trimer etc. **Figure 4** shows that the saturation curve is shifted to the right for higher energy transfer efficiencies E, which greatly affects interpretation of the saturation assay. In several experiments using a small E approximation, it was observed that saturation assay data lay under the theoretical saturation curve (Mercier et al., 2002; Ramsay et al., 2004; Goin and Nathanson, 2006). The shift was interpreted as a presence of a monomeric fraction in the receptor pool, although high E could be responsible for the shift.

BRET COMPETITION ASSAY

In an attempt further to confirm the existence of oligomeric complexes, a competition assay can be performed. In this assay, the concentration of untagged receptor is increased over the concentration of donor and acceptor tagged receptors (Ayoub et al., 2002; Devost and Zingg, 2004; Vrecl et al., 2006; Achour et al., 2011). The BRET signal is expected to decrease if untagged receptors compete with tagged receptors for binding in complexes. Following the

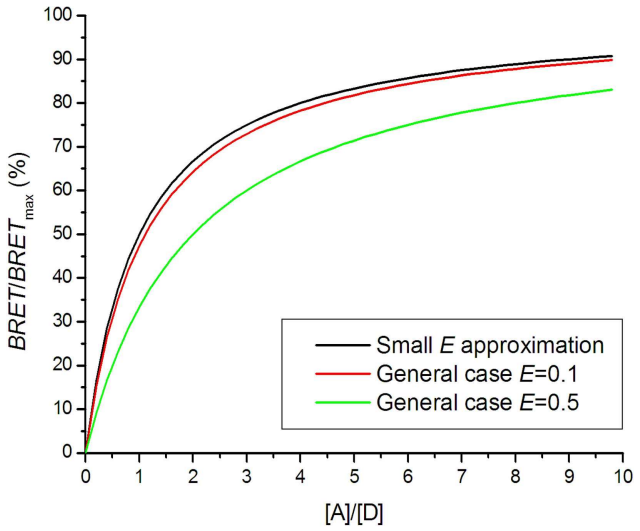


FIGURE 4 | Bioluminescence resonance energy transfer saturation curves for dimers with different energy transfer efficiencies *E*.

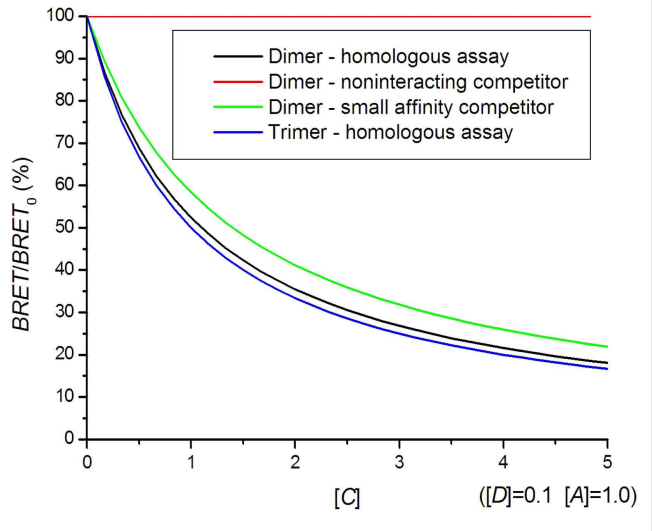


FIGURE 5 | Bioluminescence resonance energy transfer competition curves. In a homologous assay, the same kind of receptor is used as a competitor, whereas in a heterologous assay, a different receptor with a smaller affinity for hetero-dimer formation is used.

Veatch and Stryer approach (Veatch and Stryer, 1977), the BRET signal as a function of receptor concentration is obtained (Kubale et al., 2012):

$$\mathrm{BRET} = \frac{T}{Q} = \frac{E\,[\mathrm{AD}]}{2\,[\mathrm{DD}] + (1-E)\,[\mathrm{AD}] + [\mathrm{CD}]}, \tag{14}$$

where $[C]$ represents the concentration of untagged competitor. If all receptors form dimers and association constants are the same for AA, AD, DD, CD, AC, and CC dimers, the BRET competition curve for dimers is obtained:

$$\mathrm{BRET}_{\mathrm{Dimer}} = \frac{E\frac{[A]}{[D]}}{1 + (1-E)\frac{[A]}{[D]} + \frac{[C]}{[D]}}. \tag{15}$$

The competition curve for trimers is obtained in the same way:

$$\mathrm{BRET}_{\mathrm{Trimer}} = \frac{2E\frac{[A]}{[D]}}{1 + (1-2E)\frac{[A]}{[D]} + \frac{[C]}{[D]}}. \tag{16}$$

A high acceptor to donor concentration ratio is commonly used in BRET saturation experiments, since variations in this ratio do not influence the BRET signal as much as for $[A]/[D] = 1$. In general, the interaction with untagged receptors causes a reduction of the BRET signal following a hyperbolic curve (**Figure 5**). It can very well be distinguished whether oligomerization is present but the exact oligomerization state is difficult to assess, because the dimer and higher oligomer curves are too similar. A competition assay can be used to study hetero-dimer formation. The smaller affinity for hetero-dimer formation results in a shallower competition curve (**Figure 5**).

In contrast to the hyperbolic competition curve (Eq. 15), a linear dependence of BRET vs. competitor concentration has been proposed for dimers (Ayoub et al., 2002). In practice, a quantitative competition assay is less frequently performed than a saturation assay due to the need to quantify the untagged competitor concentration using biochemical methods (immuno-based methods, radioligand binding). Qualitative assays using single wild type receptor concentrations have been used on several occasions to assess the specificity of the interaction (Kroeger et al., 2001; Terrillon et al., 2003; Breit et al., 2004).

CONTROL EXPERIMENTS

A non-interacting receptor should be used for negative control, which is expressed at similar levels and occupies the same cellular compartment (Terrillon et al., 2003; Pfleger and Eidne, 2005). For positive control experiments, donor and acceptor species are fused together to express a constant BRET signal. This type of experiment is used to test the measurement system and compare data from different datasets (Pfleger and Eidne, 2006).

NEW RET-BASED TECHNIQUES FOR OLIGOMERIZATION STUDIES

In order to prove higher order oligomerization with more certainty, new RET-based approaches have been developed that combine two different techniques. A sequential-BRET-FRET (SRET) has been created that enables identification of hetero-oligomers formed by three different proteins (Carriba et al., 2008). In SRET, the oxidation of the Rluc substrate by a Rluc-fusion protein triggers excitation of the acceptor GFP^2 by BRET^2 and subsequent energy transfer to the acceptor YFP by FRET. SRET is the ratio between YFP and Rluc emissions. The experiment is conducted in the same way as a saturation assay, by measuring SRET with an increased YFP tagged receptor concentration.

Using the small E approximation and a pure trimer population, a SRET curve can be obtained in the same way as those for

saturation and competition assays:

$$\text{SRET} = \frac{2E_1E_2\,[M]\,[A]}{[D]^2 + [M]^2 + [A]^2 + 2\,[D]\,[M] + 2\,[D]\,[A] + 2\,[M]\,[A]}, \quad (17)$$

where $[D]$ is the Rluc tagged donor, $[M]$ is a GFP2 tagged "mediator" and $[A]$ is the YFP tagged acceptor. If the donor and mediator concentrations are kept constant and the acceptor concentration increased, a rise toward a transient maximum and a decay toward zero for higher acceptor concentrations should be observed. In experiments performed by Carriba et al. (2008) only the rising part of the SRET curve was observed. It can be assumed that higher acceptor concentrations, for which the decaying part of the SRET curve should be observed, were not tested.

Other creative approaches to detecting receptor hetero-dimerization/multiprotein complex formation include combinations of (i) bimolecular luminescence (BiLC) and bimolecular fluorescence (BiFC), (ii) BiFC and BRET, (iii) GPCR-Heteromer Identification Technology (GPCR-HIT), and (iv) complemented donor-acceptor resonance energy transfer (CODA-RET). BiLC and BiFC enable the detection of tetramer formation (Guo et al., 2008). Complementary fragments are used to reconstitute the functional protein when brought into close proximity. A BRET signal is thus produced only in a protein complex incorporating both Rluc8 fragments, which act as donors, and both Venus fragments, which act as acceptors. By increasing the acceptor concentration, it is possible to observe the BRET concentration dependence, similar to the standard saturation curve (Eq. 12).

Bimolecular fluorescence in combination with BRET is based on the ability to produce a fluorescent complex from non-fluorescent constituents if a protein–protein interaction occurs. Two receptors are fused at their C-termini with either N-terminal or C-terminal fragments of YFP, and receptor hetero-dimerization causes YFP reconstitution. If there is heterotrimerization, BRET can then be obtained when the cells also co-express the third receptor fused to Rluc (reviewed by Ferre and Franco, 2010). GPCR-HIT utilizes BRET and ligand-dependent recruitment of 7TMR-specific interaction partners (such as a β-arrestin, PKC, or G-protein) to enable 7TMR heteromer discovery and characterization (Mustafa and Pfleger, 2011; See et al., 2011). In this set up, only one receptor subtype is fused to Rluc and the second receptor subtype is untagged. A third protein capable of interacting specifically with one or both receptors in a ligand-dependent manner is fused to the YFP. The ligand-induced BRET signal indicates that activation of the untagged receptor or the heteromer results in recruitment of the YFP tagged protein to the heteromer. The recently developed CODA-RET method combines protein complementation with RET in order to study conformational changes in response to activation of a defined 7TMR heteromer. CODA-RET quantifies the BRET between a receptor heterodimer and a subunit of the heterotrimeric G-protein. It eliminates the contribution from homodimeric signaling and enables analysis of the effect of drugs on a defined 7TMR heterodimer (Urizar et al., 2011).

SUMMARY

Quantitative BRET-based techniques are extremely potent tools for investigation of membrane receptor interaction in live cells and in real time, provided they are correctly conducted and data critically interpreted. A dilution assay is a basic tool for distinguishing specific binding from random interaction and is used to set the receptor concentration range for other BRET assays. Relative affinities for homo-dimer and hetero-dimer formation can be investigated using BRET competition and saturation assays. The latter can also be used to determine the oligomerization state of the receptors, if the energy transfer efficiency is known and the correct mathematical model is used. In order unambiguously to show the formation of trimers and tetramers, the use of methods that combine different RET-based techniques seems to be more suitable.

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Receptor-Heteromer Investigation Technology and its application using BRET

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Receptor heteromerization has the potential to alter every facet of receptor functioning, leading to new pharmacological profiles with increased signaling diversity and regulation from that of the monomeric receptor, or indeed receptor homomer. An understanding of the molecular consequences of receptor heteromerization will provide new insights into the physiology and pathology mediated by receptors, expanding the possibilities for pharmacological discovery. Particularly advantageous approaches to investigate novel heteromer pharmacology utilize cell-based assay technologies that assess ligand-dependent functional responses specific to the receptor heteromer. Importantly, this allows for differentiation of heteromer-specific pharmacology from pharmacology associated with the co-expressed receptor monomers and homomers. The Receptor-Heteromer Investigation Technology (Receptor-HIT) successfully employs a proximity-based reporter system, such as bioluminescence resonance energy transfer (BRET), in a configuration that enables determination of such heteromer-specific pharmacology. Therefore, Receptor-HIT provides a simple, robust and versatile approach for investigating the elusive "biochemical fingerprint" of receptor heteromers.

Keywords: Receptor-HIT, GPCR-HIT, GPCR, RTK, heteromer, BRET, bioluminescence resonance energy transfer

INTRODUCTION

There are many types of membrane receptors that can be broadly classified into three families based on distinct mechanisms of signal transduction, namely G protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), and ionotropic receptors, which are channels that directly allow flux of ions upon activation. Additionally, there are intracellular receptors such as those binding steroids. It is well established that many of these receptors exist as oligomeric species consisting of two or more receptor subunits (Neubig et al., 2003). In many cases, dimerization or oligomerization is required for the formation of a functional receptor unit. These receptors are known as "homomeric receptors" if the constituents are the same and "heteromeric receptors" if the constituents differ (Ferré et al., 2009). RTKs are the archetypal homomeric and heteromeric receptors, as they require homo- or heteromerization for activation and signaling (Lemmon and Schlessinger, 2010). For GPCRs, classic examples of heteromeric receptors are the $GABA_B$ receptor ($GABA_BR1$-$GABA_BR2$; Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998) and taste receptors (T1R-T2R and T2R-T3R; Nelson et al., 2001, 2002; Li et al., 2002). In contrast, "receptor homomers" and "receptor heteromers" are macromolecular complexes that include two or more functional receptor units (identical or different, respectively) and display pharmacology that is distinct from that of their component receptors (Ferré et al., 2009). The concepts of GPCR homomerization and heteromerization have been described for 30 years (Fuxe et al., 2010), but have only recently become widely accepted. Furthermore, it is now clear that an array of receptor homomers and heteromers from all classes of membrane receptors exist (Liu et al., 2000, 2006; Maudsley et al., 2000; Lee et al., 2002; Nair and Sealfon, 2003; Olivares-Reyes et al., 2005; Watt et al., 2009). As both receptor homomers and receptor heteromers have the potential to attain a unique pharmacological profile, their existence adds another level of complexity to cell signaling systems. Of the two classes, receptor heteromers have been the major focus of research interest due to the numerous potential receptor combinations, as well as the difficulty in separating the pharmacology of a monomer from its homomer. The unique pharmacology associated with receptor heteromers has been termed its "biochemical fingerprint" (Ferré et al., 2009) and provides a mechanism for achieving greater signaling diversity and specificity. Receptor heteromers are therefore viewed as a new class of drug target, providing the opportunity for designing heteromer-specific/-biased drugs with improved selectivity and reduced side effects (Mustafa et al., 2010).

Investigating the pharmacological properties of receptor heteromers can be a particularly difficult process as the heteromer-specific pharmacology needs to be differentiated from pharmacology of associated monomers/homomers. Due to difficulties in investigating heteromers in native tissue, heterologous expression systems currently provide the major method to study heteromers. The first step is the identification of a heteromer, and subsequent characterization of its biochemical fingerprint. In time, this biochemical fingerprint will ideally be used to demonstrate the presence of the heteromer in native tissue. To achieve this end, it is critical that the initial cell-based assays employed are able to

robustly differentiate heteromer-specific pharmacology from that of the component receptors.

RECEPTOR-HETEROMER INVESTIGATION TECHNOLOGY

A novel technique recently developed to enable identification and pharmacological profiling of heteromers is the Receptor-Heteromer Investigation Technology (Receptor-HIT). This provides information on ligand-dependent functional responses specific to the heteromer. Receptor-HIT uses a proximity-based reporter system comprising four elements, three of which are: labeled Receptor A, untagged Receptor B, and a labeled interacting Protein C that is recruited to the heteromer in a ligand-dependent manner (See et al., 2011). This configuration is illustrated in **Figure 1** using bioluminescence resonance energy transfer (BRET), however, the approach can be applied using a variety of reporter systems including fluorescence resonance energy transfer (FRET), bimolecular fluorescence complementation (BiFC), bimolecular luminescence complementation (BiLC), enzyme fragment complementation (EFC), and the protease-cleaved transcription factor assay system known as Tango™ (Mustafa et al., 2010; Mustafa and Pfleger, 2011). Co-expression of the aforementioned elements in cells enables the signal between the label of choice on Receptor A and complementary label on Protein C to be monitored. The fourth element in the system is a ligand that, upon binding to untagged Receptor B or the heteromer, selectively modulates the recruitment of Protein C to Receptor B and/or the heteromer (See et al., 2011; Mustafa et al., 2012). Receptor-HIT is unsuitable for investigating homomers due to this receptor-selectivity requirement, but heteromers of closely related receptor subtypes where a selective agonist may be unavailable can still be assessed. This issue is overcome by additional use of an antagonist selective for Receptor A, thereby meaning that Receptor B

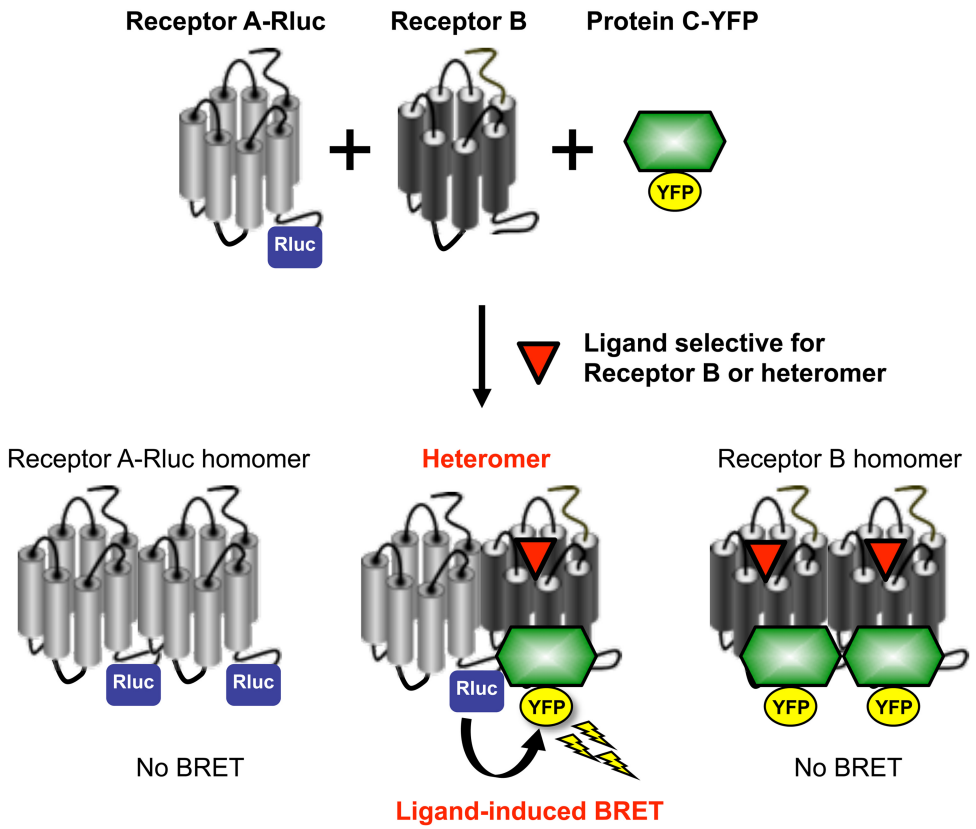

FIGURE 1 | Utilization of Receptor-HIT on the BRET platform to investigate receptor heteromerization. BRET is a biophysical phenomenon involving the non-radiative transfer of energy from a donor enzyme to an acceptor fluorophore. The donor enzyme is a variant of *Renilla* luciferase (Rluc), and energy emission results from the oxidation of its substrate coelenterazine h to coelenteramide (Pfleger and Eidne, 2006; Pfleger et al., 2006). If the acceptor fluorophore (such as yellow fluorescent protein; YFP) is correctly orientated and within 10 nm, it will be excited by the energy transferred from the donor resulting in the emission of light at a characteristic wavelength (Dacres et al., 2010). BRET is used to study protein-protein interactions through tagging one of the proteins of interest with the donor enzyme and the other protein with the acceptor fluorophore (Pfleger and Eidne, 2006). If the two proteins are in close proximity, the energy generated by the donor enzyme will be transferred to the acceptor fluorophore. The resulting BRET signal provides evidence for the two fusion proteins being in the same complex. In contrast to BRET between Rluc and YFP fused to each receptor, a specific receptor heteromer can be monitored by ligand-induced BRET due to proximity of a tagged receptor and a tagged intracellular protein used as a reporter partner (such as β-arrestin or G protein). In this system, energy transfer is measured between Receptor A-Rluc and a Protein C-YFP that interacts with the heteromer complex after selective activation of Receptor B or the heteromer itself. In addition to the induction of BRET by the Receptor B or heteromer-selective ligand providing evidence for Receptor AB heteromerization, it also identifies a biological function of the heteromer. Reprinted from Ayoub and Pfleger (2010).

and/or the heteromer are still activated selectively. Alternatively, it is possible to use a non-selective ligand if it does not modulate recruitment of Protein C to Receptor A in the absence of Receptor B (Porrello et al., 2011). Whichever approach is used, generation of a signal upon application of the ligand indicates that Protein C has been recruited to the heteromer, thereby bringing the label on Receptor A into close proximity with the label on Protein C. The signal obtained is not only indicative of the receptors being in a heteromeric complex, it also reveals an aspect of the heteromer's pharmacology through generation of ligand-dependent functional responses.

Receptor-HIT is an excellent assay for identifying and profiling heteromers as signals do not result from the homomeric or monomeric receptor populations (See et al., 2011). The ligand-dependent nature of the signal also enables screening, identification, and profiling of compounds exhibiting heteromer-specific or biased signaling (Mustafa and Pfleger, 2011; Mustafa et al., 2012).

The Receptor-HIT assay has largely been published with respect to GPCRs in the form of the GPCR-Heteromer Identification Technology (GPCR-HIT; Ayoub and Pfleger, 2010; Mustafa et al., 2010, 2012; Mustafa and Pfleger, 2011; Porrello et al., 2011; See et al., 2011), however it can also be applied to other receptors, including RTKs (Pfleger, 2011; Story et al., 2011), ionotropic receptors and steroid receptors. Consequently, there is also an extensive number of interacting partners that can be used. For example, GPCR-HIT studies can utilize G proteins or β-arrestins, whereas we have found Grb2 to be particularly amenable to Receptor-HIT assays investigating RTKs (Pfleger, 2011; Story et al., 2011).

BRET is our preferred platform for Receptor-HIT (**Figure 1**) because it can monitor protein proximity in live cells in real time at 37°C without the need for cell lysis, the assay does not rely upon proteins refolding in a complementation event to produce a readout, and no alteration of receptor function is required (Mustafa et al., 2010). The traditional configuration for studying receptor heteromers using BRET involves tagging one receptor with the Rluc enzyme, while the second receptor is tagged with the acceptor fluorophore. A particular limitation to this approach is that overcrowding of receptors in the endoplasmic reticulum or degradative compartments can lead to non-specific "bystander BRET" (Pfleger and Eidne, 2006). This is commonly addressed by employing BRET saturation assays (Mercier et al., 2002), however these are rather laborious. The ligand dependency of Receptor-HIT addresses this issue as it requires Receptor B or the heteromer to be capable of binding ligand (**Figure 1**), either because it is sufficiently mature and/or because it is appropriately localized to provide the ligand access for binding. Furthermore, although providing evidence of proximity of the two receptors, no functional information about the heteromer is revealed by saturation assays (Mustafa et al., 2012). In contrast, the use of an interacting protein also enables functional responses to be assessed, with the potential to uncover novel heteromer-specific pharmacology (Mustafa et al., 2012).

While there are advantages to using BRET as outlined above, there are also advantages to using other platforms in certain situations. For example, although EFC is not a real-time assay and requires cell lysis for signal detection, it is probably capable of achieving higher levels of screening throughput than BRET. Furthermore, assay systems like FRET and BiFC are more amenable to assessing subcellular localization if combined with confocal microscopy. However, because FRET uses a fluorophore as donor, there are issues arising from the need for external excitation. These include autofluorescence, photobleaching, cell damage, and direct acceptor excitation. Some of these issues can be addressed using time-resolved FRET (TR-FRET; Cottet et al., 2012). BiFC enables specific visualization of complemented fluorophores, and therefore the fused proteins of interest, but this is not a real-time assay due to a time delay while refolding occurs and once complemented, the proteins remain associated (Porrello et al., 2011). BRET is very sensitive to distance and relative donor–acceptor orientation. This is advantageous in terms of proximity specificity, however, it means that receptors could potentially form a heteromer without this being detected by BRET, and a lack of signal should be interpreted with caution (Pfleger et al., 2006). Other platforms may have a lower false-negative rate than BRET, however, the potential for higher false-positives may then need to be considered.

APPLICATION OF RECEPTOR-HIT USING BRET

Receptor-HIT has been used effectively on the BRET platform to investigate multiple established and novel heteromers. The CCR2-CCR5 and CCR2-CXCR4 heteromers that have been described by a number of studies (Mellado et al., 2001; Rodríguez-Frade et al., 2004; El-Asmar et al., 2005; Percherancier et al., 2005; Springael et al., 2006; Sohy et al., 2007, 2009) have recently been profiled in terms of dose–response curves, kinetics and Z' data using GPCR-HIT (See et al., 2011). Of particular note were the findings with the combination of CXCR4/Rluc8, β-arrestin2/Venus and CCR2. Treatment with CXCL12 (CXCR4 agonist) resulted in a relatively transient BRET signal that returned to baseline before 40 min, whereas addition of CCL2 (CCR2 agonist) resulted in a more prolonged BRET kinetic profile, indicative of CCR2 forming a complex with CXCR4. Intriguingly, treatment with a combination of CXCL12 and CCL2 resulted in a prolonged and substantially higher BRET signal than observed with either agonist alone. Possible explanations for this include β-arrestin2 recruitment being facilitated by both types of receptor complex being in active receptor conformations, or proximity of the donor and acceptor being sufficiently close to enable detection of changes in donor–acceptor distance and/or relative orientation. Either way, this observation provides good evidence for specific reporting of β-arrestin2 recruitment to the heteromer complex (See et al., 2011).

When generating dose–response curves with Receptor-HIT data, the Hill slope has been seen to alter for particular combinations depending upon whether the tagged or untagged receptor is activated, consistent with stabilization of distinct complex conformations with the different ligands. For example, with the CCR5/Rluc8, β-arrestin2/Venus and CCR2 combination, the dose–response curve with CCL2 was significantly steeper than with CCL4 (CCR5 agonist; See et al., 2011). As discussed previously, the reason for this difference is currently unclear, however, as the protein expression profile is identical in both cases and the only difference is the agonist treatment, this observation may help to shed light on the mechanism of GPCR heteromerization and/or allosterism across the complex in the future (See et al., 2011).

Receptor-HIT (in the form of GPCR-HIT) has also been used to investigate the heteromer between the angiotensin II (AngII) type 1 receptor (AT_1R) and the AngII type 2 receptor (AT_2R; Porrello et al., 2011). A number of studies have shown that the AT_2R does not couple to arrestins and does not internalize following treatment with AngII (Pucell et al., 1991; Hunyady et al., 1994; Turu et al., 2006). Our BRET data indicating a lack of β-arrestin2/Rluc8 recruitment to AT_2R/Venus are also consistent with these findings (Porrello et al., 2011). Therefore, upon co-expression of untagged AT_1R with β-arrestin2/Rluc8 and AT_2R/Venus, even though AngII can bind to both receptors, the ligand is still selective in terms of recruiting β-arrestin2 to only the untagged receptor. Therefore, the observation that a ligand-induced BRET signal results upon addition of AngII is indicative of AT_1R-AT_2R heteromerization (Porrello et al., 2011).

Receptor-HIT on the BRET platform has recently been used to characterize the novel heteromer between the α_{1A}-adrenoceptor (α_{1A}AR) and the CXC chemokine receptor 2 (CXCR2) that may play a role in prostate stroma (Mustafa et al., 2012). The Receptor-HIT studies showed that the heteromer recruits β-arrestin2 in a norepinephrine (NE)-dependent manner that can be blocked by both the α_1AR antagonist Terazosin and the CXCR2-specific allosteric inverse agonist SB265610 (**Figure 2**). This is despite the very weak β-arrestin2 interaction with α_{1A}AR monomers/homomers in transfected human embryonic kidney 293 cells (Stanasila et al., 2008), but consistent with the observation of α_{1A}AR recruiting β-arrestin2 in prostate stroma (Hennenberg et al., 2011). The specificity of this change in α_{1A}AR pharmacology with co-expression of CXCR2 was demonstrated by the lack of effect upon co-expression of CC chemokine receptor 2, vasopressin receptor 2 (V2R), or orexin receptor 1 (Mustafa et al., 2012).

