Quaternary structure of a G-protein-coupled receptor heterotetramer in complex with $G_i$ and $G_s$

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Abstract

Background: G-protein-coupled receptors (GPCRs), in the form of monomers or homodimers that bind heterotrimeric G proteins, are fundamental in the transfer of extracellular stimuli to intracellular signaling pathways. Different GPCRs may also interact to form heteromers that are novel signaling units. Despite the exponential growth in the number of solved GPCR crystal structures, the structural properties of heteromers remain unknown.

Results: We used single-particle tracking experiments in cells expressing functional adenosine $A_1$-$A_{2A}$ receptors fused to fluorescent proteins to show the loss of Brownian movement of the $A_1$ receptor in the presence of the $A_{2A}$ receptor, and a preponderance of cell surface 2:2 receptor heteromers (dimer of dimers). Using computer modeling, aided by bioluminescence resonance energy transfer assays to monitor receptor homomerization and heteromerization and G-protein coupling, we predict the interacting interfaces and propose a quaternary structure of the GPCR tetramer in complex with two G proteins.

Conclusions: The combination of results points to a molecular architecture formed by a rhombus-shaped heterotetramer, which is bound to two different interacting heterotrimeric G proteins ($G_i$ and $G_s$). These novel results constitute an important advance in understanding the molecular intricacies involved in GPCR function.

Keywords: GPCR, Heterotetramer, Heterotrimeric G protein, Single-particle tracking, BRET, Molecular modeling

Background

G-protein-coupled receptor (GPCR) oligomerization is heavily supported by recent biochemical and structural data [1–6]. Optical-based techniques are instrumental in studying the dynamics and organization of receptor complexes in living cells [7]. For instance, total internal reflection fluorescence microscopy shows that 30 % of muscarinic M1 receptors exist as dimers (with no evidence of higher oligomers) that undergo interconversion with monomers on a timescale of seconds [8]. Similarly, the $\beta_1$-adrenergic receptors ($\beta_1$-AR) are expressed as a mixture of monomers and dimers whereas $\beta_2$-adrenergic receptors ($\beta_2$-AR) have a tendency to form dimers and higher-order oligomers [9]. Moreover, the monomer-dimer equilibrium of the chemoattractant N-formyl peptide receptor at a physiological level of expression lies within a timescale of milliseconds [10]. Together, these studies in heterologous systems show that a given GPCR is present in a dynamic equilibrium between monomers, dimers, and higher-order oligomers.

Studies in a broad spectrum of GPCRs [11–14] show that these receptors may form heteromers. GPCR heteromers are defined as novel signaling units with functional properties different from homomers and they represent a completely new field of study [15]. Innovative crystallographic techniques have permitted researchers to obtain crystal structures of GPCR families A, B, C, and F, bound to either agonists, antagonists, inverse agonists or allosteric modulators; in the form of monomers or homo-oligomers; and in complex with a G protein or...
with a β-arrestin [16]. However, crystal structures of GPCR heteromers have not yet been obtained. Here, we propose a quaternary structure of a heteromer, taking into account the molecular stoichiometry and the interacting G proteins. Adenosine A₁-A₂A receptor (A₁R-A₂AR) complexes constitute a paradigm in the GPCR heteromer field because A₁R is coupled to Gᵢ and A₂AR to Gₛ that is, they transduce opposite signals in cyclic adenosine monophosphate (cAMP)-dependent intracellular cascades. First described as a concentration-sensing device in striatal glutamatergic neurons [17], the A₁R-A₂AR heteromer is thought to function as a Gₛ/Gᵢ-mediated switching mechanism by which low and high concentrations of adenosine inhibit and stimulate, respectively, glutamate release [17, 18]. The structural basis of this switch is key to understanding heteromer function and the biological advantage behind the GPCR heterimerization phenomenon. Here, we have devised the molecular architecture of the adenosine A₁R-A₂AR heteromer in complex with G proteins using a combination of microscope-based single-particle tracking, molecular modeling, and energy transfer assays in combination with molecular complementation. The results point to A₁ and A₂A receptors organizing into a rhombus-shaped heterotetramer that couples to Gᵢ and Gₛ. The overall structure is very compact and provides interacting interfaces for GPCRs and for G proteins.

Results and discussion

Reciprocal restriction of adenosine receptor motion in the plasma membrane

To examine the dynamics of A₁R-A₂AR heteromers in the plasma membrane of a living cell, the motion of the receptors tagged with fluorescent proteins (A₁R-green fluorescent protein [GFP] or A₂AR-R-mCherry) was measured by real-time single-particle tracking (SPT) (Fig. 1).

Examples of fluorescent images and individual particle trajectories are shown in Additional file 1: Figure S1. Analysis of data corresponding to 500 A₁R-GFP particles showed a linear relationship between the mean square displacement (MSD) versus time lag in the trajectories of up to 1600 single fluorescent particles (Fig. 1a, c). This is typical for Brownian diffusion, indicating a lack of restrictions in A₁R-GFP motion. Co-expression of A₂AR-R-mCherry (Fig. 1b) led to a reduction in the lateral mobility of A₁R-GFP, which became confined to plasma membrane regions of 0.461 ± 0.004 μm in diameter. Its diffusion coefficient decreased from 0.381 ± 0.002 μm²/s to 0.291 ± 0.003 μm²/s (p = 0.002, one-tailed t-test).

Similarly, A₁R-GFP also decreased the A₂AR-R-mCherry diffusion coefficient from 0.317 ± 0.002 μm²/s to 0.143 ± 0.005 μm²/s (p < 0.0001) (Fig. 1d–f). A₂AR moved within a confinement zone of 0.941 ± 0.007 μm in diameter that was reduced to 0.360 ± 0.001 μm (p < 0.0001) when both receptors were co-expressed. We conclude from these mobility comparisons that reciprocally restricted motion of the individual receptor particles must be due to A₁R-A₂AR receptor-receptor interactions.

