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REVIEW

Structure and function of adenosine receptor heteromers

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Abstract

Adenosine is one of the most ancient signaling molecules and has receptors in both animals and plants. In mammals there are four specific receptors, A_1, A_{2A}, A_{2B}, and A_3, which belong to the superfamily of G-protein-coupled receptors (GPCRs). Evidence accumulated in the last 20 years indicates that GPCRs are often expressed as oligomeric complexes formed by a number of equal (homomers) or different (heteromers) receptors. This review presents the data showing the occurrence of heteromers formed by A_1 and A_{2A}, A_{2A} and A_{2B}, and A_{2A} and A_3 receptors highlighting (i) their tetrameric structural arrangements, and (ii) the functional diversity that those heteromers provide to adenosinergic signaling.

Keywords G-protein-coupled receptors GPCRs · Signaling · Signal transduction · Cell surface functional unit

Introduction. The four adenosine receptors

Adenosine is an endogenous purine nucleoside, obtained by the breakdown of adenosine triphosphate (ATP) and constituted by an adenine attached to a ribose sugar via a glycosidic linkage. ATP is one of the main metabolites in living organisms on Earth and there exist cell surface receptors for its degradation product, adenosine. Accordingly, adenosine receptors have been traced back to pre-Cambrian speciation. A nice and informative review on the evolutionary origins of purines as signaling molecules in plants and animals was provided in 2009 by Burnstock and Verkhratsky [1]. The actions of adenosine in mammals are multiple, and one or more of the four specific adenosine receptors (AdoRs) are found in every single cell.

The purinergic system consists of two types of receptors, those for nucleosides or P1 and those for nucleotides or P2. While P2 receptors may be ionotropic or metabotropic, four P1 adenosine receptors are all class A members of the G-protein-coupled receptor (GPCR) superfamily, which is the most populated in the human proteome [2]. AdoRs were first identified by means of pharmacological assays. In principle those first characterized were named as A_1 and A_2, and the difference was that A_1 was coupled to G_i proteins whereas the A_2 was coupled to G_s proteins. A second receptor that displayed low affinity for adenosine was coupled to G_s and this lead to define A_{2A} as the high-affinity receptor and A_{2B} as the low-affinity receptor [3–7]. A fourth receptor with differential selectivity for agonists and Gi-coupling was identified and named as A_3.

Structure of adenosine receptors

Cloning of adenosine receptors was a breakthrough that came from the development of a novel approach [8, 9] leading, among others, to the discovery of four orphan receptors with homology to α_1 and $ß_2$ adrenergic and serotonin 5-HT_{1A} receptors. In fact, authors used degenerate primers corresponding to consensus sequences of two transmembrane

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segments for PCR using cDNA obtained from mRNA isolated from the thyroid dog gland. The set of experiments lead to the discovery of 4 open reading frames with high sequence homology, they were named as RDC1, RDC4, RDC7 and RDC8 [9], D referring to dog and C to clone. Later on, it was confirmed that the clones were indeed coding for functional GPCRs and, finally, RDC1, RDC4, RDC7, and RDC8 were deorphanized by evidence of being the cDNAs coding for the four AdoRs. Sequences for AdoRs cDNAs for several species, including human, are available in data bases. Figure 1a shows a 93.2% of sequence identity between human and dog $A_{2A}R$, with only few non identical amino acids. Figures 1a, b are useful to compare the four sequences coding for the human AdoRs. $A_{2A}R$ and $A_{2B}R$ are located in the same branch of the phylogenetic tree, A_3R is the most distant sequence from these two receptors and A_1R being in between. The crystal structure of $A_{2A}R$ was one of the first reported for the GPCR family [10]. Today, there are more than fifty structures of A_1R and $A_{2A}R$ bound to agonists, to antagonists, and to coupled G protein [11]; all of them displaying the common features of class A GPCRs. All these structures share the common architecture of seven transmembrane domains (TMs, which serves to name this family of proteins as 7TM receptors) connected to each other with three extracellular (ECL) and three intracellular loops (ICL). The N-terminal end is extracellular and relatively short and the cytoplasmic C–terminus contains an α-helix (Hx8) parallel to the cell membrane (Fig. 1c). Figure 1c shows the superimposition of the structures of A_1R and $A_{2A}R$ in the inactive state. The most striking difference between both receptors is the conformation of their ECL2s. ECL2 residues of A_1R conforms a longer helix as compared to the $A_{2A}R$, extending in a conformation almost perpendicular to the plane of the membrane [12]. This difference is due to the existence of different cysteine bridges that are variable among AdoRs [13]. In particular, the $A_{2A}R$ contains a disulfide bridge that tethers ECL2 helix to ECL1, which is absent in the A_1R. Whereas the size of the other loops and TM domains are quite similar among AdoRs, a major difference resides in the size of the C-terminal domain (Fig. 1c). Unfortunately, the structure of the C-terminal domain of most GPCRs (except rhodopsin) remains unknown since it has been removed in most of the crystals. However, the C-tail contains important regulatory sites that enable interactions with intracellular signaling effectors and is very relevant in the formation of GPCR oligomers (see later). Notably, the C-terminal end of the $A_{2A}R$ is between 82 and 88 amino acids longer than that of the other three members of AdoRs (Fig. 1c).

