Cell-surface protein-protein interaction analysis with time-resolved FRET and snap-tag technologies: application to GPCR oligomerization

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Cell-surface proteins are important in cell-cell communication. They assemble into heterocomplexes that include different receptors and effectors. Elucidation and manipulation of such protein complexes offers new therapeutic possibilities. We describe a methodology combining time-resolved fluorescence resonance energy transfer (FRET) with snap-tag technology to quantitatively analyze protein-protein interactions at the surface of living cells, in a high throughput–compatible format. Using this approach, we examined whether G protein–coupled receptors (GPCRs) are monomers or assemble into dimers or larger oligomers—a matter of intense debate. We obtained evidence for the oligomeric state of both class A and class C GPCRs. We also observed different quaternary structure of GPCRs for the neurotransmitters glutamate and γ-aminobutyric acid (GABA): whereas metabotropic glutamate receptors assembled into strict dimers, the $GABA_B$ receptors spontaneously formed dimers of heterodimers, offering a way to modulate G-protein coupling efficacy. This approach will be useful in systematic analysis of cell-surface protein interaction in living cells.

Cell-cell communication involves cell-surface proteins such as receptors, cell adhesion molecules, channels and transporters. Among these proteins, the G protein–coupled receptors (GPCRs) form the largest family of membrane signaling molecules and represent the major target for drug development[1]. Although these 7–transmembrane helix proteins can activate heterotrimeric G proteins in a monomeric form[2–5], their possible assembly into larger complexes[6,7] provokes much interest. GPCRs may not only oligomerize but also associate with other membrane proteins such as channels, enzymes, other receptor types and transporters. Such complexes are proposed to allow faster signaling, specific cross-talks or specific responses. However, such organization of GPCRs remains a matter of intense debate[3, 8–10]. Even if such oligomers exist, their stoichiometry—that is, dimers versus higher-order oligomers—is not known.

Today resonance energy transfer technologies are widely used to validate the proximity of proteins in living cells[11,12]. These approaches are based on the fusion of fluorescence resonance energy transfer (FRET)-compatible GFP variants, or luciferase and GFP for bioluminescence resonance energy transfer (BRET). However, the fusion proteins are often overexpressed in transfected cells such that FRET can occur within intracellular compartments where proteins accumulate, making it difficult to demonstrate that resonance energy transfer results from a direct interaction of proteins at the cell surface.

Another limitation of the commonly used resonance energy transfer techniques is the low signal-to-noise ratio resulting from the intrinsic fluorescence of cells and the overlap between the emission spectra of FRET donors and acceptors. The time-resolved FRET (TR-FRET) approach based on the use of europium cryptate as a donor and Alexa Fluor 647 (Molecular Probes), DY-647 (Dyomics) or d2 (a fluorophore developed by Cisbio to be an optimal acceptor for homogeneous TR-FRET technology; htrf technology from Cisbio) as acceptors offers a much higher signal-to-noise ratio for two main reasons. First, the long lifetime of the europium allows measurement of FRET emission when all natural fluorophores have switched off[13] (**Supplementary Fig. 1a** online), and second, this donor fluorophore has a very limited emission at 665 nm where the acceptor emission is measured[13] (**Supplementary Fig. 1b**).

The limitations imposed by receptor fusion overexpression and cellular autofluorescence can be overcome by the use of antibodies labeled with TR-FRET donors and acceptors. This methodology has been used to validate the existence of GPCR oligomers at the surface of living cells[14–16], but the

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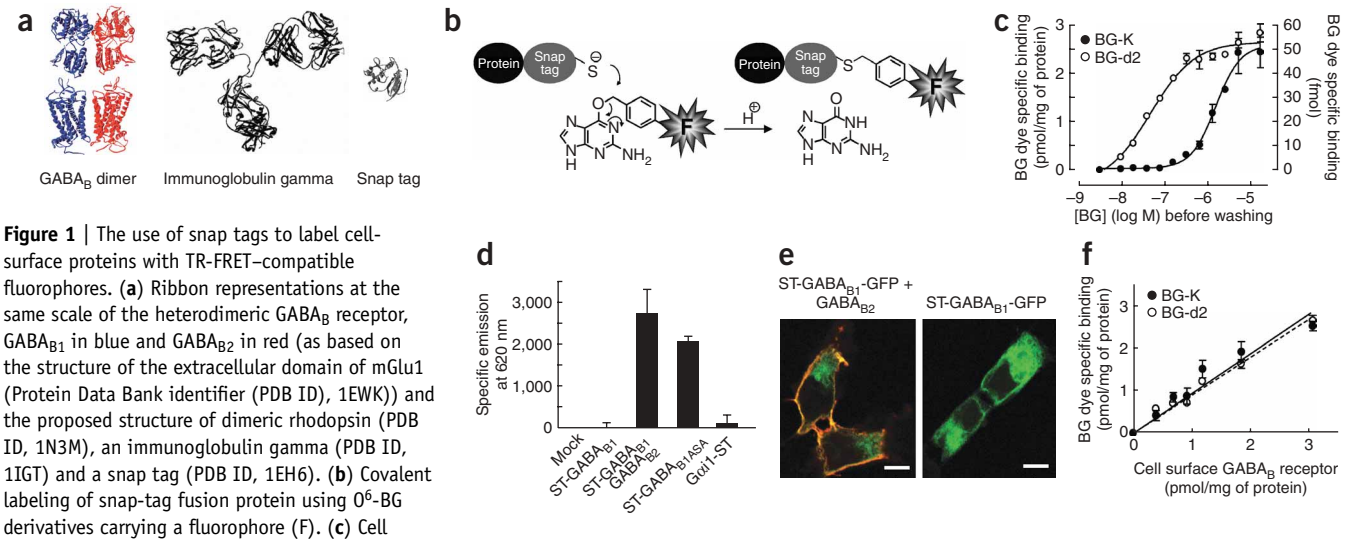