The ligand-dependent nature of Receptor-HIT enables it to report on, albeit without differentiating between, constitutive and dynamic heteromers (Mustafa and Pfleger, 2011). The α_{1A}AR-CXCR2 complex is an example of a constitutive heteromer that exhibits novel pharmacology revealed by the ligand dependency of Receptor-HIT (Mustafa et al., 2012). Indeed, BRET

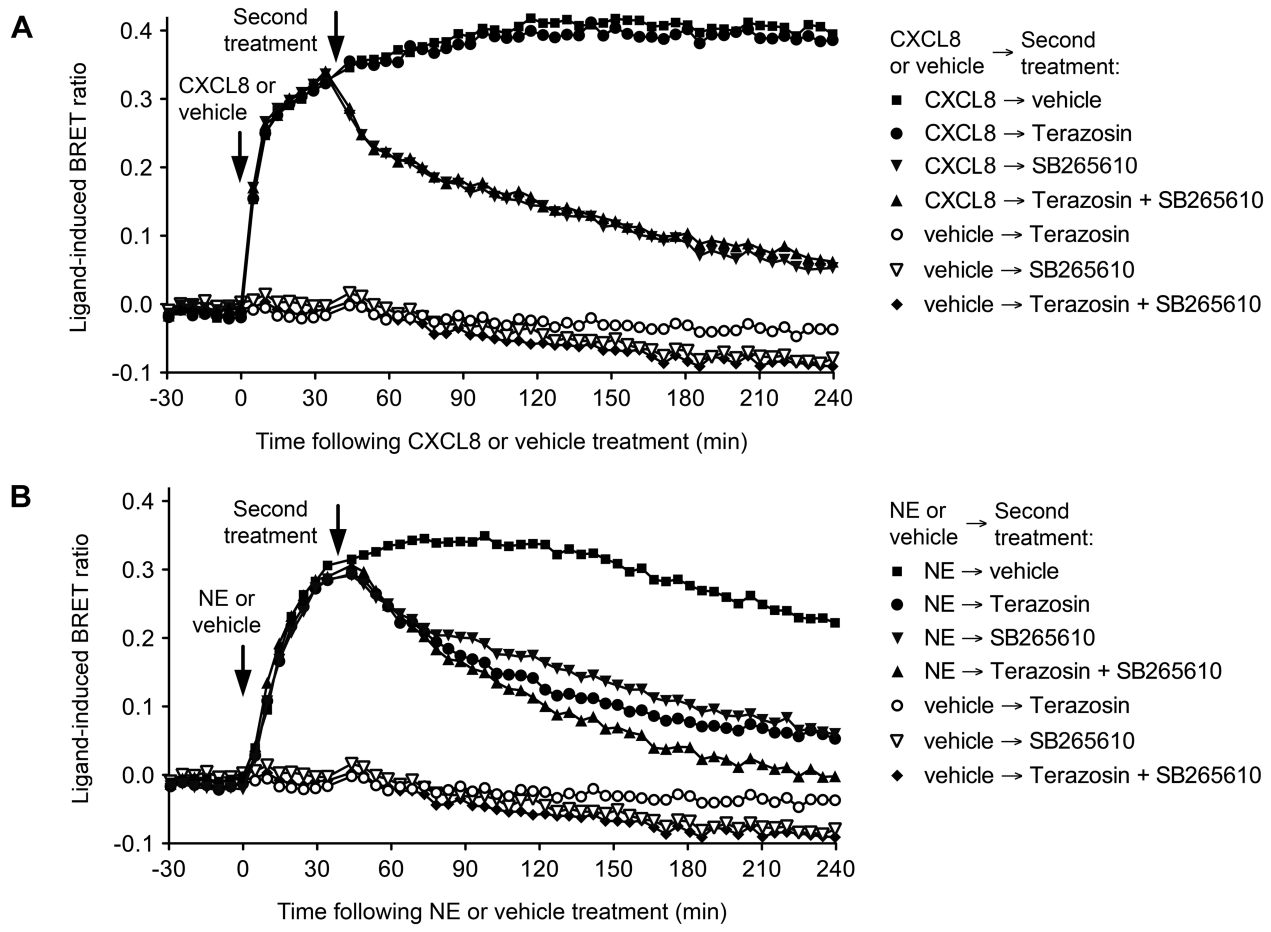

FIGURE 2 | Use of Terazosin (α_1AR antagonist) and SB265610 (CXCR2 inverse agonist) to interrogate β-arrestin2 recruitment to the α_{1A}AR-CXCR2 heteromer. Extended BRET kinetic profiles were generated for the CXCR2/Rluc8, β-arrestin2/Venus and α_{1A}AR combination in HEK293FT cells by treating with CXCL8 or vehicle **(A)** or NE or vehicle **(B)** ~30 min before a second treatment with vehicle, 10 μM Terazosin, and/or 10 μM SB265610. Data are representative of three independent experiments. This research was originally published in Mustafa et al. (2012). Copyright © 2012 the American Society for Biochemistry and Molecular Biology.

saturation assays indicated strong specific BRET signals between α_{1A}AR/Rluc8 and both CXCR2/Venus and V2R/Venus, however, the functional change in α_{1A}AR pharmacology revealed by GPCR-HIT was only observed with CXCR2 and not with V2R. This indicates that proximity between GPCRs does not necessarily result in a functional effect of one receptor on another, and further demonstrates the ability of Receptor-HIT to unmask specific heteromer functionality (Mustafa et al., 2012).

Receptor-HIT is also able to investigate the functionality of apparent dynamic receptor interactions, even though it is unable to determine the dynamics *per se*. This is seen for the heteromer between the glucagon-like peptide-1 receptor (GLP-1R) and the gastric inhibitory polypeptide receptor (GIPR; Schelshorn et al., 2012). Using the combination of GLP-1R-Rluc8 and YPet-β-arrestin2 in the absence and presence of GIPR, dose–response data were generated indicating that expression of GIPR partially inhibited GLP-1-induced recruitment of YPet-β-arrestin2 proximal to GLP-1R-Rluc8. This inhibition was overcome by co-treatment with GIP (Schelshorn et al., 2012). The authors of this study suggested a model to explain their GPCR-HIT data, whereby formation of the heteromer occurs as a consequence of low affinity binding of GLP-1 to the GIPR in addition to the GLP-1R. The heteromer is proposed to recruit β-arrestin2 less well in comparison with the GLP-1R monomer/homomer. Co-treatment with GIP is therefore suggested to compete off the GLP-1 from GIPR, resulting in the heteromer being dissolved and allowing improved GLP-1-induced recruitment of β-arrestin2 to GLP-1R (Schelshorn et al., 2012).

Receptor heteromers are complexes with unique pharmacology that are likely to be expressed in a distinct tissue-specific manner. This makes them exciting new prospects as drug targets, with the goal of developing drugs with improved selectivity and reduced side effects. Indeed, the concept of biased signaling is now well established and applies as readily to receptor heteromers as it does to monomers/homomers (Mustafa et al., 2010). Therefore, heteromerization provides enormous opportunities for identifying ligands with heteromer-selective and/or heteromer-biased pharmacology, such as that observed with Labetalol acting at the α_{1A}AR-CXCR2 heteromer (Mustafa et al., 2012).

Screening is an essential step in the identification of lead compounds, and consequently there is a need to develop heteromer assays that are compatible with this process. An assay's suitability for screening can be gauged by its Z' value; Z' values >0.5 indicate assays that are highly suitable (Zhang et al., 1999). The potential of Receptor-HIT as a screening assay has been demonstrated with the CCR2-CCR5 and CCR2-CXCR4 chemokine receptor heteromers for which a Z' value of 0.68 was generated with both combinations (See et al., 2011). The Z' value for the α_{1A}AR-CXCR2 heteromer (Mustafa et al., 2012) was 0.87 (See and Pfleger, unpublished observations). Unlike profiling, screening with Receptor-HIT is a two-step process. Using BRET for example, compounds are firstly screened with the Receptor-HIT configuration of Receptor A-Rluc, Receptor B and Protein C-YFP (**Figure 1**). This may generate hits for ligands that bind to Receptor A, Receptor B (when in a heteromer) or the heteromer specifically. These hits are then rescreened in parallel with the configurations Receptor A-Rluc with Protein C-YFP, and Receptor B-Rluc with Protein C-YFP. This will then enable differentiation between ligands that bind to Receptor A directly, ligands that bind to Receptor B directly, and those that are heteromer-selective (revealed by a lack of signal in the latter two assay configurations). Furthermore, the generation of dose-response curves for the different configurations can enable shifts in potency as well as efficacy to be evaluated. Comparison of different signaling pathways can also reveal compounds exhibiting biased signaling as a consequence of heteromerization (Mustafa et al., 2012).

Finally, the amenability of Receptor-HIT for identifying heteromers or profiling/screening compounds is dependent upon the existence of a suitable "Protein C" for a particular receptor-receptor combination (**Figure 1**). For example, β-arrestin2 is a particularly good interacting partner for most GPCRs, and when it is not, G protein can often be utilized instead. It is also important not to make assumptions in terms of Protein C selection, as heteromerization can change the pharmacological profile in a manner that changes interactions with Protein C. This is illustrated by the findings with the α_{1A}AR and the distinct β-arrestin2 recruitment profile when forming the α_{1A}AR-CXCR2 heteromer (Mustafa et al., 2012).

CONCLUDING REMARKS

The formation of receptor complexes has a significant impact on cellular signaling. Receptor heteromers are of particular interest due to the unique biochemical profile they attain through heteromerization. Characterization of a heteromer's biochemical fingerprint in heterologous expression systems is the first step to identifying the function of the heteromer in native tissue. Receptor-HIT is a novel cell-based approach for identifying and profiling heteromers that provides information on ligand-induced functional responses specific to the heteromer. The approach is highly versatile, allowing for simple and yet robust heteromer characterization. Utilizing a platform such as BRET, Receptor-HIT is a powerful tool that enables a deeper understanding of the molecular and physiological relevance of heteromers to be revealed.

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BRET and time-resolved FRET strategy to study GPCR oligomerization: from cell lines toward native tissues

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The concept of oligomerization of G protein-coupled receptor (GPCR) opens new perspectives regarding physiological function regulation. The capacity of one GPCR to modify its binding and coupling properties by interacting with a second one can be at the origin of regulations unsuspected two decades ago. Although the concept is interesting, its validation at a physiological level is challenging and probably explains why receptor oligomerization is still controversial. Demonstrating direct interactions between two proteins is not trivial since few techniques present a spatial resolution allowing this precision. Resonance energy transfer (RET) strategies are actually the most convenient ones. During the last two decades, bioluminescent resonance energy transfer and time-resolved fluorescence resonance energy transfer (TR-FRET) have been widely used since they exhibit high signal-to-noise ratio. Most of the experiments based on GPCR labeling have been performed in cell lines and it has been shown that all GPCRs have the propensity to form homo- or hetero-oligomers. However, whether these data can be extrapolated to GPCRs expressed in native tissues and explain receptor functioning in real life, remains an open question. Native tissues impose different constraints since GPCR sequences cannot be modified. Recently, a fluorescent ligand-based GPCR labeling strategy combined to a TR-FRET approach has been successfully used to prove the existence of GPCR oligomerization in native tissues. Although the RET-based strategies are generally quite simple to implement, precautions have to be taken before concluding to the absence or the existence of specific interactions between receptors. For example, one should exclude the possibility of collision of receptors diffusing throughout the membrane leading to a specific FRET signal. The advantages and the limits of different approaches will be reviewed and the consequent perspectives discussed.

Keywords: G protein-coupled receptor, fluorescence, FRET, time-resolved FRET, BRET, fluorescent ligand, oligomer

INTRODUCTION

The analysis of the molecular mechanisms underlying cellular processes reveals the existence of very complicated molecular networks. Each of them is likely to constitute a platform to integrate information. Membrane proteins such as G protein-coupled receptors (GPCRs) are probably one of the first molecular integrators upon cell stimulation. They lead to the activation of one or various signaling pathways depending on the binding of full or biased agonists. Indeed, GPCRs interact with G proteins and/or proteins such as β-arrestins. Their integrating capacities are even larger than expected since, during the last two decades, GPCRs like other membrane proteins such as tyrosine kinase receptors or ionic channels, have been shown to have the propensity to oligomerize (Salahpour et al., 2000; Terrillon and Bouvier, 2004; Milligan, 2010; Lohse et al., 2012).

The emergence of the GPCR oligomerization concept is challenging at different levels and consequently remains a controversial issue. The first difficulty regards molecular and mechanistic aspects. The ability of one GPCR to interact with identical or different GPCRs to form respectively homomers or heteromers opens fascinating perspectives in terms of receptor functioning. However, as experiments have been performed on numerous receptors models, no unifying mechanism regarding size of the oligomers, their stability for example, seems to exist.

The second level regards physiology: the concept has essentially been studied on receptors expressed in heterologous expression systems and various parameters (level of expression, expression of chimeric receptors, and localization of receptors) can deeply impact receptor oligomerization. Whether the data can be extrapolated to physiological context is crucial. Moreover the exact role of GPCR oligomers is far from being well understood and the

Abbreviations: BRET, bioluminescent resonance energy transfer; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; RET, resonance energy transfer; SLP, self-labeling protein; TR-FRET, time-resolved fluorescence resonance energy transfer; YFP, yellow fluorescent protein.

impact of GPCR oligomerization remains to be established in (patho-)physiology.

Various experimental strategies have been used to study GPCR oligomers: binding experiment, biochemical, or biophysical approaches. Among them, those based on resonance energy transfer (RET) are probably the ones which exhibit the best resolution to conclude to direct receptor interactions. Variants of energy transfer strategies have been developed to explore specific aspects of GPCR oligomerization. In a first part, major findings regarding GPCR oligomerization are briefly reviewed and in the following sections, characteristics, advantages and drawbacks of the various RET approaches have been examined.

GPCRs FORM OLIGOMERS

As mentioned above, the concept of GPCR oligomerization emerged two decades ago. The demonstration of the direct specific interaction between two proteins is far from being trivial. Various pharmacological- and biochemical-based data have been brought to support the concept of GPCR oligomerization. First, complex curve profiles illustrating ligand binding on GPCRs have been reported for various receptors and have been interpreted as evidence for the existence of GPCR complexes (Mattera et al., 1985; Wreggett and Wells, 1995; Armstrong and Strange, 2001; Rios et al., 2001; Albizu et al., 2006; Springael et al., 2006; Birdsall, 2010), although other hypotheses can also be considered (Chabre et al., 2009). Second, functional trans-complementations upon chimeric or mutated receptor co-expression have been reported for adrenergic α2 and muscarinic M3 (Maggio et al., 1993), angiotensin 2 AT1 (Monnot et al., 1996), and histamine H1 (Bakker et al., 2004) receptors. Third, co-immunoprecipitation experiments illustrating the existence of GPCR complexes have been reported for many receptors, for example for β2 adrenergic (Hebert et al., 1996), δ opioid (Cvejic and Devi, 1997), and metabotropic glutamate mGlur5 (Romano et al., 1996) receptors. Regarding co-immunoprecipitation data, it has been mentioned that they are dependent on the experimental conditions used, and notably on the nature of the detergent and that the functional unit is the monomer (Chabre and le Maire, 2005). Finally, disruptions of GPCR complexes by sequences corresponding to VI and/or VII transmembrane domains (Hebert et al., 1996; Ng et al., 1996) have been reported. All these data, although not definitive, support the idea that GPCRs can form complexes. However, they cannot prove the existence of direct interaction between receptors. Moreover they raise questions regarding the size, the stability, the regulation, and of course the role of such complexes. Biophysical strategies and more specifically RET approaches have been developed to investigate these questions.

SIZE OF GPCR COMPLEXES

Size of complexes is variable depending on the GPCR model. Metabotropic glutamate receptors (mGluRs) form dimers, covalent disulfide bridges connecting the extracellular domains of two receptors (Pin et al., 2003) and no higher order oligomers were found (Doumazane et al., 2011). By contrast, $GABA_B$ receptors have the propensity to form heterotetramers (Maurel et al., 2008; Comps-Agrar et al., 2011a). Regarding receptors of class A, large complexes are been considered for rhodopsin (Fotiadis et al., 2003; Fotiadis et al., 2006), and α1b receptor (Lopez-Gimenez et al., 2007); receptors tetramers have been reported for dopamine receptors (Guo et al., 2008) and monomers for neurokinin 1 receptor (Meyer et al., 2006). It is noteworthy that experimental conditions and more specifically receptor density, have been suspected to impact receptor oligomerization (Chabre et al., 2009).

STABILITY OF OLIGOMERS

First only considered as monomer and then as stable oligomers, GPCR monomers and higher order oligomers have now been reported to co-exist. For example, co-existence of monomers and dimers and equilibrium between these two forms has been described for M1 muscarinic (Hern et al., 2010) and *N*-formyl peptide (Kasai et al., 2011) receptors. Transient interactions between β1 receptors have also been described, while stable interactions were observed between β2 receptors (Dorsch et al., 2009). Whether this equilibrium is regulated by ligand binding remains an open question since, depending on the receptor considered, contradictory results were reported: for example, muscarinic M1 receptors dimerize upon antagonist binding (Ilien et al., 2009) or is insensitive to antagonists (Goin and Nathanson, 2006); somatostatin receptor 2 dimer dissociates upon agonists binding (Grant et al., 2004) and absence of ligand effect has been reported on numerous GPCR models (for example, Albizu et al., 2010).

ROLE OF OLIGOMERS

The role of receptor oligomerization is probably the most crucial question but is nonetheless quite poorly characterized. For few receptors, GPCRs oligomerization has been described as essential for receptor trafficking. It has been well illustrated for the $GABA_B$ receptor for which the targeting of $GABA_{B1}$ subunit is possible only if it interacts with $GABA_{B2}$ subunit (Pagano et al., 2001). Homodimerization of β2 adrenergic receptor has also been reported to play a major role in exporting receptor from endoplasmic reticulum to the cell surface (Salahpour et al., 2004). Moreover the selective activation of V1a or V2 receptors when engaged in an heterodimer determines the internalization pattern of the receptor (Terrillon et al., 2004).

Oligomerization also plays key roles in receptor activation. Once again this has been well illustrated by the $GABA_B$ obligatory heterodimer model. The $GABA_{B1}$ subunit is responsible for orthosteric ligand binding whereas the $GABA_{B2}$ subunit activates G proteins (Kaupmann et al., 1998; Galvez et al., 1999, 2000; Duthey et al., 2002). The generalization of such a crosstalk to other heteromer models (oligomers of different kinds of receptors) remains to be established. It is noteworthy that heterodimerization can impact the pharmacological profile of both receptor. This has been reported for example for opioid receptors (Jordan and Devi, 1999). In the case of homomers (oligomers of identical receptors), their role is not as clear. A few hypothesis and models have shown the possibilities offered by such complexes (Durroux, 2005; Han et al., 2009; Rovira et al., 2010). However, their relevance *in vivo* still remains to be established as homomer formation could impair GPCR function (White et al., 2007; Arcemisbéhère et al., 2010; Comps-Agrar et al., 2011a), although the opposite has also been established (Pellissier et al., 2011). Identifying oligomeric

complexes and understanding how oligomerization can modify receptor signaling is crucial in pharmacology and drug discovery as it can provide unique targets and new ways to specifically address pathologies (Fribourg et al., 2011).

EXISTENCE OF OLIGOMERS IN NATIVE TISSUES

Most of the experiments regarding receptor oligomerization have been performed on receptor expressed in cell line and whether the results can be extrapolated to receptors *in vivo* remains to be established. Oligomerization of mGluRs and $GABA_B$ receptor has been widely accepted. Regarding class A receptors, oxytocin receptor oligomer has been reported in mammary gland in lactating rate (Albizu et al., 2010). Functional trans-complementation of mutant receptors in the absence of functional wild-type receptors in mice (Rivero-Müller et al., 2010; Vassart, 2010) strongly suggests LH receptor oligomerization *in vivo*. Hetero-oligomerization *in vivo* has also been suspected for various GPCR pairs although direct interactions between receptors were not formally demonstrated (González-Maeso et al., 2008; Albizu et al., 2011).

PRINCIPLE OF RESONANCE ENERGY TRANSFER

In the 1990s, the most popular experimental approaches to demonstrate receptor oligomerization were Western blot and co-immunoprecipitation assays, although false positive interactions can sometimes be observed. These techniques have proved the participation of both proteins to the same complex but not a direct interaction between two receptors.

Only a very few experimental approaches offer a spatial resolution high enough to conclude to a real interaction. Experiments based on RET principle are probably the most adapted to demonstrate a proximity between two proteins. Indeed, RET, formalized by Theodor Förster in the middle of the 20th century, consists in a non-radiative energy transfer occurring between two partners, one being considered as the donor the other as the acceptor (Förster, 1948), which have to fulfill three conditions. First, donor and acceptor should present energy compatibility, i.e., donor emission spectrum and acceptor excitation spectrum should overlap. Second, the donor and the acceptor should present compatible orientation; the transfer is maximal when the donor and acceptor transition dipole moments are parallel and minimum (equal to 0) when they are perpendicular. Finally, energy transfer can take place only if the two partners are in proximity. The efficiency of the transfer is inversely proportional to the sixth power of the distance.

$$E = \frac{{R_0}^6}{{R_0}^6 + r^6}$$

where R_0 is the distance corresponding to 50% energy transfer efficiency. Although R_0 depends on the spectral compatibility of the two species and their alignment, it is generally in the range of 30–60 Å. Therefore, because of the spatial resolution offered by RET strategies, RET signals are often interpreted as resulting from direct interactions between partners. Of note, other techniques such as classic microscopy approach and even high-resolution microscopy do not exhibit such high resolutions; they are usually greater than 250 and 30 nm, respectively, and therefore can only provide evidence of receptor co-localization.

Developing an efficient RET-based assays requires to focus on various aspects. First, obtaining a high signal-to-noise ratio is crucial. Different factors can impact this ratio: (i) the overlap of the excitation and emission spectra of the donor and the acceptor. This results in the need to resort to indirect measures of the actual RET, for example, by correcting the measured signal of possible bleed-through and fluorescence contamination. It requires various mathematical operations (Zheng et al., 2002), resulting in a significant decrease of the signal-to-noise ratio; (ii) autofluorescence of the medium and/or the biological preparation and light scattering by cells or membrane preparation often deeply impact the signal-to-noise ratio.

The second aspect regards the labeling of the protein of interest. Initially, experiments were often performed on purified proteins and labeling was achieved via chemical approaches. Performing similar experiments in a cellular context required novel labeling strategies. This has often been carried out by molecular engineering strategies, i.e., by fusing fluorescent proteins to the protein of interest. Various mutants of the natural green fluorescent protein (GFP) or other fluorescent proteins have been engineered and exhibit fluorescence at various wavelengths.

As a solution to these issues, two major strategies have been developed in the last decade: bioluminescent resonance energy transfer (BRET; **Figure 1A**) and time-resolved fluorescence resonance energy transfer (TR-FRET; **Figure 1B**). Interestingly, the use of these two techniques goes beyond the strict GPCR dimerization framework and many aspects of the GPCR life cycle can be analyzed with these approaches.

BRET STRATEGY

Briefly, BRET is based on the use of a bioluminescent protein, commonly luciferase from *Renilla reniformis* (Rluc), as donor. Therefore, RET occurs without light excitation of the sample leading to a very low background signal, the excitation being chemically triggered (**Figure 1A**). BRET has been optimized along the last two decades and its different implementations (**Figure 1A**) have been recently reviewed (Ayoub and Pfleger, 2010). Indeed, Coelenterazine h was first used as substrate of Rluc and yellow fluorescent protein (YFP) as acceptor. Because of the overlap of the donor and acceptor spectra, a second version of BRET (BRET2) has been developed with Coelenterazine 400a (also known as DeepblueC) as substrate for Rluc and GFP as acceptor and displays a better spectral resolution. However, it also exhibits rapid decay kinetics of the substrate and a weak sensitivity because of a low quantum yield when using Rluc (Hamdan et al., 2005; Pfleger et al., 2006). More recently, eight mutations were introduced in the native Rluc to give Rluc8 which shows a fourfold increase in light output (Loening et al., 2006). It can be used in combination either with GFP2 (with Coelenterazine 400a as substrate) or with YFP or a mutant red fluorescent protein (mOrange; Bacart et al., 2008; De et al., 2009; with Coelenterazine as substrate), offering various possibilities to perform BRET with the same donor.

The development of BRET strategy, widely used to characterize receptor interactions, has played a major role in the evolution of the GPCR oligomerization concept (Achour et al., 2011). Interestingly, BRET has also been convenient to show that some receptors

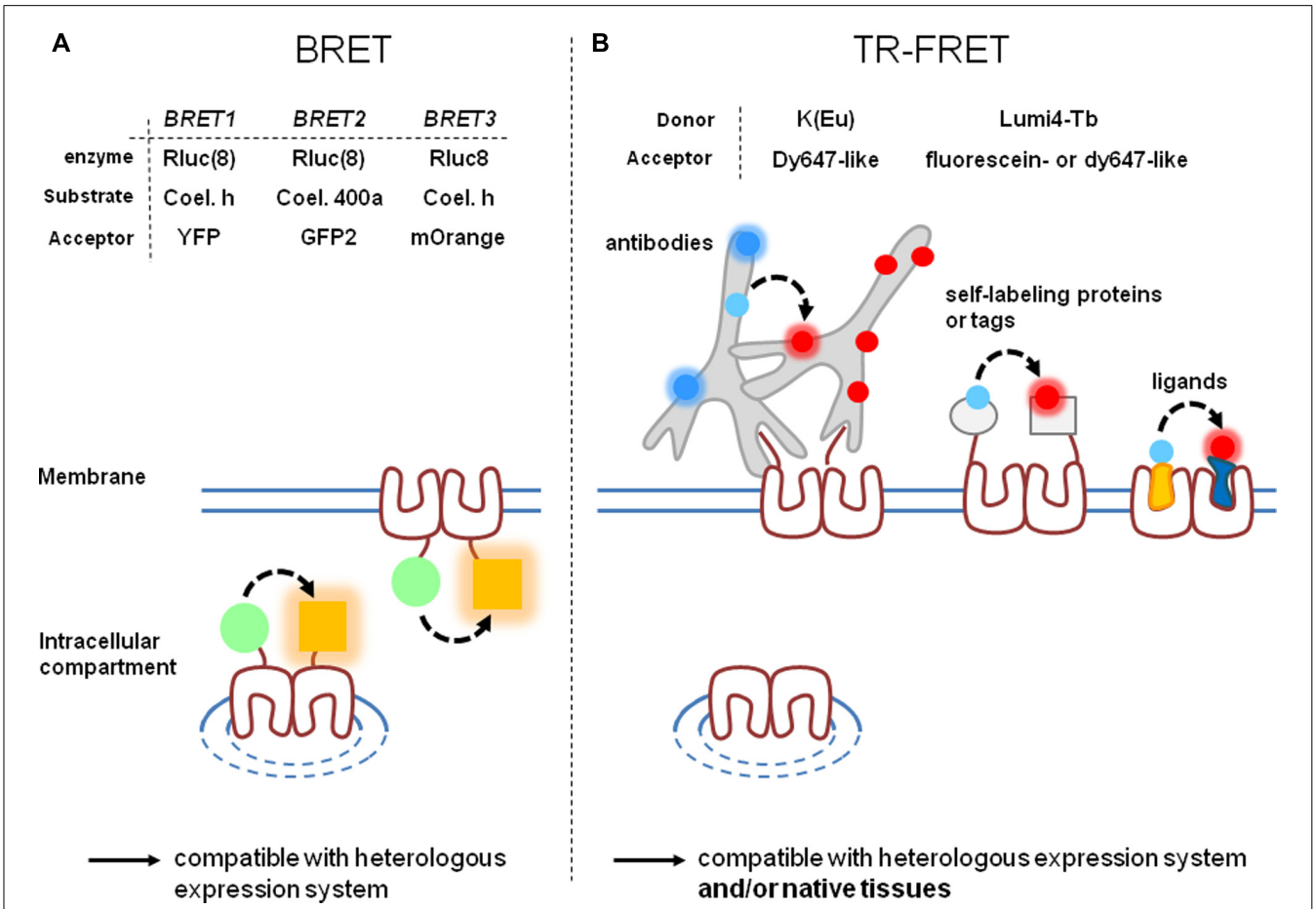

FIGURE 1 | Comparison of BRET and time-resolved FRET approaches. BRET **(A)** and TR-FRET **(B)** techniques are actually the most widely used RET techniques since they offer high signal-to-noise ratio. However, they present different characteristics: labeling strategies are often simpler with BRET than with TR-FRET. **(A)** Three variants of BRET depending on the substrate/enzyme complex donor (green circle) and on the acceptor (orange square) have been developed. BRET gives the opportunity to label intracellular or cell-surface targeted receptors. Variants of TR-FRET depending on the fluorescent carrier have been developed. **(B)** TR-FRET opens the possibility to discriminate receptor targeted to the cell surface from those trapped in intracellular compartments. TR-FRET is also more adaptable to different cellular contexts and is the only one to be compatible with receptors expressed in a native context. [Rluc(8), Rluc or Rluc8; Coel. h, Coelenterazine h; Coel. 400a, Coelenterazine 400a (also known as DeepblueC)].

such as vasopressin and oxytocin receptors (Terrillon et al., 2003) assemble in oligomers early during their synthesis in the endoplasmic reticulum. Additionally, a combination of bioluminescence and fluorescence complementation and RET strategies have been used to demonstrate that at least four dopamine D2 receptors are located in close molecular proximity in living mammalian cells, consistent with D2 receptor tetramerization (Guo et al., 2008).

