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

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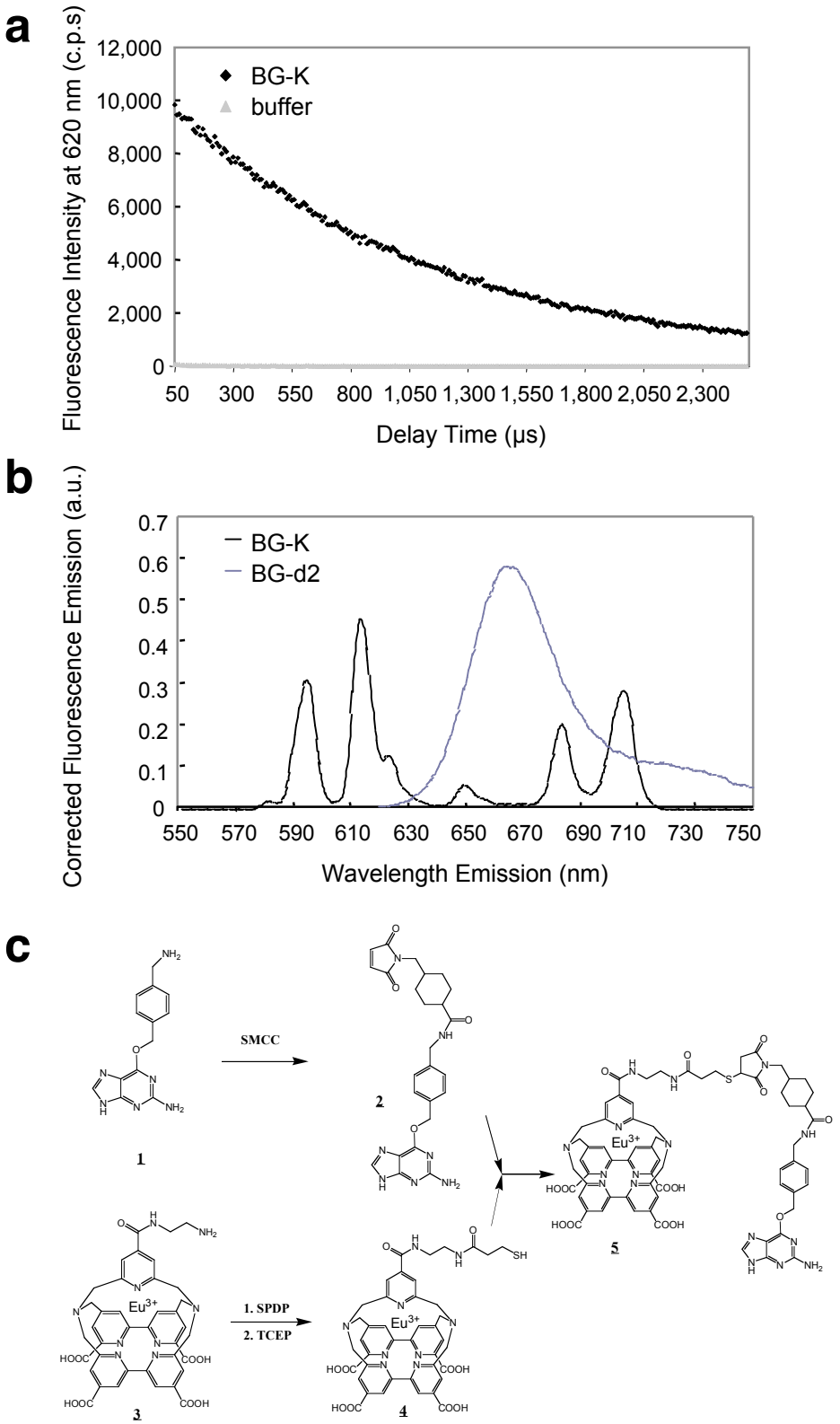
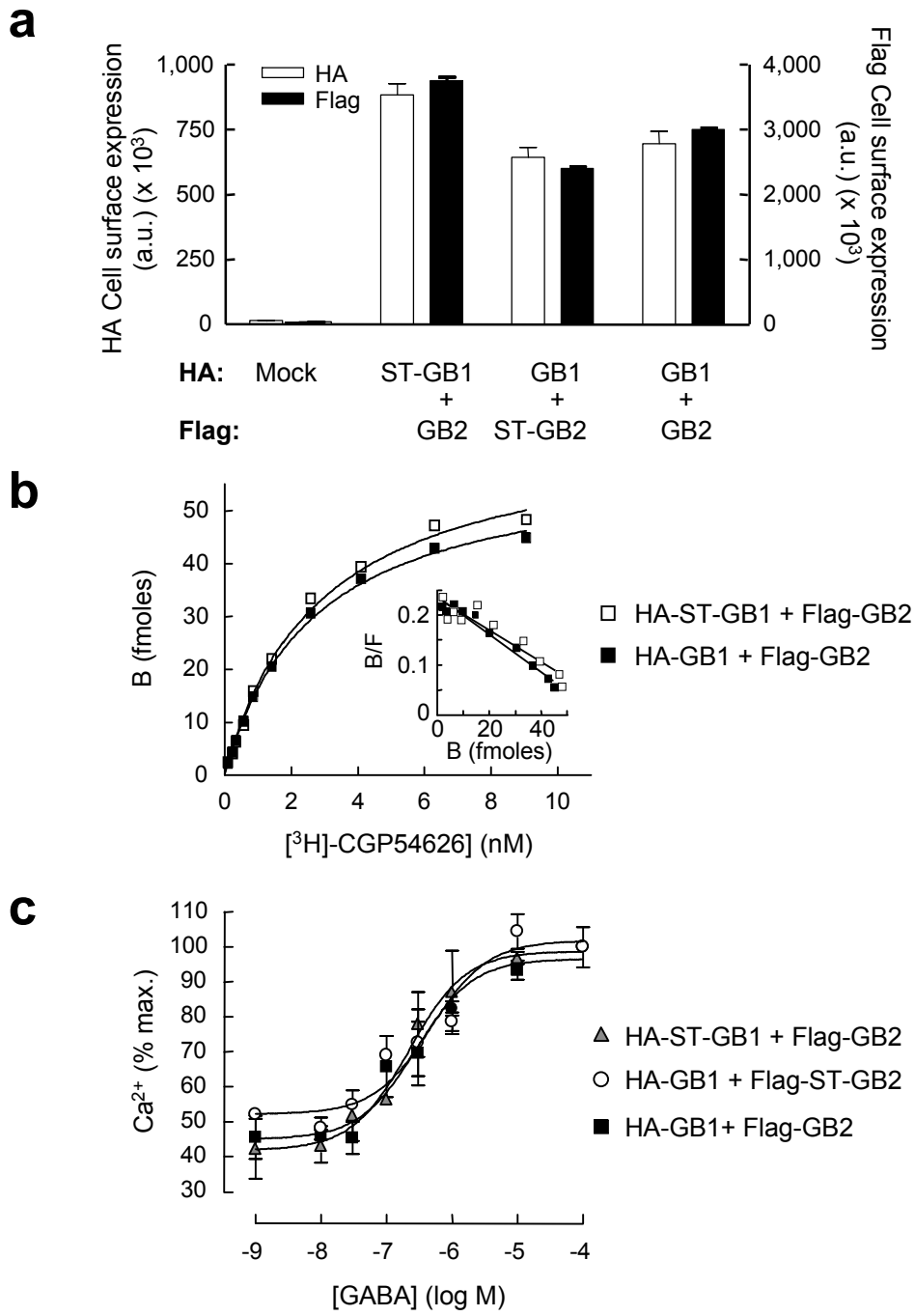
Supplementary figures and text:

Supplementary Figure 1



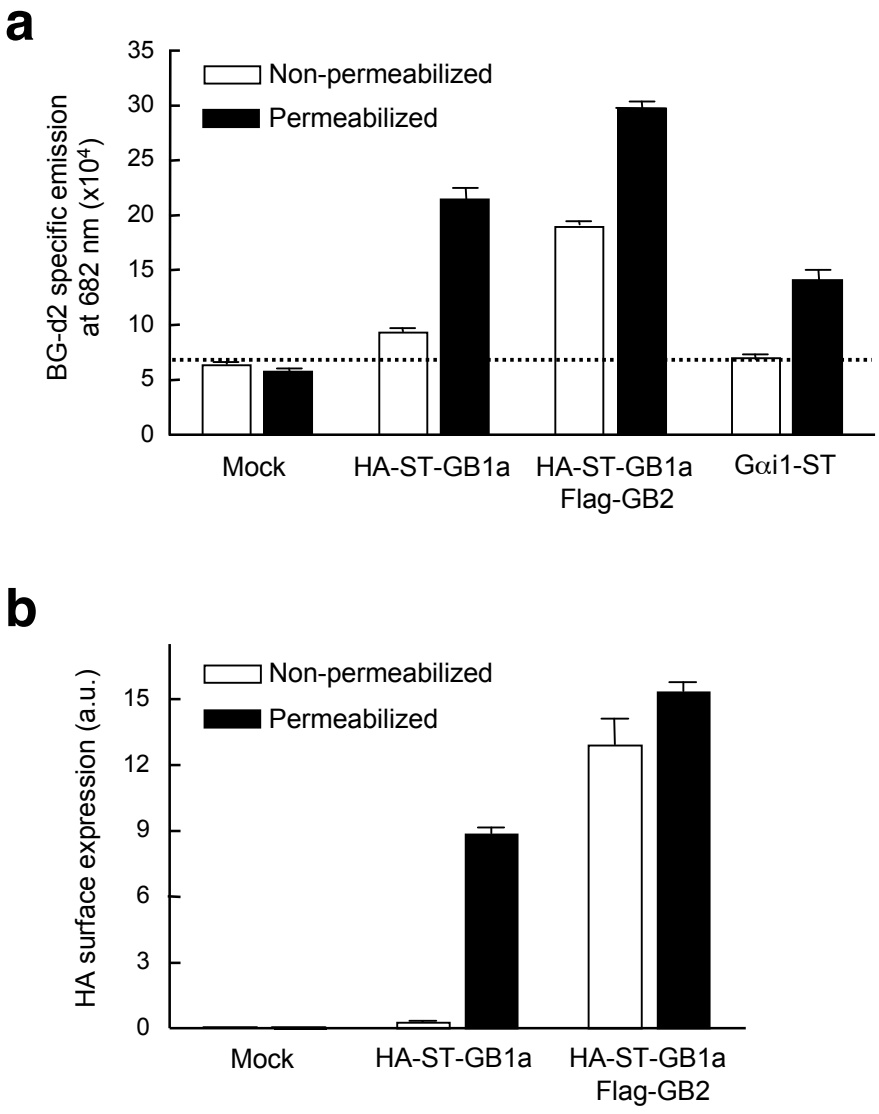
Supplementary Figure 1. Properties of the europium cryptate and synthesis of the BG-K. **(a)** Light emission intensity of BG-K at 620 nm, 50 to 2,500 µs after laser excitation at 337 nm. **(b)** Emission spectra of BG-K (black line) and BG-d2 (grey line) after excitation at 337 nm and 640 nm, respectively. **(c)** Scheme describing the synthesis of BG-K (5) used in this study.

