The Specificity of Downstream Signaling for A1 and A2AR Does Not Depend on the C-Terminus, Despite the Importance of This Domain in Downstream Signaling Strength

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Abstract: Recent efforts to determine the high-resolution crystal structures for the adenosine receptors (A1R and A2AR) have utilized modifications to the native receptors in order to facilitate receptor crystallization and structure determination. One common modification is a truncation of the unstructured C-terminus, which has been utilized for all the adenosine receptor crystal structures obtained to date. Ligand binding for this truncated receptor has been shown to be similar to full-length receptor for A2AR. However, the C-terminus has been identified as a location for protein-protein interactions that may be critical for the physiological function of these important drug targets. We show that variants with A2AR C-terminal truncations lacked cAMP-linked signaling compared to the full-length receptor constructs transfected into mammalian cells (HEK-293). In addition, we show that in a humanized yeast system, the absence of the full-length C-terminus affected downstream signaling using a yeast MAPK response-based fluorescence assay, though full-length receptors showed native-like G-protein coupling. To further study the G protein coupling, we used this humanized yeast platform to explore coupling to human-yeast G-protein chimeras in a cellular context. Although the C-terminus was essential for Ga protein-associated signaling, chimeras of A1R with a C-terminus of A2AR coupled to the A1R-specific Ga (i.e., Ga1 versus Gas). This surprising result suggests that the C-terminus is important in the signaling strength, but not specificity, of the Ga protein interaction. This result has further implications in drug discovery, both in enabling the experimental use of chimeras for ligand design, and in the cautious interpretation of structure-based drug design using truncated receptors.

Keywords: yeast pheromone response; G protein-coupled receptors; adenosine receptor; C-terminus; G protein; receptor chimera

1. Introduction

G-protein coupled receptors (GPCRs) are the largest family of membrane proteins, with over 800 genes in humans [1]. GPCRs are characterized by seven alpha-helical transmembrane domains and bind to extracellular molecules, activating downstream signaling responses inside the cell. GPCRs are found in eukaryotic systems from yeast to mammals and aid in essential functions; in yeast, they mediate the mating pheromone response pathway [2]. Because of their membrane localization and the ability to produce intracellular changes, they are desirable targets for therapeutics, with approximately 40% of drugs on the market targeting these receptors [3,4].
Biophysical characterization and high-resolution structure determination are routinely used for GPCR drug design and discovery and require heterologous expression and purification [5–7]. Most receptors are not expressed in heterologous systems at mg/L concentrations required for this structural characterization [8]. Therefore, additional strategies like truncations to remove unstructured regions, thermostabilization via point mutations, and chimeras with thermostable proteins have been utilized to improve expression and crystallization (for example, see modifications for adenosine receptors in Table 1). However, these modifications may change receptor activity and function.
Table 1. List of adenosine receptor high-resolution structures. Agonists are underlined.

| Receptor | Year | Resolution (Å) | Expression Host | Ligand | Modification | Reference |
|----------|------|----------------|-----------------|--------|--------------|-----------|
| A2AR     | 2008 | 2.6            | S. frugiperda    | ZM241385 | X             | Jaakola et al. [9] |
|          | 2011 | 2.7            | S. frugiperda    | UK-432997 | X             | Xu et al. [10] |
|          | 2011 | 2.6–3          | Trichoplusia ni  | Adenosine, NECA | X             | Lebon et al. [11] |
|          | 2011 | 3.3–3.6        | S. frugiperda    | Caffeine; ZM241385, XAC | X             | Dore et al. [12] |
|          | 2012 | 2.7–3.1        | P. pastoris      | ZM241385 | X             | Hino et al. [13] |
|          | 2012 | 3.27–3.34      | S. frugiperda    | Novel compounds | X             | Congreve et al. [14] |
|          | 2012 | 1.8            | S. frugiperda    | ZM241385 | X             | Liu et al. [15] |
|          | 2015 | 2.6            | Trichoplusia ni  | CC261680 | X             | Lebon et al. [16] |
|          | 2016 | 1.72–2.2       | Trichoplusia ni  | ZM241385 and 4 novel compounds | X             | Segala et al. [17] |
| A2AR     | 2016 | 3.4            | Trichoplusia ni  | NECA    | X             | Carpenter et al. [18] |
|          | 2016 | 1.9–2.5        | S. frugiperda    | ZM241385 | X             | Batyuk et al. [19] |
|          | 2017 | 3.5            | S. frugiperda    | Novel compound | X             | Sun et al. [20] |
|          | 2017 | 3.2            | S. frugiperda    | ZM241385 | X             | Martin-Garcia et al. [21] |
|          | 2017 | 2.8            | S. frugiperda    | ZM241385 | X             | Melnikov et al. [22] |
|          | 2017 | 2.2–2.8        | Trichoplusia ni  | Theophylline, caffeine; PSB36 | X             | Cheng et al. [23] |
|          | 2017 | 1.7–2.14       | Trichoplusia ni  | ZM241385 | X             | Weinert et al. [24] |
|          | 2018 | 2.35           | S. frugiperda    | ZM241385 | X             | Broecker et al. [25] |
|          | 2018 | 2.51           | P. pastoris      | ZM241385 | X             | Eddy et al. [26] |
|          | 2018 | 1.87–3.1       | Trichoplusia ni  | Theophylline, ZM241385, Vipadenant, LIAA47070, Tozadenant and 2 novel compounds | X             | Rucktooa et al. [27] |
|          | 2018 | 2.6–2.9        | P. pastoris      | UK-432997 | X             | White et al. [28] |
|          | 2018 | 4.11           | Trichoplusia ni  | NECA    | X             | Garcia-Nafria et al. [29] |
|          | 2019 | 4.2            | S. frugiperda    | ZM241385 | X             | Martin-Garcia et al. [30] |
|          | 2019 | 2.25           | S. frugiperda    | ZM241385 | X             | Shimazu et al. [31] |
|          | 2019 | 1.85           | S. frugiperda    | ZM241385 | X             | Ishchenko et al. [32] |
|          | 2020 | 1.92–2.13      | Trichoplusia ni  | Novel Ligands | X             | Jespers et al. [33] |
|          | 2020 | 2              | S. frugiperda    | ZM241385 | X             | Lee et al. [34] |
|          | 2020 | 2              | Trichoplusia ni  | AZD4635 | X             | Borodovsky et al. [35] |
|          | 2020 | 1.8–2          | S. frugiperda    | ZM241385 | X             | Ibara et al. [36] |
| A1R      | 2017 | 3.2            | S. frugiperda    | DU172   | X             | Glukhova et al. [37] |
|          | 2017 | 3.3            | Trichoplusia ni  | PSB36   | X             | Cheng, et al. [33] |
|          | 2018 | 3.6            | Trichoplusia ni  | Adenosine and DU172 | X             | Draper-Joyce et al. [38] |
Adenosine receptors are a GPCR subfamily of four receptors (A₁R, A₂AR, A₂BR and A₃R) that recognize the natural ligand adenosine, an important energy metabolite [39,40]. Adenosine is produced in tissues under stressful conditions like ischemia or hypoxia or energy “demand-supply” imbalance [41,42]. All four adenosine receptor subtypes provide critical protection under stressful conditions and, therefore, are therapeutic targets for Parkinson’s disease, Alzheimer’s disease, cardiovascular diseases, and many others [43]. Multiple crystal structures of A₂AR have been resolved with bound agonists or antagonists (Table 1). All structures reported for the adenosine receptors contain a C-terminal truncation, except a recently published cryo-EM structure of A₁R [38].

The C-terminus of A₁R is 34 amino acids long, whereas the A₂AR C-terminus is relatively long with 122 amino acids. The two crystal structures of A₁R contain a truncation from residues 311 and 316. Most crystal structures of A₂AR contain a truncation from residue 316 (A₂ARΔ316R), corresponding to only 26 amino acids out of the 120, or approximately 20% of the total A₂AR C-terminus. The long C-terminus of A₂AR has been hypothesized to be involved in receptor expression [44–46], interactions with other signaling partners [47,48], oligomerization [49] and receptor turnover [50,51]. However, previous studies have suggested that the A₂ARΔ316R has native-like signaling [52–54] and native-like ligand binding [52,55]. In addition, the absence of the canonical cysteine for palmitoylation (position 309) in A₂AR has been noted to potentially add flexibility to bind interaction partners [48]; however, the truncation does not appear to alter its desensitization or turnover, as the critical Thr298 is still present [52,56].

Receptor chimeras have been used traditionally to understand the role of the receptor domains in improving functional expression, ligand recognition, and G-protein coupling and specificity, and the ability to produce downstream signaling [46,57–60]. In our previous study [44], we created an adenosine A₁/A₂A receptor chimera to improve membrane localization and expression in yeast for A₁ receptor (A₁R) variants and reported exceptional yields of the active receptor compared to parental A₁R expressed in any host system to date. In that study, plasma membrane trafficking of A₂AR and A₂AΔ316R were similarly efficient, while trafficking of the A₁/A₂AR chimera was improved relative to wild-type A₁R, with resulting improvements in radioligand binding activity, as measured in whole cells [44].

