Adenosine A\(_{2A}\) receptor ligand recognition and signaling is blocked by A\(_{2B}\) receptors

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ABSTRACT

The adenosine receptor (AR) subtypes A\(_{2A}\) and A\(_{2B}\) are rhodopsin-like G\(_{s}\) protein-coupled receptors whose expression is highly regulated under pathological, e.g. hypoxic, ischemic and inflammatory conditions. Both receptors play important roles in inflammatory and neurodegenerative diseases, are blocked by caffeine, and have now become major drug targets in immuno-oncology. By Förster resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), bimolecular fluorescence complementation (BiFC) and proximity ligation assays (PLA) we demonstrated A\(_{2A}\)-A\(_{2B}\) AR heteromeric complex formation. Moreover we observed a dramatically altered pharmacology of the A\(_{2A}\)AR when co-expressed with the A\(_{2B}\)AR (A\(_{2B}\) ≥ A\(_{2A}\)) in recombinant as well as in native cells. In the presence of A\(_{2B}\)ARs, A\(_{2A}\)-selective ligands lost high affinity binding to A\(_{2A}\)ARs and displayed strongly reduced potency in cAMP accumulation and dynamic mass redistribution (DMR) assays. These results have major implications for the use of A\(_{2A}\)AR ligands as drugs as they will fail to modulate the receptor in an A\(_{2A}\)-A\(_{2B}\) heteromer context. Accordingly, A\(_{2A}\)-A\(_{2B}\)AR heteromers represent novel pharmacological targets.

INTRODUCTION

Adenosine receptors (ARs) are G protein-coupled receptors (GPCRs) activated by the nucleoside adenosine. Four subtypes designated A\(_1\), A\(_{2A}\), A\(_{2B}\) and A\(_3\)ARs exist. A\(_1\) and A\(_3\)ARs preferentially couple to G\(_{i/o}\) proteins mediating inhibition of adenylate cyclase (AC) activity, while A\(_{2A}\) and A\(_{2B}\) receptors couple to G\(_{s/olf}\) proteins leading to AC activation and subsequent increase in cAMP formation [1]. In addition, A\(_{2B}\) and A\(_3\)ARs were shown to couple to G\(_{q}\) proteins which results in phospholipase C activation followed by a rise in inositol trisphosphate levels mediating intracellular calcium release [2–3]. The A\(_{2A}\)AR is expressed in high density in the caudate-putamen, and at low levels in most other brain regions. In the periphery, the A\(_{2A}\)AR is highly expressed in cells of the immune system and blood platelets, and at lower levels in many other cells and organs [4]. The A\(_{2A}\)AR is broadly expressed but mostly at moderate to low levels. A\(_{2A}\) and A\(_{2B}\)ARs are the most closely related AR subtypes with an overall sequence identity of 58% and a similarity of 73% [5]. They are co-expressed on many different cell types and in various organs and tissues, e.g. in heart [6], myeloid cells [7], T-cells [8], blood platelets [9], brown and white adipocytes [10], and in many tumors, e.g. neuroendocrine tumors [11], ovarian cancer [12], and prostate cancer.
The expression of $A_{2A}$ and $A_{2B}$ARs and their relative proportion can be markedly altered under pathological conditions [14]. For example, increased $A_{2A}$AR expression is observed in the brains of patients suffering from neurodegenerative diseases [15], in multiple sclerosis and in amyotrophic lateral sclerosis [16–17]. Upon activation of T-lymphocytes the $A_{2A}$AR is considerably upregulated [18]. On the other hand, the expression of $A_{2B}$ARs can be drastically increased in a hypoxia-inducible factor-(HIF1α-) dependent manner under hypoxic conditions, e.g. in inflamed or ischemic tissue, in tumors and cancer cells [19–20]. Hypoxia induction leads to a decrease in $A_{2A}$AR expression while increasing $A_{2B}$AR expression in human umbilical vein endothelial and bronchial smooth muscle cells. Pharmacological responses of $A_{2A}$/A$_{2B}$AR agonists were significantly altered in these cells [21]. The well investigated $A_{2A}$AR subtype, the so-called “high-affinity $A_{2A}$AR receptor”, is typically activated by relatively low (nanomolar) concentrations of adenosine, mediating potent anti-inflammatory and immunosuppressant as well as hypotensive and anti-psychotic effects [22]. In contrast, activation of the $A_{2B}$AR subtype, the “low-affinity $A_{2A}$AR”, requires high, micromolar adenosine concentrations for activation [4]. Extracellular adenosine levels can rise from basal values of around 100 nM by up to 100-fold reaching concentrations of around 10 μM under pathological, i.e. hypoxic, ischemic or inflammatory conditions [1, 4, 23]. Cell death can lead to the formation of large amounts of extracellular adenosine through enzymatic degradation of released ATP by ectonucleotidases (CD39, CD73), e.g. in solid tumors [24]. Both anti- as well as pro-inflammatory effects have been associated with the $A_{2B}$AR [25], and the reasons for these contradictory results have remained obscure. The physiological significance of the $A_{2B}$AR subtype is scarcely understood so far. During the last decade it has become well accepted that GPCRs are able to form di- or oligomeric assemblies of identical or distinct receptor monomers [26]. Most of these complexes have been detected in transfected living cells using well accepted biophysical techniques such as resonance energy transfer (bioluminescence and Förster resonance energy transfer, BRET and FRET) or bimolecular fluorescence complementation (BiFC) assays [27–28]. Proximity ligation assays (PLA) have been developed for identifying receptor heteromers in native cells and tissues [29]. Heteromer formation may modulate receptor pharmacology such as the affinity and potency of ligands or G protein coupling and signaling [30–31]. Recently, structural models of GPCR oligomers associated with G proteins have been built [32], and the development of heteromer-selective receptor ligands is becoming a promising new research area [33]. The $A_{2A}$AR was reported to form homomeric receptor complexes as well as heteromers with several other GPCRs including dopamine D$_1$ and D$_2$, cannabinoid CB$_1$, nucleotide P2Y$_1$ and P2Y$_2$ and A$_1$ARs [1]. Especially $A_{2A}$-D$_2$ heteromeric receptor complexes have been intensively studied since they play a significant role in Parkinson’s disease [34]. However, homo- or heteromer formation of the $A_{2A}$AR subtype has not been demonstrated up to now. Based on the frequent co-expression of the closely related AR subtypes $A_{2A}$ and $A_{2B}$, and considering the up-regulation of the $A_{2A}$AR subtype and the up- or down-regulation of the $A_{2A}$AR under many pathological conditions, the question arises if both receptors could form heteromers and whether this might affect their pharmacology and signaling. Here we demonstrate that $A_{2A}$-$A_{2B}$AR heteromers are formed in living cells by employing FRET, BRET and PLA, and their presence in native tissue was confirmed. Heteromer formation was found to be independent of the presence of agonists or antagonists, and does not require the long C-terminus of the $A_{2A}$AR. Importantly, we demonstrate that $A_{2A}$-$A_{2B}$ heteromerization is the reason for drastically altered pharmacology, in particular for the $A_{2A}$AR, which is completely blocked by the presence of $A_{2B}$AR protein. These results can now help to explain many unexpected or previously misinterpreted observations. They will be of high relevance for recently started drug development programs targeting $A_{2A}$ or $A_{2B}$ARs, in particular in neurodegenerative diseases and immuno-oncology.

RESULTS

FRET, BRET and BiFC experiments

FRET is a powerful technique for measuring protein-protein interactions in living cells [35]. To investigate a possible $A_{2A}$-$A_{2B}$AR interaction, FRET experiments were performed in Chinese hamster ovary (CHO-K1) cells transiently transfected with fusion proteins of green fluorescent protein variant 2 (GFP$^2$) and enhanced yellow fluorescent protein (EYFP) attached to the C-terminus of the receptors [36] (Figure 1A–1C). The previously described $A_{2A}$AR-homodimer and the fusion protein GFP$^2$-EYFP were employed as positive controls showing FRET efficiencies of 0.23 and 0.44, respectively, similar to those previously reported (A$_{2A}$AR homodimer: 0.28, GFP$^2$-EYFP: 0.52) (Figure 1A, 1B) [37]. The fusion protein GFP$^2$-EYFP displayed a high FRET efficiency due to the very close proximity of donor and acceptor as a result of the short linker between both fluorophores. A clear FRET signal with an efficiency of 0.16 was observed in the co-transfected cells indicating the formation of $A_{2A}$-$A_{2B}$ heteroreceptor complexes (Figure 1A). The pair $A_{2A}$AR and GABA$_A$ receptor [36–37] was employed as a negative control; it showed a very low FRET signal demonstrating the specificity of the observed interactions (Figure 1A–1B). To gain insight into the potential $A_{2A}$-$A_{2B}$ heteromer interface, the C-terminal tail of the $A_{2B}$AR was removed and the resulting construct $A_{2B}$AR-EYFP was studied in FRET experiments as an acceptor fluorophore in combination with the $A_{2B}$GFP$^2$ donor.
fluorophore. The results indicated that the A<sub>2A</sub>-AR that was lacking the C-terminal domain was still fully able to form heteromers with the A<sub>2B</sub>-AR (FRET efficiency 0.24, Figure 1B) suggesting that different receptor domains, possibly helical domains, have to be involved in heteromer formation.