Supplementary Figure 2



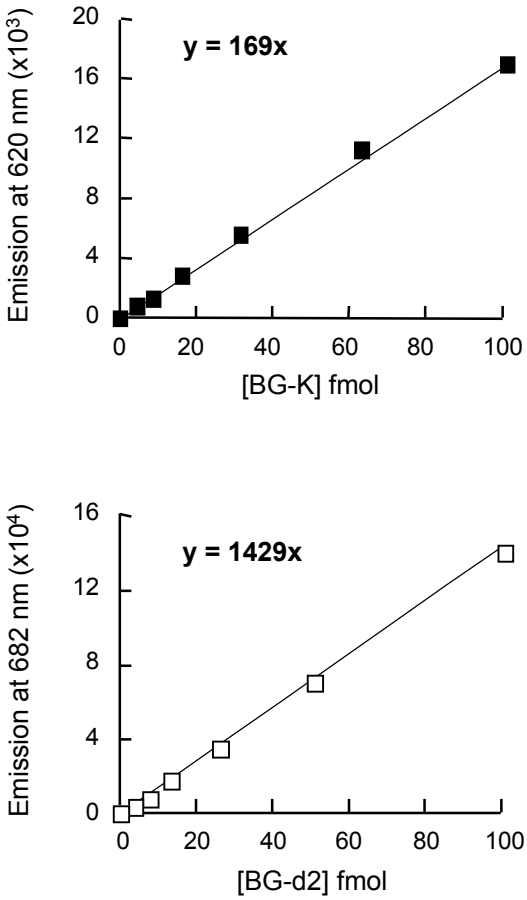
Supplementary Figure 2. Functional characterization of ST-$GABA_{B1}$ and ST-$GABA_{B2}$.
(a) Anti-HA (white columns) and anti-flag (black columns) ELISA performed on intact cells expressing the indicating subunits. **(b)** Saturation binding experiments using [^{3}H]-CGP54626 on intact cells expressing either $GABA_{B1}$ and $GABA_{B2}$ (black squares) or ST-$GABA_{B1}$ and $GABA_{B2}$ (open squares). **(c)** Ca^{2+} signals generated by increasing concentrations of GABA in cells expressing ST-$GABA_{B1}$ and $GABA_{B2}$ (grey triangles), $GABA_{B1}$ and ST-$GABA_{B2}$ (open circles) or $GABA_{B1}$ and $GABA_{B2}$ (black squares). Data are means ± s.e.m. of triplicate determinations from typical experiments.