Yeast shares many functionally exchangeable proteins involved in the GPCR signaling pathway with higher eukaryotes [2,61], has served as a useful microbial platform for rapid ligand screening and leads the development of orphan GPCRs [62]. The most researched GPCR-mediated pathway in yeast is responsive to the presence of peptide mating pheromones that regulate metabolism related to mating. Activated receptors catalyze dissociation of Gpa1, the yeast G protein, activating a mitogen-activated protein kinase (MAPK) cascade, which has been used as a unique platform to study human GPCR signaling [63]. In contrast, the presence of multiple GPCRs and Gα proteins in native mammalian systems can confound the results from downstream signaling assays. Yeast provides a relatively simple and inexpensive platform without the complexities of multiple GPCRs, receptor promiscuity, and crosstalk that occurs in native mammalian hosts [64,65].

Engineered yeast strains with modification to the native MAPK-based signaling pathway to report on ligand-mediated downstream signaling from human GPCRs (Figure 1A) were obtained both from the Broach laboratory [66] and the Dowell laboratory at GlaxoSmithKline [67]. In these yeast strains, the last five amino acids of native yeast Gα (Gpa1) were replaced with the last five amino acids residues from a human Gα to yield native-like GPCR-Gα interactions. This replacement has been shown to be sufficient for coupling with many human GPCRs, including human A₂AR, resulting in a native-like dose response and ligand binding order preference [67]. Because of structure-based drug discovery efforts that rely on truncated receptors for in silico screening, we investigated one of the key protein-protein interactions of the C-terminus, coupling to G-protein to activate downstream signaling by utilizing this engineered yeast pheromone response pathway.
Figure 1. (A) Schematic of G-protein coupled receptors (GPCR)-mediated mitogen-activated protein kinase (MAPK) signaling cascade in yeast. In this engineered pheromone response signaling pathway, cells express a yeast/human chimeric Ga protein to enable human GPCRs to couple with the yeast signaling pathway. Upon activation of downstream signaling, cells express mCherry fluorescent protein, which acts as an indirect measure of receptor activation. (B) Diagram represents homologous recombination approach used to include the mCherry gene along with the antibiotic resistance gene for clone selection within the Fus1 locus of yeast strains.

2. Experimental Section

2.1. Materials

Adenosine receptor ligands 5′-N-ethylcarboxamidoadenosine (NECA), N6-cyclopentyladenosine (CPA) and CGS21680 were purchased from Tocris (Minneapolis, MN, USA). Forskolin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Precision Plus Protein Western C Standards was purchased from BioRad (Hercules, CA, USA). Human embryonic kidney cells (HEK-293, were obtained from ATCC (Manassas, VA, USA). Dulbecco’s modified eagle medium (DME, 11995-065), Opti-MEM I reduced serum media (31985-070), fetal bovine serum (FBS, 16000-044), Lipofectamine 2000 transfection reagent (11668-019), RIPA buffer, Halt Protease and Phosphatase Inhibitor Cocktail, mammalian expression vectors (pCEP4) and Alexa 568-donkey anti-rabbit antibody (A10042) were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). The cAMP dynamic 2 kit was purchased from Cisbio US Inc. (Bedford, MA, USA). The mouse monoclonal A2AR antibody was obtained from Santa Cruz Biotechnology (sc-32261, Dallas, TX, USA). The rabbit anti-GFP antibody (ab6556) and goat pAb to Mouse IgG HRP antibody (ab97265) were obtained from Abcam (Cambridge, MA, USA).
2.2. Strains and Culture Conditions

_E. coli_ strain DH5α was used for amplifying yeast expression plasmids and mammalian expression vectors. _E. coli_ was grown in Luria-Bertani media supplemented with 100 µg/mL ampicillin at 37 °C at 250 rpm.

All yeast strains used in this study are summarized in Table 2. Yeast strains with modified pheromone response pathway and human-yeast chimeric Gα proteins (Figure 1A) were obtained from the Broach laboratory [66] and Glaxo-Smith-Kline (GSK) [67]. These parental yeast strains were grown in YPD media (2% bacto peptone, 2% glucose, 1% yeast extract) and, depending on the fus1 transformation, supplemented with 300 µg/mL hygromycin B or 200 µg/mL G418. Yeast expression plasmids were constructed using homologous recombination in _S. cerevisiae_ strain BY4741 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and were grown in synthetic media. The synthetic media (SD or SG) was comprised of 2% dextrose or galactose, respectively, 0.67% yeast nitrogen base, citrate buffer at pH 5.4 (4.2 g/L citric acid and 14.7 g/L sodium citrate) and supplemented with amino acids and essential nutrients as described by Burke and colleagues [68]. Uracil was omitted from this media (SD-ura or SG-ura) to select for plasmid-containing cells. Yeast was grown in culture tubes and multiwell plates at 30 °C at 275 rpm.

| Yeast Strain | G Protein | Last 5 Amino Acids at C-Terminal | Equivalent Human Gα |
|--------------|-----------|---------------------------------|---------------------|
| MMY12, BY4741 | Gpa1 | KIGII**COOH** | GPA1 (yeast) |
| MMY14 | Gpa1-Gaq(5) | EYNLY**COOH** | GNAQ, GNA11 |
| MMY16 | Gpa1-Ga16(5) | EINL**COOH** | GNA15, GNA16 |
| MMY19 | Gpa1-Gat(125) | DMQL**COOH** | GNA12 |
| MMY20 | Gpa1-Gat(135) | QLMQL**COOH** | GNA13 |
| MMY21 | Gpa1-Ga14(5) | ENFLQ**COOH** | GNA14 |
| MMY22 | Gpa1-Gat(5) | GCGLY**COOH** | GNAO |
| MMY23, CY13393 | Gpa1-Gai(5) | DCGLE**COOH** | GNA11, GNA12, GNA1, GNAT2, GNAT3 |
| MMY24 | Gpa1-Gai(35) | ECGLY**COOH** | GNA3 |
| MMY25 | Gpa1-Gaz(5) | YIGLC**COOH** | GNAZ |
| MMY28, CY13399 | Gpa1-Gas(5) | QYELL**COOH** | GNAS, GNAL |

Human embryonic kidney (HEK-293) cells were maintained in growth media containing DMEM with 10% FBS at 37 °C in a 5% CO2 incubator. Transient transfections were performed by seeding cells on day 0 to be approximately 70% confluent on day 1. On day 1, cells were transfected using 10 µL Lipofectamine 2000 reagent, and 1 µg DNA in 2 mL Opti-MEM reduced serum media (per 25 cm² flask). On day 2, the media was replaced by FBS supplemented DMEM media, and used for experimentation on day 3, approximately 36 h post-transfection. The cAMP accumulation assay described below (Section 2.6) was performed on cells with a passage number lower than 25.

2.3. Yeast Genomic Transformation

To develop a fluorescence-based assay to measure the downstream signaling response in yeast following ligand binding, monomeric Cherry fluorescent protein (mCherry) [69] was introduced into the _FUS1_ locus under control of the _FUS1_ promoter. To this end, overlapping fragments were first assembled in yeast using a homologous recombination as described below. The fragment consisted of the mCherry fluorescent protein and hygromycin resistance gene _hphMx6_ or kanamycin resistance gene _KanR2_ with the translation elongation factor 1 promoter and terminator (pTEF and TEFt). The fragment was flanked with approximately 300 base pairs of the Fus1 promoter and Fus1 terminator to aid in genomic recombination. The Fus1 promoter and Fus1 terminator sequences were amplified from BY4741 using colony PCR. The mCherry protein and pTEF-hphMx6-TEFt fragments were amplified from the pBS35 plasmid, while the pTEF-KanR2-TEFt fragment was amplified from the pBS7 plasmid (Figure 1B). Both pBS7 and pBS35 were obtained from the Yeast Resource Center at the University of Washington. The fragments were assembled in BY4741 using a homologous recombination using
pRS316 as a template. Fragment assembly was verified using Sanger sequencing (Operon, Louisville, KY, USA). The resulting fragment was then amplified using PCR and transformed into yeast using the protocol from Gietz and Woods [70]. Colony PCR was used to confirm successful genomic integration. Partial sequencing confirmation of final clones was obtained for some of the transformants.

### 2.4. Subcloning and Plasmid Construction

A set of yeast expression plasmids (Table 3) containing a GPCR and C-terminal protein tags, necessary for adenosine receptor detection and quantification, was constructed using a homologous recombination in BY4741 as described previously [44]. The plasmid contains a galactose (pGAL1-10) promoter, a pre-pro leader sequence (PP) [71] for targeting to the secretory pathway and the CYC1 terminator (CYC1t) [44]. For fluorescence microscopy, the GPCRs were C-terminally tagged for easier detection of protein expression with monomeric Citrine fluorescent protein (mCitrine) [44,57,58,72]. Sequencing was used to confirm the correct gene sequence for the constructs (Operon, Louisville, KY, USA).