Therapeutic potential of adenosine receptors

Worth mentioning that the strong effect on the mammalian heart of adenine compounds were reported in late twenties [14]. About 30 years later, Berne's lab identified adenosine as the key adenine compound [15]. Soon, the potential for the treatment of heart failure due to the vasodilator properties of adenosine was proven [16]. It is known that these effects are mainly mediated by adenosine receptors in which high hopes were deposited when the first drugs acting on adrenergic (beta-blockers) and histamine (H_2 antagonists) receptors were approved. Until recently only adenosine was approved as a therapeutic drug and subject to restrictions derived from Emergency Room (ER) use. In fact, adenosine

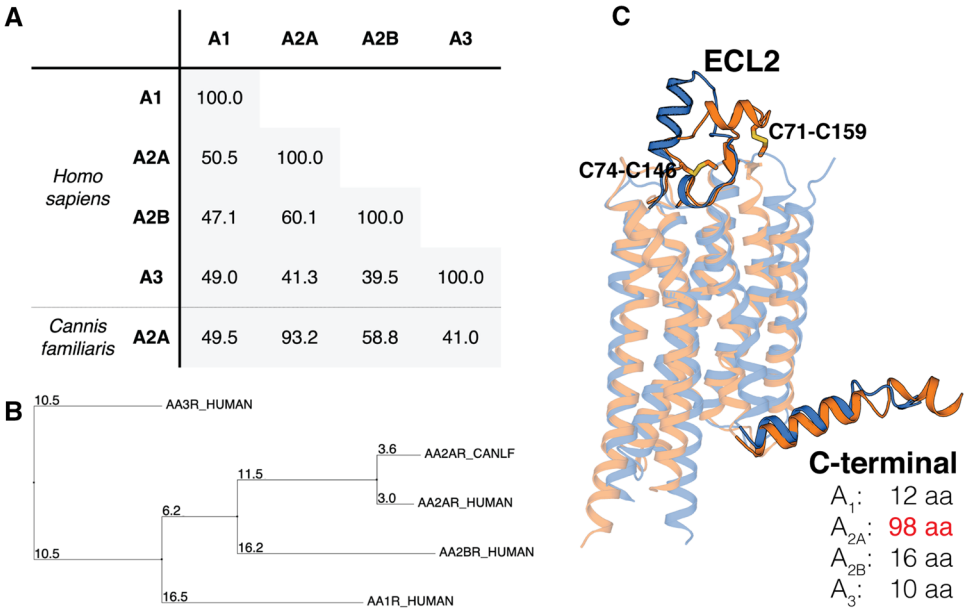

Fig. 1 Comparison of the four human AdoRs and $A_{2A}R$ from *Cannis Lupus Familiaris*. **a** Percent identity matrix obtained from the multiple sequence alignment calculated with the Clustal Omega algorithm (https://www.ebi.ac.uk/Tools/msa/clustalo/). **b** Neighbour joining phylogenetic tree using the percent identity distance (**a**), as calculated with Jalview (https://www.jalview.org/). **c** Superimposition of the crystal structures of inactive A_1R (PDB id 5UEN) and $A_{2A}R$ (2YDO). Major structural differences are highlighted such as disulphide bridges, the orientation of ECL2 and the length of the C-terminal domain. [12] Adapted from reference

boluses (first name given: *Adenocard*™) are administered with great success to critically ill patients suffering from paroxysmal tachycardia. The next approval by the US Food and Drug Administration was Regadenoson in 2008, a selective $A_{2A}R$ agonists that is a coronary vasolidator, marketed as *Lexiscan*™, as used not for therapy but for diagnostics. Istradefylline, a selective $A_{2A}R$ antagonist, used as adjunctive therapy in Parkinson's disease, was marketed as *Nouriast*™ in Japan in 2013 and as *Nourianz*™ in the United States in 2019. These recent approvals have regained the interests in the development of drugs acting on adenosine receptors.

Receptor–receptor interactions: homomers and heteromers

The potential formation of interactions between a number of equal (homomers) or different (heteromers) GPCRs was suggested by Fuxe and Agnati based on the regulation of neurotransmission by neuropeptides [17–22]. Confirmation arrived at the end of the 20th and beginning of the twenty-first century when molecular research on GPCRs disclosed novel properties resulting from the formation of these complexes. Receptor–receptor interactions lead to new functional units whose properties are different from those displayed by individually acting receptors (see below).

It is not possible to establish beforehand the domain(s) (TMs, ICLs, ECLs, or/and C-terminal) of two interacting GPCRs that contribute to the formation of the homo/heterodimer. In other words, the receptor interface at which the monomer–monomer interactions occur cannot be predicted by either sequence analysis or computer simulations. A recently developed tool (http://lmc.uab.es/dimerbow/) reports the packing of GPCRs in crystal structures [23]. DIMERBOW is both a database and a web tool that allows browsing the complete repertoire of potential GPCR dimers derived from available 3D structures, i.e., DIMERBOW allows finding the most reliable template to model GPCR homomers.