Stoichiometry of A₁ and A₂A receptor heterocomplexes

The stoichiometry of the fluorescent receptors on the cell surface can be calculated from the brightness distribution of the individual particles [19] (see “Methods”). In cells expressing A₁R-GFP, we found the majority of clusters to consist of either two (~47 %) or four (~34 %) receptors, and clusters with one or three receptors were scarce (~10 % and ~9 %, respectively) (Additional file 2: Figure S2A and black bars in Additional file 2: Figure S2C). In the case of A₂AR-R-mCherry, the stoichiometry analysis showed that the clusters mostly expressed trimers (45 %), with dimers (29 %) and tetramers (12 %) the second and third most common populations (Additional file 2: Figure S2D and black bars in Additional file 2: Figure S2F). Remarkably, this stoichiometry for either A₁ or A₂A receptors was altered when the partner receptor was also expressed. In cells co-expressing A₁R-GFP and A₂AR-R-mCherry, the dimer population increased (57 % for A₁R-GFP and 49 % for A₂AR-R-mCherry, blue bars in Additional file 2: Figures S2C, F) and became the predominant species (Additional file 2: Figures S2B, C, E, F).

In order to focus the analysis on heteromer complexes, we identified clusters containing both receptors (individual yellow dots in Fig. 1g, displaying both GFP and mCherry fluorescence). In ~1000 analyzed co-localized clusters that consisted of a mixture of A₁-GFP and A₂AR-Cherry (yellow dots in Fig. 1g), we found a similar high amount of dimers of A₁R (75 %, left panel in Fig. 1h and green bar in Fig. 1i) and A₂AR (74 %, right panel in Fig. 1h and red bar in Fig. 1i). Trimers and tetramers of A₁R, and monomers and tetramers of A₂AR, were in the minority or negligible (see Fig. 1h, i). In summary, given that the percentage of dimers of either A₁R-GFP or A₂AR-R-mCherry in the yellow dots (which show co-localization of the two receptors) was similar and high (~75 %), the heterotetramer containing two A₁Rs and two A₂ARs must have been the most predominant species. To our knowledge, this is the first stoichiometry data for a GPCR heteromer in living cells.

Arrangement of G proteins interacting with A₁ and A₂A receptors

Monomeric GPCRs are capable of activating G proteins [20]. However, recent findings suggest that one GPCR homodimer bound to a single G protein may be a common functional unit [21]. Thus, an emerging question is how G proteins couple to GPCR heteromers. Because A₁R selectively couples to Gᵢ and A₂AR to Gₛ [22], the
working hypothesis was that both $G_i$ and $G_s$ proteins may couple to the $A_1R$-$A_{2AR}$ heterotetramer. To test this hypothesis, we used bioluminescence resonance energy transfer (BRET) assays [23]. In agreement with the SPT experiments (see above), homodimers and heterodimers were detected by BRET assays in cells expressing $A_1R$ fused with Renilla luciferase ($A_1R$-Rluc) or yellow fluorescent protein ($A_1R$-YFP) (Fig. 2a), $A_{2AR}$-Rluc and $A_{2AR}$-YFP (Fig. 2b), or $A_1R$-Rluc and $A_{2AR}$-YFP (Fig. 2e). Neither $A_1R$-Rluc nor $A_{2AR}$-YFP interacted with the ghrelin receptor 1a fused to YFP (GHS1a-YFP), used as a control as a protein unable to directly interact with these adenosine receptors (Fig. 2a, b). In order to test the presence of the two $G$ proteins in the heterotetramer, we transfected cells with minigenes that code for peptides blocking either $G_i$ or $G_s$ binding to GPCRs [24]. In addition, cells were treated with pertussis or cholera toxins that catalyze ADP-riboseylation of $G_i$ or $G_s$. Clearly, treating cells with pertussis toxin, or expressing the minigene-coded peptide that blocks $\alpha_i$ coupling, reduced the value of BRET$_{max}$ for $A_1R$-$A_{2AR}$ homodimers (Fig. 2a) and for $A_1R$-$A_{2AR}$ heterodimers (Fig. 2e) but not for $A_{2AR}$-$A_{2AR}$ homodimers (Fig. 2b). This indicates that $G_i$ is coupled to $A_1R$ in both the homodimer and the heterodimer. Similarly, blocking $G_s$-receptor interaction using cholera toxin or a minigene-coded peptide that blocks $\alpha_s$ coupling reduced BRET$_{max}$ for $A_{2AR}$-$A_{2AR}$ homodimers (Fig. 2b) and for $A_1R$-$A_{2AR}$ heterodimers (Fig. 2e) but not for $A_{2AR}$-$A_{2AR}$ homodimers (Fig. 2a). Interestingly, BRET curves showed sensitivity to both cholera and pertussis toxins in cells expressing either $A_1R$-Rluc-$A_1R$-YFP and $A_{2AR}$ (Fig. 2c) or $A_{2AR}$-Rluc-$A_{2AR}$-YFP and $A_1R$ (Fig. 2d). Functionality of constructs and controls in cells expressing minigenes, and in cells expressing the ghrelin GHS1a receptor instead of one of the adenosine receptors, are shown in Additional file 3: Figure S3. To further confirm that $G_i$ binds $A_{2AR}$ in the receptor heteromer, the energy transfer between Rluc fused to the N-terminal domain of the $\alpha$-subunit of $G_i$ ($G_i$-Rluc) and $A_{2AR}$-YFP was analyzed in
Fig. 2 Influence of G proteins on A₁R and A₂AR homodimerization and heterodimerization. BRET saturation curves were performed in HEK-293T cells 48 h post-transfection with (a, c) 0.3 μg of cDNA corresponding to A₁R-Rluc and increasing amounts of A₁R-YFP (0.1–1.5 μg cDNA) or GHS1a-YFP (0.25–2 μg cDNA) as negative control (a, purple line), without (a) or with (c) 0.15 μg of cDNA corresponding to A₂AR; (b, d) 0.2 μg of cDNA corresponding to A₂AR-Rluc and increasing amounts of A₂AR-YFP (0.1–1.0 μg cDNA) or GHS1a-YFP (0.25–2 μg cDNA) as negative control (b, purple line), without (b) or with (d) 0.5 μg of cDNA corresponding to A₁R (e) 0.3 μg of cDNA corresponding to A₁R-Rluc and increasing amounts of A₂AR-YFP (0.1–1.0 μg cDNA); and (f) 0.5 μg of cDNA corresponding to A₁R (except control blue curves that were obtained in cells not expressing A₁R), 2 μg of cDNA corresponding to Gᵢ-Rluc, and increasing amounts of A₂AR-YFP (0.1–0.5 μg cDNA). In panels a, b, and e, cells were also transfected with 0.5 μg of cDNA corresponding to the Gᵢ-related (orange curves) or Gₛ-related (blue curves) minigenes. Cells were treated for 16 h with medium (black curves), with 10 ng/ml of pertussis toxin (green curves), or with 100 ng/ml of cholera toxin (red curves) prior to BRET determination. To confirm similar donor expressions (approximately 100,000 bioluminescence units) while monitoring the increase in acceptor expression (10,000–40,000 fluorescence units), the fluorescence and luminescence of each sample were measured before energy transfer data acquisition. MiliBRET unit (mBU) values are the mean ± standard error of the mean of four to six different experiments grouped as a function of the amount of BRET acceptor. In each panel (top) a cartoon depicts the proteins to which Rluc and YFP were fused and the presence or not of partner receptors and/or Gₛ or Gᵢ proteins [schemes in c to f are not intended to illustrate on stoichiometry because the predominant form in cells expressing the two receptors was the heterotetramer containing two A₁ and two A₂AR receptors (see "Results")].
cells co-expressing or not co-expressing A₁R (Fig. 2f). A hyperbolic BRET curve was observed in the presence of A₁R, but not in its absence, indicating that G₁ and G₅ are bound to their respective receptor homodimers within the A₁R-A₂AR heteromer.