Figure 1 | The use of snap tags to label cell-surface proteins with TR-FRET–compatible fluorophores. (**a**) Ribbon representations at the same scale of the heterodimeric $GABA_B$ receptor, $GABA_{B1}$ in blue and $GABA_{B2}$ in red (as based on the structure of the extracellular domain of mGlu1 (Protein Data Bank identifier (PDB ID), 1EWK)) and the proposed structure of dimeric rhodopsin (PDB ID, 1N3M), an immunoglobulin gamma (PDB ID, 1IGT) and a snap tag (PDB ID, 1EH6). (**b**) Covalent labeling of snap-tag fusion protein using O^6-BG derivatives carrying a fluorophore (F). (**c**) Cell surface–specific binding with indicated concentrations of BG-K or BG-d2 on ST-$GABA_{B1}$ coexpressed with $GABA_{B2}$. Data are expressed as the number of BG-K or BG-d2 fixed to the cells, as deduced from the amount of fluorescence (**Supplementary Fig. 4**) per well (right scale) or per milligram of protein (left scale). (**d**) Specific BG-K labeling of mock-transfected COS-7 cells, and cells expressing the ST-$GABA_{B1}$ alone (ST-$GABA_{B1}$) or with $GABA_{B2}$ (ST-$GABA_{B1}$: $GABA_{B2}$), ST-$GABA_{B1}$ mutated in its intracellular retention motif (ST-$GB1_{ASA}$), and the Gαi1 subunit fused to a snap tag (Gαi1-ST). (**e**) Confocal imaging of HEK 293 cells overexpressing a ST-$GABA_{B1}$-GFP fusion alone or together with $GABA_{B2}$ and labeled with BG-d2. Scale bars, 5 μm. (**f**) Amount of d2 and K fluorophores specifically bound to cells expressing various amounts of ST-$GABA_B$ receptors at the cell surface. The specific number of cell-surface receptors (*B*max) was determined by Scatchard analysis using the $GABA_B$ selective radioligand antagonist [^{3}H]CGP54626. Linear regression revealed a slope (number of fluorophores per $GABA_B$ dimers) of 1.05 and 1.04 for the BG-K and BG-d2 labeling, respectively. Data in **c**, **d** and **f** are means ± s.e.m. of triplicate determinations from a representative experiment.

bivalent nature of antibodies could well stabilize large complexes. Moreover, the large size (150 kDa, 160 Å in length; **Fig. 1a**) and multiple labeling of these proteins can easily increase FRET resulting from random collision.

Here we used the newly developed snap-tag technology from Covalys to specifically label surface proteins with TR-FRET–compatible fluorophores[17,18]. A snap tag derives from the O^6-guanine nucleotide alkyltransferase that covalently reacts with benzyl guanine (BG) (**Fig. 1b**). This tag (**Fig. 1a**), two-thirds the size of GFP, can be specifically and covalently labeled with any fluorophore carried by the benzyl group of BG. By generating nonpermeant BG derivatives compatible with TR-FRET measurement, we confirmed the oligomeric assembly of both class A and class C GPCRs. Using an optimized quality control system that allows the specific labeling of a single subunit in a dimer we showed that the metabotropic glutamate (mGlu) receptors assemble into strict dimers, whereas the γ-aminobutyric acid receptor type B ($GABA_B$ receptor) can form dimers of dimers. This approach will be useful to analyze rapidly and quantitatively, in a high-throughput format, other cell-surface signaling complexes in living cells, allowing the rapid identification of molecules, antibodies or other protein partners affecting these complexes.

RESULTS

Labeling cell-surface receptors with TR-FRET fluorophores

Among the large GPCR family, the $GABA_B$ receptors are composed of two distinct subunits, $GABA_{B1}$ to which agonists bind and $GABA_{B2}$ that activates G proteins[19] (**Fig. 1a**). $GABA_{B1}$ has an intracellular retention sequence in its C-terminal tail that prevents it from reaching the cell surface unless the retention signal is masked through a coiled-coil interaction with the C-terminal tail of the $GABA_{B2}$ subunit[20,21]. Therefore, these receptors constitute an excellent model to test new approaches for quantifying protein-protein interactions at the cell surface.