#### Table 3. List of plasmids used for receptor expression in yeast and mammalian cells. Yeast expression plasmids contain an N-terminal leader sequence (PP) to improve receptor expression and trafficking to the plasma membrane [71].

| Name   | Plasmid                                                                 |
|--------|------------------------------------------------------------------------|
| ARJ001 | pRS316 pGal1-10 PP A1R mCitr cyc1                                      |
| ARJ002 | pRS316 pGal1-10 PP A1/A2A R mCitr cyc1                                 |
| ARJ051 | pRS316 pGal1-10 PP A1/A2AΔ316R mCitr cyc1                              |
| ARJ030 | pRS316 pGal1-10 PP A2A R mCitr cyc1                                    |
| ARJ057 | pRS316 pGal1-10 PP A2AΔ316R mCitr cyc1                                 |
| ARJ194 | pCEP4 A1R                                                              |
| ARJ195 | pCEP4 A1/A2A R                                                         |
| ARJ196 | pCEP4 A1/A2AΔ316R                                                      |
| CM001  | pCEP4 A2A R                                                            |
| CM002  | pCEP4 A2AΔ316R                                                          |
| ARJ073 | pRS316 pGal1-10 PP pFus1 mCherry pTEF-kanR2-tTEF Fus1 cyc1             |
| ARJ172 | pRS316 pGal1-10 PP pFus1 mCherry pTEF-hphMx6-tTEF Fus1 cyc1             |

Mammalian expression vector pCEP4 was used for expressing receptors in transiently transfected HEK-293 cells. Untagged A1R and A1/A2A R were inserted into the pCEP4 multiple cloning site between HindIII and NotI restriction enzyme sites, whereas A1/A2AΔ316R, A2A R, and A2AΔ316R were inserted between KpnI and XhoI restriction enzyme sites. Transformations of E. coli were performed by the heat shock method. Sequencing was used to confirm the correct gene sequences for the plasmids (Operon, Louisville, KY, USA).

### 2.5. Cyclic Adenosine Monophosphate Accumulation Assay

Transiently transfected HEK-293 and either non-transfected cells or cells transfected with empty plasmid (control) were incubated for 30 min in the presence or absence of ligand (DMSO only, no ligand control) at a cell density of 1000 cells/well in a white 384-well plate (Grenier Bio-One #784075, Monroe, NC, USA). Excess cells were pelleted and stored at −80 °C for subsequent Western blotting. The concentration of cAMP per well was determined using the cAMP dynamic 2 kit using a BioTek Synergy H1 Plate Reader according to the manufacturer’s protocol. Our previous study [73] has shown that adenosine deaminase (ADA) pre-treatment of cells did not alter the ligand binding or downstream signaling, and therefore the cells were not treated with ADA prior to ligand treatment while utilizing the CisBio HTRF kits. Experiments were performed in triplicate for three independent transfections. Data were analyzed as per manufacturer’s recommendation, and mean and standard
error were plotted using Prism (GraphPad, La Jolla, CA, USA). Student’s *t*-test was performed using Prism to obtain the significance of the data.

2.6. MAPK Response Signal Determination

Yeast cultures were grown overnight in SD-ura selection in 400 µL or 1 mL media in 48-well or 24-well plates (Falcon 353047 and 353078, Corning, NY, USA), respectively, at 30 °C at 275 rpm. Recombinant GPCR expression was induced by transferring 12.5 µL of overnight culture into 400 µL SG-ura. For some strains, 0.125% glucose was used to supplement the SG-ura media to improve cell growth of the engineered yeast strains. This level of glucose supplementation has been shown to result in minimal glucose-based suppression of the galactose promoter, as described previously [74]. Yeast cells expressing the receptor were imaged using Nikon A1 laser-scanning confocal microscope, as described in our earlier published study [44].

To determine signaling, all ligand stock solutions were prepared to the highest soluble concentration (typically around 40–100 mM) in dimethyl sulfoxide (DMSO), according to the recommendations of the manufacturer. Working concentrations of 5 mM ligand (50X) in DMSO were used for all yeast signaling experiments. After 24 h of GPCR expression, twelve µL of overnight culture was added to 380 µL fresh SG-ura media per well of a 48-well plate. Eight microliters of ligand or DMSO were added to each well (final DMSO concentration at 2% (v/v) per well). Although this concentration is well above the KD, a high ligand concentration has been shown previously to be needed for effective downstream signaling response in yeast [75–78]. After ligand addition, the 48-well plate was incubated at 30 °C at 275 rpm for 24 h. Adenosine deaminase treatment was not required for working with the yeast-based assay, as previous studies show this treatment does not impact downstream signaling measurements [79,80]. Similar results were obtained from 4 h incubations, but the fold change difference was not as pronounced. Fluorescence intensities of 100 µL of resulting liquid culture were measured in triplicate in a 96-well plate (Costar 3915, Corning, NY, USA) using a BioTek Synergy H1 microplate reader (Winooski, VT, USA) maintained at 30 °C. Experiments were performed for six independent transformants.

2.7. Western Blotting

Transiently transfected HEK-293 cells were scraped, pelleted, and resuspended in ice-cold 1X TE buffer (1% 1M Tris-Cl pH 7.5, 0.2% 500 mM EDTA pH 8) with protease inhibitors. Cells were sonicated with a Branson Sonifier 450 at 50% power for 30 pulses and then centrifuged at 2000 × g for 5 min at 4 °C to remove cell debris and unlysed cells. The supernatant was then centrifuged at 100,000 × g for 1 h at 4 °C to pellet cell membranes. Membranes were solubilized in 1X RIPA buffer (10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) with protease inhibitors; if necessary, membranes were sonicated again for five pulses at 50% power to break up any visible pieces of membrane. BCA assay (Pierce; Rockford, IL, USA) was performed to determine the total protein concentration of isolated membrane, using bovine serum albumin (BSA; Thermo Fisher, Waltham, MA, USA) as a standard.

Isolated HEK cell membranes were utilized for A2AR and A2ARΔ316R protein quantification via Western immunoassay. Western blotting analysis could not be performed for untagged A1R and its variant due to the lack of an effective antibody against the receptor. The untagged (non-fluorescent) receptor was used for the study to not interfere with the fluorescence-based CisBio HTRF kits. A total of 10 µg of total protein per sample was loaded onto a 12% Tris-Glycine gel and electrophoresed in SDS buffer at 125 V for 65 min. Western immunoassay was performed using adenosine A2AR mouse monoclonal IgG antibody (sc-32261, Santa Cruz Biotechnology, Dallas, TX, USA) at 1:5000 dilution, and Goat pAb to Mouse IgG HRP antibody at 1:5000 dilution. Membranes were imaged with the UVP BioSpectrum imaging system.

Yeast cell pellets (10 OD600) were resuspended in 250 µL lysis buffer (10% glycerol, 50 mM sodium phosphate, 300 mM sodium chloride, pH 8) supplemented with cOmplete EDTA-free protease
inhibitor cocktail (Roche, Indianapolis, IN, USA). An equal volume of 0.5 mm zirconia/silica beads (BioSpec, Bartlesville, OK, USA) was added to the cells and lysis was performed using a vortexer or a BeadBug homogenizer (Benchmark Scientific, Edison, NJ, USA). Cell lysates were combined with 4X Laemmli sample loading buffer supplemented with β-mercaptoethanol (Bio-Rad, Hercules, CA, USA). One OD₆₀₀ equivalent of cell lysate was loaded per well for Western blotting. Precision Plus Protein WesternC Standard (BioRad) was used as a standard to enable molecular weight estimation. Rabbit anti-GFP antibody (1:5000 dilution) and Alexa 568-donkey anti-rabbit (1:2500) was used to detect mCitrine protein-tagged receptors.

3. Results

3.1. Loss of the Cytoplasmic C-Terminus Results in Loss of Downstream Signaling

The A₂AR truncation at residue 316 (A₂Α₃16R) present in many crystal structures (Table 1) contains helix 8 and some residues of the cytoplasmic tail, and has been reported previously to have native-like affinity for the agonist NECA and antagonist ZM 241385 in mammalian and yeast systems [52,55,81]. In prior studies, we showed that the full-length and truncated A₂AR (A₂Α₃16R) receptors trafficked to the yeast plasma membrane comparably and showed similar ligand-binding ability [44].

Here, HEK-293 cells were transiently transfected with pCEP4 encoding full-length or truncated receptor in order to determine the effect of the truncation on downstream signaling. A₂AR couples to Gₛα, and thus agonist addition activates adenylyl cyclase, resulting in increased cAMP synthesis. As expected, cells transfected with empty plasmid showed negligible cAMP synthesis in the absence of ligand and remained unchanged following the addition of a selective A₂AR agonist, CGS21680 (1 µM) (Figure 2A). The presence of full-length A₂AR led to constitutive activation in the absence of ligand as well as a significant increase in cAMP levels following agonist treatment, consistent with previous studies [73]. Surprisingly, A₂Α₃16R showed no increase in cAMP levels upon agonist addition (Figure 2A). The loss of signaling for the truncated A₂Α₃16R was surprising, as the agonist-bound crystal structures have been reported to be in an active state [11,18], and the truncation localizes well to the cell surface (reported by our lab previously in [44]). This A₂AR truncation at residue 316 has been reported previously to have native-like binding to the agonist NECA and the antagonist ZM 241,385 by Magnani and colleagues [81] at 32 and 12 nM, respectively, compared to 20 and 1 nM for the full-length receptor [82]. Furthermore, we find that A₂Α₃16R also showed no constitutive activity, though this construct contains residues Y197 and Y288 and NPxxY in TM7 that have been reported to be important for binding G protein in active structures [83]. These data show that though A₂Α₃16R binds ligand, it does not activate Gₛα, suggesting the C-terminus is necessary for downstream signaling of the receptor.