Among the obtained GPCR structures, by X-ray crystallography or cryogenic electron microscopy (cryo-EM), the following contacts between protomers must be highlighted. (i) The structure of CXCR4 that shows a homodimer with an interface including TM5 and TM6 (PDB id 4DKL) [24]. (ii) The structure of μ-opioid receptor (4DKL) that shows receptor protomers associated into pairs through two different interfaces [25]. The first is via TM1, TM2 and Hx8 and the second interface comprises TM5 and TM6 (named in this manuscript as the TM5/6 interface). (iii) The structure of the turkey β_1-adrenergic receptor (4GPO and 5F8U) that also displays two dimer interfaces [26]. One interface also involves TM1, TM2 and Hx8, as in μ-opioid receptor. In contrast, the other interface engages residues from TM4, TM5 and ICL2 (TM4/5 interface). (iv) The structure of A_1R (5UEN) that reveals an interface including TM4, TM5, ICL2, and the helix of ECL2 [12]. And finally, (v) the cryo-EM structures of a full length mGlu5 receptor in the apo inactive/open (6N52) and active/closed (6N51) conformations [27] have shown that activation leads to compacting of the mGlu5 dimer, enabling the 7TM domains to reposition closer, via a TM6–TM6 interface. The co-expression of two class C GPCRs may lead to coexistence of homodimers of each receptor, as well as heterodimers [28, 29]. Unfortunately, there is not any heteromer whose structure has been experimentally solved by either X-ray or cryo-EM. The field is extremely avid for the appearance of such structures.

Structural breakthroughs have permitted to elucidate the molecular mechanisms of agonist-induced receptor activation [30]. For class A GPCRs, agonist binding at the extracellular orthosteric binding site triggers local structural changes that are transmitted to the intracellular side, leading to outward movement of TM5 and TM6 (~ 14 Å) (Fig. 2a) and the opening of an intracellular cavity that is required for the binding of the C-terminal α5 helix of the G-protein [30]. In the case of AdoRs, this mechanism of propagation of the adenosine signal [31] and the binding of G_i to A_1R [32] or G_s to $A_{2A}R$ [33, 34] seems conserved with the other members of the GPCR family. Notably, GPCR protomers forming homomers or heteromers via the TM5/6 interface, as in the crystal of the μ-opioid receptor, are locked in the inactive conformation since the opening of TM5 and TM6 for G-protein binding is not feasible (Fig. 2a). Thus, it has been proposed that this TM5/6 interface is responsible for the ability of one protomer to influence the signaling of the partner protomer [35, 36]. Interacting GPCRs in a crystal do not demonstrate the occurrence of homodimers in natural sources, but distances and arrangements are compatible with receptor–receptor interactions in the cell membrane [see [37]].

The C-terminal and the ICL3 of GPCRs also play an important role in receptor–receptor interactions. We have shown that these domains contain Ser/Thr residues that could be phosphorylated and form strong electrostatic interactions with positively charged Lys/Arg residues located at the end of TM5 or ICL3 [38, 39].

The size of a G protein is larger than the 7TM domain of a GPCR and, despite a monomeric GPCR can activate a G protein [40], it is also feasible a 2:1 (receptor:G protein) stoichiometry [37]. It is unlikely a 2:2 stoichiometry since both G proteins would crash (Fig. 2b). In the case that GPCRs can form heteromers, these could be viewed as constituted by different interacting homodimers. Of special functional significance could be those heteromers constituted by one homodimer coupled to a G_s protein and another different homodimer coupled to a G_i protein. We have proposed that such a GPCR heterotetramer would have a 2:2:1:1