We introduced a snap tag at the N-terminal end of $GABA_{B1}$ and $GABA_{B2}$ subunits. Both fusion proteins were correctly expressed and showed no alteration of their functional properties (**Supplementary Fig. 2** online). We next prepared BG derivatives carrying either europium cryptate (BG-K; **Supplementary Fig. 1c** and **Supplementary Methods** online), or the acceptor d2 (BG-d2) on the benzyl group. A clear, specific labeling could be detected with these BG derivatives when a snap tag–$GABA_{B1}$ fusion (ST-$GABA_{B1}$) was at the cell surface after coexpression with $GABA_{B2}$, or when its intracellular retention signal was mutated ($GABA_{B1ASA}$; **Fig. 1c–e**). In contrast, we did not observe specific labeling in cells expressing ST-$GABA_{B1}$ alone (**Fig. 1d**), unless the cells were permeabilized (**Supplementary Fig. 3** online). We obtained similar data with the intracellular protein Gαi1 fused to a snap tag (ST-Gαi1; **Fig. 1d**). Fluorescence imaging also confirmed that only the cell-surface snap-tag fusion proteins were labeled (**Fig. 1e**). We used specifically bound fluorescence to estimate the number of snap tags labeled under these conditions, based on the relationship between the fluorescence intensity and the number of fluorophores (**Supplementary Fig. 4** online). By comparing these values with the total amount of binding sites at the cell surface, we found that both BG derivatives labeled all cell-surface receptors over a wide range of expressed receptor concentrations (**Fig. 1f**).

Detecting cell-surface $GABA_B$ heteromers

We used the snap-tag fusion versions of $GABA_{B1}$ and $GABA_{B2}$ to examine whether snap tags could be used to detect protein-protein

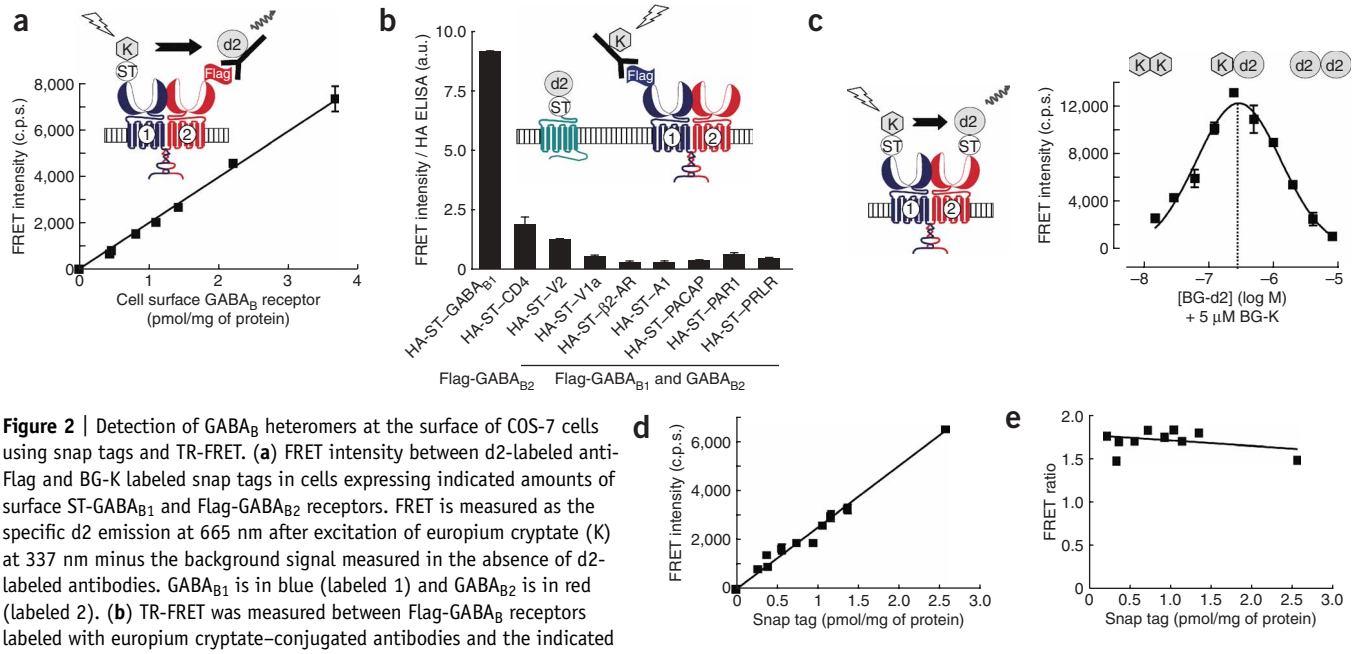

Figure 2 | Detection of GABA$_B$ heteromers at the surface of COS-7 cells using snap tags and TR-FRET. (**a**) FRET intensity between d2-labeled anti-Flag and BG-K labeled snap tags in cells expressing indicated amounts of surface ST-GABA$_{B1}$ and Flag-GABA$_{B2}$ receptors. FRET is measured as the specific d2 emission at 665 nm after excitation of europium cryptate (K) at 337 nm minus the background signal measured in the absence of d2-labeled antibodies. GABA$_{B1}$ is in blue (labeled 1) and GABA$_{B2}$ is in red (labeled 2). (**b**) TR-FRET was measured between Flag-GABA$_B$ receptors labeled with europium cryptate–conjugated antibodies and the indicated HA–snap tag fusion proteins labeled with BG-d2. Data were obtained with the same amount of snap-tag proteins at the cell surface as measured by ELISA with antibodies to the HA epitope and a constant amount of Flag-GABA$_B$ receptors. Positive control (left) was performed with cells expressing ST-GABA$_{B1}$ and Flag-GABA$_{B2}$. (**c**) FRET intensity was measured in cells expressing ST-GABA$_{B1}$ and ST-GABA$_{B2}$ with varying concentration of BG-d2 and 5 μM BG-K. (**d**) FRET intensity as a function of the amount of ST-GABA subunits at the cell surface. The number of snap tags was deduced from the Bmax of [^{3}H]CGP54626. (**e**) FRET efficacy as determined by the ratio of the specific d2 emission at 665 nm resulting from FRET, and the fluorescence intensity (at 620 nm) of the specifically bound BG-K, plotted as a function of the amount of snap tags at the cell surface deduced from the Bmax of [^{3}H]CGP54626. Data in **a** and **b** are means ± s.e.m. of triplicate determinations from a typical experiment. Data in **d** and **e** are triplicate determinations from 4 independent experiments.