Western blot analysis of membrane preparations was utilized to verify that the absence of A₂Α₃16R activity was not due to a lack of protein expression. Though interpretation of Western quantitation should be undertaken with caution, it is clear that there are no significant differences in the A₂AR and A₂Α₃16R expression in HEK-293 cells (Figure 2B), suggesting the >20-fold differences seen in cAMP signaling were not the result of expression differences. Cells transfected with pCEP control vector did not show any receptor expression via Western blot analysis (Figure 2B) and previous work from our laboratory has shown that the multiple bands (particularly evident in the monomeric form) are the result of protein glycosylation that occurs in the HEK cells [84].

We further examined the expression of full-length A₂AR and A₂Α₃16R with C-terminal tagged mCitrine fluorescent protein fusions in yeast. Expression was confirmed using Western blot analysis (Figure 3A). Previous studies from our laboratory have shown that the C-terminal fluorescent protein fusion does not impact trafficking or downstream activation of A₂AR [55,85–87], and we previously reported that full-length and truncated A₂AR (A₂Α₃16R) receptor trafficked to the yeast plasma
membrane comparably and showed similar ligand binding ability [44]. Confocal microscopy shows the localization of both full-length and truncated A2AR primarily at the plasma membrane (Figure 3B).

**Figure 2.** Transiently transfected HEK-293 cells were used to determine downstream signaling for chimeras. (A) Agonist-mediated cAMP accumulation for transiently transfected cells with A2AR and A2AΔ316R (DMSO only shown in red filled bars, 1 μM CGS21680 agonist in blue hatched bars). Data represent mean ± S.E.M. for three independent transfections performed in triplicate (* p < 0.001, Student’s t-test). (B) Representative Western blot analysis of A2AR and its truncation from transiently transfected in HEK-293, as obtained from equal protein loadings of total cell lysate or membrane fractions. HEK sample shows HEK-293 cells transfected with pCEP only compared to pCEP-A2AR. Dimer and full-length monomeric receptor are indicated by arrows in cell lysates, and multiple forms visible—particularly in the monomeric form at ~50 kDa—can be attributed to variable glycosylation. Samples of A2AΔ316R shows a smaller band visible at ~30 kDa that is likely a proteolytic product, indicated by an asterisk. Expected molecular weights for A2AR is 44.7 kDa and A2AΔ316R is 35.1 kDa, and molecular weights were determined using Precision Plus Protein Western C standards.

As described in the Experimental Section, engineered yeast strains with modification to the native MAPK-based signaling pathway [66,67] were further modified to replace the original Fus1 modification that relied on growth-dependent signaling (via His3 expression) in the Broach lab strain [66] or β-galactosidase reporter activity in the GSK strains [67] with an easily detectable fluorescence signal from monomeric Cherry (mCherry) fluorescent protein (Figure 1 and Table 2). Because the yeast G protein-coupled signaling pathway contains homologues to proteins in the human signaling pathway, engineered yeast have been used to successfully recapitulate native ligand-binding preferences and G protein-coupling for human GPCRs [66,67,79,80]. In these engineered yeast strains, mCherry is produced in the cells upon ligand-mediated downstream signal activation via human GPCR-Gα protein coupling (Figure 1A). The fold change in mCherry fluorescence in these strains can be easily compared by the addition of agonist relative to a control. As in HEK cells, upon agonist addition, there was no downstream signaling observed in the truncated A2AΔ316R as compared to the full-length receptor in the stimulatory yeast strain (Figure 3C). This observation is important as it allows us to use the yeast system to screen and validate receptor variant activity.
which are consistent with the reported values for A2A production of cAMP even in cells not expressing A2AR. A moderate reduction in cAMP signaling (72% in the presence of 10 μM forskolin) was observed using Western blot analysis with an anti-GFP antibody. Full-length receptor is indicated by an arrow. Molecular weights were estimated using Precision Plus Protein Western C standards. In our previous study, trafficking of the A1R/A2AR chimera was improved relative to wild-type A1R, with resulting improvements in radioligand binding activity to the A1-selective ligand [3H]-DPCPX, as measured in whole cells [44]. The KD for DPCPX determined for A1/A2AR and A1/A2AΔ316R were 48 and 7.3 nM, respectively [44], which are consistent with the reported values for A1R with DPCPX (0.18–6.1 nM) [89–92].

To test the role of the A2AR C-terminus in A1R signaling in mammalian cells, cAMP was measured in transiently transfected HEK-293 cells. A1R couples to Gαi/o, which inhibits activation of adenyl cyclase, so in the absence of ligand there should be minimal changes to cAMP levels, consistent with our results (Figure 4). Forskolin directly activates adenyl cyclase, which leads to stimulation of the production of cAMP even in cells not expressing A1R, so treatment with 10 μM forskolin was used to elevate the basal level of cAMP. Cells expressing A1R showed a reduction in cAMP following treatment with an A1R-selective agonist (1 μM CPA in the presence of 10 μM forskolin).

Next, we compared cAMP activation in HEK cells for wild-type A1R with the A1/A2AR and A1/A2AΔ316R chimeras. The addition of the A2AR C-terminus to A1R did not lead to constitutive activity of the receptor in the absence of a ligand (Figure 4, black filled bars); therefore, 10 μM forskolin was used to enable a basal cAMP signal. When treated with the A1R-specific agonist CPA (1 μM, in the presence of 10 μM forskolin), cells transfected with either A1/A2AR or A1/A2AΔ316R showed a moderate reduction in cAMP signaling (72 ± 10% and 67 ± 10%, respectively), consistent with the...
wild-type A1R (62 ± 3%), verifying that A1/A2AR and A1/A2AΔ316R chimeras couple to Gαi (Figure 4, red filled bars). Note that the data were normalized to cAMP levels for forskolin treated cells for each variant.

![Figure 4](image_url)

**Figure 4.** Expression of A1R or A1/A2AΔ316R chimeras in HEK-293 cells results in inhibition of cAMP production after forskolin stimulation. Inhibition of cAMP production with A1R and compared to pCEP (empty) vector controls and A1/A2AR and A1/A2AΔ316R chimeras (no ligand in black filled bars, 10 μM forskolin in blue hatched bars, 10 μM forskolin and 1 μM CPA in red filled bars). Data represent the mean ± S.E.M. for three independent transfections performed in triplicate (*p < 0.001, Student’s t-test).

To recreate a cellular library of human GPCR downstream signaling, twelve strains containing different yeast-human Gα chimeras that reproduce downstream signaling responses of human Gα proteins [67] were modified, as described in Section 3.1 above, to enable MAPK-activated increases in mCherry fluorescence. These strains can be classified into five Gα families: Gαi/o, Gαs, Gαq, Gα12 and native Gα. First, to ensure full-length human A1R showed mammalian coupling behavior in yeast, we mapped the interaction between the A1 adenosine receptors and the appropriate Gα in the engineered Gα chimera strains, using the non-selective high-affinity adenosine receptor family agonist, NECA (100 μM, Figure 5A). A1R showed a signaling response upon agonist addition with the inhibitory Gα family (Gαi1, Gαi3, Gαo and Gαz) and the promiscuous Gα16 (Figure 5A), as expected for this receptor based on the mammalian preferences for inhibitory Gαs. The highest fold change between cells treated with NECA as compared to DMSO-treated cells was observed for the yeast strain expressing the Gpa1-Gαo chimera. Note that ligand levels used for these studies were well above expected Kd values; however, the use of high ligand concentration is consistent with earlier studies [67,79,80,93], and perhaps reflects the reduced ability of hydrophobic ligand to penetrate the chitosan-rich yeast cell wall and then reach the plasma membrane, resulting in an apparent reduced effective ligand concentration at the membrane.

To ensure that our results were not strain-dependent, we compared the signaling response obtained from Gpa1-Gαi1 and Gpa1-Gαs engineered yeast strains obtained from GSK to those modified from those of the Broach laboratory. Consistent with those from the GSK laboratory strains (Figure 5A), A1 receptors maintained their native Gα coupling-specificity (Figure 5B). Although both strains are derived from W303 parental yeast cells, interestingly, the Broach laboratory strains showed a higher fold change in mCherry fluorescence, as well as a reduced constitutive activity. Because of the higher fold change in fluorescence compared to the GSK strains, the Broach strains were utilized for subsequent studies.

Expression of the chimeras was observed using Western blot analysis (Figure 6A) and confocal microscopy shows the localization of both full-length and truncated A1/A2AR chimeras to the plasma membrane (Figure 6Bii and iii) as compared to wild-type A1 receptor (Figure 6Bi). The downstream signaling response was evaluated in the inhibitory (Figure 6C) and stimulatory (Figure 6D) yeast reporter strains, and both chimeras showed coupling with the inhibitory yeast strain, similar to wild-type A1R,
though the $A_1/A_2\Delta 316R$ chimera showed reduced MAPK signaling via lower mCherry levels than the full-length $A_1/A_2R$ chimera. No signaling response was obtained in the stimulatory yeast strains for the $A_1R$ variants (Figure 6D). This observation suggests that the presence of the $A_2AR$ C-terminus does not affect the interaction of the chimeric $A_1/A_2A$ receptors with the native-like inhibitory $G\alpha$ and that it does not become non-selective, i.e., by binding to all $G\alpha$ proteins. This observation is consistent with previously published work with canine $A_1R$ and $A_2R$, where an $A_1R$ chimera showed native coupling with $G\alpha\iota$ [60]. We also determined the dose-dependent mCherry fluorescence response for $A_1R$ and the $A_1/A_2AR$ chimera and found that they were statistically equivalent when the non-specific agonist, NECA, was added (Figure 6E).