BRET is another biophysical technique that can be utilized to detect protein-protein interactions by measuring energy transfer from a bioluminescence donor to a fluorescent acceptor [35]. To confirm a direct A<sub>2A</sub>-A<sub>2B</sub>-AR interaction, BRET experiments were performed in living CHO-K1 cells transiently expressing fusion proteins

Figure 1: Biophysical assays using A<sub>2A</sub> and A<sub>2B</sub>-ARs fused to FRET donor and acceptor. (A) FRET efficiencies were calculated by a sensitized emission method in living CHO cells transiently transfected with the different plasmids. Data are means ± SEM of 4–5 independent experiments performed in duplicates. The one-way ANOVA with Dunnett’s post-hoc test showed significant differences in A<sub>2A</sub>-GFP² + A<sub>2A</sub>-EYFP (positive control) or A<sub>2B</sub>-GFP² + A<sub>2A</sub>-EYFP versus the negative control (A<sub>2A</sub>-GFP² + GABA<sub>B</sub>R²-EYFP), **p < 0.01. As an internal control the fusion protein GFP²-EYFP was used. (B) FRET efficiencies determined in CHO cells transiently transfected with the different plasmids. The same controls were used as shown in (A). Data are means ± SEM of 5 independent experiments performed in duplicates. The one-way ANOVA with Dunnett’s post-hoc test showed significant differences between A<sub>2A</sub>-GFP² + A<sub>2A</sub>-EYFP vs 405 nm 510 nm 535 nm 405 nm 510 nm 535 nm
consisting of a receptor (A\textsubscript{2A}, A\textsubscript{2B}, D\textsubscript{2}, or GABA\textsubscript{B2}) and Rluc (Renilla luciferase) or the fluorescent protein EYFP attached to the C-terminus (Figure 2A–2D). Three kinds of experiments were performed as shown in Figure 2A, 2C and 2D. For BRET saturation curves cells were co-transfected with a constant amount of cDNA for Rluc-receptor constructs and increasing concentrations of cDNAs for EYFP-receptor constructs. As a widely accepted positive control for GPCR dimers, donor/acceptor proteins having the dopamine D\textsubscript{2}R-A\textsubscript{2A}AR were used. The results showed a high BRET signal displaying a hyperbolic curve, with a BRET\textsubscript{50} value of 239 ± 40 and a BRET\textsubscript{max} value of 144 ± 6 mBU. As a negative control, donor/acceptor proteins having the A\textsubscript{2A}AR and GABA\textsubscript{B2} receptor pair were used. The combination of A\textsubscript{2A} and A\textsubscript{2B} ARs resulted in a specific BRET signal which was even higher than the positive control indicating a specific interaction of both receptors. A BRET\textsubscript{50} value of 122 ± 6 and a BRET\textsubscript{max} value of 158 ± 10 mBU were determined (Figure 2A). Subsequently, a BRET displacement study was performed in which increasing amounts of unlabeled A\textsubscript{2B}AR were added to A\textsubscript{2B}-Rluc and A\textsubscript{2A}-YFP receptors. The experiment showed a significant decrease in the BRET signal, which was dependent on the added amount of unlabeled A\textsubscript{2B}AR (Figure 2C), indicating displacement by the unlabeled receptor of the Rluc-tagged A\textsubscript{2B}AR in the heteromer. As a final step, potential effects of A\textsubscript{2A} and A\textsubscript{2B} AR agonists and antagonists on A\textsubscript{2A}-A\textsubscript{2B} heteromer formation were studied. The BRET signal after 60 min of treatment with agonists (adenosine, nonselective; NECA, nonselective; CGS-21680, A\textsubscript{2A}-selective; BAY60–6583, A\textsubscript{2B}-selective) or

![Figure 2: Biophysical assays using A\textsubscript{2A} and A\textsubscript{2B} ARs fused to BRET donor and acceptor. (A) BRET saturation curves: CHO-K1 cells were transiently co-transfected with a constant amount of A\textsubscript{2A}, A\textsubscript{2B}, or D\textsubscript{2} receptors fused to Rluc and increasing amounts of cDNA for A\textsubscript{2A} or GABA\textsubscript{B2} receptors fused to EYFP. BRET experiments were performed in duplicates for A\textsubscript{2B}-Rluc and A\textsubscript{2A}-YFP (▲) (n = 11) with a BRET\textsubscript{min} = 158 ± 10 mBU and BRET\textsubscript{50} = 122 ± 58, positive control D\textsubscript{2}-Rluc and A\textsubscript{2A}-YFP (●) (n = 15) BRET\textsubscript{max} = 144 ± 6 mBU and BRET\textsubscript{50} = 239 ± 40, and negative control A\textsubscript{2A}-Rluc and GABA\textsubscript{B2}-YFP (♦) (n = 13). (B) Schematic representation of the BRET experiments. (C) BRET competition experiments were performed (n = 4, in triplicates) in cells transfected with 1.25 µg of cDNA for A\textsubscript{2B}-Rluc, 2.5 µg of cDNA for A\textsubscript{2A}-YFP and increasing amounts of cDNA for untagged A\textsubscript{2B}ARs. The one-way ANOVA with Dunnett’s post-hoc test showed a significant decrease in the BRET signal compared to cells which were not transfected with untagged A\textsubscript{2B}ARs (green column; *p < 0.05; **p < 0.01). (D) CHO cells were transiently co-transfected with 2 µg of cDNA for A\textsubscript{2B}-Rluc and 3 µg of cDNA for A\textsubscript{2A}-YFP. Different agonists (adenosine, NECA, CGS-21680, BAY60-6583) and antagonists (PSB-603) were added and the BRET signal was measured over a time period of 60 min (n = 3, in duplicates).}
the $\text{A}_{2\text{B}}$ antagonist PSB-603 was similar to that obtained in the absence of ligands (Figure 2D), thus indicating that $\text{A}_{2\text{A}}$-$\text{A}_{2\text{B}}$ heteromer formation was not influenced by those receptor ligands.

These results were further corroborated by BiFC experiments, which provided strong evidence for a very close interaction between $\text{A}_{2\text{A}}$ and $\text{A}_{2\text{B}}$ ARs (Supplementary Figures 1, 2).

**In situ proximity ligation experiments in the rat brain**

The PLA combines the high specificity and affinity of antibodies (PLA probe) with the sensitivity of quantitative polymerase chain reactions (PCR) to detect proteins that are forming molecular complexes in native sources [38]. Initially we studied the recombinant CHO-$\text{A}_{2\text{A}}$-$\text{A}_{2\text{B}}$ cell line to investigate the receptors’ proximity (Supplementary Figure 3A–3C) and obtained small, brightly green fluorescent spots each of which represents a single $\text{A}_{2\text{A}}$-$\text{A}_{2\text{B}}$ AR heteromer (Supplementary Figure 3C). Next we performed in situ PLA focusing on the dorsal hippocampus of the rat brain (Figure 3, Supplementary Figures 4, 5) where moderate to high densities of PLA-specific clusters were found. It should be noted that the molecular layer of the dentate gyrus lacked PLA clusters, and the unspecific labeling there was similar to that observed in negative control sections obtained by omitting the primary anti-$\text{A}_{2\text{A}}$ antibody. Furthermore, few PLA positive clusters were observed in the oriens of the CA1 areas. In contrast, a high density of $\text{A}_{2\text{A}}$-$\text{A}_{2\text{B}}$ specific clusters was found in the CA3 pyramidal cell layer, mainly in perisomatic location (Figure 3), where PLA-positive clusters had diameters from 0.5–2 µm. They were present also in lower densities in the radiatum and oriens. In all these regions PLA positive clusters were also found in the neuropil. The CA1 showed a similar distribution pattern (as compared with CA3), with high dot/cluster densities within the pyramidal cell layer. An important difference was, however, the diameter range of the clusters, which appeared to be reduced in this CA1 region versus CA3 (Supplementary Figure 4).