Supplementary Figure 3



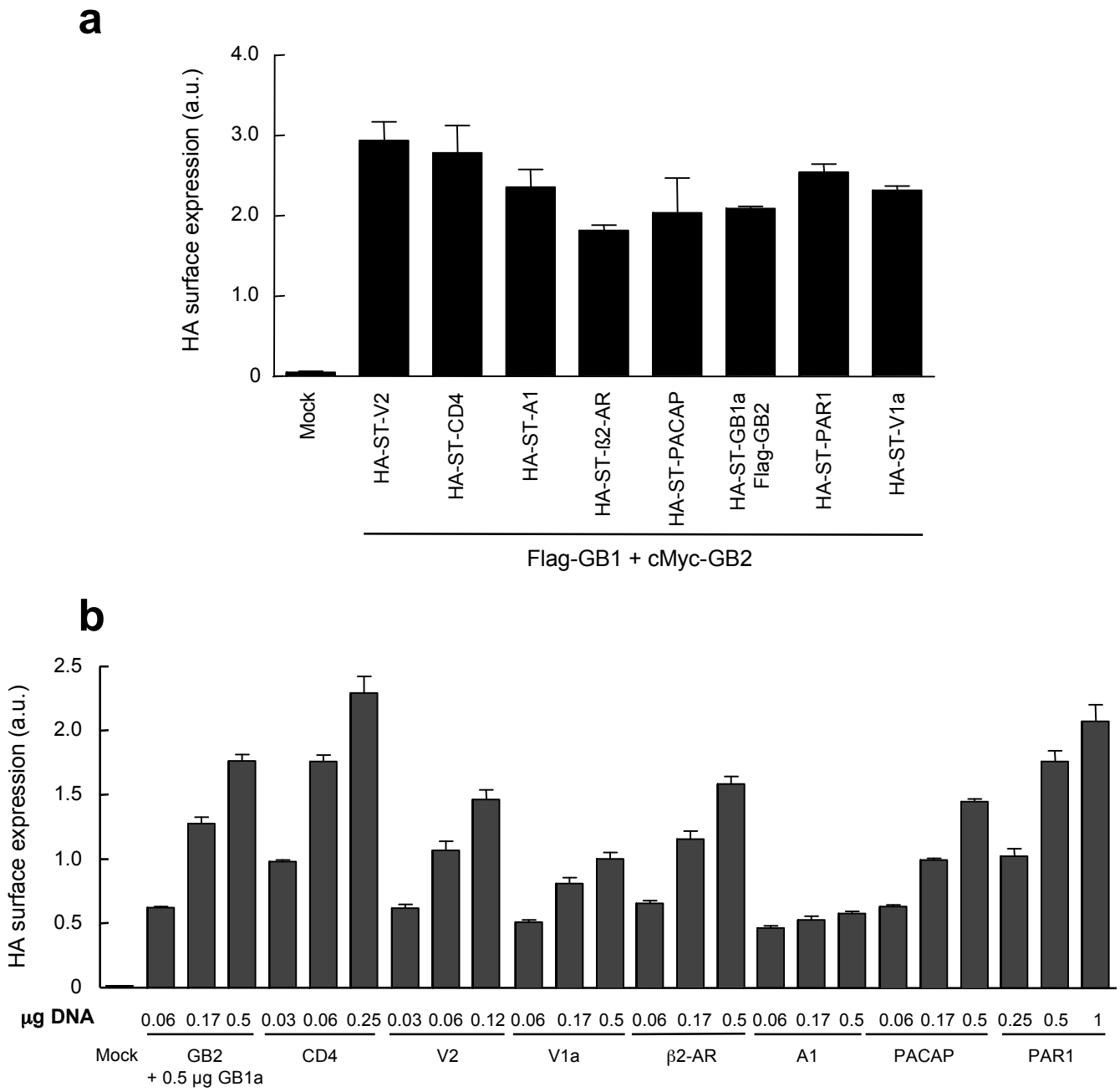
Supplementary Figure 3. Cell surface and intracellular labeling of SNAP tag fusion proteins.
(a) Fluorescence of BG-d2 bound on mock transfected cells or cells expressing Gαi1 or ST-GB1 alone or with GB2 before (white columns) and after (black columns) cell permeabilization (top panel). **(b)** Anti-HA ELISA performed on the same transfected cells to quantify the surface (non-permeabilized, white columns), and total (permeabilized, black columns) expression of GB1 (lower panel).

Supplementary Figure 4



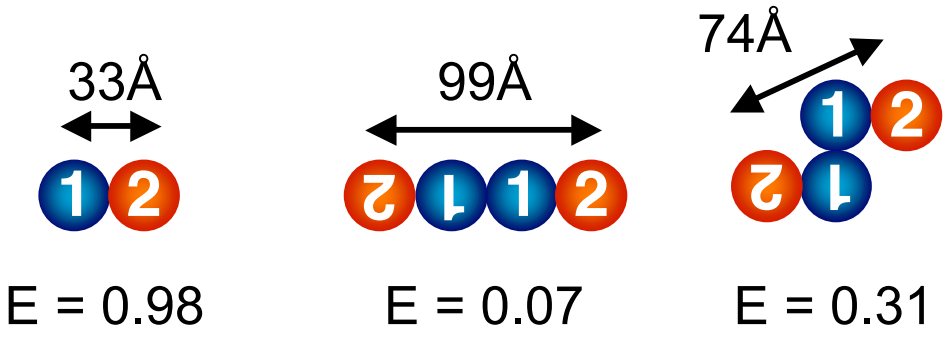
Supplementary Figure 4. Relation between fluorescence intensity and the corresponding amount of fluorophores. The fluorescence signal of the BG-K (black squares) and BG-d2 (open squares) measured at 620 nm and 682 nm respectively was represented according to the corresponding BG-fluorophores amount (fmoles per well of a 96 plate). Data are means ± s.e.m. of triplicate determinations.