**Figure 5.** Downstream signaling in engineered yeast cells enables determination of $G\alpha$ coupling preferences. Agonist (100 $\mu$M NECA, blue hatched bars) mediated downstream signaling responses as compared to control (DMSO, red filled bars) for $A_1R$ were measured in yeast expressing Gpa1p-human $G\alpha$ chimeras (mean ± S.D., for three independent experiments) for either modified GSK strains (A) or Broach laboratory strains (B).
Because of the higher fold change in fluorescence compared to the GSK strains, the Broach strains were utilized for subsequent studies. Expression of the chimeras was observed using Western blot analysis (Figure 6A) and confocal microscopy shows the localization of both full-length and truncated A1/A2AR chimeras to the plasma membrane (Figure 6Bii and iii) as compared to wild-type A1 receptor (Figure 6Bi). The downstream signaling response was evaluated in the inhibitory (Figure 6C) and stimulatory (Figure 6D) yeast reporter strains, and both chimeras showed coupling with the inhibitory yeast strain, similar to wild-type A1R, though the A1/A2ARΔ316R chimera showed reduced MAPK signaling via lower mCherry levels than the full-length A1/A2AR chimera. No signaling response was obtained in the stimulatory yeast strains for the A1R variants (Figure 6D). This observation suggests that the presence of the A2AR C-terminus does not affect the interaction of the chimeric A1/A2AR receptors with the native-like inhibitory Gαi and that it does not become non-selective, i.e., by binding to all Gα proteins. This observation is consistent with previously published work with canine A1R and A2AR, where an A1R chimera showed native coupling with Gαi/o [60]. We also determined the dose-dependent mCherry fluorescence response for A1R and the A1/A2AR chimera and found that they were statistically equivalent when the non-specific agonist, NECA, was added (Figure 6E).

**Figure 6.** Expression and downstream MAPK signaling response in yeast for A1/A2AR and A1/A2ARΔ316R chimeras show native A1R-like behavior. (A) Western blot images showing expression of mCitrine tagged receptors for full-length and truncated chimeric receptors. Precision Plus Protein Western C standards were used to determine molecular weight as indicated. The expected molecular weight of each receptor with mCitrine is as follows: A1R, 63.4 kDa; A1/A2AR-71.5 kDa and A1/A2ARΔ316R-62.3 kDa. Note that the mobilities are slightly faster than that expected by calculated molecular weight; membrane proteins have been found to run faster than expected, possibly due to their hydrophobicity or incomplete denaturation, due to the absence of sample heating [88]. (B) Representative confocal images of yeast strains showing receptor localization of A1R (i), and A1/A2AR (ii) and A1/A2ARΔ316R (iii). Cells were stained with fluorescent brightener calcofluor white M2R (F3543; Sigma-Aldrich) that binds to chitin in the yeast cell wall prior to imaging, and this stain is shown in blue, while mCitrine fluorescence is shown in yellow. Scale bar = 2 µm. (C,D) Downstream signaling response of receptor variants in inhibitory Gpa1p-Gαi1(5) (C) or stimulatory Gpa1p-Gαs(5) strain (D). A1/A2AR and A1/A2ARΔ316R produce signaling response similar to A1R in inhibitory strain; however, none of the receptors produce a response in stimulatory strain. 100 µM NECA is shown as blue hatched bars and DMSO in red filled bars. (E) Dose-response curve for A1/A2AR chimera (green squares) is similar to the native A1R receptor (black circles). Data represent the mean ± 95% C.I. for experiments performed in duplicate for three independent transformants.
4. Discussion

Since the early 1990s, the engineered yeast MAPK response pathway has been known as a useful tool to study human GPCR signaling and identify lead drug candidates by recapitulating native dose-response binding preferences [63,66,93]. Both A1R and A2AR have been shown previously to interact with yeast/human chimeric Ga protein to produce downstream signaling responses in the engineered yeast [79,80,93,94]. Here, these yeast strains were further engineered with a fluorescence reporter and also successfully captured A1R and A2AR downstream signaling via their corresponding native Ga proteins. Strains obtained from the Broach laboratory showed a higher fold change than those from the Dowell (GSK) laboratory under these conditions, although both strains were derived from parental W303 cells, indicating that strain differences outside of the FUST locus can impact the results obtained in cell-based assays.

One of the strengths of the engineered yeast system is the capability of quantifying the GPCR-Gα interaction at a common endpoint of the signaling cascade. This allows direct comparison of the strengths of the activation for different Gα biased ligands. One such study performed by Stewart et al. [93] identified a novel A1R agonist with biased specificity for Gαi vs. Gαo coupling. Efforts have been made to replicate this approach of utilizing the last five amino acids of the C-terminus of the Gα protein into a mammalian system using Gαs or Gαq as templates [95,96]. A study by Hsu and Lou [95] in HEK-293 cells tested the interaction of A1R with Gαs chimeras via a cAMP assay. The authors observed cAMP production for all Gα variants tested except Gαs, suggesting the system was not effective in capturing the specificity of the interaction of A1R with Gα proteins. The higher than native levels of Gα protein expressed (~three-fold higher than mock transfected, native HEK) [97,98] and the presence of additional GPCRs present in the HEK cells may have contributed to signal promiscuity [99]. In our study, all the signaling components in the engineered yeast were expressed under their native promoters and, perhaps as a result, the yeast cell assay more effectively captured the specificity of GPCR-Gα interaction.

The long C-terminus of the A2AR (122 amino acids) is assumed to be highly flexible and disordered; thus, crystallization of adenosine receptors has all focused on using truncated receptors. Here, we found that A2ARΔ316R resulted in no downstream cAMP signaling, and that the A2AR C-terminus did not change the G-protein coupling preference from Gαi to Gαs for the A1/A2AR variants. Our results were consistent with previously published work by Tucker et al. [60] that found that a chimera of canine A1R with a canine A2AR C-terminus showed no change in G-protein coupling behavior. The A1/A2AR chimera showed a dose-dependent MAPK response similar to the wild-type human A1R receptor in the yeast system, suggesting there was no change in G-protein coupling behavior due to the presence of the A2AR C-terminus. Taken together with our previous results of exceptional yields of the chimera that binds A1R-selective agonist [44], these data suggest that A1/A2AR could be an effective variant to study biophysical characteristics and function for the A1 receptor. Previously, we have reported that a similar A3/A2AR chimera, consisting of the A3 transmembrane helices and the A2AR C-terminus, showed native coupling with Gαi/o but not Gαs [58]. As noted in the introduction, the C-terminus of A2AR has some special properties, such as the absence of the canonical cysteine for palmitoylation (position 309 in A2AR) that may potentially add flexibility to bind interaction partners [48]. Thus, although these results are of interest for the adenosine receptors family, more experiments will need to be performed to understand the general applicability of this chimeric approach to another class A GPCR subfamily.

The A2AR C-terminus is known to interact with many accessory proteins in the GPCR signaling pathways like G protein receptor kinases and β-arrestins that aid in receptor signaling and desensitization [47,48,100], but has previously been thought to be dispensable for G-protein signaling [52,54]. Bennett et al. [53] showed that A2ARΔ316R expressed by an inducible promoter was capable of coupling to Gαs in a receptor expression-level dependent manner; however, their data were normalized, and total cAMP levels were not reported. We do see a small increase in ligand-dependent signaling for the A2ARΔ316R truncation (Figure 2A), but the signal is over twenty-fold less than wild
type A2AR, suggesting the truncation is responsible for the loss of G protein signaling. Taken together, our results highlight the role of the C-terminus for A2AR and A1R in G-protein coupling, but not in G-protein specificity.