In the polymorphic layer of the dentate gyrus (PoDG), a high density of specific $\text{A}_{2\text{A}}$-$\text{A}_{2\text{B}}$ clusters was observed in both perisomatic and neuropil position. The range of diameter size in the clusters was similar to that in the CA3 area (Supplementary Figure 5).

**Figure 3: In situ proximity ligation assay in rat hippocampus.** $\text{A}_{2\text{A}}$-$\text{A}_{2\text{B}}$AR-specific PLA clusters in the CA3 region of the dorsal hippocampus of the rat (Bregma: −3.6 mm). The sampled region is taken from the framed section of the dorsal hippocampus in the upper right corner of the figure. The microphotographs taken are based on 20 Z-scans (1 µm each). The nuclei are shown in blue. A high density of PLA positive clusters in red are visualized mainly in the pyramidal cell layer shown also in higher amplification in the panel at the lower right part of the figure. A few are indicated by arrows. The diameter range of the clusters is 0.5–2 µm. They are mainly located in a perisomatic position around the blue nuclei but also in the neuropil. A low density of specific PLA clusters is also found in the radiatum and oriens close to the pyramidal cell layer.
Pharmacological implications of A<sub>2A</sub>-A<sub>2B</sub> AR heteromer formation

To study the pharmacology of A<sub>2A</sub>-A<sub>2B</sub> AR heteromers, native as well as recombinant cell lines were investigated. Expression levels of A<sub>2A</sub> and A<sub>2B</sub> ARs were analyzed by reverse transcriptase (RT) PCR, Western blot analysis and radioligand binding studies (Supplementary Figure 6A–6E). Recombinant cell lines were prepared to control the proportion of A<sub>2A</sub> and A<sub>2B</sub> AR expression (A<sub>2B</sub> ≥ A<sub>2A</sub>, and A<sub>2A</sub> > A<sub>2B</sub>) (Supplementary Table 1).

Radioligand-receptor binding studies

Radioligand binding studies were performed using the A<sub>2B</sub>-selective antagonist radioligand [H]PSB-603 and two A<sub>2A</sub>-selective radioligands, the antagonist [H]MSX-2, and the agonist [H]CGS-21680 (Supplementary Figure 7A–7C, Supplementary Table 2). A selective radiolabeled agonist for A<sub>2B</sub> ARs is currently not available. Labeling of A<sub>2B</sub> ARs with [H]PSB-603 demonstrated high A<sub>2B</sub> expression in membrane preparations of CHO-A<sub>2A</sub> and CHO-A<sub>2A</sub>-A<sub>2B</sub> (A<sub>2B</sub> ≥ A<sub>2A</sub>) cell lines both of which displayed similar levels of A<sub>2B</sub> expression (502 and 418 fmol/mg protein, respectively). Jurkat-T (220 fmol/mg protein) and HeLa cells (80 fmol/mg protein) had lower A<sub>2B</sub> AR expression levels (Supplementary Figure 6E, Supplementary Table 2). As expected, in cells lacking significant A<sub>2B</sub> expression (CHO-K1, CHO-HA-A<sub>2A</sub>, HEK-A<sub>2A</sub>), no high-affinity binding of the A<sub>2A</sub>-selective antagonist radioligand [H]PSB-603 was observed (Supplementary Figure 7C). In cells that co-expressed both receptors (CHO-A<sub>2A</sub>-A<sub>2B</sub> cells, HeLa cells, Jurkat-T cells, native human T-lymphocytes), specific binding of [H]PSB-603 was detected, and its affinity was similar to that determined at CHO cells expressing only A<sub>2B</sub> ARs (Supplementary Figure 8A, Supplementary Table 2). Native primary human lymphocytes displayed a moderate expression level of A<sub>2B</sub> ARs, lower to that of A<sub>2A</sub> ARs. Upon activation with phytohemagglutinin (PHA), the A<sub>2B</sub> AR expression level remained virtually unaltered (Supplementary Figure 9). All radioligand binding results on A<sub>2B</sub> ARs were in agreement with the data obtained in RT-PCR and Western blot experiments (Supplementary Figure 6A–6D, Supplementary Table 2). The A<sub>2A</sub>-selective radioligands [H]MSX-2 and [H]CGS-21680 labeled A<sub>2A</sub> ARs in CHO-A<sub>2A</sub> cells and in cells expressing more A<sub>2A</sub> than A<sub>2B</sub> ARs (Figure 4, Supplementary Figure 7A, 7B, Supplementary Table 2). Native primary human lymphocytes displayed specific binding of [H]MSX-2 indicating A<sub>2A</sub> AR expression, that was significantly upregulated (by about 4-fold) upon activation with PHA (Supplementary Figure 9, Supplementary Table 2). Unexpectedly, in cells with similar or higher expression of A<sub>2B</sub> as compared to A<sub>2A</sub> ARs no high-affinity binding of either A<sub>2A</sub>-selective radioligand, [H]MSX-2 or [H]CGS-21680, was observed (Supplementary Figure 8A, 8B, Supplementary Table 2). Competition binding assays versus [H]PSB-603 were performed to determine the A<sub>2B</sub> affinity of selected agonists and antagonists while A<sub>2A</sub> affinity of compounds was determined versus [H]MSX-2. Indeed, the latter was only possible in cell lines expressing more A<sub>2B</sub> than A<sub>2A</sub> ARs since high affinity binding of the A<sub>2A</sub>-selective radioligands was abolished when A<sub>2B</sub> receptors were co-expressed (see above).

CHO-A<sub>2A</sub> and CHO-A<sub>2B</sub> cell lines displayed the expected affinities of agonists and antagonists typical for, respectively, A<sub>2B</sub> or A<sub>2A</sub> ARs (Figure 4A, 4B, Supplementary Table 3). In CHO-A<sub>2A</sub>-A<sub>2B</sub> cell membranes, which showed a similar or slightly higher expression of A<sub>2B</sub> than of A<sub>2A</sub> ARs, the agonists adenosine, NECA, and BAY60-6583 and the antagonists PSB-603 and caffeine displayed only slightly modulated A<sub>2B</sub> affinities (Figure 4A, 4B, Supplementary Table 3). We subsequently studied a T-cell line, namely Jurkat-T cells, which express similar amounts of A<sub>2A</sub> and A<sub>2B</sub> ARs, the level of which is, however, lower than in the recombinant CHO-A<sub>2A</sub>-A<sub>2B</sub> cells. The A<sub>2B</sub>-selective radioligand [H]PSB-603 displayed high affinity binding which was displaced by the A<sub>2B</sub>-selective partial agonist BAY60-6583 (Figure 4C, Supplementary Figure 8A, Supplementary Tables 2, 3). However, no high-affinity binding was observed for the A<sub>2A</sub>-selective radioligands [H]MSX-2 and [H]CGS-21680 (Supplementary Figure 8A, 8B, Supplementary Table 2), although we could clearly detect A<sub>2A</sub> AR protein expression in Jurkat-T cells (Supplementary Figure 6C). In native T-lymphocytes, isolated from healthy human blood donors, the expression of the A<sub>2A</sub> AR was higher than that of the A<sub>2B</sub> AR (Supplementary Figure 9, Supplementary Table 2). High affinity binding was then observed for the A<sub>2A</sub>-selective radioligand [H]MSX-2 as well as for the A<sub>2B</sub>-selective radioligand [H]PSB-603 (Supplementary Figure 8A, 8B, Supplementary Table 2). In HeLa cells, which natively express more A<sub>2B</sub> than A<sub>2A</sub> ARs, again, no high-affinity binding was obtained for the A<sub>2A</sub>-selective radioligands [H]MSX-2 and [H]CGS-21680 (Supplementary Figure 8A, 8B, Supplementary Table 2) although we could clearly detect the A<sub>2B</sub> AR protein in this cell line (Supplementary Figure 6D). As a next step we overexpressed the human A<sub>2A</sub> AR containing an HA tag in HeLa cells to obtain a cell line which expressed more A<sub>2A</sub> than A<sub>2B</sub> ARs (Supplementary Figure 6D, Supplementary Table 2). This led to the recovery of high-affinity binding for the A<sub>2A</sub>-selective radioligands (Figure 4D, Supplementary Table 2). NECA and CGS-21680 showed higher affinity versus the agonist radioligand than versus the antagonist radioligand, whereas the antagonist MSX-2 displayed similar affinities versus both radioligands (Figure 4D, Supplementary Table 3), results that are typical for A<sub>2A</sub> ARs [39].
cAMP accumulation assays