interaction at the cell surface. Large TR-FRET signals could be measured in cells expressing ST-GABA$_{B1}$ and Flag-GABA$_{B2}$ after labeling with BG-K and antibodies to the Flag tag (anti-Flag) that contained the d2 acceptor (**Fig. 2a**). We obtained the same result with the BG-d2 and an anti-Flag labeled with europium-cryptate (data not shown), demonstrating that TR-FRET can be used to monitor protein-protein interactions with snap-tag fusion proteins. The simplicity of the approach allowed us to examine the possible interaction of the Flag-tagged GABA$_B$ receptor with several other cell-surface snap-tag fusions (**Fig. 2b**). In six cases, we measured no notable TR-FRET signals (**Fig. 2b**) despite similar expression of all constructs (**Supplementary Fig. 5a** online), demonstrating the specificity of this assay and the very low FRET resulting from random collision of cell-surface proteins under our conditions. We measured a higher, but still low, signal with CD4 and V2 receptors. Although this is consistent with a possible interaction of these proteins with the GABA$_B$ receptor, it is likely that this interaction is not specific.

To avoid the use of antibodies, we also showed that GABA$_B$ heteromers could be detected in cells expressing ST-GABA$_{B1}$ and ST-GABA$_{B2}$, after double-labeling the cells with both BG-K and BG-d2. In that case, we defined conditions to ensure equivalent labeling of the snap tags with either fluorophore. To that end, we used a sub-optimal concentration of BG-K (5 μM) with various concentrations of BG-d2 and determined the optimal ratio of both BG concentrations that gave rise to maximum TR-FRET (**Fig. 2c**). The specific 665 nm emission signal was directly proportional to the amount of receptors at the cell surface (**Fig. 2d**). Under these conditions, the TR-FRET efficacy could be defined as the ratio between the acceptor emission and the amount of donor fluorophore linked to the receptor. Notably, the TR-FRET efficacy was constant over a wide range of receptor density at the cell surface (**Fig. 2e**), demonstrating that this FRET signal did not result from random collision of the labeled proteins but from their physical interaction.

Oligomeric state of other GPCRs and cell-surface proteins

Although class C GPCRs are well recognized as stable dimers, the possible oligomeric state of class A GPCRs is still a matter of intense debate[8,9]. Using N-terminal snap-tag versions of several GPCRs including V2 and V1a vasopressin, β2-adrenergic, A1 adenosine and thrombin (protease activated receptor 1) receptors as well as the class B GPCR for pituitary adenylyl cyclase activating polypeptide (PACAP) and CD4, a membrane receptor with a single transmembrane domain also known to form dimers, we measured large FRET signals that, in terms of efficacy, were in the same range as those observed between both subunits of the GABA$_B$ dimer (**Fig. 3a,b**). All the proteins were expressed with a similar range of expression at the cell surface (**Supplementary Fig. 5b**). The two- to threefold receptor-dependent variation observed in the FRET efficacy was compatible with distance variations between the fluorophores resulting from snap-tag fusion with the N termini of the receptors (see **Supplementary Fig. 6** online for the relation between distances and FRET efficacy). We cannot exclude the possibility that for receptors showing a lower FRET intensity, only a fraction of the receptors were associated in homodimers. However, because of the linear relationship between the TR-FRET intensity and the number of receptors at