Further, two complementary BRET experiments were performed to determine the orientation of G₁ and G₅ within the A₁R-A₂AR heterocomplex. First, Rluc and YFP were respectively fused to the N-terminal domains of the α-subunit of G₁ (α₁-Rluc) and G₅ (α₅-YFP) (Fig. 3, bar a); second, they were fused to the N-terminal domain of the γ-subunit (γ-Rluc and γ-YFP) (Fig. 3, bar b). We observed significant energy transfer between γ-Rluc and γ-YFP in cells co-expressing A₁R and A₂AR (Fig. 3, bar b) but minimal amounts in negative-control cells (Fig. 3, bars c and d). In cells expressing either A₁R or A₂AR, the energy transfer between γ-Rluc and γ-YFP was also low (Fig. 3, bars e and f), suggesting that dimers but not tetramers were the most prevalent form of surface receptors in single-transfected cells. These results in co-transfected cells corroborate the 2:2 stoichiometry obtained from analysis of the fluorescence in single particles and are consistent with G₁ and G₅ binding to these A₁R-A₂AR heterotetramers.

Molecular model of G₁ and G₅ bound to the A₁R-A₂AR heterotetramer

To identify the orientation of the G protein in the receptor homodimer, we combined energy transfer assays between α₁-Rluc (Rluc at the N-terminus of the G protein α-subunit) and A₂AR-YFP (Fig. 4a) with information on transmembrane (TM) interfaces based on crystal structures of GPCRs [3, 4], which have been recently summarized [25]. The observed high-energy transfer using α₁-Rluc and A₂AR-YFP indicated close proximity between the N-tail of the α-subunit of G₅ and the C-tail of A₂AR. Interestingly, Rluc and YFP in the “monomeric” A₂AR-G₅ complex (see “Methods”) point toward distant positions in space (Fig. 4b). Therefore, the observed BRET should occur between Rluc in the G protein α-subunit and a second A₂AR-YFP protomer. Among all described TM interfaces for receptor homodimerization (see Additional file 4: Figure S4), we propose the TM4/5 interface, which is observed in the oligomeric structure of β₁-AR [4] and in structures derived from coarse-grained molecular dynamics (MD) simulations [26]. In fact, this is the only interface that favors BRET between α₁-Rluc and a second A₂AR-YFP protomer in a homodimer (Fig. 4c). The homologous A₁R homodimer was built using the same TM4/5 interface as for A₂AR (see Additional file 4: Figure S4 and its legend).

The remaining possible TMs able to form heteromeric interfaces are TM1 and TM5/6 (Fig. 5). Both are possible inter-GPCR interfaces as observed in the structure of the μ-opioid receptor (μ-OR) [3]. To discern between these two possibilities, a bimolecular fluorescence complementation strategy was undertaken. For this purpose,
the N-terminal fragment of Rluc8 was fused to A1R (A1R-nRluc8) and its C-terminal domain to A2AR (A2AR-cRluc8), which only upon complementation can act as a BRET donor (Rluc8). The BRET acceptor protein was obtained upon complementation of the N-terminal fragment of YFP Venus protein fused to A1R (A1R-nVenus) and its C-terminal domain fused to A2AR (A2AR-cVenus). When all four receptor constructs were transfected, we obtained a positive and saturable BRET signal (BRET\textsubscript{max} of 35 ± 2 mBU and BRET\textsubscript{50} of 16 ± 3 mBU) that was not obtained for negative controls (Additional file 5: Figure S5).