Supplementary Figure 5



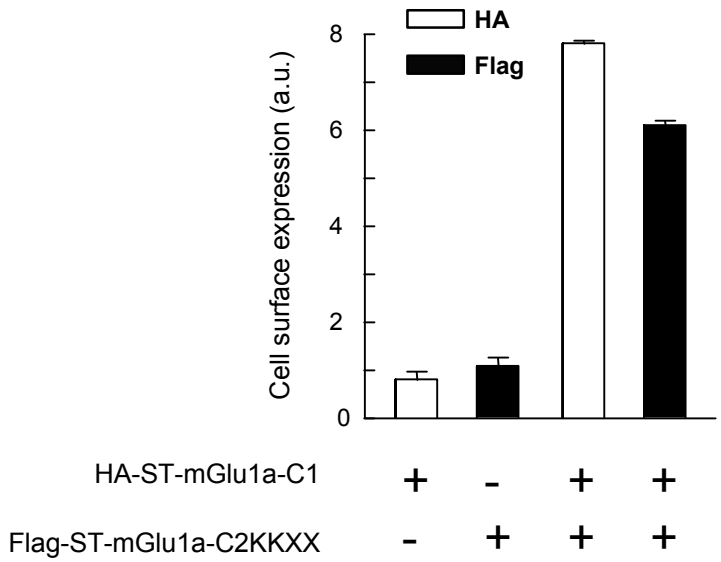
Supplementary Figure 5 . Expression level of SNAP tag fusion proteins at the cell surface. **(a)** Anti-HA ELISA performed on intact cells expressing the indicating subunits. Data were obtained with the same transfected cells as those used for Fig. 2b. **(b)** Anti-HA ELISA performed on intact cells expressing different amount of the indicating subunits. Each subunit was transfected with three different amounts of plasmids (µg). Data are those from the experiment represented in Fig. 3b.

Supplementary Figure 6



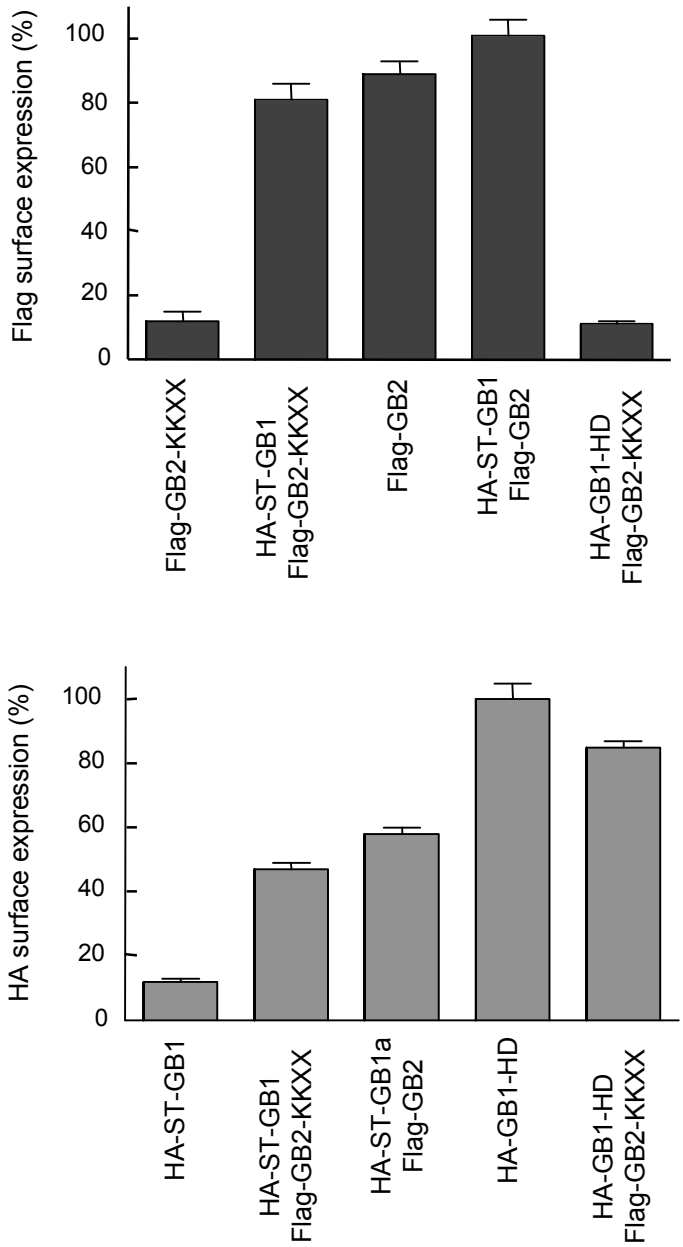
Supplementary Figure 6. Schematic representation of possible $GABA_B$ arrangements. Representation of some possible arrangements of a dimer of $GABA_B$ heterodimers, and the deduced FRET efficacy determined using the Förster equation $E=R_0^6/(d^6+R_0^6)$ were R_0 is the distance between the fluorophores giving rise to 50% FRET efficacy, and determined to be 65Å, and d is the distance between the fluorophores, assuming the distance between the fluorophores is similar to the distance between the N-terminus of the subunits. According to the presence of the snap-tag fusion, these distances are ± 20Å.

Supplementary Figure 7



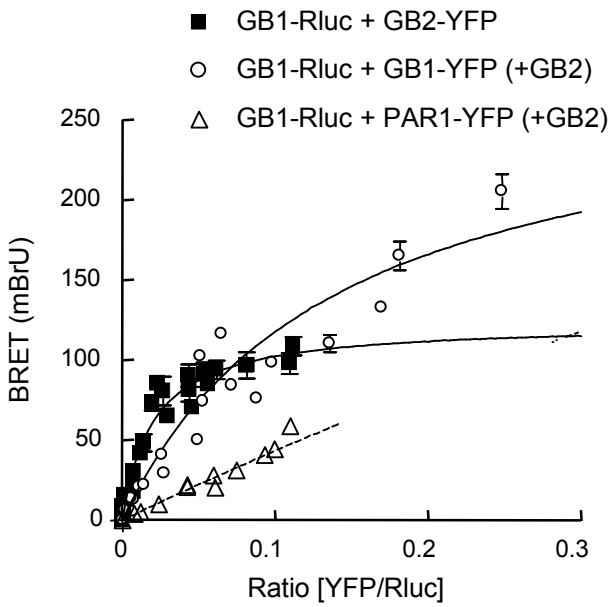
Supplementary Figure 7. Controlling the subunit composition of mGluR1.
Anti-HA (white columns) and anti-flag (black columns) ELISA performed on intact cells expressing the indicating subunits.