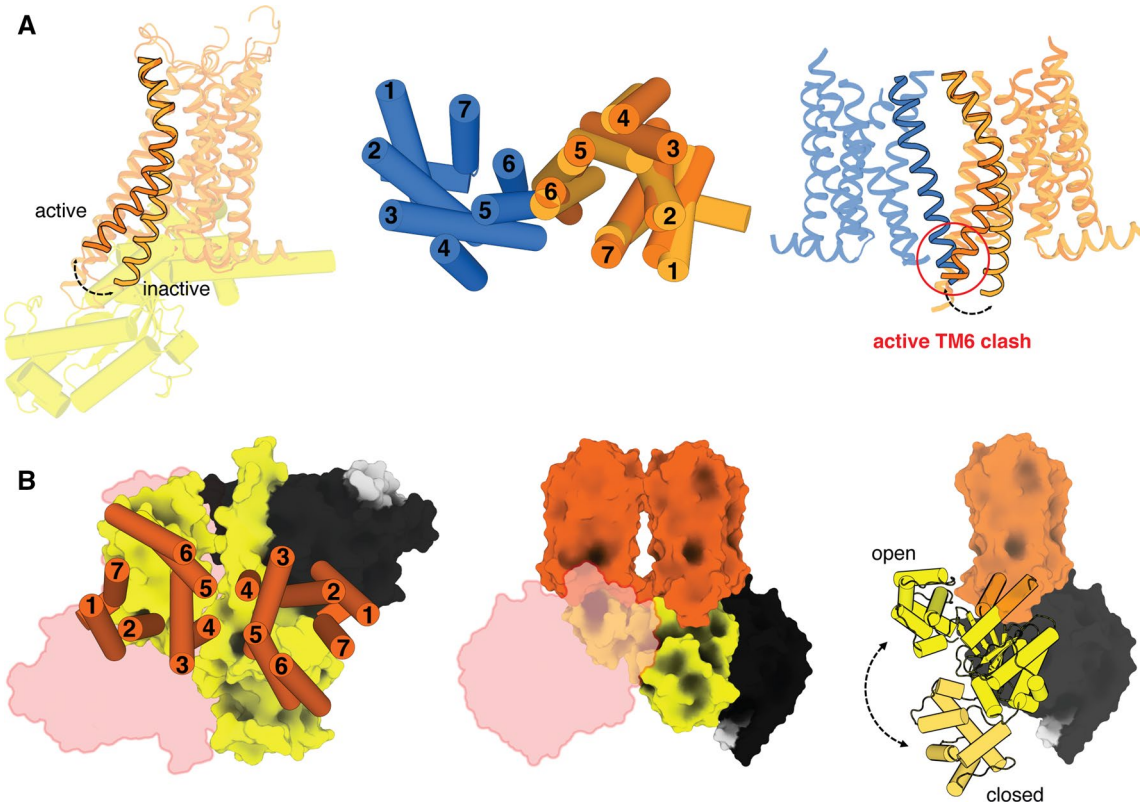

Fig. 2 Models of AdoR homo/heterodimers bound to a G protein. **a** Left panel shows the dynamical behaviour of TM6 that moves ~ 14 Å to open an intracellular cavity for the binding of the α5 helix of the α-subunit (in yellow, PDB id 6GDG) at the intracellular part. Central and right panels show an extracellular (central) and parallel to the membrane (right) views of the A_1R–$A_{2A}R$ heterodimer modelled via the TM5/6 interface. Light blue (5UEN) and light orange (3VG9) show A_1R and $A_{2A}R$ in an inactive state, respectively; dark orange shows $A_{2A}R$ in an active state (6GDG). Right panel emphasizes how opening of TM6 in an active $A_{2A}R$ is incompatible with the formation of a homo/heterodimer with the TM56 interface due to a steric clash. Adapted from reference [35]. **b** Left (extracellular view) and central (parallel to the membrane) panels show the $A_{2A}R$ homodimer modelled via the TM4/5 interface, bound to G_s (α-subunit in yellow, β- in black and γ- in white). The pink surface shows the shape of a second G_s protein bound to the second protomer of the homodimer. Clearly, there would be a steric clash between G proteins. Right panel shows the large-scale opening of the α-helical domain of the α-subunit, from the RAS domain, necessary for receptor-catalysed nucleotide exchange. The α-helical domain moves from a closed (in beige, 1AZT) to an open (in yellow, 3SN6) conformation for nucleotide exchange. This opening would also clash with the second G_s protein bound to the homodimer. Adapted from reference [37]

(receptor:receptor:G protein:G protein) stoichiometry, in which the outer protomer of the homodimers would bind both G proteins to avoid the steric clash [41, 42] (see below).

Quaternary structure of GPCR heterotetramers

Owing to the high number of GPCRs, roughly ~ 800 (~ 350 for nonsensory GPCRs) according to the recent estimates [43], the probability for casual, nonfunctional, heteromer formation is very high. This contrasts with the specificity of the interactions, namely not all GPCRs interact with each other, only few of them. As of today, no specific rules have been described to predict whether two given GPCRs form heterodimers. An online tool (http://www.gpcr-hetnet.com/) reports both experimentally determined interactions within the GPCR superfamily, as well as noninteracting GPCRs that may serve as negative controls [44]. Interactions and references appear under the "HetNet" link and lack of interactions can be searched via the "Non-HetNet" link. The database includes information on both homomers and heteromers. Thus, a series of experimental tools and techniques must be used to identify GPCR homo/heteromers [45]. Among others, in vitro proximity-based biophysical techniques; in situ proximity ligation assays to detect protein–protein interactions in native tissues; mice expressing heteromerization-deficient receptors; techniques to study specific biochemical signatures of heteromers distinct from those of the protomers, in artificial cell lines as well as native tissues; and the use of

membrane-permeable peptides that disrupts heteromeric interfaces (see below).

We have analyzed the ability of GPCRs to establish direct intermolecular interactions via bioluminescence resonance energy transfer (BRET) assays of receptor fused to the yellow fluorescent protein (YFP) and *Renilla* luciferase (Rluc). We have also developed an innovative method that permits identification of heteromers formed by three different GPCRs [46]. This method combines BRET and fluorescence resonance energy transfer (FRET) in a sequential manner (SRET). These techniques have permitted to identify that AdoRs can form homodimers [47], but it was soon noticed that they could also interact with other GPCRs to form higher order structures. The identification of AdoR heterotetramer arrangements was possible using Bimolecular Luminescence and Fluorescence complementation (BiLFC) in cells transfected with receptors fused to halves or donor/acceptor (nRluc and cRluc that only upon complementation can act as BRET donor, and nYFP and cYFP that upon complementation can act as BRET acceptor), in such a way that BRET can only be positive if correct rearrangement of the two halves occurs [37]. The experimental determination of the stoichiometry of the GPCR heterotetramer is the actual challenge. This issue may be approached by sophisticated techniques that combine imaging with mathematical analysis of fluorescence signals. FRET-based spectrometry, under controlled expression of receptors, together with simulated fluorescence intensity patterns derived from different homomer arrangements, was used to deduce that muscarinic M3 acetylcholine receptors are expressed at the plasma membrane as stable dimers and tetramers, but not as trimers, pentamers or hexamers [48]. In addition, the protomers in the homotetramer are suggested to be located at the vertices of a rhombus (from above the plane of the membrane). In the case of the A_1R–$A_{2A}R$ heteromer, we performed a single particle tracking of receptors fused to fluorescent proteins and total internal reflection microscopy (TIRFM) [37]. The analysis of fluorescence distribution led us conclude that the stoichiometry of the A_1R–$A_{2A}R$ heteromer was 2:2 (i.e., a heterotetramer formed by a combination of A_1R and $A_{2A}R$ homodimers). We also found that, unlike the $A_{2A}R$, the A_1R has a Brownian motion when individually expressed. This freedom of movement in the cell surface is lost when the $A_{2A}R$ is co-expressed, meaning that the $A_{2A}R$ restricts the movement of the partner receptor, probably due to the interaction between them.