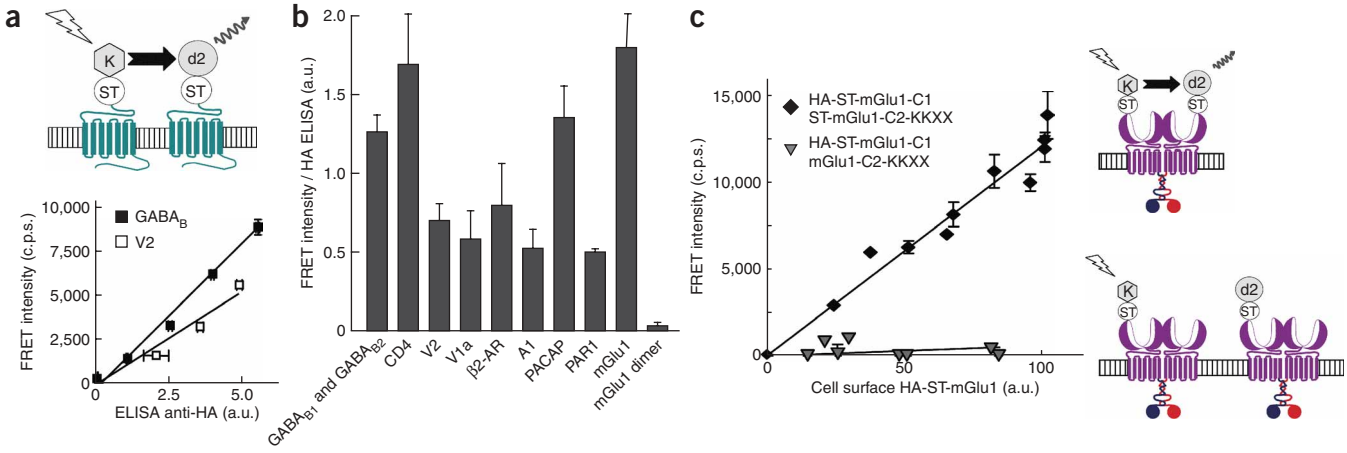

Figure 3 | Detection of cell-surface protein oligomers using snap-tag fusions and TR-FRET. (**a**) Both TR-FRET intensity and anti-HA signal in ELISA were measured for various expression levels of either $GABA_B$ or V2 vasopressin HA-ST fusions. (**b**) Experiments were conducted as in **a** for indicated cell-surface proteins, and mean TR-FRET intensity over the ELISA signal are presented. Negative control (mGlu1 dimer) was performed using a mGlu1 receptor dimer carrying a single snap tag (see **c**). (**c**) FRET intensity as a function of the amount of mGlu1 receptor at the cell surface, when both subunits are fused to snap tags (ST) or when only one subunit per dimer is labeled. To control the subunit composition of the mGlu1 receptor dimer, each subunit contained the C-terminal tail of either $GABA_{B1}$ with its natural intracellular retention signal (blue) or $GABA_{B2}$, in which an intracellular retention signal (KKXX, where X is any amino acid; here, a Lys-Lys-Thr-Asn sequence was used) was added (red). The coiled-coil interaction between the two C-terminal tails prevented intracellular retention of both proteins such that neither subunit reached the surface alone, but did so when coexpressed (**Supplementary Fig. 6**). Data are means ± s.e.m. of triplicate determinations from a representative experiment.

the cell surface, as illustrated for the V2 receptor (**Fig. 3a** and data not shown for the other receptors), the proportion of homodimers is likely to be constant over the range of expression amounts examined. These data further demonstrate that GPCRs can form dimers that are easily detected at the cell surface by this approach.

mGluR1 dimers do not form higher-order oligomers

It is still not clear whether GPCR complexes are limited to dimers or whether higher-order oligomers exist. Here we examined whether the well recognized mGlu receptor homodimers[19] can form higher-order oligomers. To that end we used the optimized quality control system that we recently developed to control the subunit composition of an mGlu dimer without affecting normal functioning of the receptor[22]. This system is based on the use of the $GABA_{B1}$ intracellular tail carrying its native intracellular retention signal (C1) and the $GABA_{B2}$ intracellular tail with the intracellular retention signal Lys-Lys-Thr-Asn inserted after the coiled-coil domain (C2). mGlu receptor subunits carrying either the C1 or the C2 tail did not reach the cell surface on their own, but did so when both subunits were coexpressed in the same cells where they could form coiled-coil interactions that masked both intracellular retention signals[22] (**Supplementary Fig. 7** online). Using this system, we could ascertain that all mGlu dimers at the cell surface carried a single snap tag allowing us, therefore, to detect any possible interaction between mGlu dimers (**Fig. 3c**). We observed no substantial signal when the snap tag was carried by mGlu1-C1 (**Fig. 3c**) or mGlu1-C2 alone (data not shown). This was in contrast to the large signal obtained when both subunits were labeled. This showed that under these conditions mGluR1 complexes were limited to strict dimers. It should be noted that these data confirmed the specificity of the GPCR dimers described above as no FRET signal could be measured between mGlu dimers despite their high level of expression at the cell surface (**Fig. 3c**).

$GABA_B$ heterodimers can form dimers of dimers

Using the snap-tag approach and optimized quality control system, we also analyzed the oligomeric assembly of the $GABA_B$ receptor. In contrast to what we observed with the mGlu1 receptor, we obtained a large TR-FRET signal in cells expressing $GABA_B$ receptors labeled only on their $GABA_{B1}$ subunit (**Fig. 4a**). This signal was close to that measured between $GABA_{B1}$ and $GABA_{B2}$. Again, the TR-FRET efficacy was constant over a wide range of $GABA_B$ receptor expression including at the physiological density of 0.5 pmol receptors/mg protein[23]. We obtained similar data with both $GABA_{B1}$ splice variants, $GABA_{B1a}$ and $GABA_{B1b}$, which differ by the presence of a pair of complement control protein modules (also known as short consensus repeats or sushi domains) at their N termini[24] (data not shown).

We observed very low TR-FRET signal when we labeled only the $GABA_{B2}$ subunits (**Fig. 4a**). This low signal did not result from a peculiar association of these subunits leading to an absence of energy transfer. First, owing to the encaging of europium, the donor dipole is not constrained, such that the low FRET cannot be due to an incompatible dipole-dipole orientation[25]. Second, when expressed alone, $GABA_{B2}$ subunits formed homodimers that could be detected using ST-$GABA_{B2}$ (**Fig. 4b**). This signal was largely inhibited by increasing the amount of $GABA_{B1}$ (**Fig. 4c**), consistent with $GABA_{B1}$ competing with $GABA_{B2}$ in $GABA_{B2}$ homodimers.