Figure 4a, b shows that the hemi-donor (A1R-nRluc8 and A2AR-cRluc8) and the hemi-acceptor (A1R-nVenus and A2AR-cVenus) moieties, placed at the C-terminus of the receptors, can only complement if A1R-A2AR heterodimerization occurs via the TM5/6 interface. The TM4/5 interface for homodimerization and the TM5/6 interface for heterodimerization give a rhombus-shaped tetramer organization (Fig. 5a). Remarkably, cell pre-incubation with either pertussis or cholera toxins decreased the BRET\textsubscript{max} by 35 % (Fig. 5c), further suggesting that both G\textsubscript{s} and G\textsubscript{i} proteins bind to the A1R-A2AR heterotetramer.

We next evaluated, using computational tools, whether the proposed A1R-A2AR heterotetramer could couple to both G\textsubscript{i} and G\textsubscript{s} proteins. Clearly, the external protomers of the proposed A1R-A2AR heterotetramer can bind to G\textsubscript{i} and G\textsubscript{s} proteins (Fig. 5d). This model positions the \(\alpha\)-subunits of G\textsubscript{i} and G\textsubscript{s} in close contact, facing the interior of the tetrameric complex, while the N-terminal \(\alpha\)-helices of \(\alpha\)i and \(\alpha\)s point outside the complex. The N-terminal \(\alpha\)-helices of the \(\gamma\)-subunits are in close proximity, facing the inside (Additional file 6: Figure S6), which explains the significant energy transfer observed between \(\gamma\)-Rluc and \(\gamma\)-YFP (Fig. 3, bar b). The model provides experimental insights into the structural arrangement of heteromers consisting of two GPCRs and coupled to two G proteins, the possibility of which has recently been discussed [25]. We used MD simulations to study the stability of this complex. Additional file 7:
Figure S7 shows root-mean-square deviations (rmsd) on protein α-carbons throughout the MD simulation, as well as key intermolecular distances among protomers and G proteins. Clearly, both the A₁R protomer bound to Gᵢ and the A₁R protomer that does not interact with it maintained a close structural similarity (rmsd ≈ 0.3 nm) relative to the initial structures. Similar results were obtained for the A₂A protomers (bound and unbound to Gₛ) (Additional file 7: Figure S7A). The fact that rmsd values of the whole system, formed by the A₁R-A₂A heterotetramer bound to Gᵢ and Gₛ, are of the order of 0.6 nm indicates that the initial structural model is maintained during the MD simulation (Additional file 7: Figure S7A). As a consequence, selected intermolecular distances among protomers and G proteins remain constant during the MD simulation (Additional file 7: Figure S7B). A key aspect in the assembly of the heterotetramer is the TM interfaces for homodimerization (TM4/5) and heterodimerization (TM5/6). Additional file 8: Figure S8B shows rmsd values of the four-helix bundle forming the TM4/5 and TM5/6 interfaces, the initial and final snapshots of these bundles, and the evolution of the A₁R-A₂A heterotetramer during the MD simulation. Clearly, the rather small structural variations of these four-helix bundles, also reflected by rmsd <0.3 nm, suggest a stable complex. Notably, the TM5/6 four-helix bundle seems more stable than the TM4/5 bundles, as shown by its lower rmsd value. Additional file 8: Figure S8B, C depicts contact maps of the TM4/5 and TM5/6 interfaces, as well as the evolution of the network of hydrophobic interactions within these interfaces during the MD simulation.
Conclusions
For more than a decade, experimental evidence has supported the occurrence of homo-oligomers and heterooligomers of GPCRs [21]. However, our basic understanding of what makes heteromers different from homomers remains unknown. Our results, studying adenosine receptors as a model heteromer, point to three important new findings. First, the predominant stoichiometry in cells expressing A1R-A2AR heteromers is 2:2; that is, a dimer of dimers (tetramer). Second, two different heterotrimeric G proteins can couple to heteromers, the overall complex constituting a functional unit. Third, the molecular orientation within the heteromer complex affords various qualitatively different interfaces; the two more relevant are the inter-protomer heteromeric interface and the inter-G-protein interface. Presumably, the two interfaces provide the key characteristic of heteromers: the ability of one protomer/G-protein complex to influence the signaling of the other. Surely, allosteric effects occurring between heteroreceptors and between Gs and Gi proteins are due to conformational changes transmitted along the intimately interacting molecules in the complex. In our controlled cell transfection system, which expressed a low density of receptors, minor species formed by monomers and trimers were found in addition to a predominance of tetromers in the plasma membrane, strongly supporting the occurrence of an in vivo dynamic distribution of receptors.

Adenosine was, from an evolutionary point of view, one of the first extracellular regulators given that it is involved in energy and nucleic acid metabolisms. Adenosine A1 and A2A receptors are expressed in almost every mammalian organ and tissue. In the heart, where adenosine plays a key role in both inotropic and chronotropic regulation, A1R-mediated cardioprotection did not occur in A2AR knockout mice, suggesting an interaction between A1 and A2A receptors. In neurons, A1 and A2A receptors show co-localization, leading to inter-receptor interactions unveiled by pharmacological treatments. For instance, Okada et al. [27] showed that cAMP-dependent protein kinase A plays a role in the regulation of hippocampal serotonin release mediated by both A1 and A2A receptors. Similarly, the control of γ-amino butyric acid transport in astrocytes was attributed to the expression of A1R-A2AR heteromers and to a specific mechanism by which the heteromer signals via Gi or Gs depending on the concentration of adenosine [28]. The structural basis of the differential signaling by the heteromer/G-protein macromolecular complex likely implies communication at the receptor-receptor level but also between Gi and Gs. Because the binding of two G proteins to a heterodimer is not feasible due to steric clashes [25], our finding that the A1R-A2AR heterotetramer may bind to both Gi and Gs provides a structural framework to interpret experimental data.