Supplementary Figure 8



Supplementary Figure 8 . Masking of a retention signal in GB2 by full-length GB1. Percent of surface expression determined as the ratio between the ELISA signals measured on intact cells over that measured after cell permeabilization of the indicated subunits. Anti-flag (top) and anti-HA (bottom) ELISA. Data are means ± s.e.m. of triplicate determinations from a typical experiment.

Supplementary Figure 9



Supplementary Figure 9. $GABA_B$ oligomers analyzed by BRET.
BRET signals was measured in cells expressing a constant amount of $GABA_{B1}$-Rluc, and i) increasing amounts of $GABA_{B2}$-YFP (filled squares), ii) increasing amounts of $GABA_{B1}$-YFP (and an excess of $GABA_{B2}$) (open circles), and iii) increasing amounts of the thrombin receptor PAR1-YFP (and an excess of $GABA_{B2}$) (open triangles). Data are means ± s.e.m. of triplicate determinations and data from 3 independent experiments were pooled.

Supplementary methods:

Plasmids and site-directed mutagenesis

Plasmids encoding the wild-type rat $GABA_{B1}$, $GABA_{B2}$ and mGlu1 subunits epitope-tagged at their N-terminus with HA and Flag after the signal peptide (SP) of the mGlu5 receptor were described previously[1]. Snap-tag sequence (obtained from the pSST26m plasmid from Covalys, Geneva, Switzerland) was subcloned in these plasmids at the level a unique MluI restriction site located in the linker downstream of the HA or flag tag. The upstream Mlu-I site was then mutated to conserve a unique Mlu-I site between the snap-tag and the $GABA_B$ coding sequence. Then, the N-terminal sequence was: (SPmGlu5)-TR-(HA or flag)-TRGS-(Snap-tag)-TR-(coding sequence).

These plasmids were then used to introduce the coding sequence of various membrane proteins in phase with the snap-tag, these include the coding sequences of rat mGlu1 (starting at A32), human V2 and V1a vasopressin, human β2-adrenergic, human A1 adenosine, rat PACAP, protease-activating 1 (thrombin receptor), and human prolactine receptors (starting at Q25) and that of human CD4 (starting at K26). The HA-GB1-HD truncated construct was made after insertion of a second Mlu-I site at the level of codons for residues 573-574 in the HA-$GABA_B$1 sequence. The fragment between the two Mlu-I sites was then removed, and a stop codon was introduced at position 875 using a Quick-Change strategy (Stratagene). The pcDNA3-Gαi1-ST construct was derived from the $G_{\alpha i1}$-Rluc fusion protein encoding plasmid previously reported[2], by replacing the coding Rluc sequence flanked by EcoR-I sites by that of the snap-tag flanked by EcoR-I sites and generated by PCR. Then, the snap-tag is introduced at position I93 and is flanked by the GNSGG and GGGNS linkers.

Cell culture and transfection

HEK-293 or COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and transfected by electroporation as described previously[3]. Culture medium, FCS, and other products used for cell culture were purchased from GIBCO/BRL/Life Technologies (Cergy Pontoise, France). Ten million cells were transfected with plasmid DNA expressing the proteins of interest as indicated in the figures and completed to a total amount of 10 μg plasmid DNA with pRK5 empty vector.

ELISA assay for quantification of cell surface expression

Cells were fixed with 4% paraformaldehyde and then blocked with phosphate-buffered saline + 1% fetal calf serum. After a 30 min incubation, the anti-HA monoclonal antibody (clone 3F10, Roche Bioscience, Basel, Switzerland) or anti-Flag-M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA), both conjugated with horseradish peroxidase, was applied for 30 min at 0.5 mg/l and cells were washed. Bound antibody was detected by chemoluminescence using SuperSignal substrate (Pierce, Rockford, IL, USA) and a Wallac Victor^2 counter (Molecular Devices, Sunnyvale, CA, USA). Validation of this assay has already been reported[1,4].

Intracellular calcium measurements

Twenty-four hours after transfection with plasmids encoding the indicated $GABA_B$ subunits and a chimeric protein Gqi9, HEK-293 cells were washed with HBSS buffer (20 mM Hepes, 1 mM $MgSO_4$, 3.3 mM Na_2CO_3, 1.3 mM $CaCl_2$, 0.1% BSA, 2.5 mM probenecid) and loaded with 1 μM Ca^{2+}-sensitive fluorescent dye Fluo-4 a.m. (Molecular Probes, Eugene, OR, USA) for 1 h at 37°C. After a wash, cells were incubated with 50 μl of buffer and 50 μl of 2 × GABA solution at various concentrations was added after 20 s of recording. Fluorescence signals (excitation 485 nm, emission 525 nm) were measured by using the fluorescence microplate reader Flexstation (Molecular Devices, Sunnyvale, CA, USA) at sampling intervals of 1.5 s for 60 s. Data were analyzed with the program Soft Max Pro (Molecular Devices, Sunnyvale, CA, USA). Dose-response curves were fitted using Prism (GraphPad software, San Diego, CA, USA).

