Cross-communication between $G_\text{i}$ and $G_\text{s}$ in a G-protein-coupled receptor heterotetramer guided by a receptor C-terminal domain

Gemma Navarro$^{1,2,3}$, Arnau Cordomí$^4$, Marc Brugarolas$^{1,2,3}$, Estefanía Moreno$^{1,2,3}$, David Aguinaga$^{1,2,3}$, Laura Pérez-Benito$^4$, Sergi Ferre$^5$, Antoni Cortés$^{1,2,3}$, Vicent Casadó$^{1,2,3}$, Josefa Mallol$^{1,2,3}$, Enric I. Canela$^{1,2,3}$, Carme Lluís$^{1,2,3}$, Leonardo Pardo$^4$, Peter J. McCormick$^{1,2,3,6}$* and Rafael Franco$^{1,2,3}$*

Abstract

Background: G-protein-coupled receptor (GPCR) heteromeric complexes have distinct properties from homomeric GPCRs, giving rise to new receptor functionalities. Adenosine receptors (A1R; A3AR) can form A1R-A3AR heteromers (A1-A3AHet), and their activation leads to canonical G-protein-dependent (adenylate cyclase mediated) and -independent ($\beta$-arrestin mediated) signaling. Adenosine has different affinities for A1R and A3AR, allowing the heteromeric receptor to detect its concentration by integrating the downstream Gi- and Gs-dependent signals. cAMP accumulation and $\beta$-arrestin recruitment assays have shown that, within the complex, activation of A3AR impedes signaling via A1R.

Results: We examined the mechanism by which A1-A3AHet integrates Gi- and Gs-dependent signals. A1R blockade by A3AR in the A1-A3AHet is not observed in the absence of A3AR activation by agonists, in the absence of the C-terminal domain of A3AR, or in the presence of synthetic peptides that disrupt the heteromer interface of A1-A3AHet, indicating that signaling mediated by A1R and A3AR is controlled by both Gi and Gs proteins.

Conclusions: We identified a new mechanism of signal transduction that implies a cross-communication between Gi and Gs proteins guided by the C-terminal tail of the A3AR. This mechanism provides the molecular basis for the operation of the A1-A3AHet as an adenosine concentration-sensing device that modulates the signals originating at both A1R and A3AR.

Keywords: C-terminal domain, GPCR, Heterotetramer, BRET, Molecular modeling

Background

Adenosine is a purine nucleoside whose relevance in the central nervous system is mainly due to its role in regulating neurotransmitter release [1]. The effects of adenosine are mediated by specific G-protein-coupled receptors (GPCRs) that are coupled to either $G_i$ or $G_s$ heterotrimeric $G_{iS}$ proteins. The endogenous adenosine acts on four receptor subtypes – $A_1R$, $A_2AR$, $A_2BR$, and $A_3R$. Convergent and compelling evidence shows that GPCRs may form complexes constituted by a number of equal (homo) or different (hetero) receptor protomers [2]. As agreed in the field, a GPCR heteromer displays characteristics that are different from those of the constituting protomers, thus giving rise to novel functional entities [3]. Adenosine receptors have been used as a paradigm in the study of receptor homo- and heteromerization. For instance, $A_1R$, which is $G_i$ coupled, and $A_2AR$, which is $G_s$ coupled, form a functional heteromer [4].
The A₁R-A₂AR heteromer (A₁-A₂AHet) is found presynaptically in, inter alia, cortical glutamatergic terminals innervating the striatum and functions as a switch that differentially senses high and low concentrations of adenosine in the inter-synaptic space. Since adenosine has higher affinity for A₁R than for A₂AR, low concentrations predominantly activate A₁R, engaging a Gₛ-mediated signaling, whereas higher adenosine concentrations also activate A₂AR, engaging a Gᵢ-mediated signaling [4]. The physiological role of such a concentration-sensing device is remarkable as it allows adenosine to fine-tune modulate the release of neurotransmitters from presynaptic terminals. However, the mechanism by which A₁-A₂AHet integrates both Gᵢ- and Gₛ-dependent signals is not yet understood. We have recently shown, using a combination of single-particle tracking experiments, bioluminescence resonance energy transfer (BRET) assays, and computer modeling, that the (minimal) functional A₁-A₂AHet/G protein unit is composed by a compact rhombus-shaped heterotetramer (with A₁R and A₂AR homodimers) bound to two different interacting heterotrimeric G proteins (Gₛ and Gᵢ) [5]. In the present study, we aim to understand the molecular intricacies underlying the signaling mediated by A₁-A₂AHet, in which (1) both receptors constituting the heteromer are activated by the same endogenous agonist and (2) is coupled to two different G proteins with opposite effects, i.e., one mediating the inhibition of the adenylate cyclase (Gᵢ) and another mediating the activation of the enzyme (Gₛ). Our data identifies a new mechanism of signal transduction and provides the molecular basis to understand the unique properties of this heteromer, in which the C-terminal tail of the A₂AR influences the Gᵢ-mediated signaling of the partner A₁R receptor.

Results
Homodimerization of A₁R and A₂AR occurs through the transmembrane (TM) 4/5 interface and heterodimerization via the TM5/6 interface in the A₁-A₂AHet

Our recently published BRET-aided computational model of the A₁-A₂AHet predicted the TM interfaces involved in homo- (TM4/5) and heterodimerization (TM5/6) [5]. To further confirm this arrangement, we used synthetic peptides with the sequence of TM domains of the A₂AR (TM1 to TM7) and the A₁R (TM5 to TM7), fused to the C-terminally penetrating HIV transactivator of transcription (TAT) peptide [6], to alter inter-protomer interactions in the A₁-A₂AHet. These peptides were first tested in bimolecular fluorescence complementation (BIFC) assays in HEK-293 T cells expressing receptors fused to two complementary halves of YFP (cYFP and nYFP) (see Methods). We detected fluorescence in HEK-293 T cells transfected with cDNAs for A₂AR-nYFP, A₂AR-cYFP, and non-fused A₁R (broken lines in Fig. 1a), indicating the formation of the A₂AR-A₂AR homodimer. Notably, in the presence of...
interference peptides, we observed a fluorescence decrease only with TM4 and TM5 of A2AR (Fig. 1a), but not with A1R TM peptides (Fig. 1a) or with peptides derived from the orexin receptor (Additional file 1: Figure S1A) used as negative controls. Further negative controls show that A2AR peptides do not alter fluorescence in HEK-293 T cells expressing A1R-nYFP and A1R-cYFP (Additional file 1: Figure S1B). These results therefore confirmed the TM4/5 interface for A2AR homodimerization in the heteromer. Similarly, we detected fluorescence in cells expressing A1R-nYFP and A2AR-cYFP (broken lines in Fig. 1b), indicating formation of the A1-A2AHet. This fluorescence was only reduced in the presence of TM4, TM5, and TM6 peptides of A2AR (Fig. 1b). The involvement of TM5/6 in the heteromer interface was also confirmed by the fact that TM5 and TM6, but not TM7, of A1R reduced fluorescence in cells expressing A1R-nYFP and A2AR-cYFP (Fig. 1b). These results reinforce our previously proposed compact rhombus-shaped arrangement of protomers in which heteromerization of A1-A2AHet occurs via the TM5/6 interface (Fig. 1f).