Methods
Total internal reflection microscopy and single-particle data analysis
Single-particle imaging and tracking were performed on a Nikon Total Internal Reflection Fluorescence (TIRF) system, as detailed in Additional file 11: Supplementary Methods. Typically, 500 readouts of a 512 × 512-pixel region, the full array of the CCD chip, were acquired. For single-particle data analysis, parameters were calculated by applying the equations described in Additional file 11: Supplementary Methods.

Cell culture and transient transfection
HEK-293T cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Thermo Fischer Scientific, Madrid, Spain) supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 5 % (v/v) heat-inactivated fetal bovine serum (FBS) (all supplements were from Invitrogen, Paisley, UK). Cells were transiently transfected with cDNA corresponding to receptors, fusion proteins, A2AR mutants, or G-protein minigene vectors obtained as detailed in an expanded view by the polylethyleneimine (PEI; SigmaAldrich, Cerdanyola del Vallès, Spain) method. Sample protein concentration was determined using a Bradford assay kit (Bio-Rad, Muenchen, Germany) or FuGENE 6 (Roche Applied Science, Indianapolis, IN, USA) and the application of 0.1–0.2 µg plasmid DNA per well. Before each experiment, cells were washed three times with 200 µL phenol red-free DMEM.

Plasmids
DNA sequences encoding amino acid residues 1–155 and 155–238 of YFP Venus protein, and amino acids residues 1–229 and 230–311 of RLuc8 protein were subcloned in the pcDNA3.1 vector to obtain the YFP Venus and RLuc8 hemi-truncated proteins. The human cDNAs for adenosine receptors, A2AR and A1R, cloned into pcDNA3.1, were amplified without their stop codons using sense and antisense primers harboring unique EcoRI and BamHI sites to clone receptors into the pcDNA3.1RLuc vector (pRLuc-N1; PerkinElmer, Wellesley, MA, USA), and EcoRI and KpnI to clone A2AR, A1R, or GHS1a into the pEYFP-N1 vector (enhanced yellow variant of GFP; Clontech, Heidelberg, Germany). Gi3s cloned into the SFV1 vector, Gi3i cloned into the pcDNA3.1 vector, or Gi, cloned into the pEYFP-C1 vector were amplified
without their stop codons using sense and antisense primers harboring unique HindIII and BamHI sites to clone them into the pcDNA3.1-RLuc vector, or EcoRI and KpnI to clone Gαi into the pEYFP-N1 vector. The amplified fragments were subcloned to be in-frame with restriction sites of the pcDNA3.1RLuc or pEYFP-N1 vectors to give plasmids that expressed proteins fused to RLuc or YFP on the N-terminal end (Gαi/RLuc, Gαs/RLuc, Gαi/RLuc, Gαs/RLuc, Gαi-YFP, and Gαs-YFP) or the C-terminal end (A2aR-RLuc, A2aR-RLuc, A2aR-YFP, A2aR-YFP, and GHS1a-YFP). The human cDNAs for A1R or GHS1a were subcloned into pcDNA3.1-nRLuc8 or pcDNA3.1-nVenus to give plasmids that expressed A1R or GHS1a fused to either nRLuc8 or nYFP on Venus on the N-terminal end of the receptor (A1R-nRLuc8 and A1R-nYFP or GHS1a-nRLuc8 and GHS1a-nYFP). The cDNAs for human A2aR or GHS1a receptors were subcloned into pcDNA3.1-cRLuc8 or pcDNA3.1-cVenus to give plasmids that expressed receptors fused to either cRLuc8 or cYFP Venus on the C-terminal end of the receptor (A2aR-cRLuc8 and A2aR-cVenus or GHS1a-cRLuc8 and GHS1a-cVenus). Expression of constructs was tested by confocal microscopy and the receptor-fusion protein functionality by measuring ERK1/2 phosphorylation and cAMP production, as described previously [13, 14, 17, 29].

“Minigene” plasmid vectors are constructs designed to express relatively short polypeptide sequences following their transfection into mammalian cells. Here, we used minigene constructs encoding the carboxy-terminal 11-amino acid residues from Gα subunits of G11/2 (G1 minigene) or Gα (Gα minigene) G proteins; the resulting peptides inhibit G-protein coupling to the receptor and consequently inhibit the receptor-mediated cellular responses as previously described [24]. The cDNA encoding the last 11 amino acids of human Gα subunit corresponding to G11/2 (I K N N L K D C G L F) or Gα (Q R M H L R Q Y E L L), inserted in a pcDNA3.1 plasmid vector, were generously provided by Dr Heidi Hamm.