In addition, the binding of G proteins to a given GPCR heteromer may be detectable via BRET assays, in which fluorescence proteins are fused to the receptors and the G proteins. For instance, BRET assays led us determine that, in the absence of ligands, G_i and G_s are bound to their respective receptor homodimers within the A_1R–$A_{2A}R$ heteromer [37]. Moreover, the use of bacteria toxins led us show that signaling mediated by A_1R and $A_{2A}R$ is controlled by both G_i and G_s proteins. Pertussis toxin that only impairs G_i-mediated signaling was able to alter both A_1R- and $A_{2A}R$-mediated signaling. Analogously, cholera toxin that only impairs Gs-mediated signaling was also able to alter A_1R- and $A_{2A}R$- signaling [41]. The coexistence of G_i and G_s within the receptor heterotetrameric complex was confirmed by the use of minigenes that encode for peptides blocking the interaction of the receptor with the α subunits of G_i or G_s [49, 50]. These results with minigenes were similar to those obtained with toxins; thus, confirming that the GPCR heterotetramer was in complex with one G_s and one G_i [42].

Knowledge of the stoichiometry of the heteromer constitutes relevant information, but the knowledge of the molecular interfaces by which the protomers interact is key to understand the quaternary arrangement of the protomers in the heterotetramer. As noted, it is not possible to establish beforehand the domain(s) of two interacting GPCRs that contribute to the formation of the homo/heterodimer. Thus, the use of synthetic peptides with the sequence of TM domains of the receptor, fused to cell-penetrating peptides [51, 52], to disrupt bimolecular fluorescence complementation (BiFC) signals attained by receptors fused to two complementary halves of YFP (cYFP and nYFP) (Fig. 3a), has been very valuable to predict contacts among TM domains of interacting GPCRs. The most common strategy consists of the use peptides having a part of the sequence corresponding to a given TM segment and another part with the YGRKKRRQRRR sequence of activator of transcription (TAT) protein of the human immunodeficiency HIV-1 virus [53]. The potency of peptides containing the TAT sequence was in vivo proven by the delivery of TAT-ß-galactosidase to different mice tissues. The chimeric peptides were able to cross the blood brain barrier. To the best of our knowledge TAT-TM peptides were first used in the GPCR field by He et al., in 2011 [54]. Their results show that TAT-TM peptides can disrupt the interaction between μ and ∂ opioid receptors, whose heteromerization was reported in 2001 [55]. Other cell-penetrating sequences (SKSKSK) have been successfully used to alter the structure of rhodopsin homodimers in isolated rod outer segment membranes [56].

Figure 3b shows the influence of the TAT-TM peptides of $A_{2A}R$ and $A_{2B}R$ in bimolecular fluorescence complementation (BiFC) assays [57]. Fluorescence is detected if cells are transfected with $A_{2A}R$-nYFP and $A_{2A}R$-cYFP or with $A_{2B}R$-nYFP and $A_{2B}R$-cYFP (broken lines), indicating the formation of homodimers. Notably, when these BiFC assays were performed in the presence of TM peptides, fluorescence complementation was significantly reduced, both $A_{2A}R$ and $A_{2B}R$, in the presence of TM4 and TM5. These experimental results indicate the TM4 and TM5 forms part of a symmetric TM45 interface for $A_{2A}R$ and $A_{2B}R$ homodimers. Fluorescence was also detected