The fluorescence decrease induced by TM4 A2AR peptide indicates that the correct homomerization is requisite for A1-A2AHet formation and/or that the TM4 peptide interferes with interactions of the TM4 of the external protomer of the A2AR homodimer with the internal protomer of the A1R homodimer (Fig. 1f) [5]. Next, we evaluated whether receptor activation, by the A1R-selective agonist N6-cyclopentyladenosine (CPA), the A2AR-selective agonist 4-

The complex formed by Gs, Gi, and the A1-A2AHet as a signal transduction unit

In order to test the ability of Gs and Gi proteins to interact with the A1-A2AHet, we used BRET assays [7]. Cells were transfected with cDNAs of A1R-nYFP and A2AR-cYFP, which only upon complementation can act as a BRET acceptor (YFP), and Renilla luciferase (Rluc) as a BRET donor fused to either G i (G i-Rluc) or G s (G s-Rluc). We observed significant energy transfer (Additional file 1: Figure S1C), indicating that G i and G s are bound to their respective receptors in the A1-A2AHet.

Next, we tested whether the A1-A2AHet can signal through Gi- and G i-dependent pathways by measuring cAMP levels in cells expressing both A1R and A2AR. The A1R-selective agonist CPA (100 nM, a concentration producing maximal effect), which was unable to modify cAMP levels in the absence of forskolin (Additional file 1: Figure S2A), decreased forskolin-induced cAMP due to its Gi coupling, and the A2AR-selective agonist CGS21680 (100 nM, a concentration producing maximal effect) increased cAMP due to a G s coupling (Fig. 3a, control), indicating that both receptors signal via their cognate G protein. We performed the same experiments in cells treated with pertussis (PTX) or cholera (CTX) toxins, which impair G i- and G s-mediated signaling, respectively, and in cells transfected with minigenes that encode for peptides blocking the interaction of the receptor with the α subunits of G i or G s [8]. As expected, we observed blockade of CPA-induced cAMP decrease by either PTX (Fig. 3a) or the G i-specific minigene (Fig. 3b), and blockade of CGS21680-induced cAMP increase by CTX.

**Fig. 2** Effect of interference peptides on the A1-A2AHet structure determined by proximity ligation assay (PLA) confocal microscopy images (superimposed sections) in which A1-A2AHets appear as red spots. HEK-293 T cells expressing A1R and A2AR were treated for 4 h with medium (control) or 4 μM of indicated TM peptides of A2AR; cell nuclei were stained with DAPI (blue); scale bars: 10 μm
Strikingly, PTX or G\textsubscript{i}-specific minigene (blocking G\textsubscript{i}-receptor interaction) also blocked the CGS21680-induced cAMP increase (Fig. 3a, b). Moreover, CTX or the G\textsubscript{s}-specific minigene (blocking G\textsubscript{s}-receptor interaction) also blocked the CPA-induced cAMP decrease (Fig. 3a, b). Control experiments using these agonists in cells expressing only A\textsubscript{1}R or A\textsubscript{2}AR did not show any crossover effect with either toxins or minigenes (Additional file 1: Figures S2B, C, E, F). These results demonstrate that both A\textsubscript{1}R- and A\textsubscript{2}AR-mediated signaling in the A\textsubscript{1}-A\textsubscript{2}AHet are dependent on the functional integrity of both G\textsubscript{i} and G\textsubscript{s} proteins. According to this, we observed by BRET experiments that the A\textsubscript{2}AR agonist-induced interaction between A\textsubscript{1}-A\textsubscript{2}AHet and G\textsubscript{s} protein diminished in cells pre-treated with PTX (Additional file 1: Figure S1D). We hypothesize that this cross-communication could depend on the ability of \(\alpha\) subunits of G\textsubscript{i} and G\textsubscript{s} coupled to the A\textsubscript{1}-A\textsubscript{2}AHet to establish mutual interactions (see below).

To further test for a cross-communication between G proteins in the G\textsubscript{s}-G\textsubscript{i}-heterotetramer signaling unit, we resolved the real-time signaling signature by using a label-free method, based on optical detection of dynamic changes in cellular density following receptor activation [9]. The magnitude of the signaling by CPA or by CGS21680 significantly decreased when cells co-expressing both receptors were pre-treated with either PTX or CTX (Fig. 3d). This phenomenon was not observed in cells expressing only A\textsubscript{1}R (Additional file 1: Figure S2G) or A\textsubscript{2}AR (Additional file 1: Figure S2H). Again, these results indicate the simultaneous coupling of interacting G\textsubscript{s} and G\textsubscript{i} proteins within the A\textsubscript{1}-A\textsubscript{2}AHet.

Simultaneous activation of both A\textsubscript{1}R and A\textsubscript{2}AR with CPA and CGS21680 increased cAMP to similar levels to those obtained with CGS21680 alone and the signal of subunits of G\textsubscript{i} and G\textsubscript{s} coupled to the A\textsubscript{1}-A\textsubscript{2}AHet to establish mutual interactions (see below).

**Fig. 3** Receptor signaling through the A\textsubscript{1}-A\textsubscript{2}AHet. Increases in cAMP percentage accumulation with respect to Fk-stimulated (a, b) or unstimulated (c) cells. A\textsubscript{1}-A\textsubscript{2}AHet-expressed cells pre-treated with medium, PTX (10 ng/mL overnight) or CTX (100 ng/mL for 1 h) before adding medium, forskolin (Fk, 0.5 \(\mu\)M), CPA (100 nM) plus/minus forskolin, CGS21680 (100 nM) plus/minus forskolin, or CPA + CGS21680. b Same assays in the absence or presence of 0.5 \(\mu\)g of cDNA corresponding to G\textsubscript{i}- or G\textsubscript{s}-\(\alpha\) subunit-related minigenes. Mean ± SEM (7 experiments/group). One-way ANOVA followed by Bonferroni’s post-hoc test in panels a, b showed a significant effect over basal in samples treated with CGS21680 or over forskolin in samples treated with CPA; in panel c, a significant effect is seen over basal (*\(P< 0.05\), ***\(P< 0.001\)). d The dynamic mass redistribution analysis was plotted as pm shifts versus time (Representative experiment, performed in triplicate). e, f Distances between the C\(\alpha\) atoms of Arg90 (\(\alpha\)AH domain) and Glu238 (Ras domain) of G\textsubscript{i} (in yellow), Asn112 (\(\alpha\)AH) and Asn261 (Ras) of G\textsubscript{s} (green), Arg90 (\(\alpha\)AH) and Asn112 (\(\alpha\)AH) (dark red), and between the center of masses of the binding sites of the G\textsubscript{i}-unbound A\textsubscript{1}R and G\textsubscript{s}-unbound A\textsubscript{2}AR protomers (black) obtained from two independent molecular dynamics (MD) simulations of A\textsubscript{1}-A\textsubscript{2}AHet in complex with G\textsubscript{i} and G\textsubscript{s} in which \(\alpha\)AH was modelled in the closed conformation (Additional file 1: Figure S6C) and \(\alpha\)AH was modelled in closed (e) or open (f) conformation. The computed distances are depicted as double arrows in the adjacent schematic representations. Representative snapshots of the models are shown. Code: G\textsubscript{i}-bound A\textsubscript{1}R/red, G\textsubscript{i}-unbound A\textsubscript{1}R/orange, G\textsubscript{s}-bound A\textsubscript{2}AR/light green, G\textsubscript{s}-unbound A\textsubscript{2}AR/dark green, \(\alpha\), \(\beta\), and \(\gamma\) of G\textsubscript{s}/G\textsubscript{i} in dark gray/light blue/purple, respectively, TM4/light blue, TM5/gray, \(\alpha\)iAH/green, and \(\alpha\)sAH/yellow. g MD simulations could not be performed for open conformations of \(\alpha\)AH and \(\alpha\)AH due to steric clash.
co-activated receptors was inhibited by both PTX and CTX (Fig. 3c). Therefore, A₁R agonist was able to decrease forskolin-induced cAMP (Fig. 3a, b) and yet was unable to decrease A₂AR-mediated increases of cAMP (Fig. 3c). Consequently, when both receptors are co-activated in the heterotetramer, only the A₂AR-mediated, but not the A₁R-mediated signaling occurs. This finding was confirmed in label-free experiments, showing that receptor co-activation with CPA and CGS 21680 did not increase the time-response curve with respect to the activation with CGS 21680 alone (Fig. 3d green and yellow lines, respectively).