**Energy transfer assays**

For BRET and complementation BRET assays, HEK-293T cells were transiently cotransfected with a constant amount of cDNA encoding for proteins fused to RLuc, nRLuc8, or cRLuc8, and with increasing amounts of the cDNA corresponding to proteins fused to YFP, nYFP Venus, or cYFP Venus (see figure legends). To quantify protein-YFP expression or protein-reconstituted YFP Venus expression, cells (20 μg protein) were distributed in 96-well microplates (black plates with a transparent bottom) and fluorescence was read in a FLUOstar OPTIMA Fluorimeter (BMG Labtechnologies, Offenburg, Germany) equipped with a high-energy xenon flash lamp, using a 10 nm bandwidth excitation filter at 400 nm reading. Protein fluorescence expression was determined as the fluorescence of the sample minus the fluorescence of cells expressing the BRET donor alone. For BRET measurements, the equivalent of 20 μg of cell suspension were distributed in 96-well microplates (Corning 3600, white plates; Sigma) and 5 μM coelenterazine h (Molecular Probes, Eugene, OR, USA) was added. After 1 min for BRET or after 5 min for BRET with bimolecular fluorescence complementation, the readings were collected using a Mithras LB 940 that allows the integration of the signals detected in the short-wavelength filter at 485 nm (440–500 nm) and the long-wavelength filter at 530 nm (510–590 nm). To quantify protein-RLuc or protein-reconstituted RLuc8 expression, luminescence readings were also performed 10 min after adding 5 μM coelenterazine h. The net BRET was defined as [(long-wavelength emission)/(short-wavelength emission)] – Cf, where Cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the donor construct expressed alone in the same experiment. BRET is expressed as miliBRET units (mBU; net BRET × 1000).

**Computational model of the A1R-A2aR tetramer in complex with Gα and Gβ**

The crystal structure of inactive A2aR [PDB:4EIY] [30] was used for the construction of human A2aR [UniProt:P29274] and A1R [UniProt:P30542] homology models using Modeller 9.12 [31]. These receptors share 51 % of sequence identity and 62 % of sequence similarity, excluding the C-terminal after helix 8. Intracellular loop 3 (ICL3) of A2aR (Lys209–Gly218) and A1R (Asn212–Ser219) were modeled using Modeller 9.12 [31] using ICL3 of squid rhodopsin [PDB:2Z73] as a template. The C-terminus tails of A1R, containing 16 amino acids (Pro311–Asp326), and of A2aR, containing 102 amino acids (Gln311–Ser412), were modeled as suggested for the oxoeicosanoid receptor (OXER) [32] (see Additional file 9: Figure S9 for details). The N-terminus of A1R and A2aR were not included in the model. The “active” conformations of A1R bound to G1 and A2aR bound to Gα were modeled using the crystal structure of β2-AR in complex with Gβ [PDB:3SN6] [33]. The globular β-helical domain of the α-subunit was modeled in the “closed” conformation [34], using the crystal structure of [AlF₄]–activated Gα [PDB:1AGR]. The location of YFP [PDB:2RH7] attached to the C-tail of A2aR was determined as suggested for the OXER [32] (see Additional file 9: Figure S9 for details). RLuc [PDB:2PSD] and YFP were fused to the to the N-terminus of the α-subunits and γ-subunits of Gα and Gβ by a covalent bond. The structures of adenosine receptor oligomers were modeled via the TM4/5 interface for homodimerization, using the oligomeric structure of the β1-AR [PDB:4GPO] [4], or via the TM5/6 interface for
heterodimerization, using the structure of the μ-OR [PDB:4DKL] [3]. The G<sub>12</sub>-bound AR and G<sub>50</sub>-bound A<sub>2A</sub>R protomers were rotated 10° to avoid the steric clash of the N-terminal helix of G<sub>12</sub> and G<sub>50</sub> with the C-terminal helix (Hx8) of G<sub>12</sub>-unbound A<sub>2A</sub>R and G<sub>50</sub>-unbound AR, respectively. This computational model, without RLuc and YFP, was placed in a rectangular box containing a lipid bilayer (814 molecules of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine - POPC -) with explicit solvent (102,973 water molecules) and a 0.15 M concentration of Na<sup>+</sup> and Cl<sup>-</sup> (1762 ions). This initial complex was energy-minimized and subsequently subjected to a 10 ns MD equilibration, with positional restraints on protein coordinates. These restraints were released and 500 ns of MD trajectory were produced at constant pressure and temperature (see Additional file 10: Movie M1). Computer simulations were performed with the GROMACS 4.6.3 simulation package [35], using the AMBER99SB force field as implemented in GROMACS and Berger parameters for POPC lipids. This procedure has been previously validated [36].

Availability of data and materials

The crystal structures 4EIY, 2Z73, 3SN6, 1AGR, 2RH7, 2PSD, 4GPO, and 4DKL are available from PDB (http://www.rcsb.org). All other relevant data are within the paper and its Additional files.

### Additional files

**Additional file 1: Figure S1.** Examples of receptor trajectories in HEK-293T cells. Images of cells expressing A-R-GFP (A) and of particular trajectories of A-R-GFP-containing (B) or A<sub>2A</sub>-R-mCherry-containing (C) particles. (TIF 1164 kb)

**Additional file 2: Figure S2.** Graphical description of the stoichiometry of A-R-GFP, A<sub>2A</sub>-R-mCherry or both A-R-GFP and A<sub>2A</sub>-R-mCherry. The fluorescence intensity signal distribution (gray area) detected for more than 7000 independent observations is given for HEK-293T cells expressing A-R-GFP (A), A<sub>2A</sub>-R-mCherry (D), or both A-R-GFP and A<sub>2A</sub>-R-mCherry (B, E). The stoichiometry analysis was performed for A-R-GFP (A, B) and A<sub>2A</sub>-mCherry (D, E). Curves approximately delineating the amount of monomers, dimers, trimers, and tetramers for A-R-GFP (C) expressed alone (black bar) or in the presence of A<sub>2A</sub>-mCherry (blue bar) or for A<sub>2A</sub>-mCherry (F) expressed alone (black bar) or in the presence of A-R-GFP (blue bar) was calculated by stoichiometry analysis from results shown in A, B, D, and E. (TIF 455 kb)