Confocal imaging

HEK-293 cells were transfected with the indicated plasmids as described above. Snap-tag labeling was performed with 1 μM BG-d2. Coverslips were mounted with Gel / Mount (Biomeda, Foster City, CA). Confocal imaging was performed with a Plan-Apochromat 63 × / 1.4 oil objective and Immersol 518F (Carl Zeiss, Jena, Germany). GFP was excited at 488 nm and detected through a 505 - 530 nm band pass filter. d2 was excited at 633 nm and detected through a 650 nm long pass filter. Pinholes were adjusted to yield optical slices of < 0.5 nm.

Binding assay

Cells were incubated with increasing concentrations of radioactive tracer (0.48 nM to 10 nM of [^{3}H]-CGP54626, a radioactive antagonist of $GABA_B$ receptor) for 4 hours at 4°C. For each concentrations of tracer, non specific binding was determined by addition of GABA (1mM). After incubation, cells were washed with Tris-KREBS buffer (20 mM Tris pH 7.4, 118 mM NaCl, 5.6 mM glucose, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.7 mM KCl, 1.8 mM $CaCl_2$) in order to eliminate the excess of free radioactive ligand. Cells were then lysed using NaOH at 0.1 M for 10 min and transfered in flasks containing scintillant (OptiPhase Supermix, Perkin Elmer). Radioactivity was counted on a Beta counter Cobra (Hewlett Packard). Fitting parameters for saturation experiments were determined using a non-linear curve-fitting routine to the Hill equation B = Bmax [L] / ([L] + Kd) where Bmax is the maximal binding, L is the concentration of labeled ligand and Kd the equilibrium dissociation constant for the labeled ligand.

BG-K synthesis

The O^6-(4-Aminomethyl-benzyl)guanine **1** (0.6 mg, 2.2 µmol) (supp Fig. C) was dissolved in 450 µl of 100 mM phosphate buffer pH7 and 50 µl of dimethylformamide, 4.3 µmol of SMCC (Succinimidyl4-[N-maleimidomethyl]cyclohexane-1-carboxylate) dissolved in 220 µl of acetonitrile were added. After 90 min reaction at room temperature the HPLC (Chromolith gradient A detection 280 nm) showed consumption of the starting guanine derivative (t_R = 6.2 min) apparition of a new peak (t_R = 18.2 min) and some residual SMCC (t_R = 19.6 min). The reaction mixture was acidified with 300 µl of 1% aqueous TFA and the purified by HPLC using the above conditions, the fraction containing the maleimide derivative **2** were evaporated to dryness and co-evaporated with water (vacuum-centrifuge), then dissolved in acetonitrile / water mixture (2:8, v/v) for UV quantitation (ε_{285} nm = 12,000 $M^{-1}.cm^{-1}$). Yield 0.77 µmol. ES+: $(M+H)^+$ = 490.3 , $(M+Na)^+$ = 490.3 , ES-: $(M-H)^-$ = 488.4.

The Eu⊂PBBP-NH_2 cryptate **3** [US Patent 7,087,384] (5.5 mg, 4 mmol) in 1.8 ml of 100 mM phosphate buffer pH 7 (t_R = 9.4 min , gradient B), was treated with SPDP (N- succinimidyl 3-(2-pyridyldithio) propionate) (8 µmol) and after 90 mn TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) (9 µmol) was added. After 10 min the reduction was complete and the thiolated cryptate **4** (t_R = 8.3 min) was purified over HPLC, the relevant fractions were evaporated to dryness. Compound **4** (3 µmol) was dissolved in 1.6 ml of 100 mM HEPES buffer pH 6.5 and the maleimido-benzylguanine **2** (in 0.2 ml of HEPES and 0.2 ml ACN) was

added. After 30 mn HPLC analysis (gradient B) showed the formation of a new peak (t_R = 12.8 min), the reaction mixture was acidified with 1% TFA and immediately purified using the same gradient conditions. The title compound **5** (BG-K) was collected and the fractions were evaporated to dryness, co-evaporated with water to remove any residual TFA, and the residue dissolved in ACN / 20 mM TEAB (Triethylammonium hydrogen carbonate) (1:1 , v/v) quantified by UV absorbance (ε_{320} = 24,000 $M^{-1}.cm^{-1}$) and stored as 100 nmol aliquots evaporated to dryness (vacuum-centrifuge) in eppendorf tubes. Yield 1.1 μmol based on the maleimido-benzylguanine. ES+ : $(M-2H)^+$ = 1487.5, $(M-2H+TFA)^+$ = 1601.6 , $(M-2H+2TFA)^+$ = 1715.6. Calc. for $C_{66}H_{64}EuN_{16}O_{14}S$ = 1489.37.