On a different note, BRET assays have also been developed to detect various signaling pathway activations. These assays are based on the occurrence of protein interactions consecutive to receptor activation such as G protein or β-arrestin recruitment. Moreover, as mentioned above, the different mutants of Rluc can be associated to various acceptors allowing multiplexing of multicolor BRET. This opens the path for concomitant monitoring of various independent biological processes in living cells (Breton et al., 2010). Lastly, BRET methods present the advantage of being compatible with kinetics measurements since signals can be recorded for up 30 min.

Despite good signal-to-noise ratio and the simplicity to label receptors (Rluc or fluorescent proteins are generally fused to receptor C-terminus), BRET strategies suffer of at least two main drawbacks. First, BRET signals do not discriminate between receptors targeted to the cell surface from those retained inside the cell (**Figure 1A**). Therefore, the BRET signal reflects the behavior of all mature (targeted to the cell surface or internalized) and non-mature receptors. Second, all BRET experiments are based on chimeric receptors expressed in heterologous expression systems. Receptor over-expression and mis-targeting can potentially impact the relevance of the results, especially when BRET is used to prove receptor heterodimerization. Therefore, BRET is not adapted to study receptors expressed in their native context except by using knock-in strategies.

TIME-RESOLVED FRET STRATEGY

Time-resolved FRET is another relevant RET method to study GPCR oligomerization. It is based on receptors labeled with

lanthanides and more specifically with terbium and europium. Lanthanides exhibit long-lasting light emission because of electronic dipole transitions that are formally forbidden. Therefore this photoluminescence is strictly speaking not fluorescence nor phosphorescence since it does not involve singlet-to-singlet or triplet-to-singlet transition (Selvin, 2002). For this reason it should be called lanthanide resonance energy transfer. However, the variations of RET signal in function of the distance between donor and acceptor with lanthanides is similar to those with classic fluorophores, the reason why it has been assimilated to FRET.

Two types of cages have been developed to complex lanthanides and enable the labeling of the receptor of interest: (i) chelates display high affinity for europium and terbium ions but the complexation is reversible and can be impacted by the presence of other ions such as Mn^{2+}, Mg^{2+}, or Ca^{2+}; (ii) cryptates, by contrast, offer a greater stability since terbium and europium cannot be released after complexation. An example of structure of cryptate, Terbium cryptate (Lumi4-Tb), is illustrated in **Figure 2A**. Importantly, chelates and cryptates are not just lanthanide carriers but play two other roles. First, they influence the lanthanide fluorescence properties. Indeed they play the role of an antenna since they absorb light and transfer the energy to the lanthanide. This is essential since lanthanides exhibit very weak absorbance (10^4-fold lower than a classic fluorophore; Selvin, 2002). Moreover the nature of this cage can also impact the emission spectra of the complex. Second, the cage protects lanthanides from quenching by water molecules (Selvin, 2002).

TIME-RESOLVED FRET EXHIBIT HIGH SIGNAL-TO-NOISE RATIO

The high signal-to-noise ratio provided by TR-FRET strategy is due to various parameters (Mathis, 1995; Bazin et al., 2002).

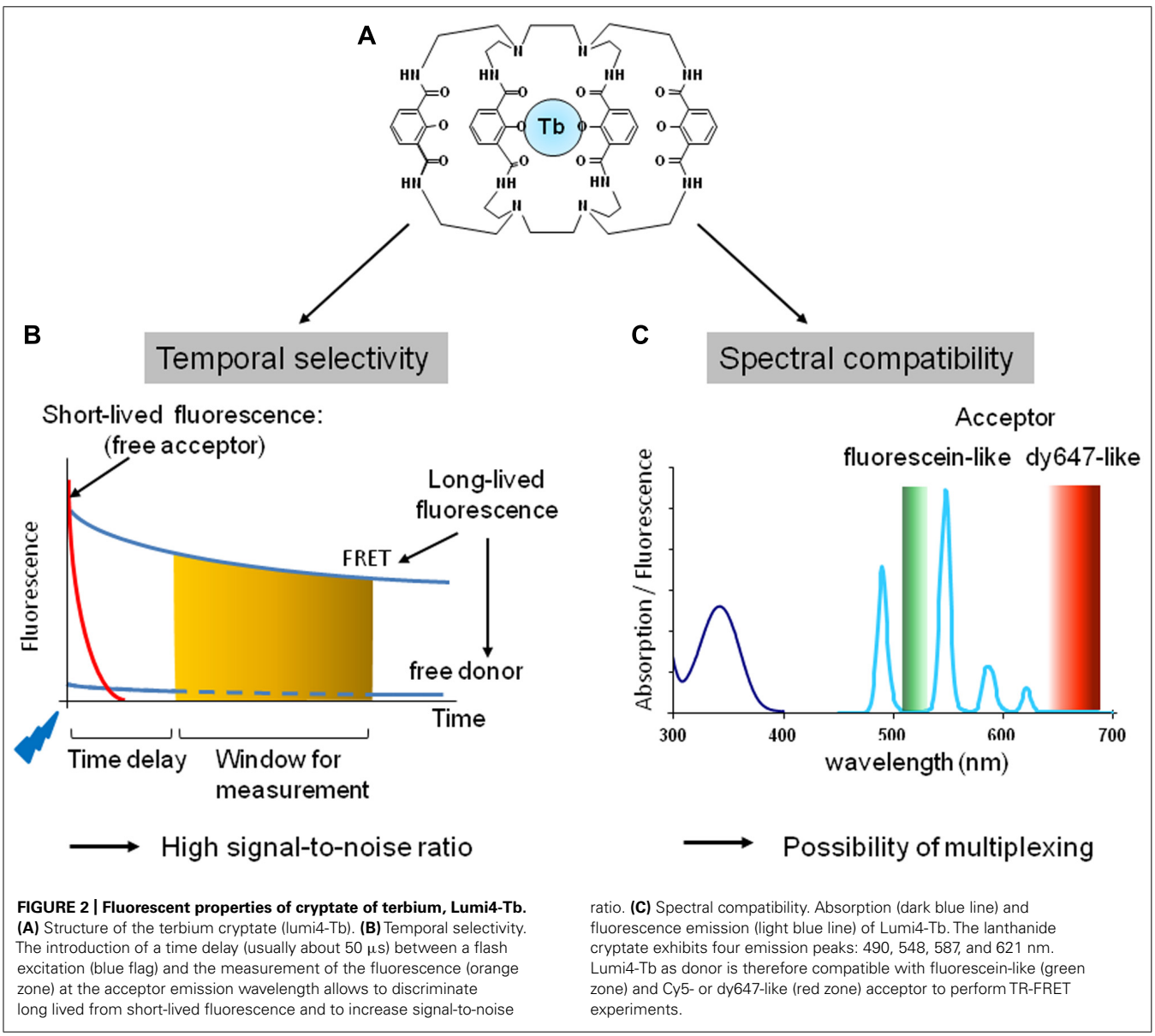

FIGURE 2 | Fluorescent properties of cryptate of terbium, Lumi4-Tb. **(A)** Structure of the terbium cryptate (lumi4-Tb). **(B)** Temporal selectivity. The introduction of a time delay (usually about 50 μs) between a flash excitation (blue flag) and the measurement of the fluorescence (orange zone) at the acceptor emission wavelength allows to discriminate long lived from short-lived fluorescence and to increase signal-to-noise ratio. **(C)** Spectral compatibility. Absorption (dark blue line) and fluorescence emission (light blue line) of Lumi4-Tb. The lanthanide cryptate exhibits four emission peaks: 490, 548, 587, and 621 nm. Lumi4-Tb as donor is therefore compatible with fluorescein-like (green zone) and Cy5- or dy647-like (red zone) acceptor to perform TR-FRET experiments.

Temporal selectivity

Upon excitation, lanthanide fluorescence half time is in the range of 1 ms while it is in the range of few nanoseconds for classic fluorophores. TR-FRET takes advantage of this property: the introduction of a time delay (typically around 50 μs) between the excitation and the fluorescence signal detection allows discriminating between short-lived and longer-lasting fluorescence. Therefore all short-lived fluorescence provided by the medium, the biological preparation or the direct excitation of the acceptor will be eliminated by the time delay. Only the long-lived fluorescence resulting from the donor or the acceptor engaged in a FRET process will be measured after the time delay (**Figure 2B**).

Spectral compatibility

Both europium and terbium cryptates are excited at 300–350 nm. They both exhibit an important Stoke shift and complex emission spectra with multiple fluorescent peaks. For example, europium cryptate trisbipyridine [TBP(Eu)] exhibits four major fluorescent peaks at 585, 605, 620, and 700 nm, while the europium pyridine bisbipyridine (Eu-PBP) has two major peaks at 595 and 615 nm, and two minor peaks at 680 and 705 nm. Terbium cryptate (Lumi4-Tb; Xu et al., 2011) also displays four emission peaks around 490, 550, 585, and 620 nm (**Figure 2C**). This makes europium and terbium cryptates compatible with deep red Cy5- or dy647-like fluorophores to perform FRET. Moreover because of the emission peak around 490 nm, terbium-cryptate is also compatible with fluorescein-like fluorophore as acceptor. By contrast to FRET or BRET strategies based on CFP/YFP or Luciferase/YFP pairs, respectively, europium and terbium cryptate fluorescence are particularly low at the acceptor emission wavelength leading to a reduced bleed through and thus a high signal-to-noise ratio.

Orientation dependence

By contrast to BRET or FRET performed with classic fluorophores, the dependence of TR-FRET to the relative orientation of the fluorophore is very weak because the lanthanide emission is not polarized. The relative orientation of the acceptor cannot impact the R_0 more than 12% due to the random orientation of the lanthanide cryptate donor (Selvin, 2002).

LABELING OF PROTEIN OF THE INTEREST

A second aspect to consider is the method used to label receptors of interest. Depending on the method, labeling can be complete or not, covalent or not, compatible with homogeneous condition or not, bulky or not. All these parameters can have a direct impact on the efficiency of RET and on the detected signal-to-noise ratio (**Figure 1B**).

Non-covalent labeling of chimeric receptor with fluorescent antibodies

Early TR-FRET-based strategies consisted in incubating cells expressing receptors of interest with primary fluorescent antibodies conjugated either to lanthanide cryptates or to classic fluorophores (as donors and acceptors, respectively). Specific antibodies for GPCRs with high affinity are difficult to obtain, so antibodies against epitotes such as hemagglutinine, FLAG, 6-Histine, or cMyc, fused to the N-terminus of the receptor have generally been used.

This method has been successfully used to monitor δ-opioid homomers using cMyc- and FLAG-tagged receptors (McVey et al., 2001) indicating the presence of the complex at the cell surface. By contrast, no cMyc-δ-opioid receptor/FLAG-β2-adrenoreceptor-GFP heteromer can be detected using the same approach, despite the presence of the receptors at the cell surface. However, co-expression of δ-opioid receptor-eYFP and β2-adrenoreceptor-*Renilla* luciferase construct resulted in a small BRET signal upon addition of Coelenterazine. This result has been interpreted as the existence of intracellular heteromer complex which are not targeting to the cell surface, illustrating the importance of discriminating cell surface targeted complexes from those retained inside cells.

Since this study, similar results have showed dimerization for numerous receptors targeted at the cell surface: α1A and α1B-adrenergic (Carrillo et al., 2004; Ramsay et al., 2004), CXCR1 and CXCR2 (Wilson et al., 2005), histamine H1 and H4 (Bakker et al., 2004; van Rijn et al., 2006), vasopressin V1a and V1b (Albizu et al., 2006; Orcel et al., 2009), and various types of mGluRs (Kniazeff et al., 2004; Goudet et al., 2005; Hlavackova et al., 2005; Rondard et al., 2006; Brock et al., 2007).

It is noteworthy that two protocols can be used to analyze receptor homodimerization. On the one hand, identical receptors may be fused to two different tags to label each with a specific antibody conjugated either with the donor or the acceptor. The relative expression of one receptor to the other has to be optimized. On the other hand, the receptors may be fused to a single tag and then labeled statistically with a mix of antibodies conjugated either to the donor or the acceptor. In this last condition one should determine the labeling kinetics and concentration to use for each antibody to get a balanced labeling.

Using this antibody-based approach on differentially tagged receptors, several studies have validated the existence of heteromeric complexes, including the $GABA_{B1}$–$GABA_{B2}$ (Maurel et al., 2004), alpha2A–adenosine A1 (Ciruela et al., 2006), and CXCR1–CXCR2 (Wilson et al., 2005) heteromers.

Advantages and drawbacks. The antibody strategy to label receptors presents strong and weak points. First, tags fused to the receptors are generally small (6–12 or 15 residues), therefore their impact on the overall conformation of the receptor is generally low, especially if placed at the N-terminus of the receptor. Moreover antibodies available for classic tags such as 6Histidine, FLAG, hemagglutinine, cMyc usually keep good affinities for the tags when fused to the N-terminus. Antibodies when exhibiting high affinities can be used at concentrations lower than 10 nM. This allows carrying out experiments in homogeneous conditions, i.e., without separating the antibody free fraction (not bound onto the tagged receptor) from the bound fraction. Experiments are thus simpler to perform.

Second, antibodies are large and not permeant molecules. Therefore their binding is only possible on cell surface receptors allowing discrimination of cell surface targeted receptor. However, it has recently been shown that similar TR-FRET experiments can also be performed on mildly permeabilized cells expressing C-terminus tagged receptors (Ayoub et al., 2010). The size of the antibodies can also be considered as a weak point since they generate important steric hindrance in the vicinity of the

receptors. This can potentially be prejudicial for the binding of at least two antibodies, especially on class A receptors which usually display shorter N-terminus than class B and C GPCRs. Moreover, because antibodies are approximately three times larger than GPCRs, FRET signal between antibodies have to be cautiously interpreted as receptor oligomerization.

Finally antibodies can carry several fluorophores. This has often been considered as an advantage since it increases the fluorescence intensity of GPCR labeling either with donor or acceptor antibodies. However, it does not necessarily increase the signal-to-noise ratio. Moreover, since labeling of antibodies with donor or acceptor fluorophores are usually random, no optimization of the position of fluorophores on antibodies is possible.

Finally, remarks must be made concerning the binding of antibodies to tagged receptor. First, labeling of receptors by antibodies is reversible and time to reach the binding equilibrium can be long depending on receptors. For example, it can exceed 4 h at 4°C for HA tagged $GABA_B$ receptor (Maurel et al., 2004). Second, saturation of receptor labeling with antibodies can require high concentration, not compatible with homogeneous conditions. Third, ligand binding onto their cognate receptor can modify receptor conformation and therefore impact the access of antibodies to their epitope. Therefore variations of FRET could not reflect variation in the dimerization process but rather a modification of the affinity of antibodies for their epitope. Finally, antibodies are bivalent proteins, and although it has not been reported yet, one cannot exclude that they may artificially drive dimerization of non-interacting receptors (Maurel et al., 2008).

To conclude, TR-FRET strategies with fluorescent antibodies are interesting approaches exhibiting good signal-to-noise ratio. They allow the specific study of receptors targeted to the cell surface. However, their size and their non-covalent binding to receptors undoubtedly constitute a limitation to their use.

Covalent labeling of chimeric receptors

Various strategies developed during the past 10 years are based on the fusion of receptors to various peptides, either a self-labeling protein (SLP; also improperly called suicide enzyme) or a sequence recognized by enzymes (**Figure 1B**). TR-FRET experiments have also been performed on purified mutated receptors in which a reactive cysteinyl residue has been introduced.

Fusion of receptor to a self-labeling protein. Several approaches consist in fusing a SLP to the N-terminus of the receptor and providing fluorescent substrates. SLPs can catalyze the transfer of a fluorescent group from the substrate onto itself. For example, the SNAP-tag protein (23 kDa, i.e., two-thirds of GFP), derived from the DNA repairing enzyme O6-alkylguanine-DNA alkyltransferase (or AGT) transfers the fluorescent benzyl group from a fluorescent benzyl guanine substrate to label itself (Juillerat et al., 2003, 2005; Keppler et al., 2003, 2004; Gronemeyer et al., 2006; **Figure 3A**). Mutations have been introduced in the native protein to reduce its size, increase its reactivity, and decrease its ability to bind DNA. Nevertheless, in permeabilized conditions, labeling of the native protein cannot be excluded and that might slightly increase background emission (Gronemeyer et al., 2005; Juillerat et al., 2005).

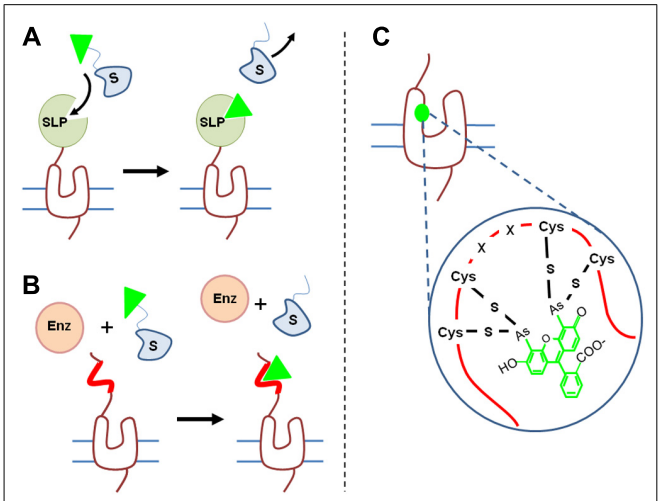

FIGURE 3 | Strategies to covalently label GPCRs. (A) SLP generally fused to the N-terminus of GPCRs catalyze the transfer of one fluorescent group (green triangle) from the substrate to itself. Various self-labeling proteins such as SNAP-tag, CLIP-tag, or HaloTag have been used to analyze receptor oligomerization. **(B)** Enzyme-based labeling: cells expressing tagged receptors are incubated in the presence of an enzyme such as AcpS and fluorescent substrate. The enzyme (Enz) catalyzes the transfer of one fluorescent group (green triangle) from the substrate to a specific tag incorporated into the receptor sequence (red line). **(C)** FlASH and ReASH strategies consists in introducing into the GPCR sequence a tetracysteine sequence (-C-C-X-X-C-C-) which reacts with fluorescent arsenical derivatives.

The efficiency of these strategies has been validated since 100% of the receptor is labeled with the fluorophore (Maurel et al., 2008; Comps-Agrar et al., 2011b). It is noteworthy that until now lanthanide derived fluorescent substrates are not permeant, therefore only receptors targeted to the cell surface, presenting an extracellular SNAP-tag will be labeled. By contrast other substrates such as tetramethyl rhodamine derivatives are permeant, allowing intracellular protein labeling (Gautier et al., 2009). Other SLP-tags have been developed [e.g., CLIP-tag with benzyl cytosine (Gautier et al., 2008), HaloTag (33 kDa) with HaloTag ligands (Zhang et al., 2006)] allowing the labeling of different receptors with reduced cross-reactivity. As mentioned above, these tags are generally fused to the N-terminus of the receptors since their fusion in the receptor extracellular loops generally induces greater conformational modifications.

These strategies have all been used to investigate GPCR oligomerization in various contexts. Several studies have included in their analysis SNAP- or CLIP-tag labeling on different GPCR models to point out their oligomerization (Maurel et al., 2008; Albizu et al., 2010; Ward et al., 2011). Incubation of cells expressing tagged-receptors in the presence of donor- and acceptor-derived substrates leads to the labeling of receptors with one donor or one acceptor fluorophore. The existence of a TR-FRET signal indicates proximity between receptors and has been interpreted as receptor dimerization.

Moreover, the absence of impact of agonist and/or antagonist binding on receptor oligomerization as reported for vasopressin, oxytocin and dopamine receptors (Albizu et al., 2010), suggest

the stability of the interaction between receptors, or at least, that the equilibrium between monomers and oligomers, if any, is not affected by ligand binding. Whether this result could be generalized to other receptors remains to be established but it seems consistent with previous data (Terrillon et al., 2003).

This method has also been used to go one step further and demonstrate that $GABA_B$ receptor forms higher order oligomers (Maurel et al., 2008). Significant TR-FRET signals have been recorded between $GABA_{B1}$ and $GABA_{B2}$ subunits and also between two $GABA_{B1}$ subunits if co-expressed with $GABA_{B2}$ subunit. By contrast a weak TR-FRET signal between two $GABA_{B2}$ subunits when expressed with $GABA_{B1}$ subunits has been reported. These results indicate the formation of $GABA_{B1/B2}$ tetramers, $GABA_{B1}$ subunit constituting the interface between the two $GABA_{B1}$/$GABA_{B2}$ dimers (Maurel et al., 2008; Comps-Agrar et al., 2011a). This result is in accordance with previous microscopy studies suggesting oligomeric complex of rhodopsin in native disk membrane (Fotiadis et al., 2003) or with combined bioluminescence/fluorescence complementation and energy transfer (Lopez-Gimenez et al., 2007; Carriba et al., 2008; Guo et al., 2008), suggesting that GPCRs can form larger oligomers.

SNAP- and CLIP-tag labeling have also been associated to study receptor heterodimerization. This strategy has been well exemplified by the analysis performed on mGluRs. Doumazane et al. (2011) have reported the existence of heterodimers between mGluR 1 and 5 on the one hand or between mGluR 2, 4, 7, and 8 on the other hand. No significant TR-FRET signal was observed between receptors of these two groups. Regarding receptors of class A, using the SNAP-tag and CLIP-tag strategy, orexin OX1 and cannabinoid CB1 receptors have been shown to oligomerize and the hetero-complex appears to be more sensitive than orexin homo-oligomers to orexin A regulation (Ward et al., 2011).

Fusion of receptor to sequence recognized by enzyme. A second approach consists in introducing a sequence recognized by an enzyme into the receptor of interest. The enzyme will catalyze the transfer of a fluorescent group from a fluorescent substrate to the sequence introduced onto the receptor (**Figure 3B**). For example phosphopantetheinyl transferase (PPTase) called acyl-carrier protein synthase (AcpS) will transfer the phosphopantetheinyl group of coenzyme-A to the acyl-carrier protein (ACP), a 8.7-kDa sequence added to the receptor. Once again, the sequence should be accessible to the enzyme and thus located on the extracellular side (Monnier et al., 2011). Such a sequence is much smaller than SNAP-tag or HaloTag and therefore its insertion into the receptor sequence may be less disturbing.

This strategy has been used in association with the SNAP-tag method to perform $GABA_B$ subunits orthogonal labeling and study receptor transactivation (Monnier et al., 2011). It has also been used with classic fluorophores to study class A neurokinin NK1 receptor oligomerization (Meyer et al., 2006).

Receptor labeling via introduction of reactive cysteinyl residue. Introduction of fluorophores onto receptors can be achieved by using cysteinyl residue reactivity. Because cysteinyl group are often present in protein sequences, such a labeling can only be performed on purified receptor and not on receptor in their membrane context to avoid a large non-specific labeling. Labeling of receptor with Lumi4-Tb can be achieved by incubating purified receptors with maleimide derivatives (Rahmeh et al., 2012) and receptors can be labeled at one specific position by introducing a cysteine at this position and by mutating all other reactive cysteines. Labeling with acceptor fluorophore can be achieved by using FlAsH and ReAsH methods. It consists in introducing a tetracysteine sequence (two cysteine pairs separated by two amino acid residues, CCXXCC) which exhibits a high affinity for green or red fluorescent arsenical derivatives (Ju et al., 2004; Zürn et al., 2010; **Figure 3C**). Thanks to the combination of these two labeling methods, Rahmeh et al. (2012) have demonstrated conformational modification of vasopressin V2 receptor upon agonist, partial agonist or inverse agonist binding.

Advantages and drawbacks. The above covalent labeling strategies offer a wide range of advantages. First, covalent labeling constitutes an interesting alternative to antibodies. It induces a lesser steric hindrance than antibody labeling. As mentioned above this is particularly interesting for class A receptors which generally have N-terminus shorter than class C receptors. Second, various "colors" can be used on the same fused receptor construction by changing the fluorophore linked to the substrate. Receptor homomerization can then be simply studied by incubating cells expressing one receptor with two different fluorescent substrates. Optimization of the labeling requires comparing kinetics of labeling with the different substrates. Third, receptor labeling is irreversible and faster than antibody labeling since 1 h is sufficient to label 100% of the receptors (Maurel et al., 2008). It is noteworthy that more reactive SNAP- and CLIP-tag mutants have recently been developed to get a faster labeling of receptors (Sun et al., 2011). Fourth, TR-FRET methods combined to efficient labeling strategies are convenient to follow receptor conformational modification as shown by Rahmeh et al. (2012) on purified receptors. Conformational changes can also be monitored by SNAP- and CLIP-tag labeling with classic fluorophores to develop sensors of different molecules such as sulfonamides (Brun et al., 2009; Monnier et al., 2011). These techniques have proven to be compatible with cellular assays (Brun et al., 2011). Therefore, the development of a sensor to follow ligand induced receptor conformational modifications or intracellular protein binding is potentially achievable.

These strategies to perform orthogonal labeling require some optimization steps. One essential step is the determination of substrate concentrations to label receptors. Using higher concentrations accelerates the kinetics of receptor labeling but also increase the cross-reactivity of substrates for SLPs (e.g., benzylcytosine presents a cross-reactivity to SNAP-tag). Finally, high concentrations of substrates are not compatible with experiments performed in homogeneous conditions. To get around this problem, one interesting alternative consists in using a substrate conjugated to both a fluorophore and a quencher. The probe becomes highly fluorescent only upon reacting with the SLP (Sun et al., 2011).

Getting positive and negative control to demonstrate receptor oligomerization is an essential point for the validation of this concept. The negative control is probably the most difficult to get. First, FRET can be observed if the expression of the partners is high enough to get random collision of receptor diffusing at the cell surface. Therefore, variation of the receptor expression level can be at the origin of inconsistence between published data. For example, using the same receptor labeling approach, Doumazane et al. (2011) reported the absence of heterodimerization between mGluRs mGluR2 and 5, while a significant signal has been observed by Delille et al. (2012). One important criterion to conclude to the specificity of the interaction is that FRET efficiency should be constant and independent of the level of receptor expression. An alternative control can be to verify the saturation of the FRET signal when the expression of the acceptor is increased and the expression of the donor is kept constant.

Finally, all these strategies, by contrast to BRET, enable the distinction between tagged receptors targeted to the cell surface from those trapped inside cells. On the other hand, they are not applicable to study wild-type receptors expressed in native tissues.

Non-covalent labeling of wild-type receptor

As mentioned above, receptor oligomerization potentially opens new perspectives regarding GPCR functioning. However, the concept needs to be validated in a native context and not only on receptors expressed in cell lines. Indeed, various biases could impair the relevance of FRET data obtained in cell lines. The exact impact of using chimeric receptors instead of wild type receptors, of high receptor expression levels, or of different receptor targeting depending on the cell line used, is difficult to evaluate. Thus, the validation of results in a native context is important. This is even more crucial regarding GPCR heteromerization. The demonstration of the existence of heteromers in a cell line can be potentially relevant only if in native tissues receptors are at least expressed at the same time, in the same cell and in the same subcellular compartment. However, native contexts impose constraints since the receptor sequence, the level of expression or the targeting cannot be modified.

Two strategies can be used to label receptors in native tissues. First, antibodies have been considered to fluorescently label endogenous receptors. However, they are large molecules generating steric hindrance and getting specific and high affinity antibodies again GPCRs has proven difficult. These two reasons make antibodies not necessarily the best tools for demonstrating direct receptor interactions. Antibodies produced by Camelids could be a good alternative to conventional antibodies since they are much smaller (17 vs. 150 kDa). Moreover they can recognize different epitopes usually not recognized by conventional antibodies and notably clefts such as ligand binding pockets or enzyme active sites (De Genst et al., 2006; Harmsen and De Haard, 2007). Therefore, besides their small size, they open new perspectives in term of molecular recognition and specificity.