These results revealed a close proximity of the $GABA_{B1}$ subunits in $GABA_B$ heterodimers, but not so for $GABA_{B2}$ subunits. This is not consistent with a random clustering or an accumulation of $GABA_B$ heterodimers into microdomains, or with a dissociation-reassociation of the subunits at the cell surface because in those cases similar FRET should be observed between $GABA_{B2}$ subunits and between $GABA_{B1}$ subunits. This is more consistent with a specific organization of the $GABA_B$ heteromers into at least dimers of dimers, interacting via the $GABA_{B1}$ subunit. This model is compatible with the FRET efficacies measured between the different

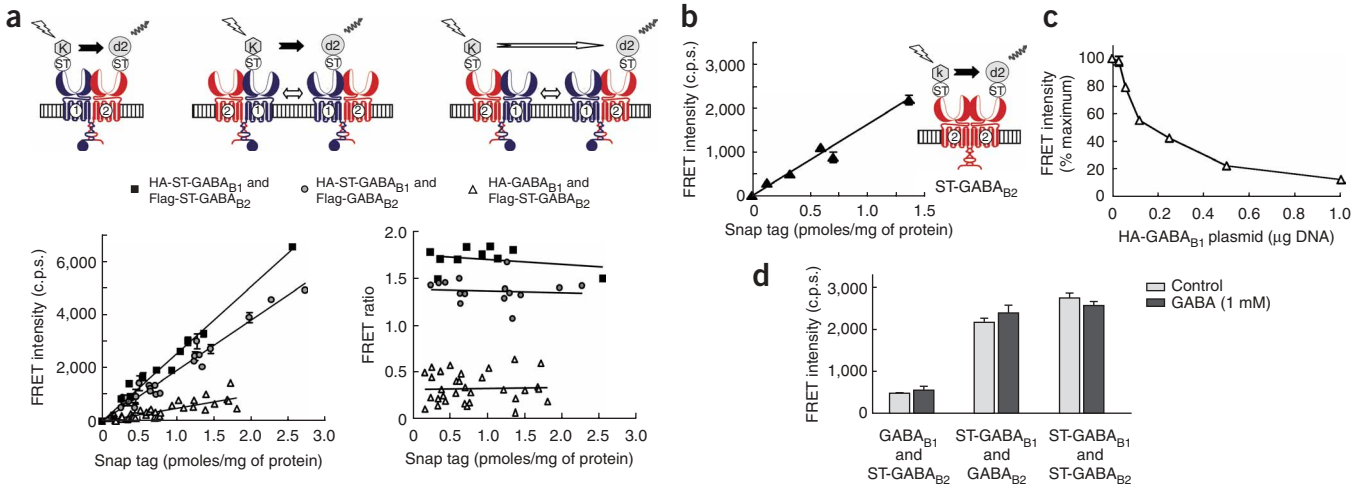

Figure 4 | Detection of $GABA_B$ dimers of dimers at the cell surface. (**a**) FRET intensity (left) and FRET efficacy (right) measured in COS-7 cells expressing various amounts of the $GABA_B$ receptor combinations illustrated in the top schemes: when both subunits carry a snap tag, when only $GABA_{B1}$ carries a snap tag or when only $GABA_{B2}$ carries a snap tag. FRET intensity is plotted as a function of the amount of snap tags at the cell surface deduced from the Bmax of [^{3}H]CGP54626. Data are means ± s.e.m. of triplicate determinations from 4–6 independent experiments. (**b**) FRET intensity as a function of the amount of ST-$GABA_{B2}$ expressed alone. (**c**) FRET between ST-$GABA_{B2}$ subunits as a function of the amount of transfected $GABA_{B1}$ plasmid. (**d**) FRET intensity measured between snap tag–labeled subunits measured under control condition or after a 5-min stimulation with GABA. Data in **b**–**d** are ± s.e.m. of triplicate determinations from a typical experiment.

subunits. Indeed, the R_0 (the distance between the fluorophores giving a 50% FRET efficacy) for the FRET pair used is 65 Å, giving rise to a FRET efficacy of more than 90% according to the Förster equation for a fluorophore distance of 35 Å, corresponding to the distance between the N termini of the two subunits (**Supplementary Fig. 6**). In contrast, a FRET efficacy lower than 20% was calculated for fluorophores more than 80 Å apart, a distance between $GABA_{B2}$ subunits compatible with $GABA_B$ dimers interacting via their $GABA_{B1}$ subunits only (**Supplementary Fig. 6**).

Such a general quaternary structure and organization of the $GABA_B$ oligomer was not influenced by receptor activation as GABA stimulation did not change the TR-FRET signal measured between any subunits of the oligomer (**Fig. 4d**).