A<sub>2A</sub> and A<sub>2B</sub> ARs are coupled to G<sub>s</sub> proteins, activating adenylate cyclase. Thus, cAMP accumulation assays were performed at CHO cells stably expressing the A<sub>2A</sub> AR, the A<sub>2B</sub> AR, or both. Adenosine increased cAMP accumulation with an EC<sub>50</sub> value of 174 nM in CHO cells expressing the “high affinity” A<sub>2A</sub> AR, and with an EC<sub>50</sub> value of 12,500 nM in CHO cells expressing the “low-affinity” A<sub>2B</sub> AR. The CHO-A<sub>2A</sub>-A<sub>2B</sub> cell line co-expressing both subtypes showed virtually the same EC<sub>50</sub> value (13,100 nM) as the cell line only expressing A<sub>2B</sub> ARs (Figure 5A, Supplementary Table 4). The metabolically more stable adenosine analog NECA displayed a similar behaviour. The potent A<sub>2A</sub>-selective agonist CGS-21680 (EC<sub>50</sub> 16.6 nM in CHO-A<sub>2A</sub> cells) was inactive in CHO-A<sub>2A</sub>-A<sub>2B</sub> cells (EC<sub>50</sub> > 10,000 nM). In contrast, the A<sub>2B</sub>-selective partial agonist BAY60-6583 (EC<sub>50</sub> 165 nM in A<sub>2B</sub> AR-expressing cells) had similar potency in the A<sub>2A</sub>-A<sub>2B</sub>-coexpressing cell line (EC<sub>50</sub> 193 nM) (Figure 5A, Supplementary Table 4). Antagonist potencies were determined by measuring concentration-response curves for the non-selective agonist NECA in the presence or absence of different antagonists, and K<sub>B</sub> values were calculated. The A<sub>2A</sub>-selective antagonist PSB-603 and the non-selective antagonist caffeine showed very similar K<sub>B</sub> values at CHO-A<sub>2A</sub>-hA<sub>2B</sub> (PSB-603, K<sub>B</sub> 0.673 nM; caffeine, K<sub>B</sub> 9,900 nM) as at CHO-A<sub>2B</sub> cells (PSB-603, K<sub>B</sub> 0.358 nM; caffeine, K<sub>B</sub> 15,600 nM) (Figure 5B, Supplementary Table 4).

We next employed Jurkat-T cells as a native cell line expressing similar amounts of A<sub>2A</sub> and A<sub>2B</sub> ARs. These cells behaved like the CHO-A<sub>2A</sub>-A<sub>2B</sub> cell line displaying moderate potency for the physiological agonist adenosine and the structurally related agonist NECA. Again, CGS-21680 was inactive at concentrations up to 10 µM, while the A<sub>2B</sub>-selective antagonist PSB-603 and the nonselective antagonist caffeine displayed K<sub>B</sub> values in the same

![Figure 4](https://example.com/figure4.png)

**Figure 4: Affinities of AR agonists and antagonists in different cells expressing A<sub>2A</sub> and A<sub>2B</sub> ARs.** (A) Competition binding experiments of agonists versus 1 nM [H]MSX-2 at A<sub>2A</sub>-expressing membranes, and versus 0.3 nM [H]PSB-603 at A<sub>2B</sub>- and at A<sub>2A</sub>-A<sub>2B</sub> AR-expressing membranes of CHO cells. The two-tailed t-test showed significant differences. ***p < 0.001, **p < 0.01, n = 2–3, see also Supplementary Table 3. (B) Competition binding experiments of AR antagonists versus 1 nM [H]MSX-2 at A<sub>2A</sub>-expressing, and versus 0.3 nM [H]PSB-603 at A<sub>2B</sub>- and at A<sub>2A</sub>-A<sub>2B</sub> AR co-expressing CHO cell membranes. The two-tailed t-test showed significant differences. ***p < 0.001, **p < 0.01, n = 3, see also Supplementary Table 3. (C) Competition binding experiments of AR agonists and antagonists versus 0.3 nM [H]PSB-603 at Jurkat-T and HeLa cell membranes, n = 3–6, see also Supplementary Table 3. (D) Competition binding experiments agonists and antagonists versus 5 nM [H]CGS-21680, and versus 1 nM [H]MSX2, respectively, at HeLa cell membranes recombantly overexpressing A<sub>2A</sub> ARs. The two-tailed t-test showed significant differences. ns: not significant, ***p < 0.001, n = 3–4, see also Supplementary Table 3.
range as those determined at CHO-A\_2B\_cells (Figure 5C, Supplementary Table 4).

**cAMP assays at HEK-A\_2A\_cells transiently transfected with increasing amounts of A\_2B\_AR**

Next, the effect of the expression of different amounts of A\_2B\_ARs on A\_2A\_AR pharmacology was studied. To this end human embryonic kidney (HEK293T) cells stably expressing the human A\_2A\_AR were transfected with human A\_2B\_AR cDNA to transiently express increasing amounts of A\_2B\_ARs. The A\_2B\_selective agonist BAY60-6583 (100 nM) showed a significant increase in cAMP accumulation with increasing amounts of transiently transfected A\_2B\_ARs confirming functionality of the A\_2B\_AR (Figure 6A). In contrast, when stimulating the cells with the A\_2A\_selective agonist CGS-21680 (100 nM), a significant decrease in cAMP accumulation with increasing amounts of the transiently transfected A\_2B\_AR was observed suggesting an inhibition of the A\_2A\_AR.

![Figure 5: cAMP accumulation in recombinant CHO cell lines and in Jurkat-T cells.](image-url)

(A) pEC\_50 values of AR agonists in cAMP accumulation assays in CHO-A\_2A, CHO-A\_2B, and CHO-A\_2A\_A\_2B cells. The one-way ANOVA with Dunnett’s post-hoc test indicated significant differences. ns: not significant, **p < 0.01, n = 2–4, see also Supplementary Table 4. The A\_2A\_AR agonist CGS-21680 showed only a negligible signal in CHO-A\_2A\_A\_2B cells at concentrations of up to 100 µM. (B) pK\_B values for AR antagonists in cAMP accumulation assays in CHO-A\_2A, CHO-A\_2B, and CHO-A\_2A\_A\_2B cells with agonist stimulation by NECA. The one-way ANOVA with Dunnett’s post-hoc test showed significant differences. ns: not significant, **p < 0.01, n = 4–6, see also Supplementary Table 4. (C) pEC\_50 values of agonists and pK\_B values of antagonists determined in cAMP accumulation assays at Jurkat-T cells (n = 3, see also Supplementary Table 4).
Figure 6: cAMP determination in cells coexpressing variable proportions of A\textsubscript{2A} and A\textsubscript{2B} ARs. (A) Stimulation of cAMP accumulation induced by 100 nM of the A\textsubscript{2B}-selective partial agonist BAY60-6583 at HEK-A\textsubscript{2A} cells transiently transfected with increasing amounts of cDNA for A\textsubscript{2B}AR (0.25–1.5 µg). The basal cAMP level, i.e. HEK-A\textsubscript{2A} cells only with medium but without the agonist, was set at 100%. The one-way ANOVA with Dunnett’s post-hoc test showed significant differences. ns: not significant, \(^{**}p < 0.01\) (\(n = 2\), in triplicates). (B) Stimulation of cAMP accumulation induced by 100 nM of the A\textsubscript{2A}-selective agonist CGS-21680 at HEK-A\textsubscript{2A} cells transiently transfected with increasing amounts of cDNA for A\textsubscript{2B}AR (0.25–1.5 µg). Basal cAMP, i.e. HEK-A\textsubscript{2A} cells only with medium but without the agonist, was set at 100%. The one-way ANOVA with Dunnett’s post-hoc test showed significant differences. ns: not significant, \(*p < 0.05\), \(^{**}p < 0.01\) (\(n = 2\), in triplicates). (C) Stimulation of cAMP accumulation induced by a combination of 100 nM of the A\textsubscript{2A}-selective agonist CGS-21680 and of 100 nM of the A\textsubscript{2B}-selective partial agonist BAY60-6583 at HEK-A\textsubscript{2A} cells transiently transfected with increasing amounts of cDNA for A\textsubscript{2B}AR (0.25–1.5 µg). Basal cAMP, i.e. HEK-A\textsubscript{2A} cells only with medium but without the agonist, was set at 100%. The one-way ANOVA with Dunnett’s post-hoc test showed significant differences. ns: not significant, \(^{**}p < 0.01\) (\(n = 2\), in triplicates).
through the A$_{2B}$AR (Figure 6B). Stimulation of the cells with a combination of both receptor subtype-selective agonists (100 nM each), showed a significant increase in cAMP accumulation with increasing amounts of transiently transfected A$_{2A}$AR additionally suggesting a dominant role for the A$_{2B}$AR (Figure 6C).