snap-tag labeling with TR-FRET compatible fluorophores

Twenty four hours after transfection, cells (100,000 cells per well of a 96 Greiner CellStar well plate) were washed with DMEM 10% FCS pre-warmed at 37°C. Then, cells were labeled one hour at 37°C, 5% CO_2 with different concentrations of derivatized benzyl guanine (BG-K or BG-d2) in DMEM 10% FCS. After labeling, cells were washed four times with Tris-KREBS buffer (20 mM Tris pH 7.4, 118 mM NaCl, 5.6 mM glucose, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4,7 mM KCl, 1.8 mM $CaCl_2$) and the signal recorded under 100 μL of Tris-Krebs per well. The emission signal from the cryptate was recorded at 620 nm on a time-resolved fluorimeter (RubyStar, BMG Labtechnologies, Champigny-sur-Marne, France) after an excitation at 337 nm by a nitrogen laser and the emission signal from the d2 was recorded at 682 nm on an Analyst reader (Molecular Devices) using a 640 nm excitation. Finally, the specific fluorescence signal was determined by substracting the total fluorescence signal from the cells expressing the cell surface snap-tag protein with the non specific signal from mock transfected cells.

TR-FRET between snap-tag and antibodies labeled with the indicated fluorophores

After snap-tag labeling with BG-K (see above), cells (100,000 cells per well of a 96 Greiner CellStar well plate) were incubated in Tris-Krebs with 2nM of anti-flag antibodies conjugated with d2 (means of 3.5 fluorophores per anti-flag M2 antibody), overnight at 4°C. Finally, the FRET signal was measured at 665 nm between 50 and 450 μs after laser excitation at 337 nm, without washing out the unbound antibodies (homogeneous format). Assay signals were expressed by the Δ665 = (total signal recorded at 665 nm) – (background at 665 nm). The background signal corresponds to snap-tag cells labeled with BG-K only (without antibodies).

Similar background values were obtained with 2 nM anti-flag-d2 and an excess of unlabeled anti-flag antibodies (1 μM). Similar experiments were conducted with 2 nM anti-HA conjugated with d2. A similar protocol was used to measure TR-FRET with BG-d2 labeling and europium cryptate-conjugated anti-flag or anti-HA antibodies. In those cases, the negatives correspond to the europium cryptate-conjugated anti-flag or anti-HA antibodies alone.

TR-FRET between two snap-tags

Twenty four hours after transfection, cells were washed one time with 100 μL of complete DMEM medium and then incubated one hour at 37°C, 5 % CO2 with a mixture of BG-K and BG-d2. The optimal concentration ratio was obtained for 5 μM of BG-K with 0.5 μM of BG-d2. After the labeling cells were washed four times with Tris-Krebs and the signal recorded on a Rubystar plate reader. Here, the Δ665 represents the FRET signal recorded on BG-K/BG-d2 labeled cells from which the signal recorded on the same cells labeled with BG-K and a cold BG diluted at the same concentration than the BG-d2 was subtracted.

Determination of the R_0 for the FRET between BG-K and BG-d2. R_0, the Förster radius, is defined as the distance at which the FRET process is 50 % efficient. R_0 between the europium cryptate PBP and d2 was determined to be 65.4 Å using the following formula:

$$R_0 = \left(J \times 10^{-3} \times k^2 \times n^{-4} \times Q_D\right)^{1/6} \times 9730$$

Previous to R_0 determination, J, the Förster integral overlap, was determined to be 8.7 10^{-10} $cm^6.M^{-1}$ using the following formula:

$$J = \frac{\int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int F_D(\lambda) d(\lambda)}$$

where $F_D(\lambda)$, the relative fluorescence intensity of the donor at wavelength λ and $\varepsilon_A(\lambda)$, the molar extinction coefficient of the acceptor at wavelength λ were determined between 570 nm and 720 nm. To determine F_D at each wavelength λ, the fluorescence emission spectrum of the europium cryptate PBP, previously diluted at 5 μM in a phosphate buffer pH = 7 containing 0.1% BSA, was acquired on a LS50B spectrofluorimeter (Perkin Elmer). $F_D(\lambda)$ was calculated by dividing the fluorescence intensity obtained at each wavelength by the value of the total fluorescence emitted between 570 nm and 720 nm. $\varepsilon_A(\lambda)$ was obtained for each wavelength λ using the following formula:

$$\varepsilon_A = \frac{O.D._{(\lambda)}}{O.D._{(649nm)}} \times 239{,}000$$

Where the ε_A value at 649nm was considered to be equal to 239,000 $M^{-1}.cm^{-1}$. The optical density at each wavelength, O.D., was obtained through the acquisition of an absorption spectrum of d2 previously diluted at 6 µM in a phosphate buffer pH = 7 containing 0.1 % BSA. Spectrum acquisition was done on a DU800 spectrophotometer (Beckman Coulter).

Q_D, the luminescence quantum yield of the europium in the absence of the acceptor d2, was previously determined to be 0.48 (unpublished data).

The other parameters needed to perform a R_0 determination were determined according to Selvin and Hearst[5]. k^2, the dipole orientation factor was assumed to be 2/3, n, the medium refractive index, was fixed at 1.33.

Supplementary references

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