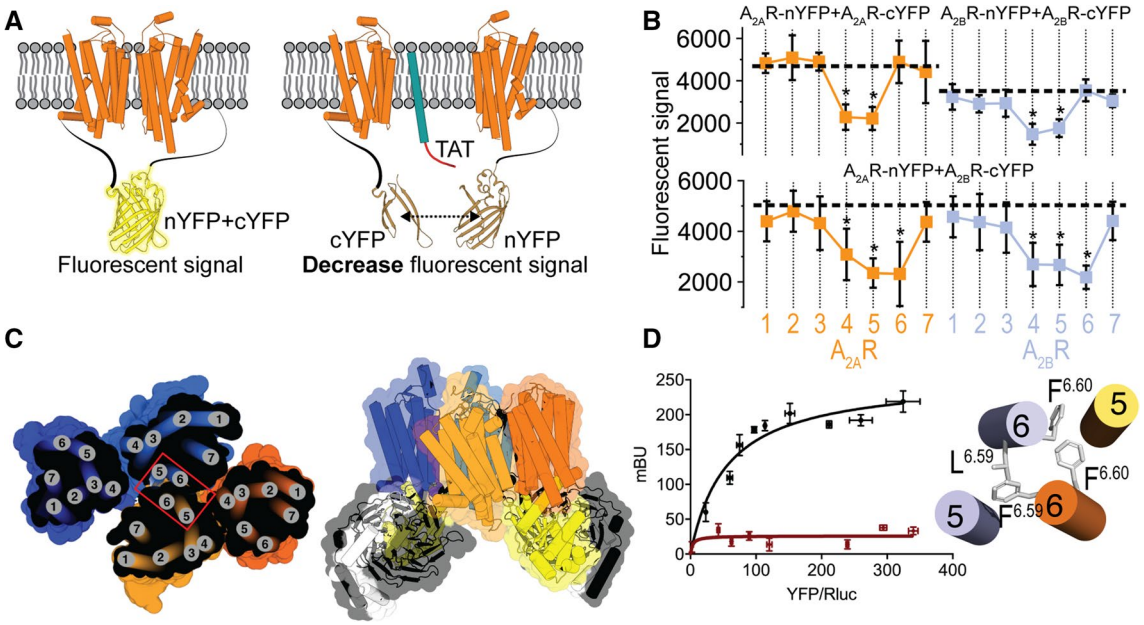

Fig. 3 Model of an AdoR heterotetramer. **a** Scheme of the effect of interference TAT-tagged peptides on AdoR homo/heterodimers (exemplified with $A_{2A}R$ fused to nYFP, amino acids 1–155 of 5OXC, and cYFP, amino acids 156–231 of 5OXC). The formation of AdoR homo/heterodimers permits the complementation of nYFP and cYFP so that a fluorescent signal is detected. TAT-tagged peptides mimicking TM helices of AdoRs might disrupt the interface between AdoR homo/heterodimers with a decrease of the fluorescent signal. **b** BiFC experiments in cells transfected with $A_{2A}R$-nYFP and $A_{2A}R$-cYFP, $A_{2B}R$-nYFP and $A_{2B}R$-cYFP, and $A_{2A}R$-nYFP and $A_{2B}R$-cYFP, as representative pair of putative AdoR heteromers. Cell were treated with medium (broken lines) or indicated TM peptides. *Represents significantly lower values of fluorescence signal as compared to control values. **c** Computational model of the $A_{2A}R$–$A_{2B}R$ heterotetramer, as representative pair of putative AdoR heteromers (see text), build using the experimental interfaces predicted in panel B. Homodimerization is via the TM4/5 interface and heterodimerization is via a TM5/6 interface. Light colors show inactive receptors and dark colors show active, G protein-bound, receptors. G-proteins are colored as in Fig. 2. **d** $A_{2A}R/A_{2B}R$ BRET signal in native receptors (black line) and in mutant receptors (red line) of amino acid residues predicted in silico (side panel). The location of these amino acids in the heterotetramer is shown by the red rectangle in Fig. 3c. (57) Adapted from reference

in cells expressing $A_{2A}R$-nYFP and $A_{2B}R$-cYFP (broken line), indicating the formation of $A_{2A}R$–$A_{2B}R$ heteromers. In this case fluorescence was only significantly reduced in the presence of TM5 and TM6 of both $A_{2A}R$ and $A_{2B}R$, suggesting a TM5/6 interface for heterodimerization. Figure 3c shows a representative molecular model of a conserved (see below) heterotetramer of AdoRs with a TM4/5 interface for homodimerization and a TM5/6 interface for heterodimerization. This model has been constructed from (i) the known crystal structures of A_1R or $A_{2A}R$ (see above) or homology models for the other AdoRs; (ii) the structural details of TM interfaces observed in the crystal structures (see above) of the β_1-adrenergic receptor [26] for the TM4/5 interface or the μ-opioid receptor [25] for the TM5/6 interface, together with molecular dynamics simulations and (iii) the known crystal structures of the complexes (see above) between A_1R and G_i [32] or $A_{2A}R$ and G_s [33, 34] or homology models for the other AdoRs. The existence of these interfaces implies two internal interacting protomers of the heterodimer and two external protomers to which the C-terminal α5 helix of G_i or/and G_s binds. The external protomers of the homodimer have been chosen for G protein binding because the internal protomers are locked in the inactive conformation due to the TM5/6 interface and G-protein binding is not feasible (see above and Fig. 2).

Mutations in TM domains can also be used to determine the interacting interfaces between protomers. The approach of inserting Cys residues to force formation of disulfide bridges that could be detected by electrophoresis led to apparently reliable results [58]. The detection of disulfide-bridged dimers permitted the identification of the TM6-TM6 interface in the active state of the mGlu5 dimer before the release of the cryo-EM structure [59]. We have mutated key amino acids in the TM5/6 interface of the $A_{2A}R$–$A_{2B}R$ heteromer to confirm the role of these TMs [57]. Notably, among the extensive network of conserved hydrophobic and aromatic interactions observed in the TM5/6 interface [60, 61], Ala-mutation of only two amino acids of this network in each protomer (Phe6.59 and Phe6.60 of $A_{2A}R$ and Leu6.59 and Phe6.60 of $A_{2B}R$) blunted the BRET signal between heteromers (Fig. 3d).

Combining the different approaches with the effect of TAT-TM peptides in molecular complementation and in

receptor function, it has been established that the molecular interfaces in both A_1R–$A_{2A}R$ and $A_{2A}R$–$A_{2B}R$ heteromers are similar. Homodimerization of receptors occur through the TM4/5 interface and heterodimerization via the TM5/6 interface (Fig. 3). Can this be a general rule for other adenosine receptor heteromers? Time will tell.