**Dynamic mass redistribution assays**

Finally, dynamic mass redistribution (DMR) assays providing a holistic readout were performed in HEK293T cells transiently transfected with different ratios of A$_{2A}$ and A$_{2B}$AR cDNA. The A$_{2A}$-selective agonist BAY60-6583 (100 nM) displayed a time-dependent increase in the signal with increasing amounts of transiently transfected A$_{2B}$AR confirming the presence of functional A$_{2B}$ARs (Figure 7A). In contrast, when stimulating the cells with the A$_{2A}$-selective agonist, CGS21680 (100 nM), a time-dependent decrease in signal was observed with increasing amounts of transiently transfected A$_{2B}$AR suggesting an inhibition of the A$_{2A}$AR-mediated DMR signal through the A$_{2B}$ARs (Figure 7B). Stimulation of the cells with a combination of both, A$_{2A}$ and A$_{2B}$AR agonist (100 nM each), showed a significant increase in signal with increased amounts of transiently transfected A$_{2B}$AR, but not with increased amounts of A$_{2A}$AR, additionally suggesting within the heteromer a dominant role for the A$_{2B}$AR (Figure 7C).

**DISCUSSION**

Adenosine is an important signaling molecule, and the A$_{2A}$ and A$_{2B}$AR subtypes are established (A$_{2A}$) or potential (A$_{2B}$) drug targets. Both receptors are G protein-coupled, but the A$_{2B}$AR requires much higher adenosine concentrations to be activated than the A$_{2A}$AR, and its physiological role remains enigmatic. In the present study we demonstrate that A$_{2A}$ARs physically interact with A$_{2A}$ARs forming A$_{2A}$-A$_{2A}$ heteromers. This interaction leads to new signalling properties as observed in cAMP experiments at Jurkat-T and further native and recombinant cells. Although A$_{1}$, A$_{2A}$ and A$_{2B}$ homomeric ARs and several heteromers of A$_{1}$ and A$_{2A}$ARs had previously been described [1, 40–41], neither homo- nor heteromers of A$_{2B}$ARs have been unambiguously demonstrated so far. Our study was motivated (i) by the fact that A$_{2A}$ and A$_{2B}$ARs are frequently co-expressed; (ii) by the observation that A$_{2B}$AR pharmacology appears to be strikingly different than expected in a number of A$_{2A}$-A$_{2B}$ co-expressing cells and tissues, (iii) by the still unknown physiological role of the “low-affinity” A$_{2B}$ARs; (iv) by contradictory results on pro- or anti-inflammatory effects mediated by the A$_{2A}$AR, and (v) by the observation that A$_{2A}$ and A$_{2B}$ARs upon activation – although they are both G protein-coupled – often show opposite effects [25]. A$_{2A}$-A$_{2B}$ heteromerization had previously been postulated to be required for high A$_{2B}$AR expression, and the C-terminus of the A$_{2A}$AR has been suggested to play an important role in this respect [42]. However, we could not confirm the published results. In fact, high A$_{2B}$AR expression is observed in many cancer cells including those which express lower amounts of A$_{2A}$ARs [12–13, 43–45]. Nevertheless, co-immunoprecipitation experiments on A$_{2A}$-A$_{2B}$ co-expressing HEK293 cells had provided the first indication of a possible existence of A$_{2A}$-A$_{2B}$ heteromers [42]. In the present study, by applying several complementary techniques, we provide compelling evidence that A$_{2A}$ and A$_{2B}$ form heteromeric di-/oligomers in recombinant as well as in native cells and tissues. FRET experiments confirmed specific interaction between both receptor subtypes (Figure 1A). So far the structure of the heteromers and their interaction surface is still unknown. In the well-characterized A$_{2A}$-A$_{2B}$ heteromer the long C-terminus of the A$_{2A}$AR is involved in heteromerization [46]. To gain insight into the A$_{2A}$-A$_{2B}$ heteromer interface, we removed the C-terminus of the A$_{2A}$AR and examined the resulting truncated receptor mutant co-expressed with the A$_{2B}$AR in FRET experiments. The observed FRET signal was comparable to the one obtained for the wt A$_{2A}$-A$_{2B}$AR heteromer (Figure 1A, 1B) indicating that the C-terminus is not involved in the formation of A$_{2A}$-A$_{2B}$ heteromers. Extensive BRET studies, including displacement experiments with untagged receptors, showed high, specific BRET signals for the A$_{2A}$-A$_{2B}$AR pair (Figure 2A, 2C). Incubation of cells with various agonists, adenosine (nonselective), NECA (nonselective), CGS-21680 (A$_{2A}$-selective), BAY60-6583 (A$_{2B}$-selective), or antagonists (PSB-603, A$_{2B}$-selective) did not lead to a significant change of the signal (Figure 2D). This means that A$_{2A}$-A$_{2B}$AR heteromer formation is independent of the presence of A$_{2A}$ or A$_{2B}$AR ligands and stable in the presence of ligands. Furthermore, BiFC experiments in which part of the EYFP sequence is attached to the A$_{2A}$, the other part to the A$_{2B}$AR, resulted in positive signals. These data convincingly underscore that both receptors are directly interacting, and that an A$_{2A}$-A$_{2B}$ hetero- or -oligomer must have been formed (Supplementary Figures 1, 2). A$_{2A}$-A$_{2B}$ heteromer formation in CHO-A$_{2A}$-A$_{2B}$ cells was confirmed by yet another technique, proximity ligation assays (PLA), using specific modified primary antibodies. The positive PLA signals obtained in living CHO-A$_{2A}$-A$_{2B}$ cells, but not in non-transfected CHO cells, indicated close proximity of both receptors in the CHO cell line that coexpress them (Supplementary Figure 3). Thus, all conducted biophysical and biochemical techniques, namely FRET, BRET, BiFC and PLA studies, provided evidence that A$_{2A}$ and A$_{2B}$ARs can form heteromers in living cells. Importantly, the existence of A$_{2A}$-A$_{2B}$ heteromers in native tissues was confirmed by using the PLA approach (Figure 3, Supplementary Figures 4, 5) thus proving their physiological relevance. Remarkably, heteromers were detected in sections from hippocampus of the rat. Receptor
pharmacology was altered in cells expressing the two receptors and, therefore, also expressing A<sub>2A</sub>-A<sub>2B</sub> heteromers, as compared to that in cells expressing only one receptor subtype. This was initially observed in a recombinant system investigating CHO-A<sub>2A</sub>, CHO-A<sub>2B</sub> and CHO-A<sub>2A</sub>-A<sub>2B</sub> cells, the latter of which had higher A<sub>2B</sub> than A<sub>2A</sub> AR expression as assessed by RT-PCR and Western blot analysis. In cell lines only expressing A<sub>2A</sub> or A<sub>2B</sub>ARs, radioligand binding studies provided the expected affinities for standard ligands, i.e. high affinity binding of the selective A<sub>2A</sub>AR ligands CGS-21680 (agonist) and MSX-2 (antagonist), but lacking affinity for the A<sub>2B</sub>-selective ligands BAY60-6583 (partial agonist) and MSX-2 (antagonist). In cell lines coexpressing variable proportions of A<sub>2A</sub> and A<sub>2B</sub>ARs, radioligand binding studies provided the expected affinities for standard ligands, i.e. high affinity binding of the selective A<sub>2A</sub>AR ligands CGS-21680 (agonist) and MSX-2 (antagonist), but lacking affinity for the A<sub>2B</sub>-selective ligands BAY60-6583 (partial agonist) and MSX-2 (antagonist). In cell lines coexpressing variable proportions of A<sub>2A</sub> and A<sub>2B</sub>ARs, radioligand binding studies provided the expected affinities for standard ligands, i.e. high affinity binding of the selective A<sub>2A</sub>AR ligands CGS-21680 (agonist) and MSX-2 (antagonist), but lacking affinity for the A<sub>2B</sub>-selective ligands BAY60-6583 (partial agonist) and MSX-2 (antagonist).