A second strategy based on fluorescent ligands presents several advantages (**Figure 1B**). Ligands are usually smaller molecules, especially regarding GPCRs of class A and C, and can exhibit high affinities for GPCRs. They are therefore potentially suitable to study receptor oligomerization insofar as their fluorescent derivatives maintain high affinities for their cognate receptors. First attempts to demonstrate GPCR oligomerization with fluorescent ligands have been performed on luteinizing hormone and somatostatin receptors (Roess et al., 2000; Patel et al., 2002). However, the sensitivity of the approach was insufficient because of a low signal-to-noise ratio. TR-FRET strategy based on fluorescent ligands represents an interesting alternative method. This has been carried out for peptidic ligands; vasopressin and oxytocin antagonist and agonist derived with lanthanide cryptate as donor (Albizu et al., 2007, 2010) and d2, dy647, and fluorescein (Durroux et al., 1999; Terrillon et al., 2003) as acceptors were synthesized. Surprisingly, this strategy has also been successfully adapted to smaller bioamine ligands. Indeed one could have predicted that adding fluorophores bigger in size than the ligands, such as lanthanide cryptates, should deeply impact the affinity of the latter. The syntheses of lanthanide cryptate labeled derivatives of *N*-(*p*-aminophenethyl) spiperone (NAPS) and (±)-4′-amino-2-(*N*-phenethyl-*N*-propyl)-amino-5-hydroxytetralin (PPHT), respectively antagonist and agonist of the dopamine D2 receptor have recently been reported (Albizu et al., 2010). Both ligands exhibit affinities in the 5 nM range for the dopamine D2 receptor. These data are very encouraging since they strongly suggest that development of lanthanide cryptate derived ligands is achievable with a large range of ligands.

TR-FRET strategy based on fluorescent ligands has been validated on V1a and V2 vasopressin receptors, on oxytocin and dopamine receptors with at least five sets of fluorescent ligands (Albizu et al., 2010). It has been shown that TR-FRET signal is not observed on mock cells, abolished in the presence of an excess of unlabeled ligand and that its variation in function of donor/acceptor ratio follows a bell-shaped cure. Therefore these data support that TR-FRET is dependent on the receptor expression and the occupancy of the binding sites with fluorescent ligands, demonstrating the specificity of the TR-FRET signal. It has also been observed that TR-FRET signal obtained with fluorescent agonists is weaker than with fluorescent antagonists. This result has been related to the negative cooperative binding of agonists, in contrast to antagonists, and strongly supports that TR-FRET signal does not result from random collision of receptor diffusing at the cell surface. Indeed when considering the collision hypothesis of monomeric receptors, agonists or antagonists should lead to the same TR-FRET signal for the same level of receptor occupancy.

Similar experiments have been carried out on oxytocin receptors expressed in the mammary gland of lactating rat and consistent results have been obtained proving the existence of oxytocin receptor homodimers in this tissue. Moreover, experiments performed on tissues patches clearly indicate the targeting of receptor dimer to the cell surface. These data validate the fluorescent ligand-based TR-FRET strategy to prove the existence of receptor oligomers in native tissues.

Advantage and drawbacks. A large set of fluorescent ligands has been synthesized for numerous GPCRs. Whether the fluorescent derivatives will exhibit high affinity for their cognate receptor remains to be established but the example of D2 dopamine ligands proves that the development of such ligands is feasible. The

sensitivity of the technique is dependent on the affinity of the ligand for its receptor. Ligands exhibiting affinity in the nanomolar range are suitable for such experiments since experiments can be carried out in a 96-well plate format and in homogeneous conditions. The absence of washing steps makes the experiments very simple to perform (Cottet et al., 2011) and more reproducible. Moreover, the TR-FRET kinetics can simply be performed and the time to reach equilibrium is easily determined. In the case of low affinity fluorescent ligands or strong negative cooperative binding of one ligand between two binding sites, one could have expected to perform FRET measurements after washing steps. However, dissociation kinetics of ligands can be rapid and therefore incompatible with such a protocol. Finally, because several examples of negative cooperative binding of agonist have been reported (Urizar et al., 2005; Albizu et al., 2006; Springael et al., 2006; Han et al., 2009), it seems more relevant to use fluorescent antagonists to get a double labeling of binding sites within a dimer. It underlines that FRET signal is strongly dependent on cooperative binding mechanisms between ligands.

The approach has been validated on one receptor expressed in a native context, the oxytocin receptor expressed in mammary gland of lactating rat. Indeed this receptor model is interesting since it is highly expressed in this tissue. Whether the method is applicable to tissues expressing receptors at a lower density remains to be established.

Data must be interpreted with caution. Indeed the absence of FRET signal is not necessarily a proof of oligomer absence. It can also be explained by the binding of only one ligand because of a high negative cooperative binding or because of the existence of hetero-oligomers.

CONCLUSION AND PERSPECTIVES

RET techniques have provided very interesting experimental solutions to study receptor complexes. Indeed, the resolution of RET approaches is <10 nm, far below all conventional optical microscopy techniques. A significant RET signal has thus been interpreted as direct interactions between receptors while conventional microscopy can only conclude to receptor co-localization. Are all RET approaches equivalent to study receptor oligomerization? Certainly not as BRET and TR-FRET are significantly more sensitive with a higher signal-to-noise ratio, and both techniques provide the possibility to perform multiplexing. BRET offers the simplicity of receptor labeling performed by bioengineering techniques. This is a strong advantage but also a disadvantage since it is impossible to distinguish the receptors targeted to the surface or trapped inside the cell. Moreover, although various pairs of donor/acceptor have been developed, all the donors are derived from Rluc. Whether the development of a different and smaller luminescent donor is conceivable remains an open question. TR-FRET displays a larger panel of tools for receptor labeling. Regarding the labeling step, TR-FRET is a little bit more complicated. Antibodies are large molecules inducing a steric hindrance which can be, on some receptor models, prejudicial for observing signals of large amplitude. Covalent labeling techniques offer some advantages with much smaller tags but they generally need additional labeling and washing steps. TR-FRET based on fluorescent ligands is an interesting alternative since, to our knowledge, it is the only technique that can be applied to wild-type receptors expressed in a native context. This constitutes a breakthrough because the validation of the concept of GPCR oligomerization in physiology is crucial.

During the last 20 years, RET techniques became very popular for GPCR oligomerization studies. What are the perspectives for BRET and TR-FRET in the next decade? One major aspect is probably their use in microscopy. Both techniques have been adapted to microscopy constraints (Coulon et al., 2008; Rajapakse et al., 2010). Of note, many assays based on BRET or TR-FRET approaches have been developed in the last decades to measure ligand binding (Ilien et al., 2003; Tahtaoui et al., 2005; Albizu et al., 2007; Zwier et al., 2010), second messenger production (Trinquet et al., 2006), receptor internalization (Zwier et al., 2011), or protein recruitment such as β-arrestins (Angers et al., 2000; **Figure 4**). A number are compatible with high throughput screening (**Figure 4;** Boute et al., 2002). The adaptation of BRET and TR-FRET to microscopy opens new perspectives since both techniques will be compatible with high content screening. Because TR-FRET based on fluorescent ligands is convenient to study receptor oligomerization in native tissues, one can expect that further development of the techniques will allow the study of the role of receptor homomers and heteromers in real life.

Others strategies have recently emerged to investigate GPCR oligomerization. Time-resolved fluorescence anisotropy approach is based on the energy transfer between two identical fluorescent proteins, for example two YFPs. The transfer of energy results in a decrease in the polarization of the fluorescence emission. This approach has recently been used to study 5HT-1A receptor oligomerization (Paila et al., 2011). Other studies have combined high-resolution microscopy and single particle tracking (Hern et al., 2010; Kasai et al., 2011). As such, they open new perspectives

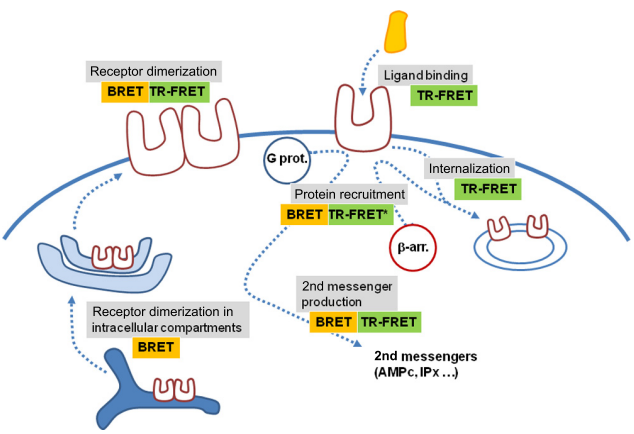

FIGURE 4 | BRET and time-resolved FRET assays application. Various BRET and TR-FRET assays have been developed to measure ligand binding, receptor activation through protein recruitment or second messenger production, receptor dimerization or receptor trafficking. Most of them are compatible with high-throughput screening. Recent developments have shown that these techniques are also potentially compatible with high-content screening, opening new perspectives in the use of RET approaches (*means that TR-FRET experiments were performed on mildly permeabilized cells expressing C-terminus tagged receptors).

since kinetics of GPCR complex dissociation can be monitored. However, high-resolution microscopy techniques on live cells do not yet display a resolution compatible with a definitive identification of GPCR complexes as oligomers. Fast, three-dimensional super-resolution imaging of live cells has recently been described when labeling light chain of clathrin fused to SNAP-tag and a resolution of 30 nm has been reported (Jones et al., 2011). This resolution is thus at least threefold greater than FRET resolution while FRET technique resolution is better than 10 nm. Therefore these techniques offer a complementary point of view to study GPCR oligomerization.

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Unraveling receptor stoichiometry using BRET

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The first and arguably most important question that could be asked about the biology of any protein is: does it function alone? Cell surface receptors present special problems for stoichiometric analysis because, being located within lipid bilayers, they are often very hydrophobic, which means that once isolated they can exhibit a strong tendency to aggregate. A very welcome development, therefore, has been the advent of *in situ* methods for probing receptor organization, the most important of which are presently based on resonance energy transfer. Our first bioluminescence resonance energy transfer (BRET) experiments were, however, inconclusive since both monomeric and dimeric receptors gave high levels of energy transfer (James et al., 2006). It was only with the application of theoretical principles first developed for (Fung and Stryer, 1978; Wolber and Hudson, 1979), and then used in (Kenworthy and Edidin, 1998), Förster resonance energy transfer (FRET) experiments that we could use BRET to confidently distinguish between monomers and dimers.

We were very keen to test G protein-coupled receptors (GPCRs) using the new approach given the great interest in these important proteins forming constitutive oligomeric complexes (Angers et al., 2000; Ramsay et al., 2002; Babcock et al., 2003). This seemed unlikely to us firstly because, structurally, GPCRs are ideally configured for functioning autonomously (Meng and Bourne, 2001) and, secondly, because functional autonomy explains the remarkable evolutionary success (Schiöth and Fredriksson, 2005) of this very large family of receptors. We were initially ignorant of the extent to which BRET was used to buttress the "GPCRs as oligomers" concept (Pfleger and Eidne, 2005), but when our initial analyses of human β_2-adrenergic (β_2AR) and mouse cannabinoid (mCannR2) receptors yielded the "BRET signatures" of monomers (James et al., 2006), we had to confront this body of data. The resulting controversy (Bouvier et al., 2007; James and Davis, 2007a,b; Salahpour and Masri, 2007) seems to have prompted the development of other, more complicated approaches. Here, we describe our experiences using BRET and briefly consider the merits of these alternative approaches.

ONCE IS NOT ENOUGH

Like all resonance energy transfer-based methods, BRET is based on the principle of non-radiative energy transfer (Förster, 1948). In this case excitation energy is passed from a luminescent donor (luciferase) to a fluorescent acceptor protein, typically a modified variant of green fluorescent protein (GFP) such as yellow fluorescent protein or GFP[2]. Many early studies of surface receptors, particularly GPCRs, employed "conventional" BRET assays developed for analyzing interacting soluble proteins, in which donor- and acceptor-fused receptors are expressed at a single, fixed ratio, and BRET efficiency ($BRET_{eff}$) is measured as relative to controls (Angers et al., 2000; Ramsay et al., 2002; Babcock et al., 2003). These early studies were largely unanimous in concluding that the receptors in question form homo- and hetero-oligomeric interactions and were significant in establishing the oligomeric GPCR paradigm (Pfleger and Eidne, 2005). We initially used this assay to determine whether an immune protein, CD80, forms dimers at the cell surface as implied by our crystal structure (Ikemizu et al., 2000), and were pleased to see strong energy transfer in our first experiments. However, the closely related protein, CD86, which is a monomer, also yielded high levels of energy transfer – as much as 25% of the levels obtained for covalent homodimers (James et al., 2006). We suspected that this was "background" energy transfer arising from random interactions within the membrane, a view strengthened by analysis of a second monomer, CD2. We concluded that conventional BRET assays could be problematic for measuring receptor organization in membranes because, within the crowded two-dimensional plane of the cell membrane, the signal arising from random interactions can reach significant levels.

THEORETICAL WORK-AROUNDS

Theoretical considerations (Fung and Stryer, 1978; Wolber and Hudson, 1979; Kenworthy and Edidin, 1998) have predicted that the dependence of FRET on total and relative donor and acceptor concentrations differs systematically for specific and non-specific energy transfer. Applied to BRET in "type 1" experiments, total protein concentration is held constant and the acceptor/donor ratio increased by replacing donors with acceptors (**Figure 1A**; James et al., 2006). In this context, $BRET_{eff}$ for monomers is independent of the acceptor/donor ratio above a certain threshold because donors always experience the same "acceptor environment." For oligomers, however, replacing donors with acceptors reduces the fraction of donor–donor complexes, converting them into BRET-productive pairs and increasing $BRET_{eff}$. In "type 2" experiments (**Figure 1B**; James et al., 2006) total protein density is varied at constant acceptor/donor ratio. For monomeric proteins $BRET_{eff}$ varies linearly with total surface density for low expression levels, tending to zero at very low densities. Conversely, for constitutive oligomeric proteins $BRET_{eff}$ is largely constant because expression itself is generally reliant on oligomerization. However, at high densities, $BRET_{eff}$ increases due to random

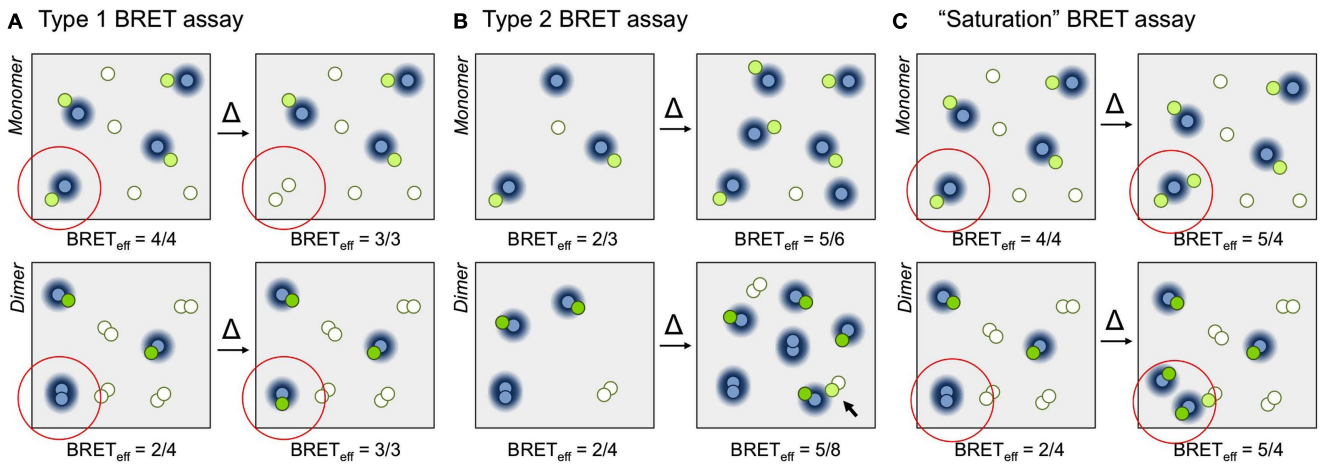

FIGURE 1 | Principles of BRET assays. (A) In a type 1 BRET assay the acceptor/donor ratio is increased but surface density is kept constant. The increase in acceptor/donor ratio is obtained by exchanging a donor for an acceptor (the change is indicated within the red circle). For simplicity, BRET$_{eff}$ is defined here as the ratio of the numbers of fluorescent acceptors and luminescent donors. In the examples shown, for the monomer (top) BRET$_{eff}$ *is unchanged* (3/3 versus 4/4), whereas for the dimer (bottom) the ratio *increases* from 2/4 to 3/3 as the fraction of productive dimers increases. **(B)** In a type 2 BRET experiment, the acceptor/donor ratio is kept constant and surface density is varied, in this case by a factor of two. Due to the increase in monomer density (top) the likelihood of random collisions increases, and BRET$_{eff}$ *increases* from 2/3 to 5/6. For constitutive dimers (bottom), however, BRET$_{eff}$ is largely *unchanged*, increasing from 2/4 to 5/8, because the likelihood of dimerization doesn't change. Only random interactions of dimers (arrow) contribute to increases in BRET$_{eff}$ (in reality, of course, these contributions will be significantly smaller than the effects of dimerization). **(C)** In the "saturation" BRET assay, the acceptor/donor ratio is increased by keeping donor numbers constant and increasing the numbers of acceptors. In the examples shown, BRET$_{eff}$ for monomers (top) and dimers (bottom) *both increase* upon addition of one or two extra acceptors, respectively (from 4/4/ to 5/4 for the monomer, and from 2/4 to 5/4 for the dimer). This is due to the increased random interactions of monomers, and increased formation and random interactions of dimers. We expect assays in which BRET$_{eff}$ always increases to be more easily misinterpreted than assays in which changes in BRET$_{eff}$ vary systematically with receptor stoichiometry. Fluorescing and non-fluorescing acceptor molecules are shown as green and white circles, respectively, and donors as blue circles. The BRET-permissible area surrounding donors is represented as a blue halo.

interactions of the oligomers within the membrane. For this reason it is inappropriate to draw any conclusions from the gradient of the slope for BRET$_{eff}$ versus expression level as, e.g., in Ramsay et al. (2002).

Using these new types of BRET experiments we readily distinguished well-known monomeric and dimeric Type I membrane proteins, and even confirmed that CD80 forms apparently transient dimers at the cell surface, as implied by analytical ultracentrifugation (Ikemizu et al., 2000). Applied to two GPCRs, β_2AR and mCannR2, these assays yielded the unambiguous "BRET signatures" of monomers (James et al., 2006). We also showed that the GABAβ receptor, a *bona fide* GPCR dimer, gave data characteristic of dimers and that transfer of the cytoplasmic domain of GABAβR2 to β_2AR converted monomer-like into dimer-like behavior. As expected, since β_2AR and other GPCRs were widely believed to form homo- and hetero-dimers (reviewed in Bouvier, 2001), these findings were controversial (Bouvier et al., 2007; James and Davis, 2007a,b; Salahpour and Masri, 2007).

ALTERNATIVE ASSAYS

Broadly speaking there is now consensus that conventional, single-ratio BRET experiments are inadequate to the task of assigning receptor stoichiometry. However, although type 1 and 2 BRET and FRET experiments are done occasionally (e.g., Kenworthy and Edidin, 1998; Meyer et al., 2006), these approaches are not widely used. Instead, the so-called BRET "saturation" assay first used in 2002 (**Figure 1C**; Mercier et al., 2002) remains popular (Contento et al., 2008; Ayoub and Pfleger, 2010). In this approach, donor numbers are kept constant and acceptor expression systematically increased. Under such conditions BRET$_{eff}$ for a monomeric protein is linearly related to acceptor expression level, whereas for oligomers the relationship is hyperbolic. The problem therefore becomes one of distinguishing between two increasing signals, which we would expect to be more difficult than distinguishing between increasing versus non-increasing signals, as in type 1 BRET assays (James et al., 2006). The problem becomes more acute for transient oligomers whose signals emerge from monomer/dimer equilibria, which is particularly relevant now that GPCRs are being claimed to transiently dimerize (Hern et al., 2010; Lambert, 2010; Kasai et al., 2011).

A second, newer assay, the "BRET competition" assay, presents subtler problems. In this assay, untagged "competitor" receptors are co-transfected with acceptor- and donor-tagged proteins, leading to reduced BRET$_{eff}$ for oligomers and unchanged BRET$_{eff}$ for monomers (Veatch and Stryer, 1977). In our experience, expression of untagged competitors often reduces expression of their tagged equivalents (Felce et al., unpublished data), including monomer control proteins, reducing BRET$_{eff}$ artifactually. In BRET competition assays of GPCR homo- and heterodimerization (e.g., Terrillon et al., 2003; Guo et al., 2008), reduced energy transfer in the presence of untagged competitors is always observed, yet the issue of surface density is never addressed. Such approaches have their place but the absolute levels of tagged protein must be factored in to avoid ambiguity.

CONTROL PROBLEMS

An important factor complicating some BRET experiments is the heterogeneity of protein distribution, emphasizing the importance of the careful choice of controls. The cell membrane is a highly complex environment (Kusumi et al., 2011), and evidence is mounting that complex regulatory processes may control the localization and movement of integral membrane proteins, including GPCRs (Meyer et al., 2006; Nikolaev et al., 2010; Weigel et al., 2011). The potential for proteins to be localized to different areas of the cell surface, or to have different constraints on their trafficking, has important implications for data interpretation. This applies especially to "irrelevant" controls, which should have similar hydrodynamic diameter to the protein of interest but be sufficiently unrelated to not form specific associations (Angers et al., 2000; Mercier et al., 2002). However, such proteins may not be similarly localized at the membrane. For example, if the control protein exhibits strong association with the cytoskeleton but the protein of interest does not, $BRET_{eff}$ will be lower in the control experiment than it would be if the two proteins co-localized but randomly interacted. Similarly, control proteins may be expressed at different total densities or have different stoichiometries, adding further complications. Without knowing their behavior and expression characteristics in detail, it is difficult to select appropriate controls.

Approaches in which acceptors are recruited to donor-tagged proteins of interest are especially dependent on control choice. In "Third-party BRET" (Kuravi et al., 2010), a membrane-associated acceptor is chemically recruited to an untagged receptor of interest and $BRET_{eff}$ increases if the untagged receptor is a dimer that brings with it a donor-tagged receptor, the goal being to avoid the complication of varying expression levels. However, if the receptors are co-localized but do not interact, then acceptor/untagged receptor dimerization could recruit the acceptor to an area of greater donor concentration, increasing $BRET_{eff}$ without genuine association. Similar arguments apply to GPCR-Heteromer Identification Technology (GPCR-HIT; Pfleger, 2009; Mustafa and Pfleger, 2011). For this reason, no conclusively reliable BRET-based assay for heterodimers presently exists. Despite these difficulties, conventional (Pfleger and Eidne, 2005), saturation (Sohy et al., 2009), and competition (Terrillon et al., 2003) BRET assays have all been used to support claims for GPCR heterodimerization.

CONCLUDING REMARKS

There is now implicit agreement that single measurements of $BRET_{eff}$ are unhelpful because the contribution of random interactions to the signal is not easily discerned. Similarly, the notion that varying expression levels can also give potentially misleading changes in $BRET_{eff}$ is taking root, prompting new methods such as "Third-part BRET," which seek to control for background effects in single measurements. The problem with these approaches is their heavy reliance on negative controls, which as we have discussed are often difficult to choose. We are surprised that the relatively simple approaches involving systematic variations of the acceptor/donor ratio, or of expression level alone, are not more widely used. We emphasize once again that the key to these methods is their exclusive reliance on the measurable, intrinsic behavior of populations of receptors diffusing in the plane of the membrane, and that an important advantage is that the assays are effectively control-independent.

Overall, the question of whether or not GPCRs generally form oligomers remains unsettled. The notion that they do is driven not only by BRET experiments, but also by FRET (Albizu et al., 2010; Cunningham et al., 2012), photon-counting analyses (Kilpatrick et al., 2012), and single-molecule microscopy (Hern et al., 2010; Kasai et al., 2011). We are seeking to test our BRET-based conclusions using super-resolution imaging, and to address GPCR stoichiometry at the family level using type 1 BRET and other experiments implemented in a high throughput setting.

Despite the controversies over its use BRET still has a very bright future. New luciferases, such as Rluc2 and Rluc8 (De et al., 2007), and acceptor fluorophores, such as Venus (Kocan et al., 2008), mOrange (De et al., 2009), and Renilla GFP (RGFP; Kamal et al., 2009), are brighter and offer up the possibility of *in vivo* studies (De et al., 2009). Future developments in BRET-quantum dot (Wu et al., 2011; Quiñones et al., 2012) and BRET-FRET (Carriba et al., 2008) assays will also advance the technique. The effective resolution of resonance energy transfer methods in live cells, i.e., ~10 nm, is presently significantly better than that of *in situ* single-molecule imaging techniques, which, even in fixed cells, is limited to ~20 nm (Moerner, 2012). We think that it will be some time before BRET, rigorously applied, is surpassed as a probe of receptor stoichiometry.

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Receptor-G protein interaction studied by bioluminescence resonance energy transfer: lessons from protease-activated receptor 1

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Since its development, the bioluminescence resonance energy transfer (BRET) approach has been extensively applied to study G protein-coupled receptors (GPCRs) in real-time and in live cells. One of the major aspects of GPCRs investigated in considerable details is their physical coupling to the heterotrimeric G proteins. As a result, new concepts have emerged, but few questions are still a matter of debate illustrating the complexity of GPCR-G protein interactions and coupling. Here, we summarized the recent advances on our understanding of GPCR-G protein coupling based on BRET approaches and supported by other FRET-based studies. We essentially focused on our recent studies in which we addressed the concept of preassembly vs. the agonist-dependent interaction between the protease-activated receptor 1 (PAR1) and its cognate G proteins. We discussed the concept of agonist-induced conformational changes within the preassembled PAR1-G protein complexes as well as the critical question how the multiple coupling of PAR1 with two different G proteins, Gαi1 and Gα12, but also β-arrestin 1, can be regulated.

Keywords: BRET, PAR1, G proteins, preassembly, precoupling, protein interactions

INTRODUCTION

G protein-coupled receptors (GPCRs) constitute one of the largest cell surface receptor family, and are involved in many cellular signaling and physiological responses (Bockaert, 1991; Gether, 2000). They are encoded by the largest gene family in the mammalian genomes and they constitute the site of binding and action of a large panel of natural mediators such as hormones and neurotransmitters (Bockaert and Pin, 1999). Thus, GPCRs are known to be the target of many drugs used to treat diseases (Schlyer and Horuk, 2006). Initially, the cellular signaling via GPCRs has been thought to occur only by their interaction with and activation of several types of guanine nucleotide binding proteins or G proteins (Limbird, 1983; Bockaert et al., 1987; Gilman, 1987). However, it is now obvious that in addition to G protein-dependent signaling, GPCRs also activate G protein-independent signaling pathways (Hermans, 2003; Lefkowitz and Whalen, 2004). Furthermore, GPCRs are now known to interact with many intracellular proteins other than G proteins and these proteins play a major role in promoting and regulating GPCR signaling (Brady and Limbird, 2002).

From their discovery until now, the coupling of GPCRs to the heterotrimeric G proteins and their activation has been extensively studied. The initial model explaining their functioning has considerably evolved (Bourne, 1997; Limbird, 2004; Strange, 2008) and new concepts have emerged such as, constitutive activity and precoupling (Leff and Scaramellini, 1998; Seifert and Wenzel-Seifert, 2002), multiple coupling (Hamm, 1998; Hermans, 2003; Perez and Karnik, 2005), functional selectivity (Rajagopal et al., 2011), and the role of GPCR oligomerization (Dean et al., 2001). The initial ternary model of GPCR/G protein activation postulated that agonist binding promotes the transition of the receptor from the inactive to the active state leading to the physical association of the receptor with the heterotrimeric Gαβγ protein, allowing the exchange of bound GDP for GTP in the Gα subunit (Limbird et al., 1980; Gether and Kobilka, 1998). GTP binding stabilizes the active state of the G protein leading to the dissociation of the receptor-G protein complex allowing Gα on one hand, and Gβγ on the other hand to act on their respective effectors and initiating signal transduction (Hamm, 1998; Oldham and Hamm, 2008). Moreover, it is now accepted that ligand binding to GPCRs promotes conformational changes in the receptor leading to the transition of the receptor to its active state. This evidence come from the functional studies of the downstream signaling as well as the biochemical, biophysical, and structural analysis of GPCRs themselves (Gether et al., 1995; Vilardaga et al., 2003; Bockenhauer et al., 2011).