**Additional file 3: Figure S3.** Controls of cAMP production and BRET assays in cells expressing minigenes and in cells expressing the ghrelin GHS1a receptor instead of one of the adenosine receptors. (A,B) cAMP determination in HEK-293T cells transfected with (A) 0.3 μg of cDNA corresponding to A-R (or B) with 0.2 μg of cDNA corresponding to A-R with (control) or without 0.2 μg of cDNA corresponding to minigenes coding for peptides blocking either G<sub>12</sub> or G<sub>50</sub> binding. Cells were stimulated with 0.5 μM forskolin (Fk) or with the A-R agonist 4-(2-isooctylamino-9-(N-ethyl)β-D-ribofuranosyl-amino)-9H-purin-2-ylamino)ethylbenzenepranoic acid hydrochloride (CGS-21680) (200 nM, blue bar). Values expressed as % of the forskolin-treated cells (Fk reduces forskolin-induced cAMP levels, blue bar) or of the basal (CGS 21680 per se enhances cAMP levels, blue bar) are given as mean ± SD (n = 4–8). One-way ANOVA followed by a Bonferroni post hoc test showed a significant effect of CPA when compared with that of forskolin (red bars, **p < 0.001) or of CGS 21680 when compared to basal cAMP levels (blue bars, *p < 0.001). (C, D) BRET saturation curves were performed in HEK-293T cells transfected with (C) 0.3 μg cDNA coding for A-R-RLuc, increasing amounts of cDNA coding for A-R-YFP (0.1–1.5 μg cDNA), and 0.4 μg cDNA coding for GHS1a, or (D) with 0.2 μg of cDNA coding for A<sub>2A</sub>-R-RLuc, increasing amounts of cDNA coding for A<sub>2A</sub>-R-YFP (0.1–10 μg cDNA), and 0.5 μg cDNA coding for GHS1a. Prior to BRET determination, cells were treated for 16 h with medium (black curves), with 10 ng/ml of pertussis toxin (green curves), or with 100 ng/ml of cholera toxin (red curves). milli BRET units (mBU) are given as the mean ± SD (n = 4–6 different experiments grouped as a function of the amount of BRET acceptor). (TIF 1418 kb)

**Additional file 4: Figure S4.** Possible interfaces in A<sub>2A</sub>-R homodimers in complex with G<sub>50</sub>. In A-E, the A<sub>2A</sub>-R homodimer was modeled through TM4 using the H1-receptor structure as template (A), through TM5 using the structure of squid rhodopsin (B), through TM4/5 using the β<sub>1</sub>-receptor structure (C), and via TM5/6 (D) and TM1 (E) using the μ-OR structure. TM helices 1, 4, and 5 involved in receptor dimerization are highlighted in dark blue, light blue, and gray, respectively. A<sub>2A</sub>-R protomers bound to G<sub>50</sub> (in gray) are shown in light green, whereas G<sub>12</sub>-unbound A<sub>2A</sub>-R protomers are shown in dark green. RLuc (blue) is attached to the N-terminal α helix of G<sub>50</sub> and YFP (yellow) is attached to the C-terminal domain of the G<sub>50</sub>-unbound A<sub>2A</sub>-R proton (light green). It is important to note that the position of YFP is highly dependent on the orientation of the long and highly flexible C-tail of A<sub>2A</sub>-R (102 amino acids, Gin311–Ser412), which was modeled as described for the OXER2 [32] (see Additional file 9: Figure S9 for details). Despite these limitations, we can crudely estimate the approximate distances between the center of mass of RLuc and YFP as 4.6, 10.1, 6.5, 11.6, and 8.3 nm for panels A–E, respectively. Thus, among all these possible dimeric interfaces, only the molecular models depicted in panels A (TM4 interface) and C (TM4/5 interface) would favor the observed high-energy transfer between GHS-RLuc and A<sub>2A</sub>-R-YFP (Fig. 4a in main paper). However, there is a steric clash between the N-terminal helix of G<sub>50</sub> and the dark-green proton in the TM4 interface. Accordingly, we have modeled A<sub>2A</sub>-R homodimerization via the TM5/5 interface. Unfortunately, similar experiments with cells transfected with G<sub>50</sub>-Luc and A<sub>2A</sub>-R-YFP could not be accomplished because of a lack of receptor expression (not shown); it is likely that the shorter C-tail of A<sub>2A</sub>-R (16 amino acids, Pro311–Asp326) could not accommodate YFP in the presence of G<sub>50</sub> in the right three-dimensional structure. The A<sub>2A</sub>-R homodimer was built using the same TM5/5 interface as for A<sub>2A</sub>-R. (TIF 3135 kb)

**Additional file 5: Figure S5.** BRET assays in cells expressing fusion proteins containing hemi-RLuc and hemi-Venus moieties fused to adenosine receptors or containing the ghrelin GHS1a receptor instead of one of the adenosine receptors. (A) Saturation BRET curve in HEK-293T co-transfected with 1.5 μg of the two cDNAs corresponding to A-R-RLuc8 and A<sub>2A</sub>-R-RLuc8 and with increasing amounts of cDNAs corresponding to A-R-Venus and A<sub>2A</sub>-R-Venus (equal amounts of the two cDNAs). BRET<sub>max</sub> was 35 ± 2 mBU and BRET<sub>50</sub> was 16 ± 3 mBU. BRET in cells expressing cRLuc8 instead of A-R-RLuc gave a linear, non-saturating signal. (B) Comparison of BRET responses using complementary and non-complementary pairs, or replacing one adenosine receptor with the ghrelin GHS1a (gn) receptor. Data are mean ± SD of three different experiments grouped as a function of the amount of BRET acceptor. **p < 0.001 with respect to BRET in cells expressing adenosine receptors and hemi-RLuc and hemi-Venus proteins. (TIF 398 kb)

**Additional file 6: Figure S6.** Details of the relative position of RLuc and YFP in a receptor heterotetramer interacting with two G proteins.