**Author Contributions:** A.R.J. and A.S.R. designed the research plan; A.R.J. and C.M. performed experiments; A.R.J., C.M. and A.S.R. analyzed the data; and A.R.J. and A.S.R. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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**References**

1. Hill, S.J. G-protein-coupled receptors: Past, present and future. *Br. J. Pharmacol.* 2006, 147 (Suppl. S1), S27–S37. [CrossRef]
2. Elion, E.A. Pheromone response, mating and cell biology. *Curr. Opin. Microbiol.* 2000, 3, 573–581. [CrossRef]
3. McNeely, P.M.; Naranjo, A.N.; Robinson, A.S. Structure-function studies with G protein-coupled receptors as a paradigm for improving drug discovery and development of therapeutics. *Biotechnol. J.* 2012, 7, 1451–1461. [CrossRef] [PubMed]
4. Hauser, A.S.; Chavali, S.; Masuho, I.; Jahn, L.J.; Martemyanov, K.A.; Gloriam, D.E.; Babu, M.M. Pharmacogenomics of GPCR Drug Targets. *Cell* 2018, 172, 41–54.e19. [CrossRef] [PubMed]
5. Blocker, K.M.; Britton, Z.T.; Naranjo, A.N.; McNeely, P.M.; Young, C.L.; Robinson, A.S. Recombinant G protein-coupled receptor expression in Saccharomyces cerevisiae for protein characterization. *Methods Enzymol.* 2015, 556, 165–183. [CrossRef] [PubMed]
6. Chiu, M.L.; Tsang, C.; Grihalde, N.; MacWilliams, M.P. Over-expression, solubilization, and purification of G protein-coupled receptors for structural biology. *Comb. Chem. High Throughput Screen* 2008, 11, 439–462. [CrossRef]
7. Lundstrom, K.; Wagner, R.; Reinhart, C.; Desmyter, A.; Cherouati, N.; Maguin, T.; Zeder-Lutz, G.; Courtot, M.; Prual, C.; Andre, N.; et al. Structural genomics on membrane proteins: Comparison of more than 100 GPCRs in 3 expression systems. *J. Struct. Funct. Genomics* 2006, 7, 77–91. [CrossRef]
8. O’Malley, M.A.; Mancini, J.D.; Young, C.L.; McCusker, E.C.; Raden, D.; Robinson, A.S. Progress toward heterologous expression of active G-protein-coupled receptors in Saccharomyces cerevisiae: Linking cellular stress response with translocation and trafficking. *Protein Sci.* 2009, 18, 2356–2370. [CrossRef]
9. Jaakola, V.P.; Griffith, M.T.; Hanson, M.A.; Cherezov, V.; Chien, E.Y.; Lane, J.R.; Ijzerman, A.P.; Stevens, R.C. The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science* 2008, 322, 1211–1217. [CrossRef] [PubMed]
10. Xu, F.; Wu, H.; Katritch, V.; Han, G.W.; Jacobson, K.A.; Gao, Z.G.; Cherezov, V.; Stevens, R.C. Structure of an agonist-bound human A2A adenosine receptor. *Science* 2011, 332, 322–327. [CrossRef] [PubMed]
11. Lebon, G.; Warne, T.; Edwards, P.C.; Bennett, K.; Langmead, C.J.; Leslie, A.G.; Tate, C.G. Agonist-bound adenosine A2A receptor structures reveal common features of GPCR activation. *Nature* 2011, 474, 521–525. [CrossRef] [PubMed]
12. Dore, A.S.; Robertson, N.; Errey, J.C.; Ng, I.; Hollenstein, K.; Tehan, B.; Hurrell, E.; Bennett, K.; Congreve, M.; Magnani, F.; et al. Structure of the adenosine A(2A) receptor in complex with ZM241385 and the xanthines XAC and caffeine. *Structure* 2011, 19, 1283–1293. [CrossRef] [PubMed]
13. Hino, T.; Arakawa, T.; Iwanari, H.; Yurugi-Kobayashi, T.; Ikeda-Suno, C.; Nakada-Nakura, Y.; Kusano-Arai, O.; Weyand, S.; Shimamura, T.; Nomura, N.; et al. G-protein-coupled receptor inactivation by an allosteric inverse-agonist antibody. *Nature* 2012, 482, 237–240. [CrossRef] [PubMed]
14. Congreve, M.; Andrews, S.P.; Dore, A.S.; Hollenstein, K.; Hurrell, E.; Langmead, C.J.; Mason, J.S.; Ng, I.W.; Tehan, B.; Zhukov, A.; et al. Discovery of 1,2,4-triazine derivatives as adenosine A(2A) antagonists using structure based drug design. *J. Med. Chem.* 2012, 55, 1898–1903. [CrossRef]
15. Liu, W.; Chun, E.; Thompson, A.A.; Chubukov, P.; Xu, F.; Katritch, V.; Han, G.W.; Roth, C.B.; Heitman, L.H.; IJzerman, A.P.; et al. Structural basis for allosteric regulation of GPCRs by sodium ions. *Science* 2012, 337, 232–236. [CrossRef]

16. Lebon, G.; Edwards, P.C.; Leslie, A.G.; Tate, C.G. Molecular Determinants of CGS21680 Binding to the Human Adenosine A2A Receptor. *Mol. Pharmacol.* 2015, 87, 907–915. [CrossRef]

17. Segala, E.; Guo, D.; Cheng, R.K.; Bortolato, A.; Deflorian, F.; Dore, A.S.; Errey, J.C.; Heitman, L.H.; AP, I.J.; Marshall, F.H.; et al. Controlling the Dissociation of Ligands from the Adenosine A2A Receptor through Modulation of Salt Bridge Strength. *J. Med. Chem.* 2016, 59, 6470–6479. [CrossRef]

18. Carpenter, B.; Nehme, R.; Warne, T.; Leslie, A.G.; Tate, C.G. Structure of the adenosine A(2A) receptor bound to an engineered G protein. *Nature* 2016, 536, 104–107. [CrossRef]

19. Batyuk, A.; Galli, L.; Ishchenko, A.; Han, G.W.; Gati, C.; Popov, P.A.; Lee, M.Y.; Stauch, B.; White, T.A.; Barty, A.; et al. Native phasing of X-ray free-electron laser data for a G protein-coupled receptor. *Sci. Adv.* 2016, 2, e1600292. [CrossRef][PubMed]

20. Sun, B.; Bachhawat, P.; Chu, M.L.; Wood, M.; Ceska, T.; Sands, Z.A.; Mercier, J.; Lebon, F.; Kobilka, T.S.; Kobilka, B.K. Crystal structure of the adenosine A2A receptor bound to an antagonist reveals a potential allosteric pocket. *Proc. Natl. Acad. Sci. USA* 2017, 114, 2066–2071. [CrossRef]

21. Martin-Garcia, J.M.; Conrad, C.E.; Nelson, G.; Stander, N.; Zatsepin, N.A.; Zook, J.; Zhu, L.; Geiger, J.; Chun, E.; Kissick, D.; et al. Millisecond millisecond crystallography of membrane and soluble protein microcrystals using synchrotron radiation. *IUCrJ* 2017, 4, 439–454. [CrossRef][PubMed]

22. Melnikov, I.; Polovinkin, V.; Kovalev, K.; Gushchin, I.; Shevtsov, M.; Shevchenko, V.; Mishin, A.; Alekseev, A.; Rodriguez-Valera, F.; Borschchevskiy, V.; et al. Fast iodide-SAD phasing for high-throughput membrane protein structure determination. *Sci. Adv.* 2017, 3, e1602952. [CrossRef][PubMed]

23. Cheng, R.K.Y.; Segala, E.; Robertson, N.; Deflorian, F.; Dore, A.S.; Errey, J.C.; Fiez-Vandal, C.; Marshall, F.H.; Cooke, R.M. Structures of Human A1 and A2A Adenosine Receptors with Xanthines Reveal Determinants of Selectivity. *Structure* 2017, 25, 1275–1285.e1274. [CrossRef][PubMed]

24. Weinert, T.; Olieric, N.; Cheng, R.; Brunle, S.; James, D.; Ozerov, D.; Gashi, D.; Vera, L.; Marsh, M.; Jaeger, K.; et al. Millisecond crystallography for routine room-temperature structure determination at synchrotrons. *Nat. Commun.* 2017, 8, 542. [CrossRef]

25. Broecker, J.; Morizumi, T.; Ou, W.L.; Klingel, V.; Kuo, A.; Kissick, D.J.; Ishchenko, A.; Lee, M.Y.; Xu, S.; Makarov, O.; et al. High-throughput in situ X-ray screening of and data collection from protein crystals at room temperature and under cryogenic conditions. *Nat. Protoc.* 2018, 13, 260–292. [CrossRef]

26. Eddy, M.T.; Lee, M.Y.; Gao, Z.G.; White, T.A.; Gali, L.; Popov, P.A.; Lee, M.Y.; Stauch, B.; White, T.A.; Barty, A.; et al. Structural basis for allosteric regulation of G protein-coupled receptors. *Mol. Membr. Biol.* 2018, 35, 154–162. [CrossRef][PubMed]

27. Rucktooa, P.; Cheng, R.K.Y.; Segala, E.; Robertson, N.; Deflorian, F.; Dore, A.S.; Errey, J.C.; Fiez-Vandal, C.; Marshall, F.H.; Cooke, R.M. Structures of Human A1 and A2A Adenosine Receptors with Xanthines Reveal Determinants of Selectivity. *Structure* 2017, 25, 1275–1285.e1274. [CrossRef][PubMed]

28. White, K.L.; Eddy, M.T.; Gao, Z.G.; Han, G.W.; Lian, T.; Deary, A.; Patel, N.; Jacobson, K.A.; Katritch, V.; Stevens, R.C. Structural Connection between Activation Microswitch and Allosteric Sodium Site in GPCR Signaling. *Structure* 2018, 26, 259–269.e255. [CrossRef][PubMed]

29. Garcia-Nafria, J.; Lee, Y.; Bai, X.; Carpenter, B.; Tate, C.G. Cryo-EM structure of the adenosine A2A receptor coupled to an engineered heterotrimERIC G protein. *Elife* 2018, 7. [CrossRef]

30. Martin-Garcia, J.M.; Zhu, L.; Mendez, D.; Lee, M.Y.; Chun, E.; Li, C.; Hu, H.; Subramanian, G.; Kissick, D.; Ogata, C.; et al. High-viscosity injector-based pink-beam serial crystallography of microcrystals at a synchrotron radiation source. *IUCrJ* 2019, 6, 412–425. [CrossRef]