Functional validation of $GABA_B$ dimers of dimers

To examine whether the quaternary organization of the $GABA_B$ receptor could be correlated with specific functional properties, we prevented the association between $GABA_B$ dimers using a minimal domain of $GABA_{B1}$ corresponding to the heptahelical domain ($GABA_{B1}$-HD) (**Fig. 5a**). Note that this domain is known not to activate G proteins[19]. This $GABA_{B1}$-HD competed with the full-length $GABA_{B1}$ in the dimer-dimer interaction, as illustrated by the total inhibition of the TR-FRET between ST-$GABA_{B1}$ subunits (**Fig. 5a**). In parallel, we observed an increase in the TR-FRET signal between ST-$GABA_{B1}$ and the N-terminally hemaglutinin (HA)-tagged HA-$GABA_{B1}$-HD, demonstrating that $GABA_{B1}$-HD interacted with the full-length $GABA_{B1}$ subunit (**Fig. 5a**). However, the FRET between $GABA_{B1}$ and $GABA_{B2}$ remained stable (**Fig. 5a**). It is

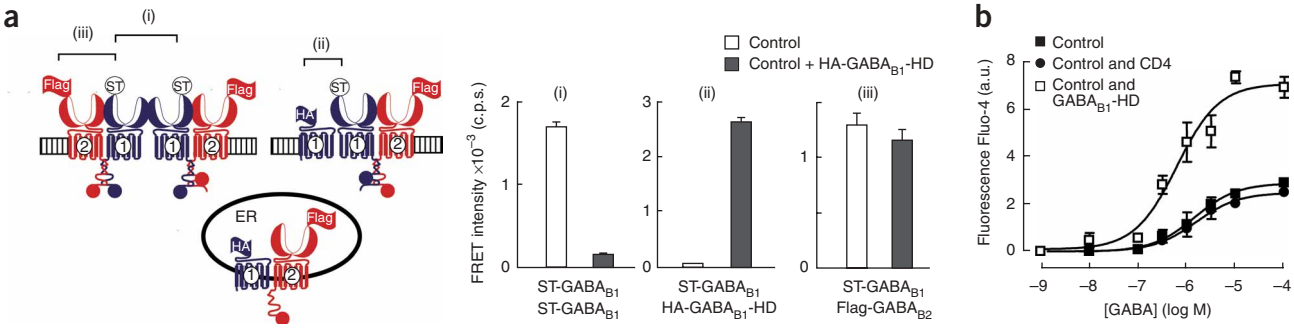

Figure 5 | Functional correlate of the association between $GABA_B$ heterodimers. (**a**) Scheme representing the various combinations of subunits coexpressed: ST-$GABA_{B1}$ (blue) with Flag-$GABA_{B2KKXX}$ (red, the KKXX motif being the intracellular retention sequence added after the coiled-coil domain), with (right) or without (left) the HA-$GABA_{B1}$-HD, corresponding to the $GABA_{B1}$ subunit deleted of both its extracellular and intracellular domains (top). ER, endoplasmic reticulum. TR-FRET intensities measured between two ST-$GABA_{B1}$ labeled with BG-K and BG-d2 (i), between ST-$GABA_{B1}$ labeled with BG-K and HA-$GABA_{B1}$-HD labeled with d2-conjugated anti-HA (ii) and between ST-$GABA_{B1}$ labeled with BG-K and Flag-$GABA_{B2KKXX}$ labeled with d2-conjugated anti-Flag (iii), in the absence (control) and in the presence of HA-$GABA_{B1}$-HD. (**b**) Ca^{2+} signals generated in HEK 293 cells expressing ST-$GABA_{B1}$ and Flag-$GABA_{B2KKXX}$ together (control), in the presence of overexpressed HA-$GABA_{B1}$-HD or in the presence of overexpressed CD4. Data are means ± s.e.m. of triplicate determinations from a typical experiment.

important to point out that we conducted this experiment using a $GABA_{B2}$ subunit carrying an endoplasmic reticulum retention signal, such that any possible heterodimers between $GABA_{B1}$-HD and $GABA_{B2}$ were retained inside the cells because the $GABA_{B1}$-HD lacks the C-terminal tail required to mask the retention signal (**Fig. 5a** and **Supplementary Fig. 8** online). We expected the absence of clear competition between full-length $GABA_{B1}$ and $GABA_{B1}$-HD for interaction with $GABA_{B2}$ because the $GABA_B$ heterodimer is strongly stabilized by (i) the coiled-coil interaction of the C-terminal tails and (ii) the direct interaction of the large extracellular domains[19], two contacts that are absent in a $GABA_{B2}$-$GABA_{B1}$-HD dimer.

Under these conditions, when most dimers of $GABA_B$ heterodimers were dissociated (**Fig. 5b**), and even though we measured the same amount of the G protein–activating subunit $GABA_{B2}$ at the cell surface (data not shown), the maximal agonist-mediated response was twice that measured under control conditions (**Fig. 5b**). We did not observe such an effect after overexpression of CD4 that did not inhibit association between $GABA_B$ dimers (**Fig. 5b** and data not shown). This brings a functional correlate to the quaternary structure of this GABA receptor and suggests that the association of $GABA_B$ dimers into dimers of dimers offers a way to modulate G-protein coupling efficacy.

DISCUSSION

Although FRET and BRET have been widely used to analyze the oligomeric state of membrane proteins, the low signal-to-noise ratio had made it difficult to use such techniques in screening assays. Moreover, it had been difficult to prove that the obtained signals originate from the cell surface. Indeed, even though a large and saturable BRET signal could be measured between $GABA_{B1}$ subunits in the presence of $GABA_{B2}$ (**Supplementary Fig. 9** online), we could not exclude that this signal originated from intracellular $GABA_{B1}$ homodimers[26]. Although imaging techniques and total internal reflection fluorescence microscopy can be used to examine FRET at the plasma membrane, such approaches are not compatible with systematic and quantitative assessments of the interaction. In contrast, the TR-FRET snap-tag technology, by allowing an easy assessment of the protein proximity at the cell surface, enabled a clear demonstration of the specificity of the interaction. Indeed, we conducted the assay in 96-well plates, and it can easily be adapted to 384-well plates as many other TR-FRET cellular assays. Notably, we observed a very low emission of the acceptor when we studied non-interacting proteins, showing that even with overexpressed proteins, very low FRET occurs because of random collision at the cell surface. This suggests that the high nonspecific 'bystander' FRET or BRET measured with GFP- or Rluc-fused membrane proteins is likely to originate from intracellular proteins.