It has been shown that the mechanism for receptor-catalyzed nucleotide exchange in G proteins involves a large-scale opening of the α-helical domain (αAH) of the α-subunit, from the Ras domain, allowing GDP to freely dissociate [10–13]. Notably, our proposed model of the A₁-A₂AHet positions the αAH and αAH domains facing each other (Fig. 3e). The fact that both Gₛ- and Gᵢ-specific toxins and Gₛ- and Gᵢ-specific minigenes affect both Gₛ- and Gᵢ-mediated coupling in the A₁-A₂AHet suggests that the proposed large-scale conformational changes of αAH domains is mutually dependent. We used molecular dynamics (MD) simulations of the A₁-A₂AHet in complex with Gₛ and Gᵢ to evaluate intermolecular distances between the αAH and αAH domains when αAH is in the closed conformation and αAH is either in the open (Fig. 3e) or in the closed conformation (Fig. 3ef). In a previous report, double electron–electron resonance (DEER) distance distributions between spin labels attached to Arg90 (αAH domain) and Glu238 (Ras domain) of Gₛ (the distance between Ca atoms is termed d[Arg90α-,Glu238α] in the manuscript) or Asn112 (αAH) and Asn261 (Ras) of Gᵢ (d[Asn112α-,Asn261α]) permitted to faithfully monitor the equilibrium within the open (distance of ~40 Å) and closed (~20 Å) conformation of the αAH domain [13]. Here, we measured the intermolecular distance between the αAH and αAH domains using Ca atoms of Arg90 of αᵢ and Asn112 of αₛ (d[Arg90αᵢ-,Asn112αₛ]). This d[Arg90αᵢ-,Asn112αₛ] intermolecular distance between αAH in the closed conformation (d[Arg90αᵢ-,Glu238αᵢ]: 11 Å, yellow line in Fig. 3e) and αAH in the closed conformation (d[Asn112αᵢ-,Asn261αᵢ]: 14 Å, green line in Fig. 3e) has an average value of 108 Å for inactive A₁-A₂AHet (Fig. 3e, dark red line). Activation of A₂AR would trigger the opening of αAH (d[Asn112αᵢ-,Asn261αᵢ]: 52 Å; Fig. 3f, green line), necessary for GDP/GTP exchange, decreasing the d[Arg90αᵢ-,Asn112αᵢ] distance between αAH and αAH to 60 Å (Fig. 3f, dark red line). Although the results are based on a single trajectory, it is unlikely that additional replicas would change, in a significant manner, the distances reported from the simulations. Moreover, the differences between the distances are so substantial that results from more simulations would not have a significant impact. We hypothesize that a similar change occurs with activation of A₁R. This indicates that both receptors can signal via their cognate G protein by opening their αAH domain. However, in the compact rhombus-shaped A₁-A₂AHet model, simultaneous opening of both αAH domains (co-activation with CPA and CGS 21680) would not be possible due to a steric clash in such open conformations (Fig. 3g). Due to this steric clash, MD simulations of this open αAH-open αAH conformation in the absence of interference peptides (see below) cannot be performed.

**Altering the heteromer interface of A₁-A₂AHet enables simultaneous Gₛ and Gᵢ signaling**

Next, we investigated whether the correct formation of the A₁-A₂AHet is a necessary condition for the crosstalk between the Gₛ- and Gᵢ-signaling units using the interference peptides (TM4, TM5 and TM6 of A₂AR, which alter receptor heterodimerization, and TM7 as a negative control). Remarkably, pretreatment of cells expressing A₁-A₂AHet with the interference peptides did not change receptor signaling when only one receptor is activated (Fig. 4a). Interestingly, in the presence of TM4, TM5 and TM6 peptides, simultaneous activation of both A₁R and A₂AR with CPA and CGS21680, respectively, allows CPA to decrease CGS21680-stimulated cAMP (Fig. 4a), in contrast to experiments in the absence of either interference peptides (Fig. 4a, control) or TM7 used as a negative control (Fig. 4a). Moreover, this decrease in cAMP accumulation in the CPA/CGS co-stimulated condition is mediated by activation of the A₁R/Gₛ pathway as, in the presence of TM peptides, a selective A₁R antagonist or the treatment with PTX blocks the CPA-induced effect (Additional file 1: Figure S2D). Thus, modification of the quaternary structure of the A₁-A₂AHet with peptides that penetrate within the heteromer interface abolishes inhibition of A₁R by A₂AR in the Gₛ-Gᵢ-heterotetramer signaling unit. These experimental results suggest that synthetic peptides inserted between A₁R and A₂AR protomers, which are not able to disrupt the heteromer as seen by PLA (Fig. 2), increase the distance between Gₛ and Gᵢ. This would allow the simultaneous opening of αAH and αAH domains for GDP dissociation. In order to verify this hypothesis, we modeled the A₁-A₂AHet with the TAT-fused peptide TM6 altering the heteromer interface between A₁R and A₂AR, in complex with Gₛ (open αAH, d[Asn112αᵢ-,Asn261αᵢ]: 56 Å; Fig. 4b, green line) and Gᵢ (open αAH, d[Arg90αᵢ-,Glu238αᵢ]: 52 Å; Fig. 4b, yellow line). Due to the insertion of TM6, the distance between the binding site of A₁R and A₂AR increases by 17 Å, from 14 Å in the absence of TM6 (Fig. 3e, f, black line) to 31 Å in the presence of TM6 (Fig. 4b, black line). This increase in the distance between heteromers also moves the
A1R and engages a Gαi-mediated signaling, which at low concentration (30 nM) binds predominantly to A1R. Due to the higher affinity for the hormone, adenosine at concentrations compatible with Gαi activation and blockade of Gαi, as depicted in the schemes of Fig. 5a. In these conditions, full active A2AR can increase cAMP over the forskolin-induced levels whilst the progressive blockade of A1R by A2AR cannot reduce cAMP accumulations. To demonstrate such blockade of A1R actions by A2AR, we performed the experiments in the presence of a peptide (A2AR TM6) that inserts into the heteromer interface (Fig. 5b). In the presence of the peptide, the device lost its concentration-sensing properties. In fact, high adenosine concentrations, in which both receptors are fully occupied and functional, led to a null response, i.e., the A2AR-mediated increase in forskolin-stimulated cAMP is counteracted by a similar Gαi-mediated decrease of cAMP. Upon heteromer structure alteration by TM6, the A2AR becomes unable to block A1R-mediated signaling.