Over the past 20 years, the question of how GPCR-G protein coupling occurs has been widely studied initially using radioligand binding (Stadel et al., 1981) and biochemical techniques (Smith and Limbird, 1981; Neumann et al., 2002) and recently through crystallographic analysis (Palczewski et al., 2000; Kobilka and Schertler, 2008) and energy transfer-based approaches, bioluminescence resonance energy transfer (BRET) and FRET (bioluminescence/fluorescence resonance energy transfer; Pin et al., 2008; Vilardaga et al., 2009; Lohse et al., 2012). BRET and FRET methods allow the determination of the proximity and/or relative orientation of two chromophores fused to the proteins being studied, such as between a GPCR and its cognate G protein, or between Gα and Gβγ subunits, directly in real-time and in live cells (Azpiazu and Gautam, 2004; Frank et al., 2005; Gales et al., 2005; Ayoub et al., 2010). As discussed in this review, these studies challenged the initial GPCR-G protein model to some extend and revealed new concepts with regard to receptor-G protein coupling as well as G protein subunit dissociation.

In this review, we focus on the recent studies using the BRET approach to investigate the physical and functional interaction between GPCRs and the heterotrimeric G proteins taking lessons from our observations on the interaction of the thrombin receptor, protease-activated receptor 1 (PAR1), with Gαi1 and Gα12 as well as β-arrestin 1 (Ayoub et al., 2007, 2009, 2010). PAR1 belongs to a particular GPCR family composed by three other subtypes, PAR2, PAR3, and PAR4, known to be activated by various and highly selective serine proteases such as thrombin, trypsin, plasmin, and the factor Xa (Cottrell et al., 2002; Hollenberg and Compton, 2002). The activation mechanism of PARs involved the cleavage of their N-terminal extremity by the protease, unmasking a new N terminus that acts as a tethered ligand, directly activating the transmembrane core of the receptor (Coughlin, 2000). Following activation, cleaved PARs are known to undergo a rapid desensitization, internalization, and degradation (Trejo, 2003). This desensitization and internalization processes involve the phosphorylation of the receptor by G protein-coupled receptor kinases and the recruitment of arrestins (Trejo, 2003). PARs have been reported to play crucial roles in a number of physiological processes such as thrombosis, vascular development, inflammation, cell proliferation, and tumorigenesis and therefore they are considered as interesting targets for the treatment of various pathologies (Coughlin, 2005). PAR1 is a prototype of the PARs family members, characterized by the diversity of its signaling pathways involving different G protein classes as well as arrestins. Indeed, PAR1 has been reported to couple to Gi/o, Gq as well as G12/13 proteins promoting multiple downstream signaling responses in various cellular models (Coughlin, 2000; Marinissen et al., 2003).

BRET TO STUDY GPCR-G PROTEIN INTERACTION

As mentioned above the initial GPCR/G protein activation model was based on elegant biochemical experiments using solubilized and purified proteins. Thus, for a long time and before the emergence of BRET and FRET techniques the detailed analysis of GPCRs and G protein activation in real-time and in live cells was very limited. Now such an analysis becomes feasible and indeed within the past 7 years a number of studies has examined the activation process of the heterotrimeric G proteins by various GPCRs, using either FRET or BRET techniques (Vilardaga et al., 2009; Lohse et al., 2012). The historically first energy transfer-based assay to study G protein activation by GPCRs was based on FRET approach using GFP variants as donor and acceptor and the pioneer study was in *Dictyostelium discoideum* using FRET between Gα and Gβγ subunits showing a direct evidence for G protein dissociation in live cells (Janetopoulos et al., 2001). Then other FRET studies on the activation and association/dissociation of the G protein subunits have been reported in yeast and various mammalian cell lines (Yi et al., 2003; Azpiazu and Gautam, 2004; Frank et al., 2005; Gibson and Gilman, 2006). These studies have reported contradictory conclusions with regard to the dissociation or non-dissociation of Gα and Gβγ subunits after receptor activation and this may depend on the GPCR-G protein pair.

Later, the investigation of the interaction and activation of GPCR-G protein complexes in real-time became possible through the measurement of FRET or BRET signals between the activating GPCRs themselves and either Gα, Gβ, or Gγ subunits (Gales et al., 2005; Hein et al., 2005; Nobles et al., 2005; Galés et al., 2006; Ayoub et al., 2007, 2010; Hasbi et al., 2007; Qin et al., 2008). These assays are based on the fusion of the energy donor and the energy acceptor with the receptor (generally on its C-terminus) and one of the G protein subunit (α or βγ at some specific position within the G protein subunit) and their co-expression and activation by the agonist (**Figure 1A**; Galés et al., 2006; Ayoub et al., 2007, 2010). Then receptor-G protein interaction and the activation of the complex are assessed either in real-time before and after agonist stimulation or after agonist preincubation depending on the model used (**Figure 1B**).

We will here illustrate such studies based on our recent finding using PAR1 and different effectors. In these studies we used proteins fused to either the energy donor *Renilla* luciferase (Rluc) or the energy acceptor yellow fluorescent protein (YFP). The energy transfer process between Rluc and YFP mainly depends on the distance between the two proteins of interest and/or their relative orientation within the protein complexes (Pin et al., 2008). Thus, the intimate interaction which is supposed to occur between GPCRs and their specific heterotrimeric G proteins constitutes an exciting field of investigation using BRET as a proximity- and conformational-based approach.

To monitor GPCR-G protein interaction and activation, three different assay configurations can be used: (i) the fusion of the receptor with YFP (Receptor-YFP) and the Gα subunit with Rluc (Gα-Rluc) in the presence of untagged Gβ and Gγ subunits (**Figure 2A**), (ii) the fusion of the Receptor-YFP and the Gβ or Gγ subunits with Rluc (Gβ/Gγ-Rluc) in the presence of untagged Gα subunit (**Figure 2B**), and (iii) the fusion of Gα subunit with Rluc (Gα-Rluc) and the Gβ or Gγ subunits with YFP (Gβ/Gγ-YFP) in the presence of untagged GPCR (**Figure 2C**). For each BRET assay configuration, the fusion proteins are transiently co-expressed in cell lines and then the basal BRET signal as well as the agonist-promoted BRET changes are measured in real-time and live cells as previously described (Ayoub et al., 2007, 2009, 2010). Therefore, real-time kinetic and dose-response analysis as well as the application of specific GPCR antagonists or agents targeting G proteins can be performed.

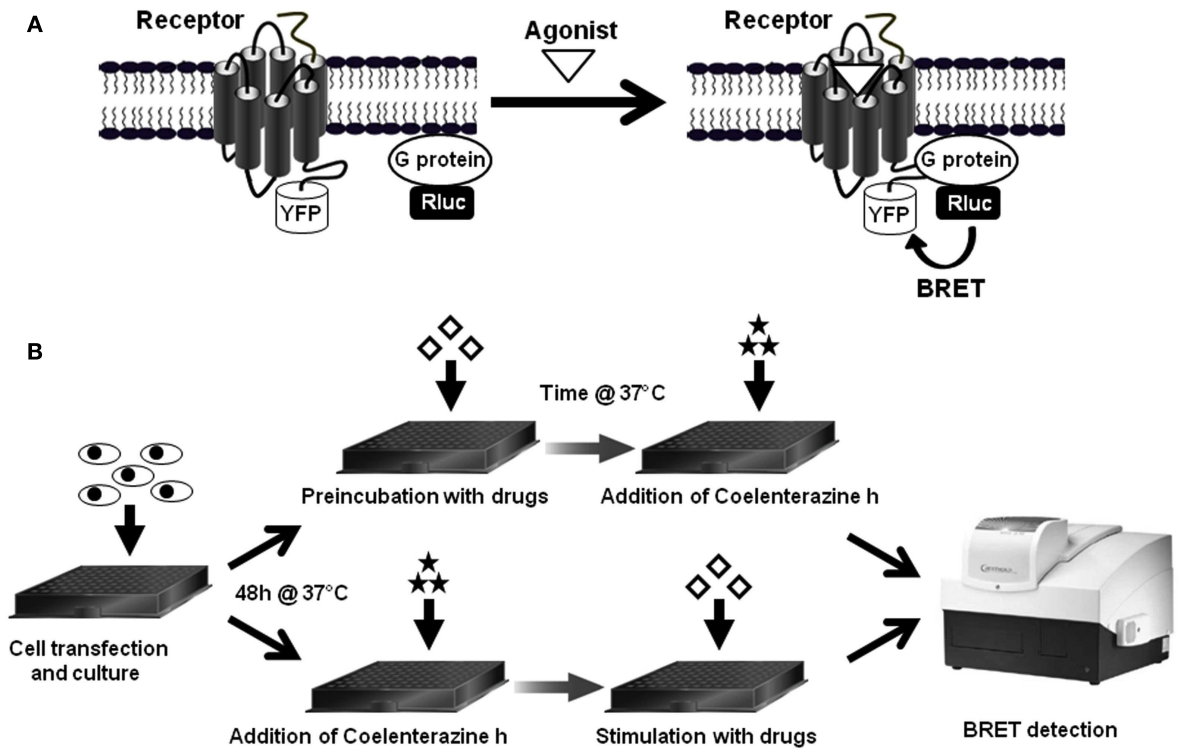

FIGURE 1 | BRET assay to study receptor-G protein interactions in live cells. (A) First, to study the interaction between a GPCR and its cognate heterotrimeric G protein, the G protein subunit (α, β, or γ) is fused to the energy donor, *Renilla luciferase* (Rluc) and the receptor is fused to the energy acceptor, YFP, and then both fusion protein are co-expressed and BRET signal is measured before and after receptor activation, as described previously (Ayoub et al., 2007, 2010). **(B)** The standard BRET protocol is based on cell transfection and culture in BRET compatible 96-well plates and then BRET assay can be performed in two different ways depending on the specificities of the model studied. In the first way, cells are first preincubated with drugs (agonist, antagonist, inhibitor etc.,) and then BRET is measured straightaway after the addition of Rluc substrate, Coelenterazine h. This method can be used for slow and sustained ligand-induced interactions, such as a stable β-arrestin recruitment or to detect irreversible BRET changes within constitutive protein complexes. The second way consists to add Coelenterazine h and measure BRET before any cell stimulation (basal BRET) and then stimulate cells with drugs in the aim to detect any rapid and transient BRET change resulted from the activation of the protein complexes. This method is recommended to detect rapid and reversible conformational changes within receptor-G protein complexes.

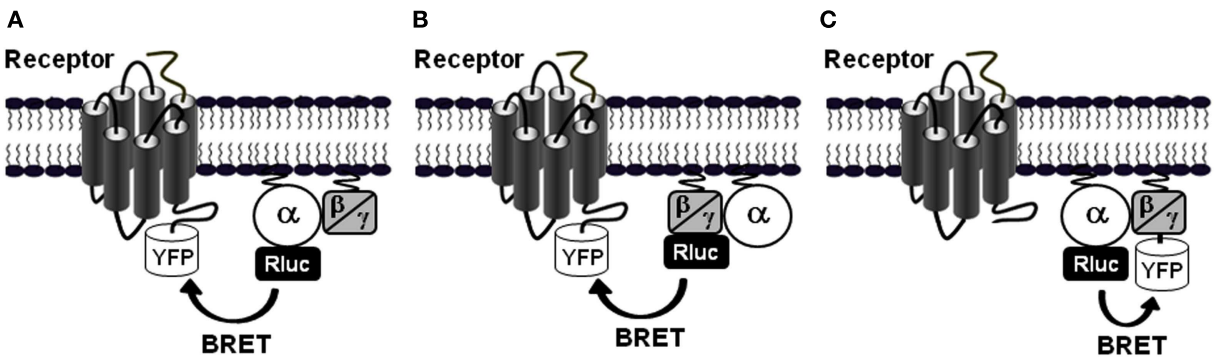

FIGURE 2 | Different BRET assay configurations to study receptor-G protein interactions. To investigate the interaction between GPCRs and the heterotrimeric G proteins and their activation in live cells using BRET, at least three configurations of the assay can be used. **(A)** BRET between the receptor fused to YFP and the Gα subunit, which can be Gαs, Gαi/o, Gαq, or Gα12/13, fused to Rluc in the presence of the untagged β and γ subunits. In this configuration, either BRET increase or decrease can be expected depending on the receptor-G protein pair and the nature of ligand-induced effect, conformational changes or G protein recruitment (Galés et al., 2006; Ayoub et al., 2010). **(B)** BRET between the receptor fused to YFP and either the Gβ or Gγ subunit fused to Rluc in the presence of a specific untagged Gα subunit which could be Gαs, Gαi/o, Gαq, or Gα12/13 (Gales et al., 2005; Ayoub et al., 2009). Similarly to the configuration A, either BRET increase or decrease can be expected depending on the receptor-G protein pair and the ligand-induced conformational changes. **(C)** BRET between a specific Gα subunit fused to Rluc and either Gβ or Gγ fused to YFP in the presence of the untagged receptor. In this configuration, in most cases a BRET decrease is expected but a BRET increase is also possible (Galés et al., 2006).

RECEPTOR-G PROTEIN PRECOUPLING/PREASSEMBLY

From the initial studies performed 30 years ago, it has become well accepted that agonist activation of a GPCR allows its physical association with a heterotrimeric G protein, promoting the GDP/GTP exchange on Gα subunit and G protein activation (De Lean et al., 1980; Limbird et al., 1980; Gether and Kobilka, 1998). It is evident that the study of receptor/G protein interaction has considerably evolved during the recent last years. However, some key questions around this subject are still unresolved and constitute a matter of debate. The main question concerns the dynamics of the receptor-G protein interaction and through the literature two different models have been proposed. The first model called "free collision coupling" model postulates that both GPCRs and G proteins diffuse freely within the plasma membrane, and only the active receptor (i.e., agonist-activated receptor) couple to and specifically activates G proteins (Leff, 1995; Hein and Bunemann, 2009; Lohse et al., 2012). This model corroborates the initial GPCR-G protein model postulating that agonist binding is the prerequisite to GPCR-G protein physical interaction and activation and once activated, the receptor and G proteins dissociate. In addition, the collision model is compatible to some degree with the ternary complex model, where agonist–receptor–G protein complex is stabilized in the absence of guanine nucleotides and GTP addition is supposed to dissociate the ternary complex. This dogma was well accepted for a long time by most of the GPCR scientists. The second model assumes that GPCRs and G proteins are "precoupled" which means here the receptor and G proteins form stable complexes regardless of the activation state of the receptor (Hein and Bunemann, 2009; Lohse et al., 2012). Thus, according to this model the agonist binding promotes receptor activation which leads to conformational changes within the pre-existing receptor-G protein complex resulting in G protein activation without physical dissociation between the receptor and G proteins. Of note, this model has been proposed to explain the high constitutive activity observed with some GPCRs (Lachance et al., 1999; Roka et al., 1999; Seifert and Wenzel-Seifert, 2002). However, other possibilities cannot be excluded such as; (i) preassociated receptors and G proteins which dissociate upon agonist-induced receptor activation, or even (ii) the separated inactive receptors and G proteins which form a stable complex only when the receptor is activated. Therefore, it is possible that GPCR-G protein coupling may be differentially structured; depending upon the specific GPCR, G protein subtype, their expression levels and the cellular system used as discussed previously (Vilardaga et al., 2009; Qin et al., 2011). This will be further discussed later in this review.

Our recent data using BRET to study the physical association of PAR1, with different G proteins revealed the existence of preassociated complex between PAR1 and Gαi1 protein (Ayoub et al., 2007, 2010), and PAR1 and Gαo (Ayoub et al., 2009) when both partners are transiently co-expressed in COS-7 cells. Indeed, a significant and saturable basal BRET signal was measured between Gαi1-Rluc and PAR1-YFP in the absence of receptor activation with thrombin or PAR1 peptide agonists. This finding was in fact not unexpected as it can be reconciled with the "precoupling" model of GPCRs but of course, the observation of a basal BRET signal between two different proteins raises the question with regard to the specificity of such a BRET signal measured under the resting condition. Our different assays and controls using BRET and also time-resolved FRET (TR-FRET) led us to accumulate several lines of evidence demonstrating the specificity of PAR1-Gαi1 association and the constitutive energy transfer signals measured. First, in contrast to the large BRET signal between PAR1 and Gαi1, no significant BRET signal could be detected between PAR1 and Gαs used a negative control and expressed at similar levels than Gαi1 (Ayoub et al., 2007, 2010). Although the absence of BRET does not mean the absence of interaction, since the lack of energy transfer can be the consequence of a perpendicular orientation of the dipoles, the absence of interaction was confirmed using a TR-FRET approach that only relies on the distance since the donor fluorophore cannot be constraint in its orientation (Pin et al., 2008). In addition, under similar conditions the basal BRET signal measured between Gα12- or Gα13-Rluc and PAR1-YFP was also very weak compared to Gαi1-Rluc (Ayoub et al., 2010) and this constitutes an interesting observation since PAR1 is also known to activate Gα12/13 proteins and this point will be discussed later. Second, the quantitative analysis of the expression levels of PAR1 and Gαi1 clearly indicated that the constitutive BRET signal measured cannot be explained by the overexpression of the BRET partners since 100,000 receptor molecules were expressed at the cell surface of COS-7 cells consistent with PAR1 expression in platelets (Ayoub et al., 2007). Third, the basal BRET signal measured between Gαi1-Rluc and PAR1-YFP was nicely saturable and blocked by the overexpression of an untagged Gαi1 protein demonstrating the specificity of the signal observed (Ayoub et al., 2007). Finally, the basal BRET signal between PAR1 and Gαi1 was also confirmed by TR-FRET developed for the first time to study GPCR-G protein interaction using Flag- and Myc-tagged proteins and antibodies conjugated with homogeneous TR-FRET-compatible fluorophores (Ayoub et al., 2010). Together, these observations rule out the possibility of an artifactual signal resulting from the overexpression of the fusion proteins because of the heterologous system used.

Furthermore, the other aspect that we addressed is the plausible link between the basal BRET measured between PAR1 and Gαi1 and any constitutive activity of the receptor-G protein complex. This is important since the precoupling model was proposed following the observation that many GPCRs display constitutive activity, a phenomena that would be consistent with the assembly of G proteins with the non-activated GPCR. We found that the constitutive BRET signal was completely insensitive to pertussis toxin (PTX) treatment which inhibits the Gαi1 protein activation (Ayoub et al., 2007, 2010), such that the assembly observed had nothing to do with G protein activation. Similarly, the basal BRET signal measured between PAR1 and Gαi1 was only partially diminished by BIM46187, a synthetic compound reported to inhibit GPCR-mediated signaling mostly by disrupting the physical association between GPCRs and the Gα subunit (Ayoub et al., 2009). Accordingly, our observation of the preassembly between PAR1 and Gαi1 was not related to any basal activity of the receptor. To clarify this issue, we considered the basal BRET observed as the consequence of a preassembly, rather than a precoupling that would instead be associated with a basal activation of the G protein.

The data obtained with PAR1 using BRET and TR-FRET are consistent with other BRET studies reporting a pre-association of

other GPCRs such as α2A-adrenergic (Galés et al., 2006), δ-opioid (Audet et al., 2008), chemokine CXCR4 and CXCR7 (Levoye et al., 2009) receptors and their cognate G proteins in the absence of receptor activation. Similar observations using other techniques to investigate the interaction between other GPCRs and G protein types have been reported including FRET between various GPCRs (adrenergic α2A, Muscarinic M4, Dopamine 2S, and Adenosine A1) with the Gαo protein (Nobles et al., 2005; Philip et al., 2007) or FRAP between the muscarininc M3 receptor and Gαq protein (Qin et al., 2011) that all support GPCR-G protein preassembly. In this context, we should also include our data using TR-FRET technique showing a preassembly of Gα12 with the serotonin 5HT2c, vasopressin V1a, and muscarinic M1 receptors, but not PAR1, in COS-7 cells (Ayoub et al., 2010). Moreover, the GPCR-Gα preassembly concept can also be supported to some extent by other studies again based on BRET and FRET between Gα and Gβγ revealing a constant proximity between the G protein subunits (Bunemann et al., 2003; Galés et al., 2006). In these studies, it has been reported that GPCR activation promotes a relative movement of βγ and α subunits associated with G protein activation which is illustrated by either a partial increase or a partial decrease in the energy transfer efficiency following the receptor activation, depending on the position where BRET/FRET chromophores are inserted (Bunemann et al., 2003; Galés et al., 2006; Gibson and Gilman, 2006). This hypothesis of a non-dissociation between Gα and Gβγ is supported by the recent report that Gαq and Gβγ are still in proximity after binding of GRK2 as observed in the crystal structure of the Gαq-GRK2 complex (Tesmer et al., 2005).

All these data on GPCR-G protein preassembly in live cells using both BRET and different FRET-based assays are in fact supported by previous biochemical studies using coimmunoprecipitation between receptors and G proteins in the absence of receptor agonists and performed in different cellular backgrounds. This is true for D2 receptor and Gαi (Senogles et al., 1987), β2-adrenergic receptor and Gαs (Lachance et al., 1999), δ-opioid receptor, and Gαi (Law and Reisine, 1997), somatostatin receptor and Gαi and Gαo (Law et al., 1993), AT2 receptor and Gαi (Zhang and Pratt, 1996), melatonin MT1 receptor, and Gαi (Roka et al., 1999). Pharmacological studies have also been useful to demonstrate a tight association between a GPCR and its G protein as shown for the serotonin 5-HT7 preassociated with Gαs even in the absence of agonist (Andressen et al., 2006). As discussed later, such a preassembly is not observed with all GPCR-G protein couples, indicating that this cannot be considered as a general phenomena involved in receptor-G protein coupling, then illustrating the importance and the specificity of such association when it can be observed.

These data then raised the question of the functional significance of the basal BRET observed between a GPCR and its target G protein. The basal BRET signal indicating a close proximity (≤10 nm) between the receptor and the G protein may result from their direct physical interaction even though we were unable to get both PAR1 and Gαi1 coimmunoprecipitated when they are co-expressed in COS-7 or HEK293 cells. This possibility is supported by our data using BIM46187 reported to inhibit GPCR-mediated signaling (Ayoub et al., 2009). Indeed, we have shown by both BRET in live cells and also FRET on purified GPCR and G proteins that BIM46187 inhibits the activation of G proteins by GPCRs by binding to the Gα subunit and thereby blocks its physical interaction with the receptor (Ayoub et al., 2009). BIM46187 partially diminished the basal BRET measured between PAR1 and Gαi1 or Gαo indicating that part of the basal BRET signal reflects the direct or indirect association between the receptor and the G proteins. The other explanation is the colocalization of PAR1 and Gαi1 in specific membrane microdomains where the limited number of G proteins are in close vicinity to the receptor, consistent with a saturable basal BRET signal. In fact, the high density of both the receptor and G proteins in such microdomains (Insel et al., 2005) may favor the energy transfer to occur between the BRET partners. This cannot be completely excluded since we observed that the treatment of cells with Methyl-β-cyclodextrin, which is known to extract cholesterol from the membrane and thereby increases membrane fluidity and disrupts microdomains, significantly increased the basal BRET signal between PAR1 and Gαi1 (unpublished data). Finally, this emerging preassembly theory to explain some specific GPCR-G protein association are also supported by a recent study reporting that large complexes forming by GPCRs, G proteins but also specific effectors are formed early in the endoplasmic reticulum (Dupre et al., 2006). Together, the data obtained with PAR1 and Gαi1 and with other GPCR-G protein pairs are therefore more consistent with the preassembly theory postulating that this specific molecular organization of GPCRs and G protein would allow a faster process of G protein activation since there is no time needed for the receptor and G protein to collide. This is nicely illustrated by our data showing a differential nature of the interaction of PAR1 with either Gαi1 (preassembly) or Gα12 (recruitment; Ayoub et al., 2010). In addition, a slow activation of Gαq by a mutant of the muscarinic M3 receptor which does not preassemble with the G protein has been reported suggesting the importance of GPCR-G protein preassembly in accelerating signaling (Qin et al., 2011). In fact, Qin et al. (2011) proposed that preassembly may have a crucial functional role in native tissues where the expression levels of GPCRs and/or G proteins is lower compared to the overexpression in cell lines. Moreover, GPCR-G protein preassembly may allow a better control of the selectivity of the signaling cascades since one would argue that a preassembled receptor has a limited availability in space (same tissue or cell) and in time (simultaneously) to interact and couple to different G proteins as we nicely demonstrated for PAR1 and Gαi1 and Gα12 (Ayoub et al., 2010). The GPCR-G protein preassembly may also control the efficiency of signaling by sequestering or limiting access to a common G protein pool as this has been shown for CXCR7 attenuating β-adrenergic-mediated Gαs/adenylate cyclase activation (Andressen et al., 2006).

AGONIST-PROMOTED CONFORMATIONAL CHANGES WITHIN THE PREASSEMBLED RECEPTOR-G PROTEIN COMPLEX

The preassembly concept raises many key questions related to the activation mechanism of the preassembled GPCR-G protein complexes and the consequences of the agonist activation on such complexes. What would be the dynamics of the receptor-G protein complexes after receptor activation? Is there any change in the complex number after activation or do GPCR and G proteins associate further or dissociate as a consequence of

agonist-promoted activation? All these questions continue to be a matter of controversy in the GPCR community and this is true for both receptor-G protein interaction and the interaction between the different G protein subunits (α, β, and γ; Hein and Bunemann, 2009; Vilardaga et al., 2009; Lohse et al., 2012). In fact, as mentioned above according to the "precoupling" model if GPCRs are preassembled with their cognate G proteins the number of G proteins available for one receptor is only one or at least very limited assuming that there is no reversible dissociation of the complex. This is determined either during the early stage of protein synthesis and/or somehow during their life in the plasma membrane of cells. However, in the "free collision" model, a receptor has a possibility to interact and activate many G proteins in different coupling/uncoupling cycles as long as the agonist is available to maintain the receptor in its active state at the cell surface.

For PAR1 and Gαi1 (and also Gαo), we observed that despite the constitutive high BRET, the activation of PAR1 with thrombin or PAR1 selective agonist peptides largely increased the BRET signal in a time- and dose-dependent manner (Ayoub et al., 2007, 2009, 2010). This agonist-increased BRET was completely blocked by PTX (Ayoub et al., 2007, 2010), protease inhibitors and PAR1 antagonist, SCH79797 (Ayoub et al., 2007), and a non-selective G protein inhibitor, BIM46187 (Ayoub et al., 2009) clearly demonstrating that agonist-induced BRET increase between PAR1 and Gαi1 reflects the activation of their preassembled complex. The EC_{50} values of thrombin (∼0.5 – 6 nM) and PAR1 selective agonist peptides (∼6 μM) measured in BRET assay are consistent with the activation of PAR1 in a functional assay (Ayoub et al., 2007). Moreover, the kinetic analysis showed a rapid and transient BRET increase between PAR1 and Gαi1 after agonist application ($t_{1/2} = 4.3 \pm 0.6$ s for thrombin; Ayoub et al., 2007, 2010). Indeed, thrombin-induced BRET increase persists for a few minutes before returning to the basal level and the decay kinetic of thrombin-induced BRET between PAR1 and Gαi1 ($t_{1/2} = 6.9 \pm 1.7$ min) paralleled the kinetic of thrombin-promoted β-arrestin 1 recruitment also measured by BRET ($t_{1/2} = 5.4 \pm 0.9$ min; Ayoub et al., 2007) or TR-FRET ($t_{1/2} = 7.5 \pm 1.5$ min; Ayoub et al., 2010) indicating the desensitization of the activated PAR1-Gαi1 complex. Furthermore, the decay of the induced BRET signal did not go beyond the basal level (Ayoub et al., 2007, 2010) further supports the concept of a preassembled receptor-G protein complex being formed even in the absence of activation and non-dissociated after receptor activation. It is important to point out here that the agonist-promoted PAR1-Gαi1 activation measured in BRET assay is still slower compared to what has been reported in the others studies (Hein et al., 2005; Galés et al., 2006) as well as what was expected for GPCR-G protein activation process. This may be due to the limitation of our BRET assay and/or the luminescence reader used for such a kinetic analysis. A difference in the activation kinetic between different GPCRs as reported between the PTH and α2-adrenergic receptors (Vilardaga et al., 2003) or a difference in the feature of the interaction observed between various GPCR-G protein pairs as we found between PAR1 and Gα12 (Ayoub et al., 2010) may be other reasonable explanations. More interestingly, BRET assay allowed us to detect the transition of PAR1-Gαi1 complex from the inactive to the active state in an agonist-dependent manner and this supports the different models implying that GPCRs and G proteins exist in at least two different states (De Lean et al., 1980; Leff, 1995).