**Figure 7: Label-free assays in cells coexpressing variable proportions of A<sub>2A</sub> and A<sub>2B</sub>ARs.** (A) Time-dependent dynamic mass redistribution (DMR) measurement induced by 100 nM of the A<sub>2B</sub>-selective partial agonist BAY60-6583 in HEK293T cells transiently transfected with a constant amount of cDNA for A<sub>2A</sub>AR and increasing amounts of cDNA for A<sub>2B</sub>AR, or vice versa, a constant amount of cDNA for A<sub>2B</sub>AR and increasing amounts of cDNA for A<sub>2A</sub>AR. (B) DMR measurement induced by 100 nM of the A<sub>2A</sub>-selective agonist CGS-21680 in HEK293T cells transiently transfected with a constant amount of cDNA for A<sub>2A</sub>AR and increasing amounts of cDNA for A<sub>2B</sub>AR, or vice versa, a constant amount of cDNA for A<sub>2B</sub>AR and increasing amounts of cDNA for A<sub>2A</sub>AR. (C) DMR measurement induced by a combination of 100 nM of the A<sub>2A</sub>-selective agonist CGS-21680 and of 100 nM of the A<sub>2B</sub>-selective partial agonist BAY60-6583 at HEK293T cells transiently transfected with a constant amount of cDNA for A<sub>2A</sub>AR and increasing amounts of cDNA for A<sub>2B</sub>AR, or vice versa, a constant amount of cDNA for A<sub>2B</sub>AR and increasing amounts of cDNA for A<sub>2A</sub>AR.
ligands BAY60-6583 (partial agonist) and PSB-603 (antagonist) at the CHO-A2A cells and vice versa for the CHO-A2B cells (Supplementary Tables 2, 3). At the CHO-
A2A-A2B cell line, however, no binding for the A2A-selective radioligands [3H]MSX2 and [3H]CGS-21680 was detected although we could clearly prove both the presence of A2A-AR mRNA and A2B-AR protein in this cell line (Supplementary Figures 8A, 8B, 6A, 6B, Supplementary Table 2) and its cell surface localization (Supplementary Figure 3C). The A2A-A2B heteromer behaved almost like an A2B-AR by displaying low affinity for adenosine and NECA (Figure 4A, 4B), high affinity for PSB-603 and no significant sign of A2B-AR activation. cAMP accumulation assays yielded results in line with the pharmacology obtained in binding studies (Figure 5A–5C, Supplementary Table 4). Moreover in cAMP assays as well as in DMR assays at HEK-A2A cells transiently transfected with increasing amounts of the A2B-AR (Figure 6A–6C, Figure 7A–7C) a clear inhibition of A2A-AR function through A2B-ARs could be demonstrated. This means that the A2B-AR signaling appears to be completely blocked in the A2A-A2B heteromer. On a molecular level, this would mean that the A2B-AR modulates the conformation of the binding site of the A2A-AR in such a way that it either loses its affinity for ligands completely, and only the A2B binding site is available for interaction with ligands, or the A2B binding site in the heteromer switches to A2B-like properties. Both would in principle be possible. In fact, the binding sites for adenosine in A2A and A2B-ARs are almost identical differing only in a single amino acid (A2A: L249, A2B: V250), and yet their affinity for adenosine is very different. It is therefore likely that the ligand binding site of A2A and A2B-ARs is readily amenable to allosteric modulation. The discovery of A2A-AR action blockade by the A2B-AR was very consistent when assayed in heterologous expression systems, in cell lines or in primary cells. Other groups had previously confirmed A2A- and A2B-AR mRNA expression in Jurkat-T cells (A2B ≥ A2A) and discovered that the selective A2A-AR agonist CGS-21680 was nearly inactive in cAMP assays [47], and also the binding of [3H]CGS-21680 (12.5 nM) to Jurkat-T cell membranes was found to be negligible [48]. These results can now be explained by taking into account our discovery that the A2A-AR is non-functional within the A2A-A2B heteromer context present in Jurkat-T cells. Our findings also provide a mechanistic basis to interpret previous results, namely the altered AR pharmacology in cells expressing both A2A and A2B-ARs with similar or higher levels of A2B (A2B ≥ A2A). In Supplementary Table 5 we have collected the appropriate literature data that may now be interpreted in the light of our main finding. For example, in the human bladder carcinoma cell line T24 the expression of A2A- and A2B receptors was demonstrated by RT-PCR (A2B > A2A). In cAMP assays NECA and adenosine were able to induce cAMP accumulation whereas the A2A-selective agonist CGS-21680 was inactive [45]. Wei et al. showed expression of all four AR subtypes in three prostate cancer cell lines by qRT-PCR and Western blot experiments, with domination of the A2B-AR (A2B > A2A). NECA and the selective A2B-AR agonist BAY60-6583, but not the A2A-selective agonist CGS-21680, concentration-dependently induced cAMP accumulation [13]. Recently, Hajiahmadi et al. demonstrated expression of all four AR subtypes in three human ovarian cancer cell lines by qRT-PCR and Western blot, in which, again, the A2B-AR was the most abundant one (A2B > A2A), and NECA but not the A2A-AR agonist CGS-21680, concentration-dependently induced cAMP accumulation [12]. In contrast, in A2A-A2B-co-expressing cell lines and tissues in which the expression level of A2B-ARs is higher than that of A2A-ARs (A2B > A2A), e.g. in T-lymphocytes from human blood, PC12 cells, or HMC-1 cells, canonical A2A and A2B pharmacology is observed (Supplementary Tables 2, 5) [8, 16, 49–50]. It is likely that A2A-A2B AR heteromers exist in these cells but in a proportion that does not prohibit activation of free A2B-ARs (that cannot be blocked by A2B-ARs). Thus, regulation of A2A- and A2B expression levels can in turn govern the pharmacological outcome of stimulation with adenosine or synthetic AR agonists. Both G-coupled A2A subtypes play important (patho)physiologic roles and are co-expressed on many cell types and tissues [6, 8, 11, 51]. Up-regulation of A2A-AR expression in different tissues can be found under various pathological conditions. For example, lymphocytes from amyotrophic lateral sclerosis (ALS) or multiple sclerosis (MS) patients show increased A2A-AR levels [16–17]. NF-kappaB was found to enhance hypoxia-driven T-cell immunosuppression via activation of upregulated A2A-ARs [52]. In a study on isolated perfused mouse heart investigating the contributions of A2A- and A2B-ARs on cardiac flow, the A2A-selective agonist CGS-21680 and the non-selective agonist NECA increased coronary flow in A2B knockout (KO) mice to a significantly higher degree than in wildtype (WT) mice [51]. The A2A-selective antagonist SCH58261 blocked NECA-induced increase in coronary flow to a higher degree in KO than in WT mice. The authors explained these discrepancies by an observed upregulation of A2A-ARs (ca. 20% as estimated by Western blot) in mesenteric arterioles of KO as compared to WT mice. However, an additional or alternative explanation could be that by deletion of A2B-ARs, heteromer formation would no longer be possible leading to a de-blocking of the A2A-ARs and thus a higher number of free receptors with typical A2A pharmacology. Several studies have led to the proposal that GPCR heteromers may constitute drug targets in their own right and that heteromers can be upregulated in disease [53]. It appears that A2A- and A2B-ARs display very high affinity for each other, and whenever they are co-expressed they would form stable heteromers with a distinct pharmacology. This implies that the A2A-A2B interface and the interactions must be unique to produce such a remarkable and persistent disappearance of A2A-AR ligand recognition and signaling through allosteric receptor-receptor modulation.
MATERIALS AND METHODS

Molecular biology

For FRET experiments the cDNAs of the human A₂₅AR and the C-terminal truncated mutant A₁₋₁ ᵃ₋ᵧ₋₁-293R were amplified by PCR using primers that delete the stop codon of the receptors and introduce EcoRI and AgeI restriction enzyme sites. The resulting PCR products were cloned in-frame with EcoRI/AgeI into the vectors pEYFP-N1 and pGFP²-N3, respectively. The cDNA of the human A₂₅AR was also cloned with EcoRI/AgeI into the vector pGFP²-N3. For a positive control a fusion protein GFP-EYFP was used. The cDNA of GFP² was amplified by PCR using primers that delete the stop codon of the protein and introduce BamHI and AgeI restriction enzyme sites. The resulting PCR product was cloned in-frame with BamHI/AgeI into pEYFP-N1 plasmid, respectively. The cDNA of the GABAᵩ₂R₂-EYFP receptor construct was a gift from Prof. Franco. For BRET experiments the cDNAs of the human A₂₅ and A₁₋₁ AR were amplified by PCR using primers that delete the stop codon of the receptors and introduce EcoRI and BamHI restriction enzyme sites. The resulting A₂₅ and A₁₋₁ AR PCR products were cloned in-frame with EcoRI/BamHI into pRluc-N2. The dopamine D₂ᵩ-pRluc-N2 receptor construct was a gift from Prof. Franco. The A₂₅AR cDNA was cloned in-frame with EcoRI/AgeI into pEYFP-N1, respectively. For BRET experiments Rluc-EYFP was used as a positive control, and Rluc alone and EYFP plasmids alone were employed as negative controls. For the positive control the cDNA of Rluc was amplified by PCR using primers that delete the stop codon of the protein and introduce EcoRI and BamHI restriction sites. The resulting PCR product was cloned in-frame with EcoRI/BamHI into pEYFP-N1 plasmid, respectively. For BRET displacement experiments, the cDNA of the human A₂₅AR was cloned with EcoRI/NotI into the pcDNA3.1(+) plasmid.