Recruitment of β-arrestin-2 by the A1-A2AHet

We used BRET assays to detect the interaction between a protomer and β-arrestin-2. Thus, cells were transfected with cDNAs of β-arrestin-2 fused to Rluc (Arr-Rluc) as the BRET donor and A1R or A2AR fused to YFP (A1R-YFP, A2AR-YFP) as the BRET acceptor. Control experiments in cells expressing only A1R-YFP or A2AR-YFP and Arr-Rluc show the ability of both receptors to recruit β-arrestin-2 (Additional file 1: Figure S3A) and the selectivity of each agonist (Additional file 1: Figure S3B). Similar experiments in cells additionally expressing non-fused A2AR (Arr-Rluc/A1R-YFP + A2AR) or non-fused A1R (Arr-Rluc/A2AR-YFP + A1R) were performed (Additional file 1: Figure S3B). Interestingly, in cells expressing Arr-Rluc, A2AR-YFP and non-fused A1R (control in Fig. 6a and Additional file 1: Figure S3B) or Arr-Rluc, A1R-YFP and non-fused A2AR (control in Fig. 6b and Additional file 1: Figure S3B), a similar degree of BRET was induced by CGS-21680 (white bars) or by CGS-21680 plus CPA (striped bars). This suggests that agonist binding to A2AR inhibits the CPA ability to stimulate β-arrestin-2 recruitment to A1R. In order to rationalize these results, we have used the recent crystal structure of rhodopsin bound to visual arrestin-1 [14] to model the A1-A2AHet complex in β-arrestin-2. The finger loop of arrestin, which adopts a short α-helix, is inserted into the intracellular cavity of the external protomer, whereas the C-domain of arrestin points towards the internal protomer of the homodimer. Figure 6c shows key intermolecular distances between the center of mass of the N- and C-domains of two arrestin molecules bound to A1R and A2AR obtained from MD simulations of A1-A2AHet in complex with Gαi and Gαs.
simulations. These data suggest that the A1-A2AHet quaternary structure permits the binding of two arrestin molecules to the external protomers of both A1R and A2AR, similarly to the simultaneous binding of G\textsubscript{i} and G\textsubscript{s} to the heterotetramer. Moreover, similar simulations of A1-A2AHet in complex with G\textsubscript{i} and β-arrestin-2 (Fig. 6d) show no steric clashes between G\textsubscript{i} (bound to A1R) and arrestin (bound to A2AR). These results suggest that sustained activation of G\textsubscript{s} (G\textsubscript{βγ} moving away from G\textsubscript{α}s to facilitate the interaction of G\textsubscript{αi} with the catalytic domain of adenylate cyclase) by agonist binding to A2AR enables β-arrestin-2 recruitment to A2AR. As stated above, within the A1-A2AHet, CPA cannot activate G\textsubscript{i} in the presence of the A2AR agonist CGS-21680 (Fig. 3) and, consequently, CPA does not trigger additional β-arrestin-2 recruitment to A1R (control in Figs. 6a, b and Additional file 1: Figure S3B).

Using the TAT-fused synthetic peptides we investigated whether the quaternary structure of the A1-A2AHet determines its putative selective A2AR-dependent β-arrestin-2 recruitment. As a negative control, we first corroborated that TM4, TM5, and TM6 peptides of A2AR do not interfere with A1R-mediated signaling (Additional file 1: Figure S3C). Pretreatment of cells expressing Arr-Rluc, A2AR-YFP and non-fused A1R (Fig. 6a), or Arr-Rluc, A1R-YFP and non-fused A2AR (Fig. 6b) with TM4, TM5, and TM6 peptides, but not in the absence of peptides (control) or with the TM7 peptide (negative control), allowed the detection of positive BRET (recruitment of β-arrestin-2) not only when cells were treated with the A2AR-selective agonist CGS-21680 (white bars), but also when treated with the A1R-selective agonist CPA (black bars) (Figs. 6a, b). Importantly, when cells expressing Arr-Rluc, A2AR-YFP, and non-fused A1R were co-activated by CPA and CGS-21680 (striped bars), BRET measurement in the presence of TM4, TM5, or TM6 peptides, but neither in the absence of peptides nor in the presence of TM7 peptide, significantly increased relative to the values obtained by the action of a single agonist (Fig. 6a). The trend is similar in cells expressing Arr-Rluc, A1R-YFP, and non-fused A2AR, but not statistically significant (Fig. 6b). These results indicate that alteration of the A1R-A2AR heteromer interface within the A1-A2AHet allows simultaneous recruitment of β-arrestin-2 to A1R and A2AR when both receptors are activated. Interference peptides abolish cross-communication of G proteins, permitting CPA to activate G\textsubscript{i} (G\textsubscript{βγ} moving away from G\textsubscript{α}s) and simultaneous recruitment of β-arrestin-2 to A2AR.

The C-terminal domain of A2AR is responsible for the dominant A2AR-mediated signaling

Despite the apparent structural symmetry of the GPCR/G protein macromolecular complex, a major difference
is the length of the intracellular C-terminal domain of adenosine receptors (16 amino acids in A1R versus 102 in A2AR). The short C-terminal tail of the A1R does not have any known specific function, while the C-terminus of A2AR, albeit dispensable for ligand binding [15], dimerization [16], and agonist induced cAMP signaling [17], influences constitutive signaling [18]. Due to the shorter C-terminus of A1R and the proposed orientation of the C-tail of A2AR toward αsAH (see Additional file 1: Figure S4a for details), as well as the proposed role of the C-terminal tail in downstream signaling cascade activation [19], we speculated that the C-terminus of A2AR could modulate the prevailing Gs-mediated signaling upon A1R and A2AR co-activation. To test this hypothesis, we engineered two A2AR mutants, one lacking most of the C-terminal end (A2ΔCTR) and another lacking the last 40 amino acids (A2Δ40R). First, we tested whether these truncated versions of A2AR could form heteromers with A1R. We observed similar BRET saturation curves in HEK-293 T cells expressing a constant amount of A1R-Rluc cDNA and increasing amounts of either A2AR-YFP, A2Δ40R-YFP, or A2ΔCTR-YFP, indicating that A2Δ40R and A2ΔCTR form heteromers with A1R (Fig. 7a; BRETmax in mU: 91 ± 3 A2AR, 99 ± 3 A2Δ40R, and 90 ± 8 A2ΔCTR). Heteromers were also detected by BiFC assays in HEK-293 T cells transfected with cDNAs for A1R-nYFP and A2ΔCTR-cYFP (Fig. 7b, dashed line). In these cells, fluorescence was reduced in the presence of TM4, TM5, and TM6 peptides of A2AR (Fig. 7b). Thus, heteromerization of A2ΔCTR with A1R occurs via the TM5/6 interface, similarly to the interaction of A2AR with A1R.