How can the rapid and transient agonist-induced BRET increase between PAR1 and Gαi1 be interpreted? Our studies and others clearly agree that such BRET changes reflect conformational changes within the preassembled receptor-G protein complex rather than a further recruitment of G proteins to the activated receptors, resulting from a change of either the distance or the orientation of the chromophores (Galés et al., 2006; Ayoub et al., 2007, 2010; Levoye et al., 2009; **Figure 3A**). First, in BRET saturation assay between PAR1 and Gαi1 no difference in the $BRET_{50}$ value, corresponding to 50% of the BRET saturation value, was observed when cells were stimulated with thrombin (Ayoub et al., 2007), consistent with an absence of any change in their relative affinity, and then arguing against an increase BRET due to further recruitment of new G proteins. However, the maximal BRET signal was largely increased consistent with a movement occurring between Rluc and YFP during activation of the preassembled complex. Second, we observed that depending on the insertion position of BRET donor and acceptor (Rluc and YFP) in the receptor and/or G protein, the BRET increase observed after PAR1 activation was not always detectable (Ayoub et al., 2007). Similarly, Galés et al. (2006) have nicely shown that depending on the position of Rluc within the G protein either an increase or a decrease in BRET signal after receptor activation was observed, not consistent with the recruitment of new G proteins (Galés et al., 2006). Third, a significant basal BRET signal between Gαi1 and a wild-type CXCR4 as well as its mutant (CXCR4-N119K), which cannot activate G proteins, has been reported, indicating that the preassembly between Gαi1 and CXCR4 does not require the activation of the G protein (Levoye et al., 2009). In parallel, the wild-type CXCR4 showed a high basal BRET which can be largely increased after receptor activation (Levoye et al., 2009). Finally, in TR-FRET assay using large fluorophore-conjugated antibodies against Flag and Myc tags, no FRET increase was measured between PAR1-Myc and Gαi1-Flag after stimulation of cells with thrombin whereas thrombin nicely and specifically promoted Gα12-Flag as well as Flag-β-arrestin 1 recruitment to the activated PAR1-Myc (Ayoub et al., 2010). Together, these observations are a demonstration that the agonist-induced BRET changes within PAR1-Gαi1 protein complex cannot be a consequence of further G protein recruitment by the activated receptor, but rather reflects a change in the relative position of the interacting proteins associated with the activation process, leading to a change of either the distance or the orientation of the chromophores (**Figure 3A**).

AGONIST-INDUCED RECEPTOR-G PROTEIN ASSOCIATION

However, some other studies have reported opposite observations with regard to the constitutive association between GPCRs and G proteins suggesting that preassembly is not a general feature of all GPCR and G protein couples. Indeed, a recent study using a variant of BRET-based assay with FKBP-Rapamycin system failed to detect constitutive BRET signal between various class A GPCRs and different G proteins (Kuravi et al., 2010). Similarly, Hein et al. (2005) have reported no specific basal FRET signal between the α2A-adrenergic receptor and Gi in the absence of receptor activation.

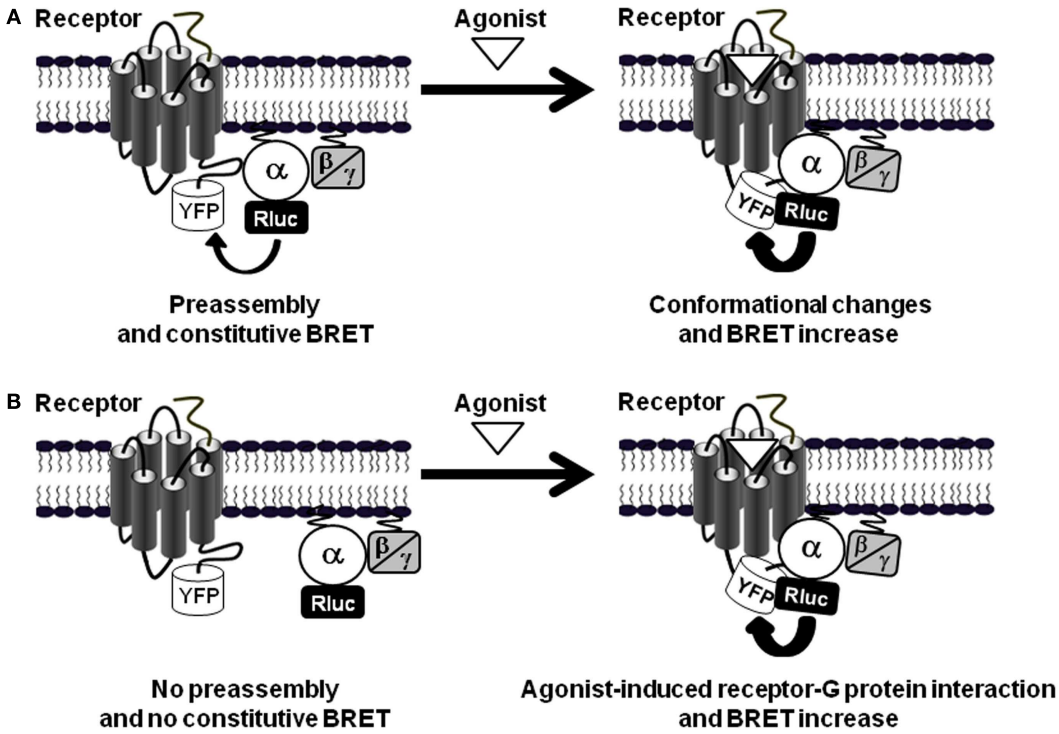

FIGURE 3 | GPCR-G protein preassembly or agonist-induced association. **(A)** In the preassembly model, as shown for PAR1-Gαi1 interaction (Ayoub et al., 2007, 2010), a basal BRET signal is measured between the receptor-YFP and Gα-Rluc fusion proteins. Agonist stimulation induces BRET changes (here indicated by an increase) without changing the receptor-G protein interaction supporting agonist-induced conformational changes within the preassembled complexes. **(B)** The other situation is illustrated by the data obtained with PAR1-Gα12 interaction (Ayoub et al., 2010) where there is no significant basal BRET signal indicating no preassembly and only receptor activation promotes receptor-G protein association and thereby induces BRET signal increase.

In this study and in agreement with the "free collision" model, the expression levels of the G proteins was found to determine the kinetics of receptor-G protein interaction (Hein et al., 2005). Moreover, Qin et al. (2011) have demonstrated that in their inactive state M3R-Gαq complexes are transient and become more stable after receptor activation. Thus, these data clearly sustain the debate around the nature of GPCR-G protein interaction and activation since they support more the "free collision" model than the "precoupling" one. These opposite observations however may have different reasons: (i) GPCRs might differ in their coupling properties due to the differences in their active conformations and the way how they activate different G proteins (Leff and Scaramellini, 1998; Hermans, 2003; Ayoub et al., 2010), (ii) the cellular model used and the expression level of GPCRs and G proteins, and of course (iii) the sensitivities of the different energy transfer-based assays or other techniques used (Lohse et al., 2012).

In this context, our data with PAR1 and its interaction with Gα12 monitored by BRET and TR-FRET have shed some light on understanding the GPCR-G protein coupling (Ayoub et al., 2010). Indeed, in contrast to the PAR1-Gαi1 pair for which we observed preassembly, we found that PAR1 and Gα12 are not preassembled in COS-7 cells as illustrated by very low basal BRET and TR-FRET signals (Ayoub et al., 2010). This was not due to the differences in the expression levels between Gαi1 and Gα12 or the position where the fluorophores were fused within the G proteins. Moreover, the short term activation (up to 2 min) of PAR1 had no effect on the basal BRET signal between PAR1 and Gα12 while a large increase was observed between PAR1 and Gαi1 as mentioned above (Ayoub et al., 2010). However, a long-term activation of PAR1 ($\geq$10–60 min) largely increased both BRET and TR-FRET signals between PAR1 and Gα12, in a time- and dose-dependent manner (Ayoub et al., 2010). Thrombin- as well as PAR1 agonist peptide-induced BRET/TR-FRET increase was specific for PAR1-Gα12 association and it was not observed with Gαs or even Gα13 also known to be activated by PAR1 in many other models. Together, BRET, TR-FRET, and coimmunoprecipitation experiments confirmed the physical recruitment of Gα12 to PAR1 in an agonist-dependent way (**Figure 3B**). In addition, the persistence of PAR1-Gα12 association under Triton X-100 condition used in coimmunorecipitation and TR-FRET assays clearly illustrates the strength of such a physical interaction. These observations suggest that the stable interaction between PAR1 and Gα12 is more compatible with the "free collision model" as observed with other GPCRs using energy-transfer approaches (Hein et al., 2005; Qin et al., 2008; Kuravi et al., 2010). However, this recruitment is very slow compared to what was really expected for GPCR activation kinetics and it is also very stable in time since it was observed even 1 h after receptor activation. Gα12 recruitment to the activated PAR1 is in fact slower than Gα12 activation monitored by p115-RhoGEF translocation to the plasma membrane (Tanabe

et al., 2004). However, the activation of the small G protein RhoA mediated by Gα12/13 proteins has also been reported to occur a few minutes after G protein activation (Tanabe et al., 2004). Unfortunately, we were unable to measure Gα12 activation by any specific readout or signaling assay in a fast enough way to compare the kinetics of Gα12 activation and recruitment to PAR1. It is then still unclear if this slow and sustained recruitment of Gα12 to the activated PAR1 is really associated with its activation in our model, or has other functional meanings. Moreover, the stability of PAR1-Gα12 complex after its formation may be explained by either a non-dissociation of the complex or many rapid association-dissociation cycles which cannot be detected by BRET and as a result a continuous BRET or TR-FRET signal. Therefore, we speculated about the significance of our BRET and TR-FRET data with Gα12 and we argued that the only reason for Gα12 to be recruited to the receptor is indeed its activation by this activated receptor. This is consistent with the strong and sustained developing effects of thrombin and PAR1 on cell morphology and proliferation where stabilizing PAR1-Gα12 interaction and maintaining their long term activation may be crucial for the control of cell proliferation, differentiation, migration, or oncogenesis (Dhanasekaran and Dermott, 1996; Riobo and Manning, 2005). However, one would exclude that the stable Gα12 association with PAR1 may inhibits subsequent receptor activation and via a sequestration process this limits the pool of Gα12 available, thereby reduces the action of Gα12-dependent signaling promoted by other GPCRs as observed with the preassembly of 5-HT7 with Gαs inhibiting this G protein to be activated by the β-adrenergic receptor (Andressen et al., 2006).

Interestingly, the agonist-promoted Gα12 recruitment is a particular feature of PAR1 and cannot be generalized to other receptors reported to couple to Gα12. Indeed, when other GPCRs such as serotonin 5-HT2c, vasopressin V1a, and muscarinic M3 receptors were tested, high constitutive TR-FRET (Ayoub et al., 2010) and BRET (unpublished data) signals was measured between Gα12 and these receptors. Agonist stimulation had no effect on the basal signals again illustrates that the preassembly or agonist-induced G protein recruitment depend on receptor-G protein pairs. Taken together with the data obtained with PAR1-Gαi1 preassembly, these observations illustrate the specificity, diversity and complexity of GPCR-G protein coupling and show how one GPCR (PAR1) can associate differently with two distinct classes of G protein (Gαi1 and Gα12; **Figure 4A**), and on the other hand how a given G protein (Gα12) is differently interacting with distinct GPCRs (PAR1 and 5-HT2c, V1aR or M3R; **Figure 4B**).

THE MULTIPLE COUPLING OF PAR1 TO GαI1, Gα12, AND β-ARRESTIN 1

It is now evident that GPCRs are able to control various physiological responses by promoting diverse signaling pathways via their coupling to different classes of G proteins and other intracellular proteins (**Figure 5**; Hamm, 1998; Hermans, 2003). As elegantly discussed by Hermans the multiple coupling abilities of GPCRs is selectively controlled and regulated at different levels (**Table 1**; Hermans, 2003) and our data on PAR1, Gαi1, Gα12, and β-arrestin 1 and others came to complement the discussion with the new concepts of preassembly and agonist-dependent G protein interaction with some specificities for PAR1.

PAR1 can be considered as an ideal model to study such a multiple coupling since it has been shown to couple to different G proteins including Gαi/o, Gαq, and Gα12/13 as well as to arrestins (Coughlin, 2000; Trejo, 2003). Indeed, our studies using BRET and TR-FRET approaches in live COS-7 cells moved one step further in understanding how the multiple coupling of PAR1 with Gαi1, Gα12, and β-arrestin 1 and its signaling can be regulated and integrated. We found two main differences between the interaction of PAR1 with Gαi1 and Gα12. The first one consists of the nature of the interaction, constitutive (for Gαi1; **Figure 3A**) or agonist-induced (for Gα12; **Figure 3B**), and consequently this results in a second difference in terms of the kinetic of their association with the receptor, rapid, and transient (for Gαi1) or slow and stable (for Gα12; **Figure 6**). Nevertheless, another interesting difference between PAR1-Gαi1 and PAR1-Gα12 coupling has been found at the molecular level. Indeed, we have shown that the last eight C-terminal residues of Gα12 are crucial for its association with PAR1 in an agonist-dependent manner whereas the corresponding region in Gαi1 does not seem to be important

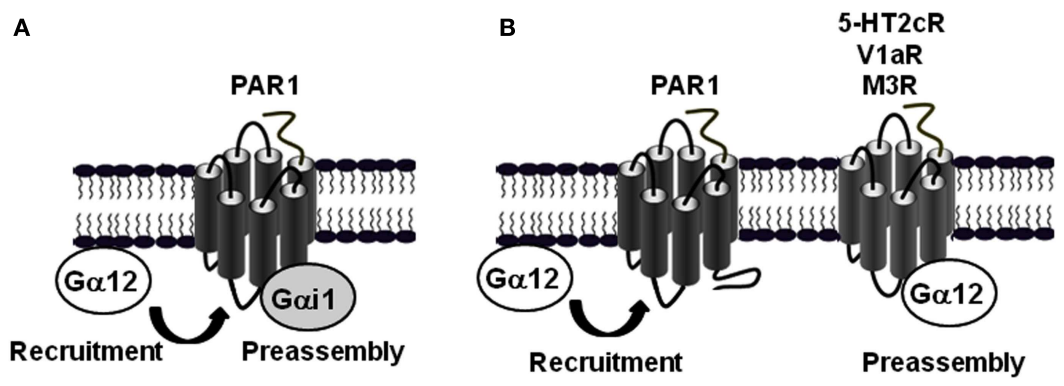

FIGURE 4 | Differential association mode of Gα12 protein with PAR1 and other GPCRs. (A) BRET and TR-FRET data demonstrated that PAR1 is preassembled with Gαi1, but not Gα12 which is only recruited after receptor activation (Ayoub et al., 2010). **(B)** In contrast to PAR1, Gα12 can be preassembled with other GPCRs such as 5-HT2c, V1aR, and M3R (Ayoub et al., 2010).

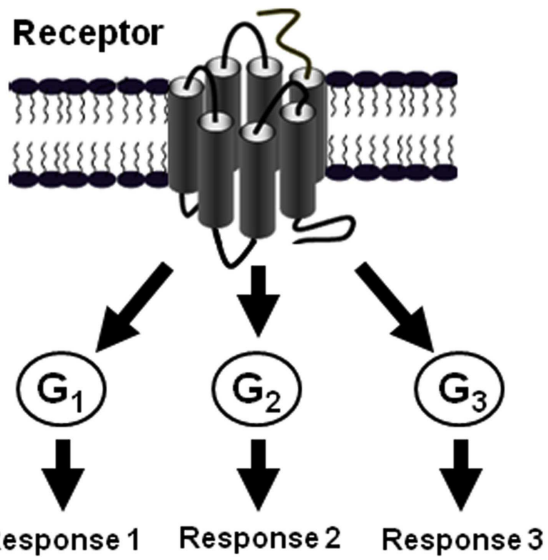

FIGURE 5 | The multiple coupling of GPCRs. The diversity of GPCR signaling as a result of the possibility of a given GPCR to couple and activate G protein of various classes (Hermans, 2003).

Table 1 | The different levels of regulation of the multiple of GPCR-G protein coupling.

Agonist	Receptor	G protein
Nature	Splice variant, RNA editing	Expression level
Potency	Receptor density	Availability
Concentration	Phosphorylation	Compartmentalization
	Palmitoylation	Regulators of G protein signaling (RGS)
	Homo- and heteromerization	Preassembly with the receptor
	Interaction with accessory proteins	
	Preassembly with G proteins	

Adapted from Hermans (2003).

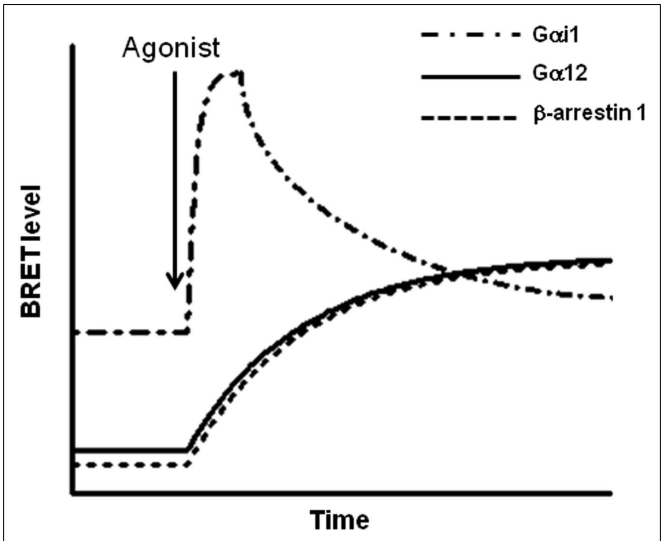

FIGURE 6 | Kinetic profiles of the interaction of PAR1 with Gαi1, Gα12, and β-arrestin 1. Based on BRET analysis, the kinetic of the interaction of PAR1 with Gαi1, Gα12, and β-arrestin 1 has been found to be interestingly different. Indeed, PAR1-Gαi1 interaction is constitutive and agonist stimulation leads to a rapid and transient transition to the activated state of the preassembled complex. The deactivation phase of the preassembled PAR1-Gαi1 complex appears to be parallel to the slow and stable recruitment of both Gα12 and β-arrestin 1 to the activated PAR1. This difference in BRET kinetic analysis clearly suggests a differential mode of association of PAR1 with these different proteins. Furthermore, the data obtained with PAR1 nicely illustrate how the multiple coupling of one GPCR to different signaling proteins can be regulated at the level of the kinetic of the interaction/activation between the receptor and the G proteins.

for preassembly. Moreover, even the mutation of the cysteine at position-4 in Gαi1, known to be crucial for PTX-induced Gαi protein inhibition, had no effect on PAR1-Gαi1 preassembly indicating a secondary role of the C-terminal region of the Gα subunit in the preassembly and suggesting the importance of other molecular determinants within the alpha subunit. Therefore, we propose that the preassembly between PAR1 and Gαi1 does not involve the C-terminal of the Gα subunit, in contrast to PAR1-Gαi1 coupling (or activation) as shown by PTX inhibiting the agonist-induced BRET increase (Ayoub et al., 2007). Furthermore, we observed that the deletion of a large part of PAR1 C-terminus completely inhibited the agonist-induced BRET increase between PAR1 and Gαi1 without affecting the high basal BRET suggesting that the C-terminus of PAR1 likely plays a role in the functional coupling of PAR1 with Gαi1, but not in their preassembly (Ayoub et al., 2007).

Together our observations led us to propose a speculative model based on our observations in COS-7 cells where the key element in PAR1-G protein interaction and regulation, when Gαi1, Gα12, and arrestins are considered in the system, is actually the existence of at least two different populations of receptors (**Figure 7**; Ayoub et al., 2010). Indeed, the first population is exclusively preassembled with Gαi1, but not Gα12, and this is supported by a basal BRET and TR-FRET signals between PAR1 and Gαi1 and the absence of any specific signal between Gαi1 and Gα12 (unpublished data). This preassembled PAR1-Gαi1 population is rapidly and transiently activated probably to control cAMP-dependent signaling in a faster way and the deactivation process which is very important appears to be parallel to the recruitment of β-arrestin 1. Interestingly, we found that PAR1 and Gαi1 do not dissociate even after β-arrestin 1 being recruited to the preassembled complex. Indeed, for the first time we were able to detect very high BRET and TR-FRET signals between Gαi1 and β-arrestin 1 when PAR1 was activated, bringing clear evidence that β-arrestin can be recruited by a receptor still associated with Gαi1 (Ayoub et al., 2010). This was observed only when β-arrestin 1 is recruited to PAR1 since a mutant of PAR1 unable to interact with β-arrestin 1 did not show any specific BRET signal between Gαi1 and β-arrestin1 despite its preassembly with Gαi1. The co-existence of PAR1, Gαi1, and β-arrestin 1, but not Gα12, in a same complex has nicely been confirmed for the first time using a multi-complex

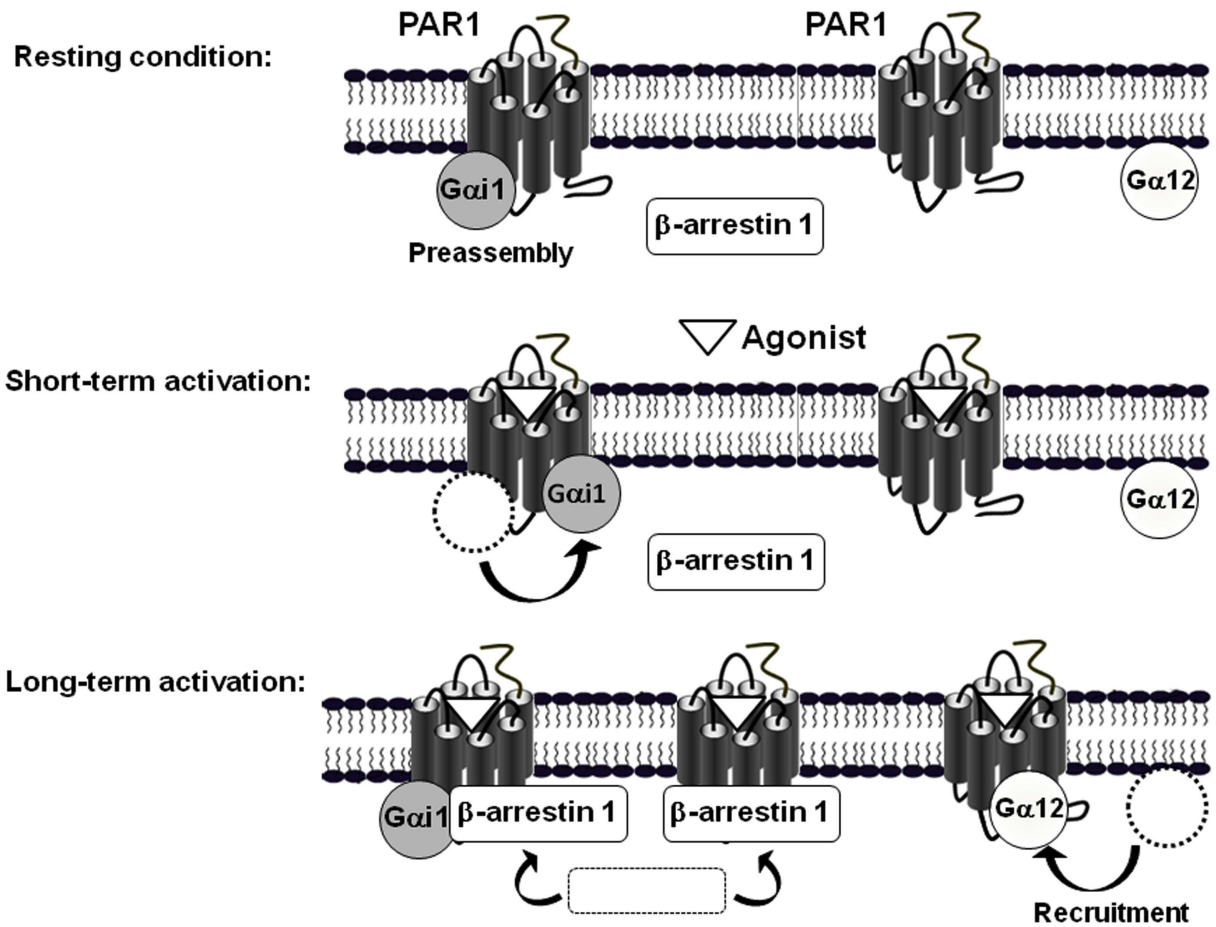

FIGURE 7 | Model of the differential mode of association of PAR1 with Gαi1, Gα12, and β-arrestin 1. The model is based on BRET observations and suggests the existence of two populations of PAR1, at least in COS-7 cells (Ayoub et al., 2010). In the absence of receptor activation, one population of PAR1 is preassembled with Gαi1 and another one would be non-associated with any of Gαi1, Gα12, or β-arrestin 1. A short term activation of PAR1 induces a rapid activation of the preassembled PAR1-Gαi1 complex characterized by a transient change in the relative position of PAR1 and Gαi1 without any change on free PAR1. In contrast, long term activation first results in the deactivation of the preassembled PAR1-Gαi1 complex and in parallel to the concomitant recruitment of both Gα12 and β-arrestin 1 to the activated PAR1. Interestingly, even though their similar kinetic of recruitment, Gα12 and β-arrestin 1 are not co-recruited to the same population of PAR1. Indeed, it has been clearly demonstrated that β-arrestin 1, but not Gα12, can be recruited by the activated PAR1-Gαi1 complex. In addition, it is not excluded that β-arrestin 1 is also recruited by the activated free PAR1. In contrast, after long term activation of PAR1-Gα12 seems to be translocated to the no assembled population of PAR1 only. *(Adapted from Ayoub et al. (2010) Differential association modes of the thrombin receptor PAR1 with Gαi1, Gα12, and β-arrestin 1. The FASEB Journal (2010), 24(9): 3522-3535).*

assay based on TR-FRET approach (Ayoub et al., 2010). Having observed that the agonist-promoted BRET increase disappeared in parallel to β-arrestin 1 recruitment, this finding indicates that the preassembled PAR1-Gαi1 complex is desensitized following β-arrestin 1 recruitment to the preassembled complex, but this does not result in a physical dissociation between PAR1 and Gαi1. Thus, our findings essentially reconcile the GPCR-G protein preassembly concluded from BRET studies in live cells. Although the preassembly may appear consistent with the "precoupling" model, we would like to point out here that our proposed preassembly model does not include basal activation of the G protein, as suggested in the precoupling model. Thus, receptor-G protein "preassembly" does not necessarily mean their "precoupling."