Transiently transfected HEK293T cells were prepared by subcloning the cDNAs of the human A₂₅ and A₁₋₁ AR with HindIII/BamHI into the vector pcDNA3.1(+). All restriction enzymes and supplements for the molecular biology were obtained at NEB (Frankfurt, Germany). DNA and RNA purification kits were obtained at Zymo Research (Freiburg, Germany) or Life Technologies (Darmstadt, Germany).

Transient transfection of CHO-K1 cells for FRET and BRET experiments

For FRET and BRET experiments CHO-K1 cells were transiently co-transfected with constant amounts of the receptor-donor DNA (e.g. A₂₅-GFP² or A₁₋₁-Rluc) and constant or increasing amounts of receptor-acceptor DNA (e.g. A₂₅-EYFP) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, USA). Moreover the receptor-donor DNAs and receptor-acceptor DNAs were transfected alone to determine their contribution to the detection channels (spectral signature). The cells were harvested 24 h after transfection and used for FRET, and BRET experiments, respectively.

FRET experiments analyzed by fluorimetry

FRET experiments were conducted as previously described [36]. 20 µg of the transfected cells (100 µl) were distributed in duplicates in a black 96-well plate with black bottom for fluorescence measurements. The fluorescence signals were detected by a Mithras LB 940 fluorimeter using a 10-nm bandwidth excitation filter at 405 nm and 500 nm and 10-nm bandwidth and 25-nm bandwidth emission filters corresponding to 510 nm and 535 nm (GFP² excitation 405 nm, emission 510 nm; EYFP excitation 500 nm, emission 535 nm). Background fluorescence signals from non-transfected CHO-K1 cells were subtracted. For all experiments the gain settings and the read time of 0.1 s were kept identical.

Quantification of FRET signal

Quantification of the FRET signals was performed as described by Elder et al. according to a sensitized emission method [54]. This method requires correction for donor bleed-through (donor fluorophore emission into the acceptor channel) and acceptor cross-excitation (direct excitation of acceptor fluorophores by donor excitation). For that, the contributions of GFP² and EYFP proteins alone to the two detection channels were measured in experiments with cells expressing only one of these proteins. The spectral signatures of the different receptors fused to either GFP² or EYFP did not significantly vary from the determined spectral signatures of the fluorescent proteins alone. The donor bleed-through correction factor (DER, measured in cells expressing only donor fluorophores) and acceptor cross-excitation factor (AER, measured in cells expressing only acceptor fluorophores) were calculated using the following formula [54]:

\[
\text{AER} = \frac{I^{DA}}{I^{AA}} \quad \text{DER} = \frac{I^{DA}}{I^{DD}}
\]

The AER is the ratio of emission into the acceptor channel when using donor excitation relative to when using acceptor excitation. The DER is the ratio of emission into the acceptor channel relative to emission into the donor channel, when using donor excitation [54]. The corrected FRET signal was then calculated according to the following formula [54]:

\[
\text{cFRET} = I^{DA} - \text{DER} * I^{DD} - \text{AER} * I^{AA}
\]

Different FRET normalisation equations can be used to determine the efficiency of the FRET signal.
Here we used the $N_{FRET}$ normalizing method according to Shyu et al., which takes changes in donor and acceptor concentrations into account [55]:

$$N_{FRET} = \frac{cFRET}{(I_{DON} \cdot I_{Acc})}$$

FRET signal analyses were done in Excel and results were displayed using GraphPad Prism 4.

**Bioluminescence resonance energy transfer (BRET) experiments analyzed by fluorimetry**

BRET experiments were conducted as previously described [36]. 20 µg of the transfected cells (100 µl) were distributed in triplicates into a black 96-well plate with black bottom for fluorescence and a white 96-well plate with white bottom for bioluminescence measurements. Signals were detected by a Mithras LB 940 fluorimeter calculating the integration for bioluminescence using filters for 440–500 nm (485 nm maximum emission of bioluminescence), and for fluorescence with a filter for 510–590 nm (530 nm maximum emission of EYFP). To confirm equal expression of Rluc and increasing expression of EYFP, for each sample bioluminescence and fluorescence was measured before starting the experiment. EYFP fluorescence was defined as the fluorescence of the sample minus the fluorescence of cells expressing only Rluc-tagged receptors. For BRET, 5 µM coelenterazine-H (PJK, Kleinblittersdorf, Germany) was added to the samples and measurements were performed after 1 min (net BRET determination) and after 10 min (Rluc luminescence quantification). Net BRET was defined as the bioluminescence of the sample minus the bioluminescence of cells expressing only Rluc-tagged receptors. BRET signals were determined by calculating the ratio of the light emitted by EYFP over the light emitted by the Rluc. milliBRET unit (mBU) is the ratio of the light emitted by the EYFP over the light emitted by the Rluc.

Signals were distributed into duplicates into a black 96-well plate with white bottom for bioluminescence measurements. Displacement studies were performed in triplicates at a constant BRET ratio, around the BRET plateau. Displacement studies were performed in triplicates at a constant BRET ratio, around the BRET plateau.

**In situ proximity ligation assay in the rat brain**

The experiments were carried out in accordance with the European Directive 2010/63/EU and were approved by the Bioethical Committee at Karolinska Institutet. Male Sprague-Dawley (derived from the licensed animal breeder Charles River, Sulzfeld, Germany), weighing between 260–310 g at the beginning of the experiment, were used. The animals ($n = 5$) were housed individually in standard plastic rodent cages (25 cm × 30 cm × 30 cm) in a colony room maintained at 21 ± 1°C and 40–50% humidity under a 12-hour light-dark cycle (lights on at 6:00 am). Rodent food and water were available ad libitum. All animals used for the *in situ* PLA neurochemical study were experimentally naive.

To study the distribution of the adenosine $A_2A$–$A_2B$ heteroreceptor complex in the rat brain the *in situ* proximity ligation assay (*in situ* PLA) was performed as described previously [56].

**Retroviral transfection**

CHO cells stably expressing HA-tagged human $A_{2A}$ or $A_{2B}$ARs, or both human $A_{2A}$ and $A_{2B}$ARs were generated using a retroviral transfection system [5]. HeLa cells stably overexpressing HA-tagged human $A_{2A}$ARs were also generated using a retroviral transfection system [5]. For generating the $A_{2A}-A_{2B}$ co-expressing cell line, GP+ env AM12 cells were co-transfected with 6.75 µg of the pQCXIP-A$_{2A}$ vector construct and 3.25 µg of VSV-G using Lipofectamine 2000. The supernatant medium containing the modified virus was filtered and transferred into a small flask of 70% confluent CHO-$A_{2B}$ cells. After infection the selection of the CHO-$A_{2A}$-$A_{2B}$ cells was started after 48 h by the addition of 10 µg/ml of puromycin.

**Cell culture**

Chinese hamster ovary cells (CHO-K1), GP+ env AM12 cells and CHO cells stably expressing human $A_{2A}$ARs, HA-tagged human $A_{2A}$AR or human $A_{2B}$AR were cultured as described previously [5, 57]. CHO cells stably co-transfected with human $A_{2A}$AR and $A_{2B}$AR were maintained in Dulbecco’s Modified Eagle Medium (DMEM-F12) medium supplemented with 10% (v/v) fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin, 800 µg/ml G418 and 10 µg/ml puromycin at 37°C and 5% CO$_2$. Human Jurkat-T cells natively expressing $A_{2A}$ and $A_{2B}$ARs were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. HeLa cells natively expressing human $A_{2A}$AR and $A_{2B}$AR were cultured in DMEM supplemented with 10% (v/v) FCS, 100 units/ml penicillin, 100 µg/ml streptomycin. HeLa cells stably overexpressing the human HA-tagged $A_{2A}$AR were cultured in the same medium with the addition of 800 µg/ml of G418. HEK293T cells were grown in DMEM supplemented with 2 mM L-glutamine, 5% (v/v) FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and
minimal essential medium non-essential amino acid solution (1:100) at 37°C in an atmosphere of 5% CO₂.
All cell culture media and supplements were obtained from (Darmstadt, Germany), Sigma-Aldrich (Munich, Germany) or Applichem (Darmstadt, Germany).