We measured cAMP production in cells expressing A1R and wild-type or truncated A2AR receptors (Fig. 7c).
Truncated A2AR were able to signal as wild-type receptors. Interestingly, the dominant Gs-mediated signaling when A1Ra and A2AR were co-activated decreased progressively with the shortening of the A2ARC-tail (Fig. 7c, striped bars). In fact, CPA inhibited CGS-21680-induced cAMP accumulation when truncated receptors were expressed, showing that, in these heteromers, A1R were functional (Additional file 1: Figure S5). Figure 7e shows a detailed view of the orientation of the C-tail (102 amino acids, Gln311-Ser412) of both A2AR protomers in the A1-A2AHet, which was modeled as suggested for the OXER [20], together with the structure of β-arrestin-2 in complex with V2 vasopressin receptor [21]. It is important to note that the exact conformation of the A2AR C-tail cannot unambiguously be determined, thus, we only predict its orientation as explained in detail in Additional file 1: Figure S4.

The fact that the C-tail of the αs-unbound A2AR protomer points toward the αsAH domain suggests that this C-tail is influencing the conformational changes required to open the αsAH, and thus controlling the balance between Gs and Gi activation. Next, we measured β-arrestin-2 recruitment by BRET assays in cells expressing A1R and wild-type or truncated A2AR receptors. In cells expressing non-fused A1R, Arr-Rluc and A2AR-YFP, A2ARΔ40R-YFP, or A2ARΔCTR-YFP, the A1R agonist CPA could increase BRET values only when the heteromer is formed with A2AR-truncated receptors. In these conditions, co-activation with CPA and CGS-21860 induced a BRET increase higher than the one obtained with CGS-21680 alone (Fig. 7d). These results indicate that the selective A2AR-dependent β-arrestin-2 recruitment in the A1-A2AHet decreases progressively with the shortening of the A2AR C-tail (Fig. 7d).
Discussion

As previously reviewed [2, 3, 22], the intercommunication between protomers of a GPCR heteromer can be observed at the level of agonist binding, ligand-induced cross-conformational changes between receptor protomers, and the binding of GPCR-associated proteins, including heterotrimeric G proteins and β-arrestins. The intercommunication between protomers is a consequence of a defined quaternary structure that is responsible for the specific functional characteristics of the heteromer. For GPCR heteromers, such as A₁-A₂AHet, constituted by receptors sensing the same hormone but producing opposite signaling effects, it is not obvious how a defined quaternary structure achieves this dual behavior. A₁-A₂AHet acts as a concentration-sensing device that allows adenosine to signal by one or the other coupled G protein (Gᵢ or Gₛ) to fine-tune modulate the release of neurotransmitters from presynaptic terminals. In the present study, we solved this question by discovering a new mechanism of signal transduction, a cross-communication between Gᵢ and Gₛ in the A₁-A₂AHet guided by the A₂AR C-terminal domain.

We have shown that cross-communication between Gᵢ and Gₛ proteins involves the formation of a GPCR heterotetramer (i.e., one homodimer of A₁R and one of A₂AR) that has a 2:2:1:1 (A₂AR:A₁R:Gₛ:Gᵢ) stoichiometry. From our data, it is deduced that the cross-talk between Gᵢ and Gₛ resides on the structural constraints surrounding the mechanism for GDP/GTP exchange, which involves the opening of the αAH domain of the α-subunit of any given G protein. We propose that cross-communication in the Gᵢ-Gₛ-heterotetramer signaling unit is a property associated with a specific quaternary structure, the compact rhombus-shaped A₁-A₂AHet (the TM4/5 interface for homodimerization and the TM5/6 interface for heterodimerization), which positions the α₁AH and α₂AH domains in close proximity, making their conformational changes mutually dependent in a way that simultaneous opening of both αAH domains would not be possible due to a steric clash in such open conformations. Alterations of this quaternary structure of the A₁-A₂AHet by insertion of synthetic peptides between A₁R and A₂AR blocks this cross-communication without disrupting the heteromer and permits simultaneous activation of Gᵢ and Gₛ in the heteromer. Since the cross-talk between Gᵢ and Gₛ resides on the structural constraints imposed by defined TM interfaces in the heteromer, it is important to note that other heterotetramers, mainly those sensing different hormones and with a different quaternary structure, might not display this cross-communication among G proteins. Moreover, although, from a structural point of view, the A₁-A₂AHet is capable to recruit not only two G proteins but also two β-arrestins, the cross-talk between Gᵢ and Gₛ, in which Gₛ activation inhibits the simultaneous activation of Gᵢ, blocks A₁R agonist-promoted arrestin recruitment. Alteration of the A₁-A₂AHet by insertion of synthetic peptides between A₁R and A₂AR facilitates simultaneous activation of Gᵢ and Gₛ and the corresponding binding of two β-arrestins to A₁R and A₂AR. Our finding that Gᵢ is dependent on Gₛ-mediated signaling strengthens the conclusion that cross-talk across G proteins is a potentially important functional property of GPCR heteromers. Remarkably, when both receptors are co-activated in this heterotetramer, only A₂AR-mediated, but not A₁R-mediated signaling occurs. We show that the ability of blunting A₁R-mediated signaling when Gₛ is engaged is dependent of the long C-terminus of the A₂AR. In the absence of A₂AR activation by agonists, or in the absence of the C-terminal domain of A₂AR, the A₁R-mediated signaling via Gᵢ is totally functional. The most straightforward hypothesis is that the opening of α₁AH parallels a movement of the C-tail to block the opening of α₂AH.

Adenosinergic signaling in mammalians is important for energy and temperature homeostasis and for neuroregulation. Multiplicity of adenosine actions is due to a balance between the expression of specific receptors and producing/degrading enzymes and to the biological diversity due to a membrane network established by the interaction among purinergic receptors [23]. Ciruela et al. [4] first identified the occurrence of heteromers formed by A₁R-Gᵢ and A₂AR-Gₛ-coupled adenosine receptors that participate in the regulation of glutamate release by neurons projecting from the cortex to the striatum. The same A₁-A₂AHet can be found in astrocytes modulating the transport of γ-amino butyric acid (GABA) [24]. Differently from the modulation of neuronal glutamate release, the A₁R-Gᵢ-coupled receptor activates and the A₂AR-Gₛ-coupled receptor inhibits the modulation of GABA transport. Under conditions of high extracellular adenosine concentrations, such as hypoxic conditions [25], the nucleoside will bind to both the high (A₁R) and the low (A₂AR) affinity receptors in the heteromer, and the predominant A₁R-mediated signaling via Gᵢ will result in counteraction of astrocytic GABA transport. Our results show that the asymmetric signaling is possible because the long C-terminus of A₂AR blunts Gᵢ-mediated signaling. We have therefore elucidated the mechanism by which the A₁-A₂AHet functions as an adenosine concentration-sensing device that can promote even opposite signaling responses depending on the extracellular concentration of adenosine. The molecular mechanism involves the C-terminal domain of the activated Gₛ-coupled A₂AR, which hinders the activation of A₁R coupled to Gᵢ.
Conclusions

Using a convergent approach including biochemical, biophysical, cell biology, and molecular biology techniques, together with in silico molecular models, we here provide the mode of action of a membrane receptor complex that responds depending on the concentration of adenosine, a hormone and a neuroregulatory molecule. The concentration sensor is a heteromer composed of four adenosine receptors (two A1 and two A2A) and two G proteins (Gi and Gs). Despite Gi sits underneath the A1 receptor dimer and Gs sits underneath the A2A receptor dimer, both G proteins do interact and are able to convey allosteric regulation depending on how the functional unit is activated. At low adenosine concentrations Gi is engaged via A1 activation without affecting/engaging Gs signaling. At higher concentrations Gs is engaged via A2A activation, and this engagement blocks Gs-mediated signaling. At low adenosine concentrations Gi is engaged via A1 activation without affecting/engaging Gs signaling. At higher concentrations Gs is engaged via A2A activation, and this engagement blocks Gi-mediated signaling. The reason why a rhombus-shaped apparently symmetric structure results in asymmetric signaling is due to the long C-terminal tail of the A2A receptor. In fact, both deletion of the C-terminal end or treatment with interfering peptides derived from the sequence of TM segments of the receptors impair allosteric cross-interaction between receptors and G proteins within the macromolecule, and the device loses its concentration sensing properties.