The second population of PAR1 would not be preassembled with any of Gαi1, Gα12, β-arrestin 1. Following PAR1 activation Gα12 is recruited to the activated receptor in slow and prolonged kinetics (**Figure 6**). In contrast to what observed with Gαi1, Gα12 recruitment ($t_{1/2} = 8.8 \pm 1.9$ min) was concomitant to β-arrestin 1 recruitment ($t_{1/2} = 7.5 \pm 1.5$ min; Ayoub et al., 2010) suggesting a co-recruitment of Gα12 and β-arrestin 1 to the same activated receptor. This is unlikely because, neither BRET nor TR-FRET signal was measured between Gα12 and β-arrestin 1 after PAR1 activation (Ayoub et al., 2010). Furthermore, in a BRET competition-based assay we found that the overexpression of β-arrestin 1 significantly reduced PAR1-Gα12 association. In contrast, when Gα12 was overexpressed the agonist-promoted β-arrestin 1 recruitment was not affected at all. In addition, a mutant of PAR1 unable to interact with β-arrestin 1 was still able to recruit Gα12 in an agonist-dependent manner and the inhibition of thrombin-promoted PAR1 internalization, which seems to

be arrestin-dependent, did not affect Gα12 recruitment. Together, these observations indicate a competitive and exclusive recruitment of Gα12 or β-arrestin 1 to the same pool of activated PAR1. In another word, Gα12 and β-arrestin 1 cannot be recruited simultaneously to the same receptor and among the free population of PAR1 molecules there are some receptor interacting with Gα12 only and others with β-arrestin 1 only but not with both. This constitutes an interesting finding which in fact raises many other questions regarding (i) the desensitization of PAR1-Gα12 complex if such a complex cannot recruit arrestins while Gα12 is there, (ii) the significance of the recruitment of β-arrestin 1 to a free PAR1 (is this population non-associated with any G protein?), and finally (iii) the molecular mechanisms involved in such a competitive and exclusive recruitment of Gα12 or β-arrestin 1. Of course this needs further investigations to be clarified.

The existence of two different populations of PAR1 is the only explanation of our BRET and TR-FRET data. This may imply that in the same cell the preassembled PAR1-Gαi1 complex and PAR1 susceptible to recruit Gα12 or β-arrestin 1 exist in different membrane domains. Our data using methyl-β-cyclodextrin support this hypothesis to some extent since we observed that treatment of cells with methyl-β-cyclodextrin largely increased both basal and agonist-induced BRET signal between PAR1 and Gαi1, but had no effect on agonist-induced Gα12 recruitment (unpublished data). The differential recruitment of β-arrestin 1 when PAR1-Gαi1 and PAR1-Gα12 complexes are compared clearly demonstrates the coexistence of at least two populations of PAR1 in COS-7 cells. Of course, the existence of these two different populations may be specific for our cellular model using transient expression in COS-7 cells, but one would hypothesize that this may also occur in native tissues. Thus, the co-expression of both Gαi1 and Gα12 proteins with PAR1 in a same cell type and at the same time, their relative expression levels and the involvement of other accessory intracellular proteins could be the major factors controlling the existence of the two populations of PAR1 and the preassembly or not with the G proteins.

Together, our studies on the physical interaction of PAR1 with Gαi1, Gα12, and β-arrestin 1 using BRET and TR-FRET approaches constitute one step further to better understand GPCR-G protein coupling and again illustrates the complexity of GPCR-G protein coupling. It nicely illustrates that many different processes can be involved in this coupling mechanism, depending on the receptor-G protein couple examined. Therefore, we conclude that the nature of the molecular association between GPCRs and G proteins characterized by either the preassembly or the agonist-dependent recruitment depends on the receptor-G protein pair and this differential association between GPCR and G proteins may constitute a novel way to control the multiple coupling of GPCRs.

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Receptor oligomerization in family B1 of G-protein-coupled receptors: focus on BRET investigations and the link between GPCR oligomerization and binding cooperativity

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The superfamily of the seven transmembrane G-protein-coupled receptors (7TM/GPCRs) is the largest family of membrane-associated receptors. GPCRs are involved in the pathophysiology of numerous human diseases, and they constitute an estimated 30–40% of all drug targets. During the last two decades, GPCR oligomerization has been extensively studied using methods like bioluminescence resonance energy transfer (BRET) and today, receptor–receptor interactions within the GPCR superfamily is a well-established phenomenon. Evidence of the impact of GPCR oligomerization on, e.g., ligand binding, receptor expression, and signal transduction indicates the physiological and pharmacological importance of these receptor interactions. In contrast to the larger and more thoroughly studied GPCR subfamilies A and C, the B1 subfamily is small and comprises only 15 members, including, e.g., the secretin receptor, the glucagon receptor, and the receptors for parathyroid hormone (PTHR1 and PTHR2). The dysregulation of several family B1 receptors is involved in diseases, such as diabetes, chronic inflammation, and osteoporosis which underlines the pathophysiological importance of this GPCR subfamily. In spite of this, investigation of family B1 receptor oligomerization and especially its pharmacological importance is still at an early stage. Even though GPCR oligomerization is a well-established phenomenon, there is a need for more investigations providing a direct link between these interactions and receptor functionality in family B1 GPCRs. One example of the functional effects of GPCR oligomerization is the facilitation of allosterism including cooperativity in ligand binding to GPCRs. Here, we review the currently available data on family B1 GPCR homo- and heteromerization, mainly based on BRET investigations. Furthermore, we cover the functional influence of oligomerization on ligand binding as well as the link between oligomerization and binding cooperativity.

Keywords: GPCRs, family B1, oligomerization, BRET, binding cooperativity

INTRODUCTION

The seven transmembrane G-protein-coupled receptors (7TM/GPCRs) comprise the largest family of membrane receptors. Through coupling to intracellular heterotrimeric G-proteins, the GPCRs mediate cellular responses to a diverse pallet of stimuli including photons, odorants, ions, nucleotides, lipids, neurotransmitters, proteases, and hormones (Bockaert and Pin, 1999). Based on structural and ligand binding criteria, several classification systems for GPCRs have been proposed, such as the A to F system (Kolakowski, 1994), the 1 to 5 system (Bockaert and Pin, 1999), and the GRAFS system (named after the first letters of the five

Abbreviations: αCGRP, α-calcitonin gene-related peptide (19–37); β-AR, β-adrenergic receptor; BiFC, bimolecular fluorescence complementation; BiLC, bimolecular luminescence complementation; BRET, bioluminescence resonance energy transfer; CALCR, calcitonin receptor; CaSR, Ca^{2+} sensing receptor; CCKAR, cholecystokinin A receptor; CCR, chemokine receptor; CFP, cyan fluorescent protein; co-IP, co-immunoprecipitation; CRH, corticotrophin-releasing hormone; CRHR1 and CRHR2, corticotrophin-releasing hormone receptor-1 and -2; CRLR, calcitonin receptor-like receptor; ECD, extracellular domain; ECL, extracellular loop; FRET, fluorescence resonance energy transfer; $GABA_BR$, γ-aminobutyric acid receptor; GCGR, glucagon receptor; GFP, green fluorescent protein; GHRHR, growth hormone-releasing hormone receptor; GIP, glucose-dependent insulinotropic polypeptide; GIPR, glucose-dependent insulinotropic polypeptide receptor; GLP-1 and GLP-2, glucagon-like peptide-1 and -2; GLP-1R and GLP-2R, glucagon-like peptide-1 and -2 receptors; GPCR, G-protein-coupled receptor; GRH, growth hormone-releasing hormone; mGluR, metabotropic glutamate receptor; PACAP, pituitary adenylate cyclase-activating peptide; PAC1, pituitary adenylate cyclase-activating peptide receptor; PTH, parathyroid hormone; PTHR1 and PTHR2, parathyroid hormone receptor-1 and -2; RAMP, receptor activity-modifying protein; *R*luc, *Renilla* luciferase; RXFP1 and RXFP2, relaxin family peptide receptor-1 and -2; SECR, secretin receptor; TR-FRET, time-resolved FRET; TSHR, thyrotrophin receptor; VIP, vasoactive intestinal peptide; VPAC1 and VPAC2, vasoactive intestinal peptide receptor-1 and -2; V1bR, vasopressin V1b receptor; YFP, yellow fluorescent protein; 7TM, seven transmembrane.

families *Glutamate*, *Rhodopsin*, *Adhesion*, *Frizzled*, and *Secretin*; Fredriksson et al., 2003). In the Kolakowski system, the GPCRs are classified into six families, A–F, of which only the families A, B, and C are found in mammalian species (Kolakowski, 1994). These families share little or no inter-family sequence homology, even though the overall morphology of GPCRs is highly conserved (Vohra et al., 2007; Kenakin and Miller, 2010). Family A (rhodopsin-like receptors) is by far the largest family of GPCRs, and most receptors in this family are characterized by very short N- and C-termini. This family includes some of the most extensively studied GPCRs: rhodopsin, the β-adrenergic receptors (β-ARs), and the opioid receptors. Family C (metabotropic glutamate receptors, mGluRs) includes, e.g., the mGluRs, the Ca^{2+} sensing receptor (CaSR), and the receptors for γ-aminobutyric acid ($GABA_BRs$). This family is characterized by a long C-terminus as well as a very long N-terminal domain often containing a "Venus fly-trap" structure responsible for ligand binding (Kristiansen, 2004; Kenakin and Miller, 2010). Family B is subdivided into the subfamilies B1 (secretin-like receptor family), B2 [adhesion family, or "long amino terminus, family B" (LNB-7TM proteins)], and B3 (Methuselah-like family) as proposed by Harmar (2001). According to this classification, the human B1 family of GPCRs includes 15 receptors, all responding to peptide hormones: the secretin receptor (SECR), the glucagon receptor (GCGR), the glucagon-like peptide-1 and -2 receptors (GLP-1R and GLP-2R), the glucose-dependent insulinotropic polypeptide receptor (GIPR), the receptors for parathyroid hormone (PTHR1 and PTHR2), the receptors for vasoactive intestinal peptide (VPAC1 and VPAC2), the pituitary adenylate cyclase-activating peptide receptor (PAC1), growth hormone-releasing hormone receptor (GHRHR), the receptors for corticotrophin-releasing hormone (CRHR1 and CRHR2), the calcitonin receptor (CALCR), and the CALCR-like receptor (CRLR; Harmar, 2001).

The natural ligands for family B1 GPCRs include secretin, glucagon, GLP-1 and GLP-2, glucose-dependent insulinotropic polypeptide (GIP), parathyroid hormone (PTH), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), growth hormone-releasing hormone (GRH), corticotrophin-releasing hormone (CRH), and calcitonin. These peptide hormones and their receptors are currently of considerable interest to the pharmaceutical industry because they are involved in the pathophysiology of human diseases, such as, e.g., diabetes (glucagon, GLP-1, GIP; Knop et al., 2009; Bagger et al., 2011; D'Alessio, 2011), osteoporosis (calcitonin, PTH; de Paula and Rosen, 2010; Verhaar and Lems, 2010), inflammation, and neurodegeneration (VIP, PACAP; Gonzalez-Rey et al., 2005). For a full list of the involvement of family B1 GPCRs in diseases, see **Table 1**.

STRUCTURE AND BINDING MECHANISM OF FAMILY B1 GPCRs

A common structural feature of the family B1 GPCRs is a relatively short C-terminus and a long N-terminal domain of 100–200 residues (George et al., 2002). The characteristic structure of the N-terminal region in family B1 includes two antiparallel β-sheets (β1, β2, β3, and β4), an N-terminal α-helix, and six cysteines that form three disulfide bridges. Furthermore, there is a conserved disulfide bridge between cysteine residues in extracellular loop 1 (ECL1) and ECL2 (Ulrich et al., 1998; Authier and Desbuquois, 2008; Kenakin and Miller, 2010). Like their receptors, the peptide ligands for the family B1 GPCRs are highly similar. They are all relatively short peptides of 27–44 amino acid residues and they are assumed to adopt an α-helical conformation during the contact with their respective receptors (Grace et al., 2004).

The N-terminal end of the receptor extracellular domain (ECD) constitutes the primary receptor–ligand interaction site that determines affinity and specificity through binding of the C-terminal portion of the ligand. This interaction constitutes the first step (step A) of ligand–receptor binding of family B1 in a model proposed by Hoare and Usdin (2001; see **Figure 1**). Subsequently, the ligand N-terminal portion interacts with the juxtamembrane domain of the receptor (step B) resulting in receptor activation (step C; Hoare and Usdin, 2001; Hoare, 2005; Tan et al., 2006).

Recently, crystal structures of ligand-bound ECDs have been determined for several family B1 GPCRs, namely GIP (Parthier et al., 2007), PTHR1 (Pioszak and Xu, 2008), GLP-1R (Runge et al., 2008), CRHR1 and -2 (Pioszak et al., 2008; Pal et al., 2010), and

Table 1 | Potential therapeutical interests of targeting family B1 GPCRs and/or their natural ligands in human diseases.

Ligand	Receptor(s)	Disease(s)	Reference
Glucagon	GCGR	Diabetes	Ali and Drucker (2009), Bagger et al. (2011), D'Alessio (2011)
GIP	GIPR	Diabetes	Knop et al. (2009)
GLP-1	GLP-1R	Diabetes	Deacon (2007), Knop et al. (2009)
GLP-2	GLP-2R	Short bowel syndrome, inflammatory bowel disease	Hornby and Moore (2011)
PTH	PTHR1 and PTHR2	Osteoporosis	de Paula and Rosen (2010), Verhaar and Lems (2010)
Calcitonin	CALCR and CRLR	Osteoporosis	de Paula and Rosen, 2010
VIP	VPAC1, VPAC2, and PAC1	Inflammation, neurodegeneration	Gonzalez-Rey et al. (2005)
PACAP	PAC1, VPAC1, and VPAC2	Inflammation, neurodegeneration	Gonzalez-Rey et al. (2005)
CRH	CRHR1 and CRHR2	Stress	Stengel and Tache (2010), Valdez (2009)
GRH	GHRHR	Dwarfism	Campbell et al. (1995)
Secretin	SECR	Gastrinoma	Ding et al. (2002)

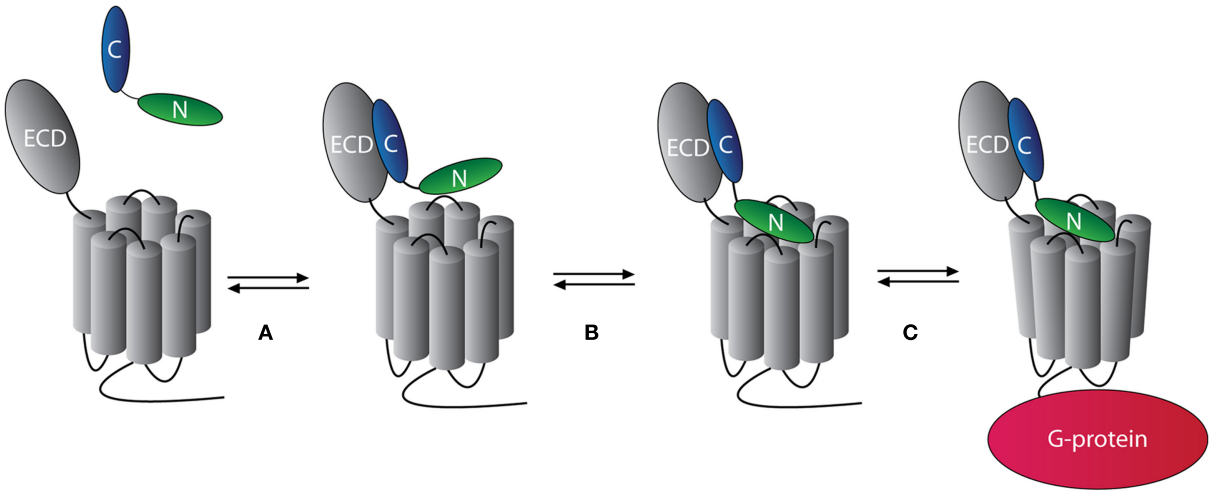

FIGURE 1 | The two-step model of peptide ligand binding to family B1 GPCRs. The binding of one ligand to one family B1 GPCR occurs in two steps. In step **(A)** the C-terminal portion of the ligand (blue) binds to the N-terminal domain of the receptor with high affinity and specificity. In step **(B)**, the N-terminal of the ligand (green) binds to the juxtamembrane domain of the receptor. This activates the receptor and mediates intracellular interaction with G-proteins [step **(C)**]. Modified from Hoare (2005).

CRLR (ter Haar et al., 2010). These structures support the binding model proposed by Hoare and Usdin (2001) in which one ligand binds to one receptor in a two-step process (**Figure 1**). However, the interaction between the N-terminal portion of the ligand and the juxtamembrane domain of the receptor may not be simplified into one single model. Based on studies on SECR, VPAC1, CALCR, and GLP-1R, an alternative model of receptor activation has been proposed, involving the exposure of a hidden epitope in the N-terminal domain of the receptor upon ligand binding that can act as an endogenous agonist ligand (Dong et al., 2006, 2008a,b).

GPCR OLIGOMERIZATION

G-protein-coupled receptors were originally thought to be functional monomers, one receptor interacting with one heterotrimeric G-protein in a 1:1 stoichiometry. Today, however, the phenomenon of GPCR homo- and/or heteromerization is widely accepted, and several studies have revealed important functional roles of GPCR oligomerization. In general, is it difficult to distinguish between dimers and higher order oligomers of GPCRs experimentally. Thus, we will refer to GPCR–GPCR interactions as oligomers throughout this review unless the specific oligomeric state has been determined.

In family C of GPCRs, oligomerization is a fundamental theme; for example, the functional $GABA_BR$ is a heteromer of the two protomers, $GABA_B1R$ and $GABA_B2R$. The $GABA_B1R$ contains the ligand binding site but is dependent on heteromerization with the $GABA_B2R$ protomer for proper transport to the plasma membrane as well as for coupling to the G-protein signaling cascade (Galvez et al., 2001; Comps-Agrar et al., 2011). The crystal structure of the ECD of another family C GPCR, the mGluR, has revealed that this receptor must be expressed on the cell surface as a homodimer in order to be functional because two mGluR protomers in combination is needed to form the ligand binding cleft (Kunishima et al., 2000).

Within family A of GPCRs there are also several examples of functionality of oligomerization (Terrillon and Bouvier, 2004) as well as examples of the involvement of oligomerization in pathophysiological conditions. The possibility that GPCR heteromerization might play an important role in pharmacological diversity was first suggested by studies on the δ- and κ-opioid receptors. Co-expression of these receptors resulted in formation of heteromers with a very low affinity for either the δ- or the κ-selective ligand alone. However, when the two ligands were combined, high affinity was restored, suggesting the occurrence of positive cooperativity (Jordan and Devi, 1999). This is very interesting in the context of drug development because it might imply that selective compounds can be designed that target specific heteromers without affecting the individual protomers (George et al., 2002). Another example comes from the study of platelets from preeclamptic hypertensive women. This study revealed that an increase in the relative number of heteromers between the AT1 receptor for the vasopressor angiotensin II and the B_2 receptor for the vasodepressor bradykinin compared to homomers of the respective receptors is involved in the pathophysiology of preeclampsia (AbdAlla et al., 2001). It should be noted though, that others have been unable to reproduce these notable findings in several cell lines (Hansen et al., 2009; See et al., 2011).

In line with these examples for family A and C GPCRs, oligomerization of family B1 GPCRs is likely to play an essential role in receptor function as well as in the pathophysiology of the diseases listed in **Table 1**. Except for the SECR, which has been extensively studied by Laurence Miller and his research group as described later, not many studies have addressed the functional issue of oligomerization in family B1 of GPCRs. However, given their importance in many severe diseases, including, e.g., diabetes which alone currently affects 346 million people worldwide (WHO, 2011), it is essential to clarify the interactions between these receptors with the prospect of developing new and

better treatments in the future. This review will focus on the current status on homo- and heteromerization of family B1 GPCRs, especially based on bioluminescence resonance energy transfer (BRET) experiments as well as the possible functional link between receptor oligomerization and binding cooperativity.

OLIGOMERIZATION IN THE FAMILY B1 OF GPCRs

METHODS USED TO STUDY OLIGOMERIZATION OF GPCRs

Different approaches have been taken to study GPCR oligomerization over the last two decades. The first GPCR oligomer was indicated by the use of co-immunoprecipitation (Co-IP) by Hebert et al. (1996). This method was used extensively in early GPCR oligomerization studies. However, methods based on resonance energy transfer between two fluorescently/luminescently tagged receptors in living cells quickly gained traction. Today, the method which is being used by far the most in GPCR oligomerization studies is BRET. The principle of this method is relying on the energy transfer between an enzyme energy donor, *Renilla* luciferase (*R*luc), and an energy acceptor usually in the form of either green fluorescent protein (GFP) or yellow fluorescent protein (YFP). In practice, an energy donor-tagged receptor is co-expressed with an energy acceptor-tagged receptor in a cell system. Upon receptor–receptor interaction, which brings the tags into a proximity of <100 Å, energy is transferred from the energy donor to the energy acceptor upon activation of the *R*luc enzyme. This energy transfer results in a measurable acceptor emission signal, the BRET signal, reflecting receptor–receptor interactions (Pfleger and Eidne, 2006). The most convincing application of this method is a BRET saturation experiment. In this setup, a constant concentration of *R*luc-tagged receptor is co-expressed with an increasing concentration of GFP-tagged receptor. For a specific receptor–receptor interaction, the BRET signal will increase with increasing amounts of energy acceptor molecules and produce a saturation curve. In contrast, a non-specific interaction will result in a linear curve, the so-called "bystander BRET," resulting from random collisions of the tagged receptors, thereby providing a negative control. Such a negative control is required for validation of a specific interaction indicated in a BRET saturation experiment (Hamdan et al., 2006).

Fluorescence resonance energy transfer (FRET) is a method based on the same principle as BRET, however, FRET is relying on the energy transfer between two fluorescent proteins, e.g., YFP and cyan fluorescent protein (CFP). As in the case of BRET, FRET has been used in numerous studies on GPCR oligomerization (Ayoub and Pfleger, 2010). Recently, the FRET-principle was improved by including long-lived energy donors such as terbium and europium in the so-called time-resolved FRET (TR-FRET). This technique has the advantage of a lower background and thus a higher signal:noise ratio as compared to normal FRET (Albizu et al., 2010). In addition, FRET is also being used to study GPCR oligomerization by co-localization investigations using confocal microscopy, a principle referred to as morphological FRET (Harikumar et al., 2006).

Bimolecular fluorescence complementation (BiFC) and bimolecular luminescence complementation (BiLC) are two other recently developed fluorescence-based techniques employed in the study of GPCR oligomerization. These techniques apply splitting either a fluorescent or luminescent protein in two parts and tagging two receptors with a part each. Upon receptor–receptor interaction, the two parts of the energy emitting protein will come in close proximity and reassemble into a functional fluorescent or luminescent protein in a spontaneous reaction (Vidi et al., 2011).

All these different techniques have limitations and should in general be combined in order to obtain convincing evidence of GPCR oligomerization. It is, however, not the goal of this review to critically compare the benefits and limitations of these methods for which we refer to reviews on the subject (Milligan and Bouvier, 2005; Kaczor and Selent, 2011). In the following section, the use of these different techniques for studying oligomerization within the family B1 of GPCRs is reviewed.

OLIGOMERIZATION OF THE SECR, THE PROTOTYPICAL FAMILY B1 GPCR

Within the family B1 of GPCRs, the SECR is by far the most thoroughly studied receptor. In 1991, SECR was the first of the family B1 receptors to be cloned (Ishihara et al., 1991). Due to the similarity in sequences and structures of later characterized receptors of this family with the SECR, the B1 subfamily of GPCRs has been named the "secretin-like receptors." Extensive studies on oligomerization properties of this prototypical family B1 GPCR have been carried out by the laboratory of Laurence Miller. SECR homomerization was indicated for the first time by morphological FRET in COS cells and further supported by energy transfer between *R*luc- and GFP-tagged SECRs in BRET studies. Furthermore, this oligomerization was shown to be necessary for the functionality of the SECR since co-expression of a misspliced SECR with wild-type (WT) SECR had a dominant negative effect on receptor function (Ding et al., 2002). Importantly, secretin regulates growth-inhibitory effects through the SECR and the misspliced SECR has been found in gastrinoma. Thus heteromerization between the two, resulting in dominant negative activity of the misspliced SECR on the WT SECR, was speculated to facilitate tumor growth (Ding et al., 2002). This work on the SECR provided the first example of the functional and physiological relevance of oligomerization within the GPCR B1 family.

Using BRET, one cannot readily distinguish between dimers and higher order oligomers. Thus, in order to investigate the oligomeric state of the SECR, Millers group in 2008 used a combination of BiFC and BRET. The results of this study showed energy transfer between two but not three of the fluorescently/luminescently tagged SECRs, indicating that the SECR forms dimers but not higher order oligomers (Harikumar et al., 2008a).

The interaction site between two SECR protomers within a dimer has also been investigated by Miller's group. By competing SECR dimer interactions measured by BRET with the co-expression of isolated SECR transmembrane (TM) sections, the interaction site of a SECR dimer was mapped to TM4 (Lisenbee and Miller, 2006). Further, by the use of mutational studies, the exact interaction site was localized to two specific residues in the lipid-exposed face of TM4, namely Gly243 and Ile247 (Harikumar et al., 2007). Subsequent mutations of these sites enabled comparative studies on ligand binding and signaling on receptor dimers versus monomers. These studies showed that SECR monomers

have similar ligand binding properties as SECR dimers. In contrast, the potency for cAMP production was decreased for the monomer, indicating that the dimeric state of the SECR is required for correct G-protein coupling (Harikumar et al., 2007). Thus, dimerization is important for optimal signaling of the SECR, thereby adding evidence to the physiological and pharmacological importance of family B1 GPCR oligomerization.

Finally, an extensive study using BRET to detect heteromerization between the SECR and other members of the family B1 GPCRs was published in 2008 (Harikumar et al., 2008b). Interestingly, this study revealed a basic constitutive interaction between SECR and almost all other tested family B1 GPCRs, including GLP-1R, GLP-2R, GHRHR, VPAC1 and -2, PTHR1 and -2, and CRLR. This indicates a possible broad communication between receptors of the family B1 GPCRs. The only receptor of this study that turned out not to interact with the SECR was the CALCR. The absence of heteromerization between these receptors was suggested to be caused by the presence of two different residues in the CALCR TM4 (shown to be the dimer interaction site for SECR) not present in any other of the tested family B1 GPCRs (Harikumar et al., 2008b; for further details, see below).

OLIGOMERIZATION OF THE GLUCAGON RECEPTOR FAMILY OF B1 GPCRs

The GCGR family branch of the family B1 GPCRs consists of the structurally related GLP-1R, GCGR, and GIPR, which are all involved in the intricate control of blood-glucose levels (Holst et al., 2011), as well as the GLP-2R.

Homo- and heteromerization studies within this subfamily have been carried out by Schelshorn et al. (2011) using the BRET technique. The most interesting finding of this study was the association of the GLP-1R and the GIPR in a heteromer complex induced by stimulation with GLP-1. This is the first indication of ligand-induced oligomerization in the family B1 GPCRs. The effect of GLP-1 on the GLP-1R/GIPR heteromer assembly seemed to be reversed by GIP stimulation, which led to dissociation of the heteromer complex. This finding was validated by BRET saturation experiments, negative controls, as well as BRET kinetic experiments where the GLP-1-induced heteromerization was found to be very fast occurring within 30 s after ligand addition. Furthermore, a functional role of the GLP-1R/GIPR heteromer was indicated. Upon co-expression of the GLP-1R with the GIPR, GLP-1R signaling was altered and the β-arrestin recruitment was reduced (Schelshorn et al., 2011). These findings indicate a functional interaction between the receptor targets of the two important incretin hormones that together regulate post-prandial blood-glucose levels by potentiating insulin secretion from the pancreatic β-cells (Holst et al., 2009).

In addition to the GLP-1R/GIPR heteromer, Schelshorn et al. (2011) also investigated homo- and heteromerization of the other members of the GCGR family B1 GPCRs. Homomerization of the GLP-1R as well as the GIPR was claimed to be found in BRET saturation experiments. Furthermore, stimulation of these homomers with GLP-1 and GIP, respectively, was found to slightly reduce the BRET signal indicating either a reduced affinity or conformational changes in the receptor homomers upon ligand binding. However, these results were not compared with a negative control nor were there any statistical evaluation supporting this theory in the publication. We have performed similar BRET saturation experiments on GLP-1R homomerization showing a saturation curve but no effect of GLP-1 stimulation (see **Figure 2A**; Roed, 2011). Several attempts to validate this specific receptor–receptor interaction by negative controls were, however, unsuccessful. Thus, it still remains to be firmly established whether GLP-1R homomerization occurs.