The A_1–$A_{2A}R$ heteromer

When we first discovered the interaction between these adenosine receptors, we could not understand the physiological meaning, as one is coupled to Gi and another to Gs. However, we were able to demonstrate that depending on the concentration of adenosine, the regulation of glutamate release by glutamatergic terminals from cortical neurons would be opposite [62]. If the receptors were independently acting the response to the endogenous agonist, adenosine, in terms of cAMP production and control of neurotransmitter release would be neutral. Later on, we noticed that the heteromer was expressed in astrocytes to mediate the regulation by adenosine of gamma-amino butyric acid uptake [63]. In summary, as schematized in Fig. 4, the heteromer acts as a sensor of adenosine concentration in such a way that there is either A_1R or $A_{2A}R$-mediated signaling. These opposite effects, G_i or G_s engagement, could not be attained by adenosine acting on individual receptors. The tetrameric structure of the heteromer in complex with the two G proteins provides the molecular basis to explain those findings [41]. Moreover, the long C-terminal domain of $A_{2A}R$, over A_1R (see above and Fig. 1), is responsible for the dominant $A_{2A}R$-mediated signaling. The flexibility of the long C-tail, the lack of this domain in crystal structures, and the fact that the C-tail may be intermingled with some other components of the macromolecular complex difficult to study its role at the molecular level. In other words, resolution of the structure of the circa 100 amino acids of the final part of the $A_{2A}R$ may not be accurate in the absence of interacting proteins. Remarkably however, it is this domain the main responsible of the heteromer property that consist of blockade of A_1R signaling when the $A_{2A}R$ is activated (see Fig. 4). Despite these limitations, this heteromer serves to understand at least one of the reasons for the differential length in the C terminus of GPCRs. We consistently found that removal of the end of the $A_{2A}R$ has the same consequences of pertussis toxin treatment. Such properties of the heteromer are lost when cells are treated with the disrupting TAT peptides, that alter the overall structure of the complex. The conclusions are that (i) the structure of the complex is well defined and (ii) when the $A_{2A}R$ is activated the C-terminal domain of the receptor is positioned to block Gi engagement [41]. As we indicated in the paper *"We identified a new mechanism of signal transduction that implies a cross-communication between G_i and G_s proteins guided by the C-terminal tail of the $A_{2A}R$. This mechanism provides the molecular basis for the operation of the A_1–A_{2A}Het as an adenosine concentration-sensing device that modulates the signals originating at both A_1R and $A_{2A}R$"* [41].

The A_{2A}–$A_{2B}R$ heteromer

The interaction between A_{2A} and A_{2B} receptors was not expected as G_s is the canonical G protein for the two receptors. Two reports have shown that the A_{2A}–$A_{2B}R$ heteromer occurs in different tissues and has provided unexpected findings [57, 64]. On the one hand, rigorous analysis performed in heterologous expression systems but also in human native cell lines show that increasing the expression of the $A_{2B}R$ results in decrease $A_{2A}R$-mediated signaling. The pharmacology of the $A_{2A}R$ is markedly altered in the presence of the $A_{2B}R$ in such a way that the high-affinity for agonists, which is a classical feature if the $A_{2A}R$, is lost. Such findings correlate with increased expression of A_{2A}–$A_{2B}R$ heteromers. It is not known which are the tissues or the physiological conditions requiring that upregulation of the $A_{2B}R$ leads to a virtual lack of $A_{2A}R/G_s$ mediated signaling [64]. In addition, these findings have implications in drug discovery. Thus, promising ligands displaying high in vitro affinity for the $A_{2A}R$ may become inefficacious in the A_{2A}–$A_{2B}R$ heteromer context. On the other hand, the heteromer itself arises as the real target in pathologies in which its expression is upregulated. It is notable that the A_{2B} adenosine receptor can couple to both G_i and G_s, and many important signaling events are mediated via G_i rather than G_s in many cell types endogenously expressing the $A_{2B}R$ receptor, although G_s is the predominant G protein in CHO cells expressing the recombinant $A_{2B}R$ [65, 66].

The prominent role attributed to the $A_{2A}R$ receptor in brown adipose tissue (BAT) [67–69] has been recently revisited after the recent discovery of A_{2A}–$A_{2B}R$ heteromers as the real mediators of the actions of adenosine in this tissue. The adenosine effects in BAT are completely dependent on the expression of $A_{2B}R$ as deduced from the use of selective antagonist or tissue from KO animals. Interestingly, the effect is also lost in animals deficient for the $A_{2A}R$ thus suggesting that heteromer formation is required [57]. It remains to be elucidated whether in BAT the heteromer is in a context that alters its functionality with respect to that found in heterologous cells in which the $A_{2A}R$ signaling is lost when the $A_{2B}R$ is present. It should be noted that the role of the C-terminal domain of the $A_{2A}R$ in the overall functionality