Methods

Cell culture and transient transfection

HEK-293 T cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and 5% (v/v) heat inactivated fetal bovine serum (all supplements were from Invitrogen, Paisley, Scotland, UK). Cells were transiently transfected with cDNA corresponding to receptors, fusion proteins, A2AR mutant constructs, or minigene vectors using polyethylenimine (Sigma-Aldrich, Cerdanyola del Vallés, Spain) as described elsewhere [7].

Expression vectors, A2AR mutants and minigenes

Sequences encoding amino acid residues 1–155 or 155–238 of YFP-Venus protein, were subcloned in pcDNA3.1 to obtain the YFP Venus hemi-truncated proteins (nYFP and cYFP). The human cDNAs for A2AR, mutant A2AR, A1R, and Gi or Gs proteins cloned into pcDNA3.1, were amplified without their stop codons using sense and antisense primers harboring unique EcoRI and BamHI sites to subclone receptors in pcDNA3.1R Luc vector (pRLuc-N1 PerkinElmer, Wellesley, MA, USA) and EcoRI and KpnI to subclone receptors in pEYFP-N1 (enhanced yellow variant of GFP; Clontech, Heidelberg, Germany), pcDNA3.1-nVenus, or pcDNA3.1-cVenus vectors. The amplified fragments were subcloned to be in-frame with restriction sites of the corresponding vectors to give the plasmids that express receptors fused to RLuc, YFP, nYFP or cYFP on the C-terminal end (A1R-RLuc, A2AR-RLuc, Gi-RLuc, Gs-RLuc, A1R-YFP, A2AR-YFP, A2ARΔ40-R-YFP, A2ARΔ40CT-R-YFP, A1R-nYFP, A2AR-nYFP, and A2AΔ40R-cYFP). Expression of constructs was tested by confocal microscopy and the receptor-fusion protein functionality by second messengers, ERK1/2 phosphorylation and cAMP production as described previously [4, 26–28]. Mutants with a deletion of aa 372 to aa 412 (A2ARΔ40R) or aa 321 to aa 412 (A2ARΔCT) on the C-terminal domain of A2AR were generated as previously described [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 11 amino acid residues from the C-terminus sequence of α subunit of Gi1/2 or Gs. The peptide coded by every minigene inhibits the coupling of the G (Gi1/2 or Gs) protein to the receptor and, consequently, it inhibits the G-protein-mediated cellular response, as previously described [8]. The cDNA encoding the last 11 amino acids of human Gs subunit corresponding to Gi1/2 (IKNNLKDCLGF) or Gi (QRMHLRQYELL), inserted in a pcDNA 3.1 plasmid vector, was generously provided by Dr. Heidi Hamm.

TAT-TM peptides

Peptides with the sequence of the TM of A1R and A2AR fused to the HIV TAT peptide (YGRKKRRQRRR) were used as oligomer-disrupting molecules (synthesized by Genmed Synthesis Inc. San Antonio, TX, USA). The cell-penetrating TAT peptide allows intracellular delivery of fused peptides [6]. The TAT-fused TM peptide can then be inserted effectively into the plasma membrane because of the penetration capacity of the TAT peptide and the hydrophobic property of the TM moieties [30]. To obtain the right orientation of the inserted peptide, the HIV-TAT peptide was fused to the C-terminus or to the N-terminus as indicated:

MEYMVYFNFFVWVLPPPLLMLYLYGKRKKRRQRRR
for TM5 of A1R,
RRRQRRKKRGYLLALIFLFAVSLPLHILNCITLF for TM6 of A1R,
ILTYIAIFLTHGNSAMNPVYAFRIYGRKKRRQRRR for TM7 of A1R,
VYITVELAIAVLAILGNVLVCWAVYGRKKRRQRRR for TM1 of A2AR,
YGRKKRRQQRRVYFVSLAADDIAVGLAIAPFAITI for TM2 of A2AR,
LFIACFVLTLTQSSIFSLAIAIYGRKKRRQRRR for TM3 of A2AR,
YGRKKRRQRRRAKGIACWVLSFAIGLTPMLGW for TM4 of A2AR.
TM4 of A2AR,
MNYMVFYFNFFACVLVPLLMLGVYLYGRKKRRQRRR
R for TM5 of A2AR,
YGRKKRRQRRRRLAIYGLFALCWPLLHINCFHTFF for
TM6 of A2AR,
LWLMYLAIVLHATNSVYNPIAYYGRKKRRQRRR
for TM7 of A2AR.
YGRKKRRQRRRRLGIWAVSLAIVMPQAAVME for
TM4 of OX1R,
SSFIVTVLAPGLMAMAYFQIFYGRKKRRQRRR
for TM5 of OX1R,
YASFIVSHLWVYANAANPIYNYFGRKKRRQRRR
for TM7 of OX1R.

**Bimolecular fluorescence complementation assay (BiFC)**

HEK-293 T cells were transiently transfected with equal amounts of the cDNA for fusion proteins of the hemi-truncated Venus (1 μg of each cDNA). At 48 h after transfection, cells were treated for 4 h at 37°C with medium or TAT peptides (4 μM) before plating 20 μg of protein in 96-well black microplates (Porvair, King's Lynn, UK). To quantify reconstituted YFP Venus expression, fluorescence at 530 nm was read in a FluoroStar 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 fluorescence of the sample minus the fluorescence of cells not expressing the fusion proteins (basal). Cells expressing receptor-cVenus and nVenus or receptor-nVenus and cVenus showed similar fluorescence levels than untransfected cells.

**Bioluminescence resonance energy transfer (BRET)**

HEK-293 T cells were transiently transfected with a constant amount of cDNA for Rluc fusion proteins and increasing amounts of cDNA for YFP fusion proteins. At 48 h after transfection, 20 μg of cell suspension were plated in 96-well black microplates for fluorescence detection or in 96-well white microplates for BRET readings and Rluc quantification. YFP fluorescence at 530 nm was quantified in a Fluoro Star Optima Fluorimeter as described above. BRET signal was collected 1 min after addition of 5 μM coelenterazine H (Molecular Probes, Eugene, OR, USA) using a Mithras LB 940. The integration of the signals detected in the short-wavelength filter at 485 nm and the long-wavelength filter at 530 nm was recorded. To quantify protein-RLuc expression, luminescence readings were also performed after 10 minutes of adding 5 μM coelenterazine H. The net BRET is 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 milli-BRET units (net BRET × 1000). To calculate maximum BRET (BRETmax) from saturation curves, data were fitted to a nonlinear regression equation, assuming a single-phase saturation curve with GraphPad Prism software (San Diego, CA, USA).