Schelshorn et al. (2011) also indicated homomerization of the GCGR as well as the GLP-2R by BRET studies. However, these

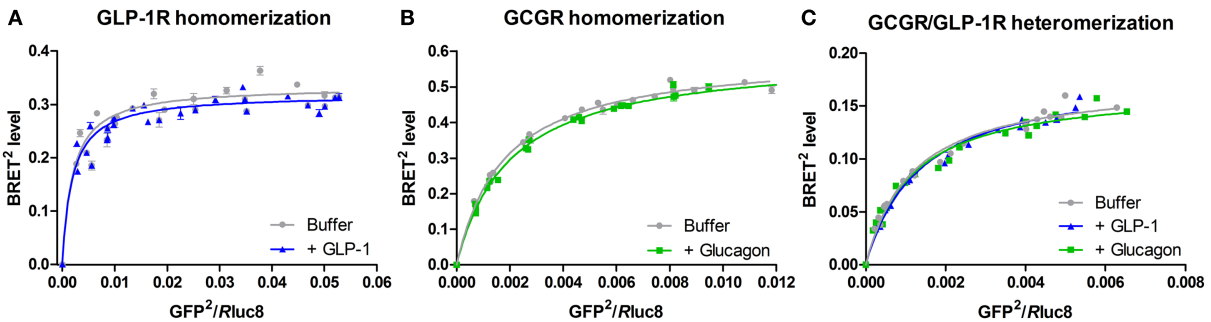

FIGURE 2 | Bioluminescence resonance energy transfer saturation studies on homo- and heteromerization of GLP-1R and GCGR. These BRET studies were performed as $BRET^2$ saturation assays, which uses a spectrally improved form of the luciferase enzyme substrate (coelenterazine), DeepBlueC, as well as a modified form of the energy acceptor, GFP^2 (Pfleger and Eidne, 2006). In addition, a mutationally improved energy donor, *R*luc8, with increased stability and quantum yield was used (De et al., 2007). HEK293 cells were transiently co-transfected with a constant amount of either GCGR-*R*luc8 or GLP-1R-*R*luc8 and an increasing amount of either GCGR-GFP^2 or GLP-1R-GFP^2 and incubated for 48 h at 37˚C and 5% CO_2. Subsequently, the cells were harvested, diluted to a concentration of 1×10^6 cells/ml, and stimulated with either 105 nM GLP-1, 83.5 nM glucagon, or buffer (negative control) for 5 min at room temperature. The $BRET^2$ signal was measured upon addition of the *R*luc8 substrate, DeepBlueC in a Mithras plate reader. All data are plotted as the $BRET^2$ level as a function of the GFP^2/*R*luc8 ratio and fitted to a one-site specific binding model in GraphPad Prism. A saturating curve indicates a specific interaction between the *R*luc- and GFP^2-tagged receptor. The data represent mean ± SD of three independent experiments carried out in quadruplicates. **(A)** GLP-1R-*R*luc8/GLP-1R-GFP^2 homomerization in the presence (blue) or the absence (gray) of 105 nM GLP-1 (Roed, 2011). **(B)** GCGR-*R*luc8/GCGR-GFP^2 homomerization in the presence (green) or the absence (gray) of 83.5 nM glucagon (Orgaard, 2011). **(C)** GCGR-*R*luc8/GLP-1R-GFP^2 heteromerization in the presence of either 105 nM GLP-1 (blue), 83.5 nM glucagon (green), or buffer (gray) (Orgaard, 2011).

results were not validated by BRET saturation experiments nor by negative controls. **Figure 2B** (Orgaard, 2011) shows our own results: a BRET saturation experiment with the GCGR in the presence or the absence of glucagon. A saturation curve was observed indicating homomerization of the GCGR with no effect of ligand addition. However, again, attempts to validate these data by negative controls were unsuccessful.

Finally, heteromerization for all possible interaction pairs of the structurally related receptors of the GCGR family was investigated by Schelshorn et al. (2011), by BRET studies testing both possible combinations of *R*luc/YFP- or YFP/*R*luc-tagged receptor pairs (e.g., both GLP-1R-*R*luc/GCGR-YFP and GLP-1R-YFP/GCGR-*R*luc). A small but significant reducing effect of GLP-1 on GLP-1R/GCGR heteromerization was observed as well as a small and significant reducing effect of both glucagon and GLP-2 on GCGR/GLP-2R heteromerization. These results were, however, not reproducible upon shifting from an *R*luc/YFP-tagged to an YFP/*R*luc-tagged receptor pair. Such differences in the BRET signal upon a shift in the tagging of the receptor pair can result from (1) differences in receptor expression levels influencing the ratio of formed homomers:heteromers or (2) changes in the relative orientation between the two tags known to affect the BRET signal (Ayoub and Pfleger, 2010). Further studies supporting either of the two possibilities were not carried out and thus no conclusion on the effect of ligand stimulation of GLP-1R/GCGR as well as GCGR/GLP-2R heteromers can be drawn from this study. We have performed BRET saturation experiments on cells co-expressing GLP-1R and GCGR resulting in saturation curves (see **Figure 2C**; Orgaard, 2011). Here, no change in the saturation curve was observed upon addition of either GLP-1 or glucagon.

Thus, within the GCGR subfamily an example of the physiological importance of family B1 GPCR oligomerization has been found for the GLP-1R/GIPR heteromer. BRET experiments indicating homo- and heteromerization of the other members of the GCGR subfamily have also been attempted. However, no firm conclusions can be drawn without further investigations including proper negative controls.

OLIGOMERIZATION OF THE "NON-PROTOTYPICAL" AND "NON-GLUCAGON RECEPTOR FAMILY" B1 GPCRs

In addition to the prototypical SECR and the glucagon subfamily receptors, oligomerization among almost all other family B1 GPCRs has now been reported.

An interesting case is the PTHR1. Crystal structures of the isolated ECD of this receptor in its ligand-bound state showed a monomeric receptor with ligand binding in a 1:1 stoichiometry (Pioszak and Xu, 2008; Pioszak et al., 2009). However, in 2010 the crystal structure of the unbound PTHR1 ECD was published, surprisingly showing a dimerization between two ECDs (Pioszak et al., 2010). Interestingly, this dimerization was mediated by the receptor ECD C-termini taking up α-helical structures, similar to the helix of the ligand, PTH. The receptor C-termini bound to the binding site of the opposing PTHR1 protomer in the absence of ligand. This cross-linking is possible due to the sequence similarity between the receptor ECD C-terminal and PTH. The oligomerization of the full-length PTHR1 in the absence of PTH was confirmed by BRET saturation studies as well as morphological FRET in living cells. As predicted from the presence of receptor ECD dimerization only in the ligand unbound state found by crystallography, PTH stimulation of the receptors in BRET studies resulted in dissociation of PTHR1 oligomers. In agreement with receptor monomerization upon ligand binding and activation, the monomeric PTHR1 was found to be sufficient for coupling and activation of G-proteins. In addition, it was shown that PTHR1 oligomerization did not influence either receptor surface expression or ligand binding and signaling (Pioszak et al., 2010). Hence, PTHR1 provides an interesting example of family B1 GPCR oligomerization where constitutive presumably dimeric receptors on the cell surface are disrupted upon ligand binding, and, therefore, likely does not influence the functionality of the receptor. Interestingly, stimulation with PTH has also been shown to disrupt interactions between the SECR and both the PTHR1 and the PTHR2 (Harikumar et al., 2008b) confirming the homomer disruption effects of PTH and the full functionality of a monomeric PTHR. The effects of ligand binding to GPCRs on GPCR oligomerization within family B1 is summarized in **Table 2**.

Another example in family B1 GPCRs where oligomerization does not influence receptor function is the VPAC1 and VPAC2 receptors. These receptors were shown to interact in both homomers and heteromers by BRET studies in 2006 (Harikumar et al., 2006). Later that same year, these interactions were further supported by Co-IP studies (Langer et al., 2006). Similar to the case of PTH binding to the PTHR1, binding of VIP to the VPACs disrupted receptor–receptor interactions (Harikumar et al., 2006). This VIP-induced VPAC oligomer disruption has not yet been followed up by structural investigations as for the PTHR1. Yet, the VPAC oligomerization was shown not to affect either ligand binding, receptor signaling, or internalization. This indicates a monomeric functional VPAC despite the presence of native constitutive receptor oligomers similar to the case of the PTHR1 (Langer et al., 2006).

The PTHR1 and VPAC receptors provide examples of the family B1 GPCRs where oligomerization does not influence receptor functionality. An example of the opposite situation where oligomerization does indeed influence the function of the GPCR can be found in the binding of GHRH to the GHRHR. Oligomerization between this receptor and a splice variant has been confirmed by Co-IP and shown to decrease ligand binding. The reduced ligand binding was not caused by a decreased receptor surface expression indicating that oligomerization has a dominant negative signaling effect on the function of GHRHR (McElvaine and Mayo, 2006). Another example of dominant negative effects of GPCR family B1 oligomerization is found in the CALCR. CALCRs form constitutive homomers which are not influenced by ligand binding as investigated by Co-IP and FRET experiments (Seck et al., 2003). As in the case of the SECR, homomerization of the CALCR was found to be important for the expression and functionality of the receptor, since heteromerization with a CALCR splice variant lead to reduced signaling. Heteromerization with the splice variant receptor thus had a dominant negative effect on the function of the WT CALCR. These studies were carried out using the rabbit CALCR (rCALCR; Seck et al., 2003). Interestingly, in a later study on the human CALCR (hCALCR), BRET experiments did not show any signals significantly different

Table 2 | Oligomerization of the family B1 GPCRs and the effect of ligand binding.

Oligomer	Ligand	Ligand effect on oligomerization	Reference
HOMOMERS			
SECR/SECR	Secretin	None	Ding et al. (2002), Harikumar et al. (2006)
GLP-1R/GLP-1R	GLP-1	None	Orgaard (2011), Roed (2011), Schelshorn et al. (2011)
GIPR/GIPR	GIP	None	Schelshorn et al. (2011)
GCGR/GCGR	Glucagon	None	Orgaard (2011), Roed (2011), Schelshorn et al. (2011)
GLP-2R/GLP-2R	GLP-2	None	Schelshorn et al. (2011)
PTHR1/PTHR1	PTH	Reducing	Pioszak et al. (2010)
VPAC1/VPAC1	VIP	Reducing	Harikumar et al. (2006)
VPAC2/VPAC2	VIP	Reducing	Harikumar et al. (2006)
GHRHR/GHRHR	GRH	N/A	McElvaine and Mayo (2006)
rCALCR/rCALCR	Calcitonin	None	Seck et al. (2003)
CRLR/CRLR	Calcitonin	None	Heroux et al. (2007)
CRHR1/CRHR1	CRH	None	Kraetke et al. (2005), Young et al. (2007)
PAC1/PAC1	PACAP	N/A	Maurel et al. (2008)
HETEROMERS			
SECR/GLP-1R	Secretin	None	Harikumar et al. (2008b)
	GLP-1	None	
SECR/GLP-2R	Secretin	None	Harikumar et al. (2008b)
	GLP-2	None	
SECR/PTHR1	Secretin	Reducing	Harikumar et al. (2008b)
	PTH	Reducing	
SECR/PTHR2	Secretin	Reducing	Harikumar et al. (2008b)
	PTH	Reducing	
SECR/VPAC1	Secretin	None	Harikumar et al. (2006)
	VIP	None	
SECR/VPAC2	Secretin	None	Harikumar et al. (2006)
	VIP	None	
SECR/GHRHR	Secretin	None	Harikumar et al. (2008b)
	GRH	None	
SECR/CRLR	Secretin	None	Harikumar et al. (2008b)
	αCGRP	None	
GLP-1R/GIPR	GLP-1	Inducing	Schelshorn et al. (2011)
	GIP	Reducing	
GLP-1R/GCGR	GLP-1	None	Orgaard (2011), Roed (2011), Schelshorn et al. (2011)
	Glucagon	None	
GLP-1R/GLP-2R	GLP-1	None	Orgaard (2011), Roed (2011), Schelshorn et al. (2011)
	GLP-2	None	
GCGR/GIPR	Glucagon	None	Schelshorn et al. (2011)
	GIP	None	
GCGR/GLP-2R	Glucagon	None	Schelshorn et al. (2011)
	GLP-2	None	
GIPR/GLP-2R	GIP	None	Schelshorn et al. (2011)
	GLP-2	None	
VPAC1/VPAC2	VIP	Reducing	Harikumar et al. (2006)

αCGRP, α-calcitonin gene-related peptide (19–37); N/A, data not available.

from baseline, indicating that the hCALCR does not homomerize (Harikumar et al., 2010). From alignment of the sequences of the rCALCR and the hCALCR a residue (aa 236) in the TM4 of the hCALCR (Arg) differing from rCALCR (His) as well as from all other human family B1 GPCRs was localized. Since TM4 has been assigned the interaction interface of SECR dimers, as previously described (Harikumar et al., 2007), it was speculated whether the lack of hCALCR homomerization was caused by this change in the hCALCR TM4 sequence. In support of this, mutation of the hCALCR TM4 residue to the rCALCR residue (A263H) resulted in a significant increased BRET signal indicating hCALCR homomerization. Further confirming the TM4 as the

CALCR homomerization interface, the co-expression of the isolated hCALCR TM4 with rCALCR reduced the homomerization of rCALCR in a BRET competition experiment (Harikumar et al., 2010). The CALCR thus makes up an interesting example of family B1 GPCR oligomerization showing species-specific differences in the ability to engage in receptor–receptor interactions.

Finally, the CRHR1 has been shown to form constitutive homomers which are unaffected by ligand binding (Kraetke et al., 2005; Young et al., 2007) as well as homomers of the PAC1 and the CRLR receptors have been indicated (Kraetke et al., 2005; Maurel et al., 2008). To our knowledge, the only family B1 GPCR that has not yet been investigated for oligomerization is the CRHR2.

INTER-FAMILY OLIGOMERIZATION OF GPCRs

As mentioned, GPCR families A, B, and C share a highly conserved general morphology, but little or no inter-family sequence homology. Hence, the search for a common sequence responsible for GPCR oligomerization has so far been fruitless (Vohra et al., 2007). Even among receptors from the same family (family A), different combinations of all seven TM domains have been identified as contact points between protomers depending on the receptor examined (GPCR-OKB, 2012). This implies that the specific residues on TM4 responsible for homodimerization of the SECR (Harikumar et al., 2007) and homomerization of CALCR (Harikumar et al., 2010) may not necessarily be the same residues that are involved in homomerization between other family B1 members, or in heteromerization between the SECR or the CALCR and other family B1 GPCRs. Interestingly, as well as intra-family heteromerization has now been demonstrated to be widespread, inter-family heteromerization has also been reported to occur.

There are a couple of reports of GPCR inter-family heteromerization involving family B1 receptors. Functional heteromers, measured by their ability to induce cAMP production upon ligand stimulation, have been reported between two family A members, β_2-AR and opsin, and the family B1 member GIPR (Vrecl et al., 2006). Also, the family A vasopressin V1b receptor (V1bR) has been shown to form constitutive heteromers with the family B1 CRHR1. However, the study did not reveal any obvious effects of heteromerization on the pharmacological properties of V1bR and CRHR1 (Young et al., 2007). In line with these reports, BRET results have suggested that the family B1 GCGR is capable of forming heteromers with the family A cholecystokinin A receptor (CCKAR; Orgaard, 2011).

In addition to the ability of family B1 GPCRs to interact with other types of GPCRs, these receptors may also rely on interactions with other types of membrane proteins for proper function or in order to diversify their physiological functions. It has been shown, that the CALCR and the CRLR as well as the VPAC1 can interact with some of the receptor activity-modifying proteins, RAMP1, -2, and/or -3 thereby assembling a variety of receptor types with different specificities for endogenous peptide ligands or altered signal transduction properties (Christopoulos et al., 2003; Barwell et al., 2011).

Taken together, the accumulating evidence of various types of GPCR heteromers suggests that all GPCRs share a common propensity to heteromerize, and that tissue-specific expression patterns are probably responsible for creating a much greater diversity of GPCR signaling than would have been expected from a 1:1 GPCR:G-protein stoichiometry.

LINK BETWEEN OLIGOMERIZATION AND BINDING COOPERATIVITY IN GPCRs

As described, GPCR oligomerization can have different functional effects on, e.g., receptor expression, signaling, and ligand binding. One example of the functional effects of GPCR oligomerization is the facilitation of allosterism in ligand binding to GPCRs. According to the current knowledge, most GPCRs bind their ligands in a 1:1 ligand:receptor stoichiometry (Hoare and Usdin, 2001; Kristiansen, 2004). One example of an allosteric interaction is cooperativity, in which binding of a ligand to one receptor binding site changes the binding affinity to another receptor binding site and vice versa. This is known as reciprocity and is one of the essential features of allosteric interactions (Gregory et al., 2010). For a detailed review on allostery at GPCR oligomers, Smith and Milligan (2010). The presence of cooperativity in binding of ligands to GPCRs does not correspond to the hypothesis of a 1:1 binding stoichiometry (see **Figure 1**), since more than one binding site is required for allosteric interactions to occur. Thus, cooperativity in GPCR ligand binding constitutes a strong indication of oligomerization (although this assumption has been challenged (Chabre et al., 2009). Cooperativity can be either positive or negative depending on whether the binding of a second ligand to a second receptor binding site increases or reduces the affinity of a pre-bound ligand to the first receptor binding site.

Over the years of GPCR investigations, several examples of negative cooperativity in the binding of ligands to these receptors have been reported. Recently, the presence of this binding phenomenon has been directly linked to GPCR oligomerization.

The classical approach for detection of negative cooperativity is to monitor the dissociation of radioactively labeled ligand in the absence or presence of unlabeled ligand using an "infinite dilution" procedure, as first proposed for the insulin receptor by De Meyts et al. (1973). In this procedure, a small fraction of the surface expressed receptors are pre-occupied by ^{125}I-labeled ligand in an initial association step followed by dissociation in an infinite dilution of either buffer alone or buffer with an excess of unlabeled ligand. In practice, the "infinite dilution" should be sufficient to prevent rebinding of the tracer (De Meyts et al., 1973). If the presence of an excess of unlabeled ligand accelerates the dissociation of pre-bound ^{125}I-labeled ligand, it means that binding of the unlabeled ligand to a second binding site decreases the ligand affinity at the first binding site. This is the hallmark of negative cooperativity (Koshland, 1996).

LINK BETWEEN OLIGOMERIZATION AND BINDING COOPERATIVITY OF GPCRs

In the mid 1970s, many years before the β_2-AR was recognized as a GPCR, negative cooperativity was observed in the binding between this receptor and its ligand (Limbird et al., 1975; Limbird and Lefkowitz, 1976). When the β_2-AR was later classified as a GPCR, a receptor type believed at that time to be a monomeric entity, the earlier finding of negative cooperativity became a matter of dispute (for review, see De Meyts, 1976, 2008). Many years later, the β_2-AR was established to be a functional homomer (Hebert

et al., 1996; Angers et al., 2000), an observation that offers an explanation for the observed negative binding cooperativity. However, no direct correlation between oligomerization and negative cooperativity has yet been established for the β_2-AR. It has even been suggested that the monomeric β_2-AR can fully couple to G-protein and exhibit cooperativity in binding (Whorton et al., 2007).

A direct correlation between negative cooperativity and receptor oligomerization was reported in 2005 for the family A GPCR thyrotrophin receptor (TSHR; Urizar et al., 2005). In this study, a combination of BRET assays and radioligand binding assays revealed that the TSHR homomerizes and displays negative cooperativity in the binding of its ligand (as shown earlier, De Meyts, 1976). In line with the accumulating evidence that most, if not all, family A GPCRs may oligomerize (Terrillon and Bouvier, 2004), this led the authors to speculate that cooperativity is a general phenomenon in family A GPCRs (Urizar et al., 2005). Indeed, several recent investigations have yielded results supporting this concept. In 2005 and 2006, negative cooperativity was found in the binding of chemokines to chemokine receptor (CCR) homo- and heteromers (El-Asmar et al., 2005; Springael et al., 2006). Furthermore, in 2008, a possible link between negative cooperativity and GPCR oligomerization was indicated for the relaxin family peptide receptor 1 (RXFP1) and RXFP2 (Svendsen et al., 2008a,b). Existence of positive cooperativity has also been reported in the binding of ligands to GPCRs. An example of this is a recent study by Gomes et al. (2011) suggesting that heteromers of μ and δ opioid receptors display strong positive cooperativity in ligand binding.

LINK BETWEEN OLIGOMERIZATION AND BINDING COOPERATIVITY IN FAMILY B1 GPCRs

In family B1 GPCRs, the extensive studies of the SECR by Miller and his group have also revealed a role for negative cooperativity in binding of secretin to the SECR. After localizing the specific residues responsible for SECR homodimer formation to TM4 (Harikumar et al., 2007), these residues were mutated producing a monomeric SECR. The dimeric WT SECR was then shown to display negative cooperativity in the binding of secretin, whereas the mutated monomeric SECR had lost this ability (Gao et al., 2009). These results provide the ultimate link between negative cooperativity and GPCR oligomerization. Another example of a link between negative cooperativity and receptor oligomerization of the family B1 GPCRs is indicated for the GLP-1R and GCGRs. As described previously and indicated by BRET saturation studies in **Figure 2**, these receptors might form constitutive oligomers. In radioligand binding assays, an accelerated dissociation of pre-bound ^{125}I-labeled ligand in the presence of excess of unlabeled ligand suggested the existence of negative cooperativity in the binding between GLP-1 and the GLP-1R as well as in the binding between glucagon and the GCGR (**Figure 3**; Orgaard, 2011; Roed, 2011). Negative cooperativity in ligand binding to the GLP-1R and GCGR suggests the presence of at least two binding sites for each of the receptors. Since only one ligand bind per family B1 GPCR, as suggested by Hoare and Usdin, 2001; **Figure 1**), the presence of negative cooperativity supports the otherwise invalidated BRET saturation studies (**Figures 2A,B**; Orgaard, 2011; Roed, 2011) indicating homomerization of GLP-1R and GCGR. Thus, negative cooperativity in ligand binding to GPCRs can provide a functional support for GPCR oligomerization. Since the link between negative cooperativity and receptor oligomerization has been shown for the prototypical family B1 receptor, the SECR, as well as indicated for the GLP-1R and the GCGR, it can be speculated that this is a general phenomenon within family B1. However, this will have to be further investigated.

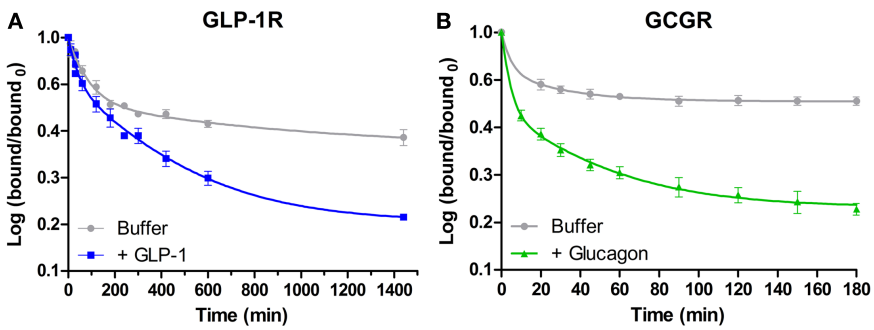

FIGURE 3 | Dissociation experiment investigating negative cooperativity in the binding of GLP-1 and glucagon to the GLP-1R and the GCGR, respectively. These dissociation assays were carried out as described by De Meyts et al. (1973) for the insulin receptor. In this procedure, a small fraction of the surface expressed receptors are pre-occupied by ^{125}I-labeled ligand in an initial association step followed by dissociation in an "infinite dilution" of either buffer of buffer containing an excess of unlabeled ligand. Accelerated dissociation of ^{125}I-labeled ligand in the presence of unlabeled ligand indicates the presence of negative binding cooperativity. The data are plotted as the logarithm of bound/bound$_0$ as a function of time in minutes and fitted to a two-site exponential decay model in GraphPad Prism. All data represent mean ± SD of three independent experiments carried out in duplicates. **(A)** Dissociation of GLP-1 from the GLP-1R. A concentration of 5×10^6 cells/ml BHK cells stably transfected with the GLP-1R were incubated with 150,000 cpm ^{125}I-labeled GLP-1 for 3 h at 15˚C. Subsequently, the unbound ^{125}I-GLP-1 was aspired and the cells were diluted 1:40 in either HEPES binding buffer (gray) or HEPES binding buffer with 167 nM unlabeled GLP-1 (blue) and incubated at 25˚C allowing ligand dissociation for up to 1440 min (24 h) (Roed, 2011). **(B)** Dissociation of glucagon from the GCGR. A concentration of 5×10^6 cells/ml BHK cells stably transfected with the GCGR were incubated with 150,000 cpm ^{125}I-labeled glucagon for 1 h at 15˚C. Subsequently, the unbound ^{125}I-glucagon was removed and the cells were diluted 1:40 in either HEPES binding buffer (gray) or HEPES binding buffer with 167 nM unlabeled glucagon (green) and incubated at 25˚C allowing ligand dissociation for up to 180 min (3 h) (Orgaard, 2011).

SUMMARY AND CONCLUSION

Here, we have reviewed the currently available data on oligomerization of the family B1 GPCRs. Studies on this family have revealed indications on homomerization of almost all members belonging to this subfamily of GPCRs. Even though not all of these studies have been as extensive as the studies on the prototypical family B1 GPCR, the SECR, the accumulating data and evidence points in the direction of homomerization as a general phenomenon for family B1 GPCRs. This is in line with the many publications on oligomerization of other classes of GPCRs (Milligan, 2001; George et al., 2002).

In addition to the increasing evidence of homomerization within family B1 GPCRs, several studies have also indicated heteromerization between different receptors of this family. The extensive study on the SECR indicating heteromerization between this prototypic receptor and almost all other family B1 GPCRs is a good example (Harikumar et al., 2008b). However, the study by Harikumar *et al.* also revealed a single family B1 GPCR that did not interact with the SECR, namely the CALCR with the lack of interaction likely caused by differences in key residues in TM4. Thus, there seems to be exceptions from the rule of a general heteromeric communication network in the family B1 GPCRs. Also, it is important to remember, that the physiological importance of such an intra- and possibly also inter-family cross-talk network for B1 GPCRs is dependent on the co-expression of the involved receptors in native tissue.

Interestingly, despite the many studies on family B1 GPCR oligomerization, no general effect of ligand binding to family B1 oligomers has been found. Indeed, most of these receptors seem to form constitutive oligomers with no effect of ligand binding (**Table 2**). Yet, examples on both the induction (GLP-1R and GIPR; Schelshorn et al., 2011) and disruption of receptor oligomerization (PTHR1; Pioszak et al., 2010 and VPAC1/2; Harikumar et al., 2006) in response to ligand binding have been indicated. This is interesting, since a common effect of ligand binding on GPCR oligomerization would be expected from the common model for the binding mechanism of ligands to family B1 GPCRs, as suggested by Hoare and Usdin (2001; see **Figure 1**).

Even though the many evidences on GPCR oligomerization cannot be neglected, the functionality of these receptor–receptor interactions is controversial and extensively debated. In family B1 GPCRs, only a few examples on GPCR oligomer functionality are available, e.g., the importance of SECR homodimerization for receptor expression and full intracellular G-protein coupling (Harikumar et al., 2007). However, family B1 GPCRs also provide examples on receptors which are fully functional in their monomeric form, such as the PTHR1 (Pioszak et al., 2010).

An important functional aspect of GPCR oligomerization is the possibility of cooperativity in ligand binding to these receptors. A strong link between negative cooperativity and GPCR oligomerization has been found for a family A GPCR, the THSR, as described previously (Urizar et al., 2005). Yet, to our knowledge, the only published data on such a link for the family B1 GPCRs is the SECR (Gao et al., 2009). In addition, we have here presented data indicating a similar link between oligomerization, as indicated by BRET experiments, and negative cooperativity, as indicated by dissociation binding experiments, for the GLP-1R and GCGR (**Figures 2A,B** and **3**; Orgaard, 2011; Roed, 2011). However, to obtain a strong direct link between these two phenomena, further studies like mutational studies and functional complementation studies are required.

In conclusion, homomerization and to some extent also heteromerization of family B1 GPCRs seem to be a general phenomenon. However, only a few studies on the functionality of these receptor–receptor interactions are currently available. The family B1 GPCRs are very important receptors since they are almost all involved in diseases affecting millions of people worldwide, such as diabetes, osteoporosis and chronic inflammation. Thus, further studies are needed to understand the importance of family B1 GPCR oligomerization and its possible role in pathophysiological conditions, in order to improve the development of treatments for these diseases.

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