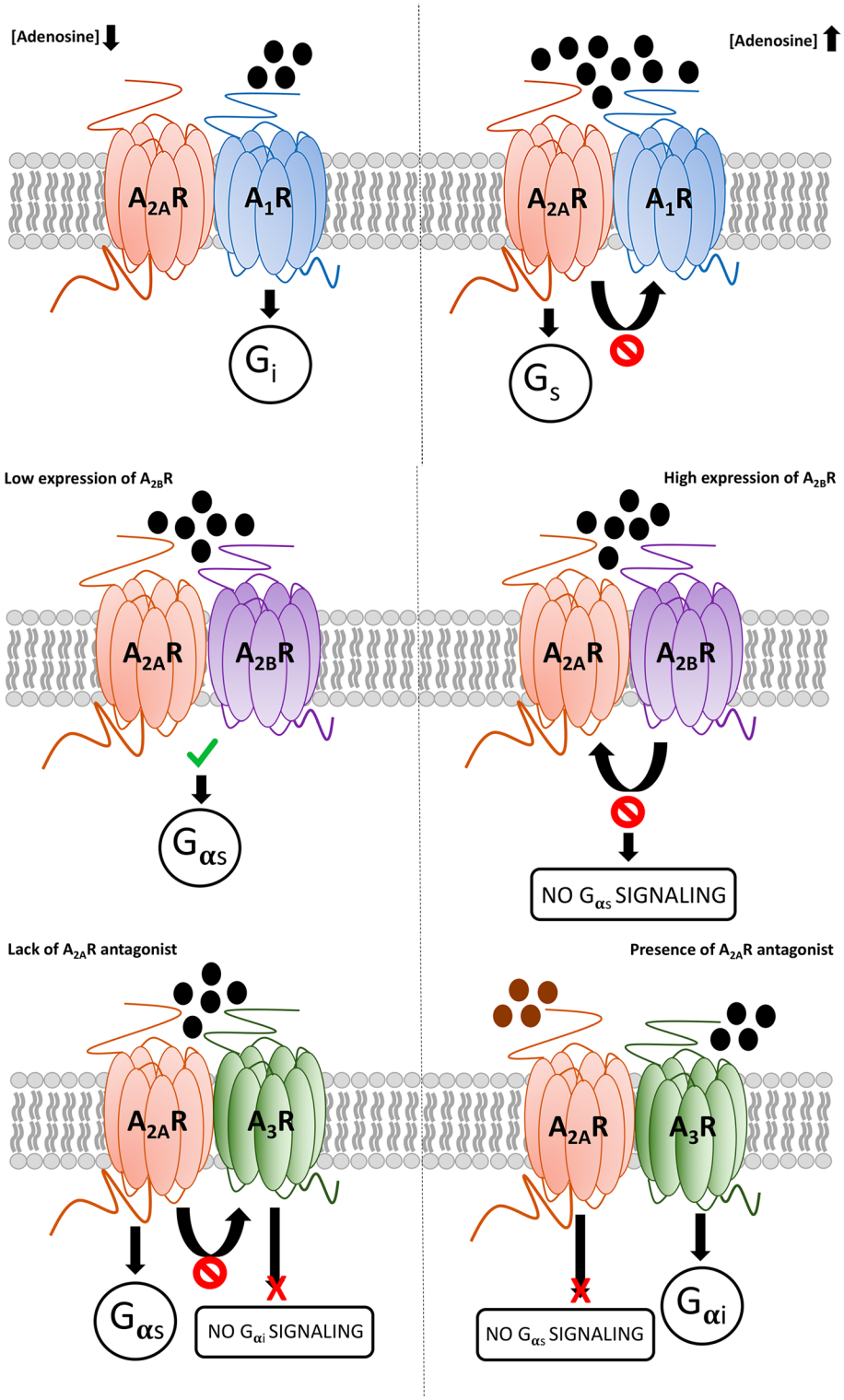

Fig. 4 Scheme summarizing the G-protein-mediated signaling output of adenosine when interacting with A_1–A_{2A}, A_{2A}–A_{2B}, or A_{2A}–A_3 receptor heteromers. Receptors are depicted as monomers for simplicity

of this heteromer is not yet addressed, although it does not affect A_{2A}–A_{2B}R heteromer formation [64].

The A_{2A}–A_3R heteromer

To our knowledge there is only one paper describing heteromer identification, expression in natural sources (primary cultures of cortical neurons) and particular functional properties. Unlike the other above described AdoR heteromers,

there is not yet any suggested structural model. The properties of this heteromer confirm that there is not any general rule and that a given heteromer has its own particularities. Despite conceptually similar to the A_1A_{2A}RHet, i.e. one receptor coupled to Gs and another to G_i, it does not seem that the heteromer acts as an adenosine concentration sensing device. Instead, the main feature in this heteromer is a marked reduction of Gi signaling originating at the A_3R receptor while A_{2A}R receptor antagonists blunted the blockade. These data are relevant to know the functional diversity provided by GPCR heteromers. In this heteromer the often-found cross-antagonism does not happen and, instead, A_{2A}R antagonists facilitate the signaling arising in the partner receptors. These findings open novel and unexpected therapeutic possibilities centered in indirectly targeting the A_3R using A_{2A}R antagonists (see Fig. 4) [70].

More adenosine receptor heteromers?

Collecting all evidence there is ground to hypothesize that all adenosine receptors interact with each other. To our knowledge, data on the potential interaction between the A_{2B} and the A_3 receptors is missing while allosteric interactions suggest potential A_1R/A_3R heteromer formation [71]. The puzzle of adenosinergic signaling will not be complete until these two interactions are addressed and information on the differential functional properties of all heteromers is available. In fact, the development of new structural methods (crystallization, NMR and Cryo-EM) capable of showing the structure of these heteromers at atomic resolution will be essential to understand the multiple potential of adenosine as a regulator and propose heteromers as possible targets of a variety of diseases.

In memoriam

Geoffrey Burnstock (May 1929-June 2020) was the discoverer of purinergic nerves, the best pharmacologist RF has ever met and a multitalented and inspiring human being.

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Author contributions RF and LP conceived the idea and made a design of the sections in the review. AC, AL, and GN searched and summarized the content of relevant papers in literature. JSM and CLT looked information to prepare the Figs. RF wrote the first draft that was edited by all authors who also approved the final version.

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Data availability Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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