Membrane preparation

The preparations of CHO and HeLa membranes recombinitely (or natively) expressing human A₂A and/or A₂B AR subtypes were performed as previously described [5]. Membranes from HEK-A₂B or A₂A cells, which were used for some of the radioligand competition binding studies, were purchased from Perkin Elmer (Waltham, USA). The preparation of Jurkat-T cell membranes was performed as follows: a Jurkat-T cell suspension was centrifuged in 50 ml falcon tubes at 200 g, 4°C, 5 min. The supernatant was discarded and the cell pellets were quickly frozen at −80°C. The defrosted pellets were then resuspended in ice-cold 25 mM Tris-buffer, 0.32 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethanesulfonylfluoride (PMSF), pH 7.4, and homogenized with an Ultra-Turrax (30 s on ice). The homogenate was centrifuged for 10 min at 1000 g at 4°C. The resulting pellets were discarded and the supernatant was centrifuged for 1 h at 48.000 g at 4°C. The resulting supernatant was discarded and the pellets were resuspended in 50 mM Tris-buffer, pH 7.4 and homogenized with a glass Teflon homogenizer on ice. The resuspended and homogenized pellets were aliquoted and stored at −80°C until use. The protein content of all membrane preparations was determined with the Lowry method [58].

Radioligand binding assays

Radioligand receptor binding experiments at membrane preparation of native (HeLa) and recombinant cells (CHO- or HEK cells recombinitely expressing A₂A and/or A₂B ARs) using the A₂B-selective antagonist radioligand [³H]PSB-603 (spec. activity: 79 Ci/mmol) to detect A₂B ARs were performed as previously described [59]. Competition binding experiments at various concentrations of [³H]PSB-603 and the non-selective antagonist caffeine were determined versus the full agonist NECA. Kᵢ-values for the A₂A-selective antagonist MSX-2, the A₂B-selective antagonist PSB-603 and the non-selective antagonist caffeine were determined versus the full agonist NECA. Kᵢ-values were calculated using the child equation [61].

Radioligand binding assays at Jurkat-T cell membranes

Competition binding experiments at Jurkat-T cell membrane preparations using the A₂B-selective antagonist radioligand [³H]PSB-603 to detect A₂B ARs were performed in a final volume of 1000 µl containing 25 µl of test compound dissolved in DMSO, 196 µl buffer (50 mM Tris-HCl, 10 mM MgCl₂, pH 7.4), 100 µl of radioligand solution in the same buffer (5 nM), and 100 µl of membrane preparation (10 to 200 µg of protein per vial, 2 U/ml ADA 20 min incubation at rt). Non-specific binding was determined in the presence of unlabeled MSX-2, or CGS-15942, respectively (10 µM), both giving identical results.

Competition binding experiments at membrane preparations of native cells (HeLa, Jurkat-T cells) or recombinant cells (CHO, HEK or HeLa recombinantly expressing the human A₂A,AR) using the agonist radioligand [³H]CGS-21680 (spec. activity: 39 Ci/mmol) were performed in analogy to described procedures [5]. The assay was performed in a final volume of 400 µl containing 4 µl of test compound dissolved in DMSO, 196 µl buffer (50 mM Tris-HCl, pH 7.4), 100 µl of radioligand solution in the same buffer (5 nM), and 100 µl of membrane preparation (10 to 200 µg of protein per vial, 2 U/ml ADA 20 min incubation at rt). Non-specific binding was determined in the presence of unlabeled MSX-2, or CGS-15942 (final concentration 10 µM); all gave identical results. All data were analyzed with GraphPad Prism, Version 4 (GraphPad Inc., La Jolla, CA).

CAMP assays

The determination of cAMP accumulation in recombinant CHO and in Jurkat-T cells was performed as previously described [5, 60]. Kᵢ-values for the A₂A-specific antagonist MSX-2, the A₂B-specific antagonist PSB-603 and the non-selective antagonist caffeine were determined versus the full agonist NECA. Kᵢ-values were calculated using the child equation [61].

Transient transfection of HEK293T cells for CAMP assays

HEK293T cells were transiently transfected with the corresponding cDNA (pcDNA3.1+-hA₂B, pcDNA3.1+-
hA<sub>2A</sub>) by the polyethylenimine (Sigma) method [62]. The determination of AR-induced cAMP accumulation in HEK293T cells transiently expressing A<sub>2A</sub> and A<sub>2B</sub> ARs was performed as previously described [62].

**Dynamic mass redistribution assays**

Dynamic mass redistribution (DMR) at HEK293T cells transfected with A<sub>2A</sub> and A<sub>2B</sub> ARs was determined as described previously [62]. In brief, 24 h before the assay, cells were seeded at a density of 7,500 cells per well in 384-well sensor microplates with 40 µl of growth medium and cultured for 24 h (37°C, 5% CO<sub>2</sub>) to obtain 70–80% confluent monolayers. Prior to the assay, cells were washed twice with assay buffer (Hank’s balanced salt solution; HBSS with 20 mM HEPES, pH 7.15) and incubated for 2 h in 40 µl per well of assay buffer in the DMR reader at 24°C. Hereafter, the sensor plate was scanned and a baseline optical signature was recorded before adding 10 µl of test compound dissolved in assay buffer containing 0.1% DMSO and DMR responses were monitored for at least 8,000 s using an EnSpire<sup>®</sup> Multimode Plate Reader (PerkinElmer, Waltham, MA, USA) by a label-free technology. Data were analyzed using EnSpire Workstation Software v 4.10.

**Abbreviations**

ADA: adenosine deaminase; AR: adenosine receptor(s); BAY60-6583: 2-[(6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-yl)sulfanylacetamide; BiFC: bimolecular fluorescence complementation; BRET: bioluminescence resonance energy transfer; BSA: bovine serum albumin; cDNA: complementary deoxyribonucleic acid; CGS-21680: (2-p-[2-carboxyethyl]phenethylamino)-5’-N-ethylcarboxamidoadenosine; CHO: Chinese hamster ovary; DAPI: 4,6-diamidino-2-phenylindole; DEPC: diethylpyrocarbonate; DMEM: Dulbecco’s Modified Eagle Medium; DMSO: dimethylsulfoxide; DPCPX: 8-cyclopentyl-1,3-dipropylxanthine; DMR: dynamic mass redistribution; EDTA: ethylenediamine tetraacetic acid; EGTA: ethyleneglycoltetraacetic acid; EYFP: enhanced yellow fluorescent protein; FCS: fetal calf serum; FRET: Förster resonance energy transfer; GFP<sup>P</sup>: green fluorescent protein variant 2; GPCR: G protein-coupled receptor; G418: geneticin; HA: human influenza hemagglutinin tag; h: human; HBSS: Hank’s balanced salt solution; KO: knock-out; KRH: Krebs-Ringer-Hepes; mRNA: messenger ribonucleic acid; MSX-2: 3-(3-hydroxypropyl)-7-methyl-8-(n-methoxystyryl)-1-propargylxanthine; NECA: 5’-N-ethylcarboxamidoadenosine; PBS: phosphate buffered saline; PEI: polyethylenimine; PMSF: phenylmethanesulfonyl fluoride; PVDF: polyvinylidene fluoride; PSB-603: 8-(4-(4-(4-chlorophenyl)piperazine-1-sulfonfyl)phenyl)-1-propylxanthine; Rluc: Renilla luciferase; RNA: ribonucleic acid; Ro 20-1724: 4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidione; RPMI: Roswell Park Memorial Institute; rt: room temperature; RT-PCR: reverse transcriptase-polymerase chain reaction; TBS: Tris buffered saline; Tris: Tris(hydroxymethyl)aminomethan.

**Author contributions**

SH designed, performed and analyzed FRET and BiFC experiments, cloned some of the receptor constructs for BRET experiments, prepared the stably transfected CHO-A<sub>2A</sub>-CHO-A<sub>2A</sub>-A<sub>2B</sub> cell lines and characterized them and performed the majority of radioligand binding and cAMP assays. GN designed, performed and interpreted the DMR studies and cAMP experiments at HEK-A<sub>2A</sub> cells. DBE designed, performed and analyzed PLA experiments at rat brains; YCA established and performed PLA experiments at CHO-A<sub>2A</sub>-A<sub>2B</sub> cells, performed Western blots at CHO-A<sub>2A</sub>-A<sub>2B</sub> and Jurkat-T cell membranes and analyzed results. BFS and BC designed, performed and analyzed BRET experiments. AD, EdF and MR prepared some of the stably transfected cell lines, characterized them and performed and analyzed cAMP experiments. SKL performed radioligand binding studies at human T-lymphocytes and analyzed the results. ACS designed PLA experiments at the CHO-A<sub>2A</sub>-A<sub>2B</sub> cell line and supervised some of the molecular biology studies. CEM, SH and RF wrote the manuscript; GN, DBE and KF contributed to writing the manuscript. RF designed and supervised some of the FRET, BRET and DMR studies. CEM designed and supervised the complete study.

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**CONFLICTS OF INTEREST**

The authors declare no competing or financial interest.

**REFERENCES**