**Proximity ligation assay (PLA)**

HEK293T cells were grown on glass coverslips and fixed in 4% paraformaldehyde for 15 min, washed with phosphate-buffered saline containing 20 mM glycine, permeabilized with the same buffer containing 0.05% Triton X-100, and successively washed with tris-buffered saline. Heteromers were detected using the Duolink II in situ PLA detection Kit (Olink; Bioscience, Uppsala, Sweden) following supplier’s instructions. A mixture of the primary antibodies (mouse anti-A2AR antibody (1:100; 05-717, Millipore, Darmstadt, Germany; RRID:AB_309931) and rabbit anti-A1R antibody (1:100; ab82477, Abcam, Bristol, UK; RRID: AB_2049141)) was used to detect A1- A2AR heteromer together with PLA probes detecting mouse or rabbit antibodies. Then, samples were processed for ligation and amplification with a Detection Reagent Red and were mounted using a DAPI-containing mounting medium. Samples were analyzed in a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with 405 nm and 561 nm laser lines. For each field of view, a stack of two channels (one per staining) and 4–6 Z-stacks with a step size of 1 μm were acquired. Images were opened and processed with Image J software (National Institutes of Health, Bethesda, MD, USA).

**cAMP determination assays**

HEK-293 T cells expressing adenosine receptors were incubated for 4 h in serum-free medium containing 50 μM zarderverine. Cells were plated in 384-well white microplates (1500 cells/well), pre-treated with toxins or the corresponding vehicle for the indicated time, stimulated with agonists for 15 min before adding medium or 0.5 μM forskolin, and incubated for an additional 15 min. cAMP production was quantified by a TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer) methodology using the LANCE Ultra cAMP kit (PerkinElmer) and fluorescence at 665 nm was analyzed on a Pherastar Flagship Microplate Reader (BMG Labtech, Ortenberg, Germany).

**Dynamic mass redistribution (DMR) assays**

The heteromer-induced cell signaling signature was determined using an EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA) by a label-free technology. Refractive waveguide grating optical biosensors, integrated in 384-well microplates, allow extremely
sensitive measurements of changes in local optical density in a detecting zone up to 150 nm above the surface of the sensor. Cellular mass movements induced upon receptor activation were detected by illuminating the underside of the biosensor with polychromatic light and measured as changes in wavelength of the reflected monochromatic light, which is a sensitive function of the index of refraction. The magnitude of this wavelength shift (in picometers) is directly proportional to the amount of DMR. Briefly, 24 h before the assay, cells were seeded at a density of 7500 cells per well in 384-well sensor microplates with 40 µL growth medium and cultured for 24 h (37 °C, 5% CO₂) to obtain 70–80% confluent monolayers. Previous to the assay, cells were pre-treated with medium or toxins as indicated and incubated for 2 h in 40 µL per well of assay-buffer (HBSS with 20 mM HEPES, pH 7.15) in the reader at 24 °C. Thereafter, the sensor plate was scanned and a baseline optical signature was recorded prior to addition of 10 µL of receptor agonist dissolved in assay buffer containing 0.1% DMSO. DMR responses were monitored for at least 8000 s and data were analyzed using EnSpire Workstation Software v. 4.10.

Computational modeling

The structural model of the A₁-A₂A-Het bound to Gₐ (closed α₃AH domain) and Gᵢ (closed α₃AH domain) was taken from our previous work [5]. This previous structural model contains a A₂₅R-based homology model of A₁R. The structure of the adenosine A₁R has recently been revealed [31], showing a remarkably similar structure (Additional file 1: Figure S6A). This structure of A₁R contains a TM4/5 dimer interface that is in close agreement with our model (Additional file 1: Figure S6B). An intermediate conformation (obtained using the g_morph tool of the GROMACS package [32]) between the closed α₃AH domain (PDB id 1AZT) and the conformation observed in the crystal structure of rhodopsin bound to β-arrestin-2 [21] (see Additional file 1: Figure S4 for details). Additional file 2: Table S1 shows the template structures used in the protein models. Modeller 9.12 was used to build these models [34]. The molecular models of A₁-A₂A-Het in complex with Gᵢ and Gₐ or β-arrestin, in the absence or presence of the TAT-fused TM6 peptide, were embedded in a pre-equilibrated box containing a lipid bilayer (~800 POPC molecules) with explicit solvent (~110,000 waters) and 0.15 M concentration of Na⁺ and Cl⁻ (~1800 ions). These initial complexes were energy-minimized and subsequently subjected to a 21 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. Computer simulations were performed with the GROMACS 4.6.3 simulation package [32], using the AMBER99SB force field as implemented in GROMACS and Berger parameters for POPC lipids. This procedure has been previously validated [35].

Additional files

Additional file 1: Figures S1–S6. Figure S1. Control experiments on the effect of interfering peptides on the A₁-A₂A-Het structure and Gᵢ and Gₐ coupling to A₁-A₂A-Het. Figure S2. Receptor signaling through A₁R and A₂₅R. Figure S3. Recruitment of β-arrestin-2 by the A₁-A₂A-Het. Figure S4. Modeling the orientation of the C-tail of A₂₅R. Figure S5. The influence of the C-terminal domain of A₂₅R in the signalling properties of the A₁-A₂A-Het in the presence of pertussis toxin. Figure S6. Modeling an A₁R homodimer and α₃AH and α₃AH in closed and open conformations. (DOCX 5727 kb)

Additional file 2: Table S1. List of target sequences and template structures used to construct the computer models of A₁-A₂A-Het in complex with Gᵢ and Gₐ. (PDF 23 kb)

Acknowledgments

We would like to thank Jasmina Jiménez for technical help (University of Barcelona). RF, PJM and LP participate in the European COST Action CM1207 (GLISTEN). Authors gratefully acknowledge the computer resources provided by the Barcelona Supercomputing Center - Centro Nacional de Supercomputación.

Funding

This study was supported by grants from the Spanish Ministerio de Economía y Competitividad (SAF2015-74627-JIN, BFU2015-64405-R and SAF2016-77830-R; they may contain FEDER funds) and by the intramural funds of the National Institute on Drug Abuse to SF. RF, PJM, and LP participate in the European COST Action CM1207 (GLISTEN).

Availability of data and materials

The crystal structures 4EIY, 2Z73, 3SN6, 4JQI, 1AZT, 1AGR, 4ZWJ, 4JQI, 2PSD, and 2HR7 used to build the presented computational models are available from PDB (http://www.rcsb.org). All other relevant data are within the paper and its Additional files 1 and 2.
Authors’ contributions
GN performed the molecular biology experiments. GN, MB, EM, and DA performed BRET experiments. AC and LP-B performed molecular modeling studies. SF, AC, VC, JM, and EIC analyzed the data. CL, LP, 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.

Ethics approval and consent to participate
Not applicable.