Within the last 10 years many studies have reported that GPCRs can form oligomers, but it was still not known whether such complexes were limited to dimers or whether higher-order oligomers could form[6,7,27]. By taking advantage of an optimized quality control system, we showed here that mGlu1 dimers cannot on their own self associate, demonstrating that a dimeric organization of these receptors is sufficient for function. Of course, one cannot exclude the possibility that, in their native environment, these mGlu dimers can associate into larger complexes through interaction with scaffolding proteins. To our surprise however, we found that the $GABA_B$ receptor heterodimer can form larger oligomers through $GABA_{B1}$ interaction. Because we did not observe close proximity between $GABA_{B2}$ subunits, it is likely that these oligomers are limited to dimers of dimers. Accordingly, as for any other GPCR homodimer, such a quaternary organization of the $GABA_B$ receptor possesses two agonist-binding sites and two possible G-protein coupling domains. Notably, this organization of the $GABA_B$ receptor can be observed over a wide range of receptor density at the cell surface, including that reported for this receptor in the brain[23]. As the receptor density is expected to be even higher in the specific microdomains in neurons, where this receptor is targeted (dendritic spines and pre-synaptic terminals), this makes it likely that what we observed here in transfected cells can also occur *in vivo*, unless specific interacting proteins absent in HEK or COS cells prevent this.

By preventing association of $GABA_B$ heterodimers, using a minimal domain of $GABA_{B1}$, we also provided functional evidence in favor of the dimer of dimers organization of the $GABA_B$ receptor. Our data are consistent with a lower G-protein coupling efficacy of the $GABA_B$ receptor when associated into dimers of dimers. Notably, such a dimerization of the $GABA_B$ heterodimer reproduces what has been recently shown for GPCR homodimers. Indeed, in GPCR homodimers, a single subunit can activate one G protein at a time[2,28–30]. As GPCR monomers can effectively activate G proteins[2–5], then two separated monomers are expected to activate more G proteins than a homodimer. This has been recently demonstrated for both rhodopsin[2] and the neurotensin1 (ref. 30) receptors. Although the stability of GPCR dimers is still debated, one cannot yet exclude the possibility that different ways exist to modulate this process *in vivo* and then to modulate G-protein coupling efficacy. It is also possible that dimerization offers a way for a simultaneous coupling to both G protein-dependent and G protein-independent pathways, but more work is required to validate this idea.

METHODS

Snap-tag labeling and TR-FRET with compatible fluorophores. Twenty-four hours after transfection of HEK 293 cells with plasmids encoding snap-tag fusion proteins, we washed the cells (100,000 per well of a Greiner CellStar 96-well plate) with DMEM and 10% fetal calf serum (FCS) and labeled them with different concentrations of BG conjugated with fluorophores (BG-K or BG-d2) for 1 h at 37 °C, 5% CO_2 in DMEM 10% FCS. We washed the cells four times with Tris-KREBS and measured the specific fluorescence signal of the BG-K and BG-d2 at 620 and 682 nm, with excitation at 337 and 620 nm, respectively (total fluorescence minus that measured with mock transfected cells).

For TR-FRET experiments between snap tags and antibodies, we labeled cells with BG fluorophores (as indicated above) and incubated them overnight at 4 °C in Tris-KREBS buffer containing 2 nM of antibodies conjugated with compatible fluorophore (donor or acceptor). We measured the FRET signal at 665 nm with a 50 µs delay after laser excitation at 337 nm using a Rubystar plate reader (BMG Labtechnologies). We calculated the FRET intensity as (total signal at 665 nm) – (background at 665 nm), where the background signal corresponds to cells labeled with the donor fluorophore alone.

To perform TR-FRET experiments between two snap tags, we incubated the cells with a mix of BG-K and BG-d2. Here the FRET signal is the signal recorded on BG-K and BG-d2 labeled cells from which we subtracted the signal recorded on the same cells labeled with BG-K and a cold BG used at the same concentration as BG-d2.

Additional methods. Descriptions of plasmid construction, cell culture and transfection, enzyme-linked immunosorbent assay (ELISA), intracellular calcium measurements, confocal imaging, binding assay, BG-K synthesis and determination of the R_0 are available in **Supplementary Methods**.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

D.M. and L.C.A. executed most of the experiments and participated in the writing of the manuscript; C.B. developed the system to control subunit composition and performed the confocal experiments; M.L.R. performed the experiments with mGlu1 receptor and the initial experiments with class A receptors; E.B. and H.B. synthesized the BG derivatives; M.A. participated in the BRET experiments; N.T. and E.T. supervised the work at Cisbio; T.D. and L.P. supervised some aspects of the work at the IGF; and J.P.P. supervised the project and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/.

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