31. Shimazu, Y.; Tono, K.; Tanaka, T.; Yamanaka, Y.; Nakane, T.; Mori, C.; Terakado Kimura, K.; Fujiiwara, T.; Sugahara, M.; Tanaka, R.; et al. High-viscosity sample-injection device for serial femtosecond crystallography at atmospheric pressure. *J. Appl. Crystallogr.* 2019, 52, 1280–1288. [CrossRef][PubMed]

32. Ishchenko, A.; Stauch, B.; Han, G.W.; Batyuk, A.; Shiraieva, A.; Li, C.; Zatsepin, N.; Weierstall, U.; Liu, W.; Nango, E.; et al. Toward G protein-coupled receptor structure-based drug design using X-ray lasers. *IUCrJ* 2019, 6, 1106–1119. [CrossRef][PubMed]
33. Jespers, W.; Verdon, G.; Azua, J.; Majellaro, M.; Keranen, H.; Garcia-Mera, X.; Congreve, M.; Deflorian, F.; de Graaf, C.; Zhukov, A.; et al. X-ray Crystallography and Free Energy Calculations Reveal the Binding Mechanism of A2A Adenosine Receptor Antagonists. *Angew. Chem. Int. Ed. Engl.* 2020. [CrossRef] [PubMed]

34. Lee, M.Y.; Geiger, J.; Ishchenko, A.; Han, G.W.; Barty, A.; White, T.A.; Gati, C.; Batyuk, A.; Hunter, M.S.; Aquila, A.; et al. Harnessing the power of an X-ray laser for serial crystallography of membrane proteins crystallized in lipidic cubic phase. *IUCrJ* 2020, 7, 976–984. [CrossRef] [PubMed]

35. Borodovsky, A.; Barbon, C.M.; Wang, Y.; Ye, M.; Prickett, L.; Chandra, D.; Shaw, J.; Deng, N.; Sachsenmeier, K.; Clarke, J.D.; et al. Small molecule AZD4635 inhibitor of A2A receptor signaling rescues immune cell function including CD103(+) dendritic cells enhancing anti-tumor immunity. *J. Immunother. Cancer* 2020, 8. [CrossRef] [PubMed]

36. Ihara, K.; Hato, M.; Nakane, T.; Yamashita, K.; Kimura-Someya, T.; Hosaka, T.; Ishizuka-Katsura, Y.; Tanaka, R.; Tanaka, T.; Sugahara, M.; et al. Isoprenoid-chained lipid EROCOC1 yields for adenosine receptor chimeras. *AIChE J.* 2020, 1867–877.e813. [CrossRef] [PubMed]

37. Glukhova, A.; Thal, D.M.; Nguyen, A.T.; Vecchio, E.A.; Jorg, M.; Scammells, P.J.; May, L.T.; Sexton, P.M.; Christopoulos, A. Structure of the Adenosine A1 Receptor Reveals the Basis for Subtype Selectivity. *Cell* 2017, 168, 867–877.e813. [CrossRef]

38. Draper-Joyce, C.J.; Khoshouei, M.; Thal, D.M.; Liang, Y.L.; Nguyen, A.T.; Furness, S.G.B.; Venugopal, H.; Baltos, J.A.; Plitko, J.M.; Daney, R.; et al. Structure of the adenosine-bound human adenosine A1 receptor-Gi complex. *Nature* 2018, 558, 559–563. [CrossRef]

39. Fredholm, B.B.; AP, I.J.; Jacobson, K.A.; Linden, J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* 2001, 53, 527–552.

40. Fredholm, B.B.; AP, I.J.; Jacobson, K.A.; Linden, J.; Muller, C.E. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors—An update. *Pharmacol. Rev.* 2011, 63, 1–34. [CrossRef]

41. McIntosh, V.J.; Lasley, R.D. Adenosine receptor-mediated cardioprotection: Are all 4 subtypes required or redundant? *J. Cardiovasc. Pharmacol. Ther.* 2012, 17, 21–33. [CrossRef] [PubMed]

42. Fredholm, B.B., Chen, J.F.; Cunha, R.A.; Svenningsson, P.; Vagueois, J.M. Adenosine and brain function. *Int. Rev. Neurobiol.* 2005, 63, 191–270. [CrossRef] [PubMed]

43. Chen, J.F.; Eltzschig, H.K.; Fredholm, B.B. Adenosine receptors as drug targets—What are the challenges? *Nat. Rev. Drug Discov.* 2013, 12, 265–286. [CrossRef] [PubMed]

44. Jain, A.R.; Stradley, S.H.; Robinson, A.S. The A2AR C-terminus provides improved total and active expression yields for adenosine receptor chimeras. *AIChE J.* 2018, 64, 4297–4307. [CrossRef]

45. Britton, Z.T. Novel Approaches to the Expression and Purification of G Protein-Coupled Receptors; University of Delaware: Newark, Delaware, 2012; p. 3526401.

46. Moriyama, K.; Sitkovsky, M.V. Adenosine A2A receptor is involved in cell surface expression of A2B receptor. *J. Biol. Chem.* 2010, 285, 39271–39288. [CrossRef] [PubMed]

47. Baltos, J.A.; Plitzko, J.M.; Danev, R.; et al. Structure of the adenosine-bound human adenosine A1 receptor-Gi complex. *IUCrJ* 2020, 8, 603 18 of 21

48. Whitton, S.; Hedley, D.; Kara, E.; Gras, A.; Iwata, S.; Ruprecht, J.; Strange, P.G.; Byrne, B. A purified C-terminally truncated human adenosine A2A receptor construct is functionally stable and degradation resistant. *Protein Expr. Purif* 2010, 74, 80–87. [CrossRef] [PubMed]

49. Palmer, T.M.; Stiles, G.L. Identification of an A2a Adenosine Receptor Domain Specifically Responsible for Mediating Short-Term Desensitization. *Biochemistry* 1997, 36, 832–838. [CrossRef] [PubMed]
53. Bennett, K.A.; Tehan, B.; Lebon, G.; Tate, C.G.; Weir, M.; Marshall, F.H.; Langmead, C.J. Pharmacology and structure of isolated conformations of the adenosine A(2)A receptor define ligand efficacy. *Mol. Pharmacol.* **2013**, *83*, 949–958. [CrossRef] [PubMed]

54. Klinger, M.; Kuhn, M.; Just, H.; Stefan, E.; Palmer, T.; Freissmuth, M.; Nanoff, C. Removal of the carboxy terminus of the A2A-adenosine receptor blunts constitutive activity: Differential effect on cAMP accumulation and MAP kinase stimulation. *Naunyn Schmiedebergs Arch. Pharmacol.* **2002**, *366*, 287–298. [CrossRef]

55. O’Malley, M.A. Expression, Purification, and Biophysical Characterization of G-Protein Coupled Receptors Expressed from Saccharomyces Cerevisiae. Ph.D. Thesis, University of Delaware, Delaware, Newark, 2009.

56. Mundell, S.; Kelly, E. Adenosine receptor desensitization and trafficking. *Biochim. Biophys. Acta* **2011**, *1808*, 1319–1328. [CrossRef]

57. Jain, A.R.; Britton, Z.T.; Markwalter, C.E.; Robinson, A.S. Improved ligand-binding- and signaling-competent human NK2R yields in yeast using a chimera with the rat NK2R C-terminus enable NK2R-G protein signaling platform. *Protein Eng. Des. Sel.* **2019**, *32*, 459–469. [CrossRef]

58. Jain, A.R.; Robinson, A.S. Functional Expression of Adenosine A3 Receptor in Yeast Utilizing a Chimera with the A2A R C-Terminus. *Int. J. Mol. Sci.* **2020**, *21*, 4547. [CrossRef]

59. Yin, D.; Gavi, S.; Wang, H.Y.; Malbon, C.C. Probing receptor structure/function with chimeric G-protein-coupled receptors. *Mol. Pharmacol.* **2004**, *65*, 1323–1332. [CrossRef]

60. Tucker, A.L.; Jia, L.G.; Holeton, D.; Taylor, A.J.; Linden, J. Dominance of G(s) in doubly G(s) G protein signaling. *Biochem. J.* **2000**, *352* Pt 1, 203–210. [CrossRef]

61. Dohlman, H.G.; Thorner, J.; Caron, M.G.; Lefkowitz, R.J. Model systems for the study of seven-transmembrane-segment receptors. *Ann. Rev. Biochem.* **1991**, *60*, 653–688. [CrossRef]

62. Huang, X.P.; Karpiak, J.; Kroeze, W.K.; Zhu, H.; Chen, X.; Moy, S.S.; Saddoris, K.A.; Nikolova, V.D.; Dohlman, H.G.; Thorner, J.; Caron, M.G.; Lefkowitz, R.J. Control of yeast mating signal transduction by a mammalian beta 2-adrenergic receptor and Gs alpha subunit. *Science* **1990**, *250*, 120–123. [CrossRef] [PubMed]

63. Saito, H. Regulation of cross-talk in yeast MAPK signaling pathways. *Curr. Opin. Microbiol.* **2010**, *13*, 677–683. [CrossRef] [PubMed]

64. Burke, D.; Dawson, D.; Stearns, T. Cold Spring Harbor Laboratory. In *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*, 2000th ed.; Cold Spring Harbor Laboratory Press: Plainview, NY, USA, 2000.