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

Publisher’s note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
1Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas, University of Barcelona, 08028 Barcelona, Spain. 2Institute of Biomedicine of the University of Barcelona (IBUB), University of Barcelona, 08028 Barcelona, Spain. 3Department of Biochemistry and Molecular Biomedicine, Faculty of Biology, University of Barcelona, 08028 Barcelona, Spain. 4Laboratorio de Medicina Computacional, Unidad de Bioestadística, Facultat de Medicina, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain. 5Integrative Neurobiology Section, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD 21224, USA. 6School of Veterinary Medicine, University of Surrey, Guildford, Surrey GU2 7AL, UK.

Received: 19 October 2017 Accepted: 22 January 2018

Published online: 28 February 2018

References
1. Thompson SM, Haas HL, Gahwiler BH. Comparison of the actions of adenosine at pre- and postsynaptic receptors in the rat hippocampus in vitro. J Physiol. 1992;451:347–63.
2. Franco R, Martinez-Pinilla E, Lancejo JL, Navarro G. Basic pharmacological and structural evidence for class A G-protein-coupled receptor heteromerization. Front Pharmacol. 2016;7:76.
3. Ferre S, Balser R, Bouvier M, Caron MG, Deva LA, Durieux T, Fuxe K, George SR, Javitch JA, Lofse MJ, et al. Building a new conceptual framework for receptor heteromers. Nat Chem Biol. 2009;5:131–4.
4. Ciruela F, Casado V, Rodrigues RJ, Lujan R, Burgueno J, Canals M, Borycz J, Ribeira N, Goldberg SR, Mallol J, et al. Presynaptic control of striatal glutamatnergic neurotransmission by adenosine A1-A2A receptor heteromers. J Neurosci. 2006;26:1980–7.
5. Navarro G, Cordona A, Zelman-Ferniak M, Brugarolas M, Moreno E, Aginaga D, Perez-Benito L, Cortes A, Casado V, Mallol J, et al. Quaternary structure of a G-protein-coupled receptor heterotetramer in complex with Gi and Gs. BMC Biol. 2016;14:26.
6. Schwarzer SR, Ho A, Vocero-Akbani A, Dowdy SF. In vivo protein transduction: delivery of a biologically active protein into the mouse. Science. 1999;285:1569–72.
7. Carriba P, Navarro G, Ciruela F, Ferre S, Casado V, Aignati L, Cortes A, Mallol J, Fuxe K, Canela EI, et al. Detection of heteromerization of more than two proteins by sequential BRET-FRET. Nat Methods. 2008;5:727–33.
8. Gilchist A, Li A, Hamn HE. G alpha COOH-terminal minigene vectors dissect heteromeric G-protein signaling. Sci STKE. 2002;2002:p11.
9. Schroder R, Merten N, Mathiesen JM, Martini L, Kudjak-Letunic A, Krop F, Blaukat A, Fang Y, Tran E, Ulven T, et al. The C-terminal tail of CRTH2 is a key molecular determinant that constrains G alpha(i) and downstream signaling cascade activation. J Biol Chem. 2009;284:1324–36.
10. Blattermann S, Peters L, Ottersbach PA, Bock A, Konya V, Weaver CD, Gonzalez A, Schroder R, Tyagi R, Luschnig P, et al. A biased ligand for OXET uncouples G alpha(i) and Gbeta(5)gamma(3) signaling within a heterotrimer. Nat Chem Biol. 2012;8:631–8.
11. Shukla AK, Manglik A, Kuse AC, Xiao K, Reis RJ, Tseng WC, Staup DP, Hilger D, Uysal S, Huang LY, et al. Structure of active beta-arrestin-1 bound to a G-protein-coupled receptor phosphoprotein. Nature. 2013;497:137–41.
12. Montague L, Kamal M, Jockers R. Asymmetry of GPCR oligomers supports their functional relevance. Trends Pharmacol Sci. 2011;32:514–20.
13. Schroder R, Merten N, Mathiesen JM, Martini L, Kudjak-Letunic A, Krop F, Blaukat A, Fang Y, Tran E, Ulven T, et al. The C-terminal tail of CRTH2 is a key molecular determinant that constrains G alpha(i) and downstream signaling cascade activation. J Biol Chem. 2009;284:1324–36.
14. Cristovao-Ferreira S, Navarro G, Brugarolas M, Perez-Capote K, Vaz SH, Fattorini G, Conti F, Llius C, Ribeiro JA, McCordick PJ, et al. A1R-A2AR heteromers coupled to Gi and G0/1 proteins modulate GABA transport into astrocytes. Pysogene Signal. 2013;9:433–49.
15. Lopes LV, Sebastiao AM, Ribeiro JA. Adenosine and related drugs in brain diseases: present and future in clinical trials. Curr Top Med Chem. 2011;11:1087–101.
16. Canals M, Marcello D, Fanelli F, Ciruela F, de Benedetti P, Goldberg SR, Neve K, Fuxe K, Aignati LF, Woods AS, et al. Adenosine A2A-dopamine D2 receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. J Biol Chem. 2003;278:46741–9.
17. Gonzalez S, Moreno-Delgado D, Moreno E, Perez-Capote K, Franco R, Mallol J, Cortes A, Casado V, Llius C, Ortiz J, et al. Circadian-related heteromerization of adenosine and dopamine D/D receptors modulates melanin synthesis and release in the pineal gland. PLoS Biol. 2012;10:e1001347.
18. Navarro G, Ferre S, Cordona A, Moreno E, Mallol J, Casado V, Cortes A, Hoffmann H, Ortz J, Canela EI, et al. Interactions between intracellular domains as key determinants of the quaternary structure and function of receptor heteromers. J Biol Chem. 2010;285:27346–59.
19. Burgueno J, Blake DJ, Benson MA, Tinsley CL, Esapa CT, Canela EI, Penna M, Pellol J, Mallol J, Mayor F Jr, Llius C, et al. The adenosine A2A receptor interacts with the actin-binding protein alpha-actinin. J Biol Chem. 2003;278:37545–52.
20. He SQ, Zhang ZN, Gnan JSU, Liu H, Zhao B, Wang HB, Li Q, Yang H, Luo J, Li ZY, et al. Facilitation of mu-opioid receptor activity by preventing delta-opioid receptor-mediated codegradation. Neuron. 2011;69:120–31.
21. Gulikova A, Thal DM, Nguyen AT, Vecchiol EA, Joord M, Scammells PJ, May LT, Sexton PM, Christopoulos A. Structure of the adenosine A1 receptor reveals the basis for subtype selectivity. Cell. 2017;168:867–77. e813
32. Pronk S, Pall S, Schulz R, Larsson P, Bjelkmar P, Apostolov R, Shirts MR, Smith JC, Kasson PM, van der Spoel D, et al. GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. Bioinformatics. 2013;29:845–54.
33. Murakami M, Kouyama T. Crystal structure of squid rhodopsin. Nature. 2008; 453:363–7.
34. Marti-Renom MA, Stuart AC, Fiser A, Sanchez R, Melo F, Sali A. Comparative protein structure modeling of genes and genomes. Annu Rev Biophys Biomol Struct. 2000;29:291–325.
35. Cordomi A, Caltabiano G, Pardo L. Membrane protein simulations using AMBER force field and berger lipid parameters. J Chem Theory Comput. 2012;8:948–58.