**Additional file 7: Figure S7.** Molecular dynamics (MD) simulation of the adenosine A<sub>2A</sub>-R heterotetramer in complex with G<sub>50</sub> and G<sub>12</sub>. (A) Root-mean-square deviations (rmsd) on protein carbons of the whole system (black solid line), of the two A<sub>2A</sub>Rs (orange and red line) and of the two G<sub>50</sub> (blue and purple line).
solid lines), of the two AγR-R (light and dark green solid lines), of Gγ (gray solid line), and of Gβγ (gray dotted line) throughout the MD simulation. This color scheme matches with the color of the different proteins depicted in the two adjacent schematic representations. (B) Intermolecular distances between the N-terminal helices of the γ-subunit of Gα and Gβ (magenta line), the N-terminal helices of the α-subunit of Gα and Gγ (gray line), the N-terminal helix of the α-subunit of Gγ, and the C-terminal helix (Hx8) of inactive AγR (orange line), the N-terminal helix of the α-subunit of Gγ, and the C-terminal Hx8 of inactive AβγR (green line), the C-terminal Hx8 of AβγR and AγγR (blue lines). These computed intermolecular distances are depicted as double arrows in the two adjacent schematic representations. (TIF 6973 kb)

Additional file 8: Figure S8. Evolution of TM5/S and TM5/S interfaces as devised from MD simulations of the adenosine A-R-AγR heterotrimer in complex with Gγ and Gβγ. (A) Representative snapshots (20 structures collected every 25 ns) of the TM domains of AγR bound to Gγ (red), Gγ-unbound AγR (orange), AβγR bound to Gβγ (dark green), and Gγ-unbound AβγR (light green). TM helices 4 and 5 are highlighted in light blue and gray, respectively. Initial (at 0 ns, transparent cylinders) and final (at 500 ns, solid cylinders) snapshots of TM interfaces are shown for homodimerization (TM5/S, within rectangles) and heterodimerization (TM5/S, within a circle) bundles. TM helices 4 (light blue), 5 (gray), and 6 (orange and green) are highlighted. (B) Root-mean-square deviations (rmsd) on protein α-carbons of the four-helix bundles forming the TM5/S interface (orange solid line), TM5/S interface of AγR (blue dotted line), and TM5/S interface of AγγR (blue solid line) throughout the MD simulation. (C) Contact maps of the TM5/S interface (rectangles in panel A) in the AγR-AγγR homodimer (left and right panels) and of the TM5/S interface (circle in panel A) in the AγR-AγγR-AγγR heterotrimer (middle panel). Darker dots show more frequent contacts. (D) Detailed view of the extensive network of hydrophobic interactions (mainly of aromatic side chains) within the TM5/S (left and right panels) and TM5/S interface (middle panel) interfaces. The amino acids are numbered following the generalized numbering scheme of Ballesteros and Weinstein (37, 38). This allows easy comparison among residues in the 7TM segments of different receptors. (TIF 4004 kb)

Additional file 9: Figure S9. Positioning YFP in the C-tail of AγR. The complex between the AγR-R protomer (in light green) and Gγ (α-subunit in dark gray and yellow, β-subunit in light gray, and γ-subunit in purple) was constructed from the crystal structure of β2 in complex with Gγ [33]. Although the exact conformation of the AγR-R C-tail (102 amino acids, Gln311–Ser412) cannot unambiguously be determined, its orientation was modeled as in the C-tail of squid rhodopsin [39], which contains the conserved amphipathic helix 8 that runs parallel to the membrane and an additional cytoplasmic helix 9. Thus, the C-tail of AγR-R expands (see solid light green line) and points intracellularly toward the N-termini of the γ-subunit as suggested for OXER [32]. The laboratory of Kostenis has shown that the C-terminal of OXER, labeled with RLuc (OXER-RLuc), gets close to the N-terminal of the γ-subunit, labeled with GFP (γ-GFP) [32]. Analogously, we propose that YFP attached to the C-tail of AγR is positioned near the N-termini of the γ-subunit (in purple). (TIF 2395 kb)

Additional file 10: Movie M1. Assembly of adenosine Aγ and Aβγ receptors in complex with two G proteins and MD simulation of the system. The assembly of Gγ and Gβγ bound to the adenosine AγR-AγγR heterotrimer was subjected to 500 ns of MD simulation in a rectangular box containing the system, the lipid bilayer, explicit solvent, and ions. AγR protomers are in orange and red, AγγR protomers in light and dark green, Gγ in white, Gβ in gray, and Gγ in purple. For easier visualization of protomer-protrimer interfaces, TM 4 and 5 are highlighted in blue and white, respectively. (MPEG 87870 kb)

Additional file 11: Supplementary methods. (DOCX 72 kb)

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
GAM, JH, GR, and JH conceived the study. GAM, SMC, GB, EM, and DA performed BRET experiments. MW-F performed single-particle tracking experiments. AC and LP-B performed molecular modeling studies. AC, VC, JM, and EIC analyzed the data. CL, LP, AJG-S, PJM, and RF designed the experiments, supervised the work in the respective laboratories and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments
We acknowledge the technical help provided by Jasmina Jiménez (CIBERNED, University of Barcelona). This study was supported by grants from the Spanish Ministerio de Ciencia y Tecnología (SAF2009-07276, SAF2010-18472, SAF2011-23813, SAF2013-48271-C2-2-R), those grants may include FEDER funds), the Max Planck-Society, the German Cancer Research Center, and the German Ministry for Education and Research (BMBF). PJM and LP participate in the European COST Action CM1207 (GLITEN). Authors gratefully acknowledge the computer resources provided by the Barcelona Supercomputing Center - Centro Nacional de Supercomputación.

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Received: 26 September 2015 Accepted: 16 March 2016

Published online: 05 April 2016

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