65. Brown, A.J.; Dyos, S.L.; Whiteway, M.S.; White, J.H.; Watson, M.A.; Marziach, M.; Clare, J.J.; Cousins, D.J.; Paddon, C.; Plumpton, C.; et al. Functional coupling of mammalian receptors to the yeast mating pathway using novel yeast/mammalian G protein alpha-subunit chimeras. *Yeast* **2000**, *16*, 11–22. [CrossRef]

66. Arnold, C.E.; Parekh, R.N.; Yang, W.; Wittrup, K.D. Leader peptide efficiency correlates with signal recognition particle dependence in Saccharomyces cerevisiae. *Biotechnol. Bioeng.* **1998**, *59*, 286–293. [CrossRef]

67. Young, C.L.; Raden, D.L.; Caplan, J.L.; Czynmek, K.J.; Robinson, A.S. Cassette series designed for live-cell imaging of proteins and high-resolution techniques in yeast. *Yeast* **2012**, *29*, 119–136. [CrossRef]

68. McGraw, C.; Yang, L.; Levental, I.; Lyman, E.; Robinson, A.S. Membrane cholesterol depletion reduces downstream signaling activity of the adenosine A2A receptor. *Biochim. Biophys. Acta Biomembr.* **2019**, *1861*, 760–767. [CrossRef]

69. Bitter, G.A.; Egan, K.M. Expression of interferon-gamma from hybrid yeast GPD promoters containing upstream regulatory sequences from the GAL1-GAL10 intergenic region. *Gene* **1988**, *69*, 193–207. [CrossRef]
75. Niebauer, R.T.; Gao, Z.G.; Li, B.; Wess, J.; Jacobson, K.A. Signaling of the Human P2Y(1) Receptor Measured by a Yeast Growth Assay with Comparisons to Assays of Phospholipase C and Calcium Mobilization in 1321N1 Human Astrocytoma Cells. *Purinergic Signal.* 2005, 1, 241–247. [CrossRef] [PubMed]

76. Price, L.A.; Kajkowski, E.M.; Hadcock, J.R.; Ozenberger, B.A.; Pausch, M.H. Functional coupling of a mammalian somatostatin receptor to the yeast pheromone response pathway. *J. Med. Chem.* 1995, 38, 1074–1079. [CrossRef] [PubMed]

77. Price, L.A.; Strnad, J.; Pausch, M.H.; Hadcock, J.R. Pharmacological characterization of the rat A2a adenosine receptor functionally coupled to the yeast pheromone response pathway. *Br. J. Pharmacol.* 1995, 115, 6188–6195. [CrossRef] [PubMed]

78. Haraguchi, K.; Shigemori, T.; Kuroda, K.; Ueda, M. Membrane-displayed somatostatin activates somatostatin receptor subtype-2 heterologously produced in *Saccharomyces cerevisiae*. *AMB Express* 2012, 2, 63. [CrossRef]

79. Bertheleme, N.; Singh, S.; Dowell, S.J.; Hubbard, J.; Byrne, B. Loss of constitutive activity is correlated with increased thermostability of the human adenosine A2A receptor. *Br. J. Pharmacol.* 2013, 169, 988–998. [CrossRef]

80. Peeters, M.C.; Wisse, L.E.; Dinaj, A.; Vroling, B.; Vriend, G.; Ijzerman, A.P. The role of the second and third extracellular loops of the adenosine A1 receptor in activation and allosteric modulation. *Biochem. Pharmacol.* 2012, 84, 76–87. [CrossRef]

81. Magnani, F.; Shibata, Y.; Serrano-Vega, M.J.; Tate, C.G. Co-evolving stability and conformational homogeneity of the human adenosine A2a receptor. *Proc. Natl. Acad. Sci. USA* 2008, 105, 10744–10749. [CrossRef]

82. de Lera Ruiz, M.; Lim, Y.H.; Zheng, J. Adenosine A2A receptor as a drug discovery target. *J. Med. Chem.* 2014, 57, 3623–3650. [CrossRef]

83. Jespers, W.; Schiedel, A.C.; Heitman, L.H.; Cooke, R.M.; Kleene, R.; Van Westen, G.; Gloriam, D.E.; Müller, C.E.; Sotelo, E.; Gutiérrez-De-Terán, H. Structural Mapping of Adenosine Receptor Mutations: Ligand Binding and Signaling Mechanisms. *Trends Pharmacol. Sci.* 2018, 39, 75–89. [CrossRef]

84. McCusker, E.C. Overcoming Expression Obstacles in Producing Functional Components of the G-Protein Coupled Receptor Pathway; University of Delaware: Delaware, Newark, 2007.

85. Niebauer, R.T.; Robinson, A.S. Exceptional total and functional yields of the human adenosine (A2a) receptor expressed in the yeast *Saccharomyces cerevisiae*. *Protein Expr. Purif.* 2006, 46, 204–211. [CrossRef] [PubMed]

86. Niebauer, R.T.; Robinson, A.S. Decreases in yeast expression yields of the human adenosine A2a receptor are a result of translational or post-translational events. *Protein Expr. Purif.* 2004, 37, 134–143. [CrossRef] [PubMed]

87. Wedekind, A.; Robinson, A.S. Optimization of the human adenosine A2a receptor yields in *Saccharomyces cerevisiae*. *Biotechnol. Prog.* 2006, 22, 1249–1255. [CrossRef] [PubMed]

88. Grisshammer, R.; Tate, C.G. Overexpression of integral membrane proteins for structural studies. *Q. Rev. Biophys.* 1995, 28, 315–422. [CrossRef] [PubMed]

89. Lohse, M.J.; Klotz, K.N.; Lindenborn-Fotinos, J.; Reddington, M.; Schwabe, U.; Olsson, R.A. 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX)–a selective high affinity antagonist radioligand for A1 adenosine receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.* 1987, 336, 204–210. [CrossRef] [PubMed]

90. Martens, D.; Lohse, M.J.; Rauch, B.; Schwabe, U. Pharmacological characterization of A1 adenosine receptors in isolated rat ventricular myocytes. *Naunyn-Schmiedebergs Arch. Pharmacol.* 1987, 336, 342–348. [CrossRef]

91. Pankevych, H.; Korkhov, V.; Freissmuth, M.; Nanoff, C. Truncation of the A1 adenosine receptor reveals distinct roles of the membrane-proximal carboxyl terminus in receptor folding and G protein coupling. *J. Biol. Chem.* 2003, 278, 30283–30293. [CrossRef]

92. Townsend-Nicholson, A.; Shine, J. Molecular cloning and characterisation of a human brain A1 adenosine receptor cDNA. *Brain Res. Mol. Brain Res.* 1992, 16, 365–370. [CrossRef]

93. Stewart, G.D.; Valant, C.; Dowell, S.J.; Mijaljica, D.; Devenish, R.J.; Scammells, P.J.; Sexton, P.M.; Christopoulos, A. Determination of adenosine A1 receptor agonist and antagonist pharmacology using *Saccharomyces cerevisiae*: Implications for ligand screening and functional selectivity. *J. Pharmacol. Exp. Ther.* 2009, 331, 277–286. [CrossRef]

94. Knight, A.; Hemmings, J.L.; Winfield, I.; Leuenberger, M.; Frattini, E.; Frenzueli, B.G.; Dowell, S.J.; Lochner, M.; Ladds, G. Discovery of Novel Adenosine Receptor Agonists That Exhibit Subtype Selectivity. *J. Med. Chem.* 2016, 59, 947–964. [CrossRef]

95. Hsu, S.H.; Luo, C.W. Molecular dissection of G protein preference using Gsalpha chimeras reveals novel ligand signaling of GPCRs. *Am. J. Physiol. Endocrinol. Metab.* 2007, 293, E1021–E1029. [CrossRef] [PubMed]
96. Conklin, B.R.; Farfel, Z.; Lustig, K.D.; Julius, D.; Bourne, H.R. Substitution of three amino acids switches receptor specificity of Gq alpha to that of Gi alpha. *Nature* 1993, 363, 274–276. [CrossRef] [PubMed]

97. Kostenis, E.; Waelbroeck, M.; Milligan, G. Techniques: Promiscuous Galpha proteins in basic research and drug discovery. *Trends Pharmacol. Sci.* 2005, 26, 595–602. [CrossRef] [PubMed]

98. Geppetti, P.; Veldhuis, N.A.; Lieu, T.; Bunnett, N.W. G Protein-Coupled Receptors: Dynamic Machines for Signaling Pain and Itch. *Neuron* 2015, 88, 635–649. [CrossRef] [PubMed]

99. O’Hayre, M.; Vazquez-Prado, J.; Kufareva, I.; Stawiski, E.W.; Handel, T.M.; Seshagiri, S.; Gutkind, J.S. The emerging mutational landscape of G proteins and G-protein-coupled receptors in cancer. *Nat. Rev. Cancer* 2013, 13, 412–424. [CrossRef] [PubMed]

100. Keuerleber, S.; Gsandtner, I.; Freissmuth, M. From cradle to twilight: The carboxyl terminus directs the fate of the A(2A)-adenosine receptor. *Biochim. Biophys. Acta* 2011, 1808, 1350–1357. [CrossRef]

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