

Original citation:

Nehmé, Rony, Carpenter, Byron, Singhal, Ankita, Strege, Annette, Edwards, Patricia C., White, Courtney F., Du, Haijuan, Grisshammer, Reinhard and Tate, Christopher G. (2017) Mini-G proteins : novel tools for studying GPCRs in their active conformation. PLoS One, 12 (4). e0175642.

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RESEARCH ARTICLE

Mini-G proteins: Novel tools for studying GPCRs in their active conformation

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Citation: Nehmé R, Carpenter B, Singhal A, Strege A, Edwards PC, White CF, et al. (2017) Mini-G proteins: Novel tools for studying GPCRs in their active conformation. PLoS ONE 12(4): e0175642. <https://doi.org/10.1371/journal.pone.0175642>

Editor: Arun Shukla, Indian Institute of Technology Kanpur, INDIA

Received: February 2, 2017

Accepted: March 29, 2017

Published: April 20, 2017

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This research was supported by the European Research Council (EMPSI 339995; R.N., A.S., C.G.T.), Heptares Therapeutics (B.C., C.G.T.), the Medical Research Council (MC_U105197215; A.S., P.E., C.G.T.) and the Intramural Research Program of the National Institutes of Health, National Institute of Neurological Disorders and Stroke (ZIA NS003016-11; C.F.W., H.D., R.G.). The funders had no role in study design, data collection

Abstract

Mini-G proteins are the engineered GTPase domains of G α subunits. They couple to GPCRs and recapitulate the increase in agonist affinity observed upon coupling of a native heterotrimeric G protein. Given the small size and stability of mini-G proteins, and their ease of expression and purification, they are ideal for biophysical studies of GPCRs in their fully active state. The first mini-G protein developed was mini-G_s. Here we extend the family of mini-G proteins to include mini-G_{oif}, mini-G_{i1}, mini-G_{o1} and the chimeras mini-G_{s/q} and mini-G_{s/i}. The mini-G proteins were shown to couple to relevant GPCRs and to form stable complexes with purified receptors that could be purified by size exclusion chromatography. Agonist-bound GPCRs coupled to a mini-G protein showed higher thermal stability compared to the agonist-bound receptor alone. Fusion of GFP at the N-terminus of mini-G proteins allowed receptor coupling to be monitored by fluorescence-detection size exclusion chromatography (FSEC) and, in a separate assay, the affinity of mini-G protein binding to detergent-solubilised receptors was determined. This work provides the foundation for the development of any mini-G protein and, ultimately, for the structure determination of GPCRs in a fully active state.

Introduction

G protein-coupled receptors (GPCRs) are integral membrane proteins that play a ubiquitous role in intercellular communication throughout the human body. Binding of an agonist at the extracellular surface of the inactive receptor results ultimately in a conformational change at its intracellular surface that allows G protein coupling [1]. The conformation of the bound G protein is altered around the nucleotide binding pocket, resulting in GDP release, binding of GTP and subsequent activation of the G protein [2]. There has been great progress in the structure determination of GPCRs in the inactive state [3] bound to antagonists which highlights common structural features of the GPCR superfamily [4], a common mode of activation [5] and a conserved mechanism for the activation of G proteins [6]. However, it is still a major challenge to determine the structures of receptors in their fully active state, which can be defined structurally as the conformation bound to a heterotrimeric G protein or pharmacologically as the high-affinity agonist

and analysis, decision to publish, or preparation of the manuscript.

Competing interests: CGT is a consultant, shareholder and member of the Scientific Advisory Board of Heptares Therapeutics. This does not alter our adherence to PLOS ONE policies on sharing data and materials. BC was also funded by a grant from Heptares Therapeutics.

binding state of the receptor. The range of GPCR structures now available allows a clear distinction between agonist-bound structures either in the inactive state [7–10], in an active-intermediate state [11–14] or in an active state [2, 15–20]. It is, however, difficult to determine structures of receptors in an active state due to the instability of activated GPCRs and receptor-bound G proteins.

Four solutions have been developed to determine the active structures of GPCRs. The first active structure determined was of opsin stabilized at low pH [21] and bound to a C-terminal fragment of the G protein transducin [22]. This methodology has not been successful for any other GPCR. The second active state structure of a GPCR was determined of the β_2 -adrenergic receptor (β_2 AR) bound to a single-chain camelid antibody (nanobody) that mimics the pharmacological effects of a heterotrimeric G protein [15]. This has been applied successfully to other GPCRs [18, 19], but currently the methodology still requires the immunization of llamas with purified receptors [23], which makes it difficult to apply to many unstable GPCRs. The third methodology was to crystallise β_2 AR with the entire heterotrimeric G protein [2]. This was a great achievement and showed for the first time how a GPCR facilitated nucleotide exchange in the G protein. However, no other structures of a GPCR coupled to a heterotrimeric G protein have been published and the resolution of the GPCR portion of the structure was poor and required the previously determined active state structure of β_2 AR bound to a nanobody to allow the model to be built [2]. The fourth methodology was based on the observation that only the GTPase domain of the $G\alpha$ subunit made significant contacts with active β_2 AR and therefore this domain was engineered and thermostabilised to generate a mini-G protein, mini- G_s [24]. Like the nanobodies, mini- G_s recapitulated all the receptor pharmacology upon coupling, but in addition, mini- G_s could couple to any G_s -coupled receptor whereas nanobodies bound to only a specific receptor. The structure of the adenosine A_{2A} receptor (A_{2A} R) coupled to mini- G_s was determined recently [20], which showed similar structural rearrangements observed in the β_2 AR- G_s complex.

The concept of mini-G proteins shows great promise for accelerating the rate of structure determination of GPCRs in their active states, from providing biophysical and pharmacological data on receptors in the active state, as well as providing a potential binding partner in co-crystallisation applications. However, there are four families of $G\alpha$ subunits (Fig 1; $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$) that show different specificities for various GPCRs [25]. Thus to be truly useful as tools in structural biology, at least one member from each family needs to be converted into a mini-G protein. Here we report the development of mini-G proteins for all the major $G\alpha$ families. We also describe five different assays that can be used to characterize the binding of the mini-G proteins to GPCRs and show in three cases that the complexes can be purified by size exclusion chromatography. The two different methodologies for generating the mini-G proteins can be applied easily to any other $G\alpha$ subunit, opening the doorway to studies on potentially any GPCR from any species.

Materials and methods

Ligands

The β_1 -adrenergic receptor (β_1 AR) agonist isoproterenol hydrochloride and inverse agonist ICI118551 hydrochloride were from Sigma-Aldrich. The adenosine A_{2A} receptor (A_{2A} R) agonist NECA and antagonist ZM241385 were also from Sigma Aldrich. Serotonin 5HT_{1B} receptor (5HT_{1B}R) agonist donitriptan hydrochloride and selective antagonist SB224289 hydrochloride were from Santa Cruz Biotechnology; the agonist sumatriptan succinate was from Cayman chemical. Angiotensin II receptor (AT_1 R) agonist angiotensin II was from Tocris. All radioactive ligands were from PerkinElmer.

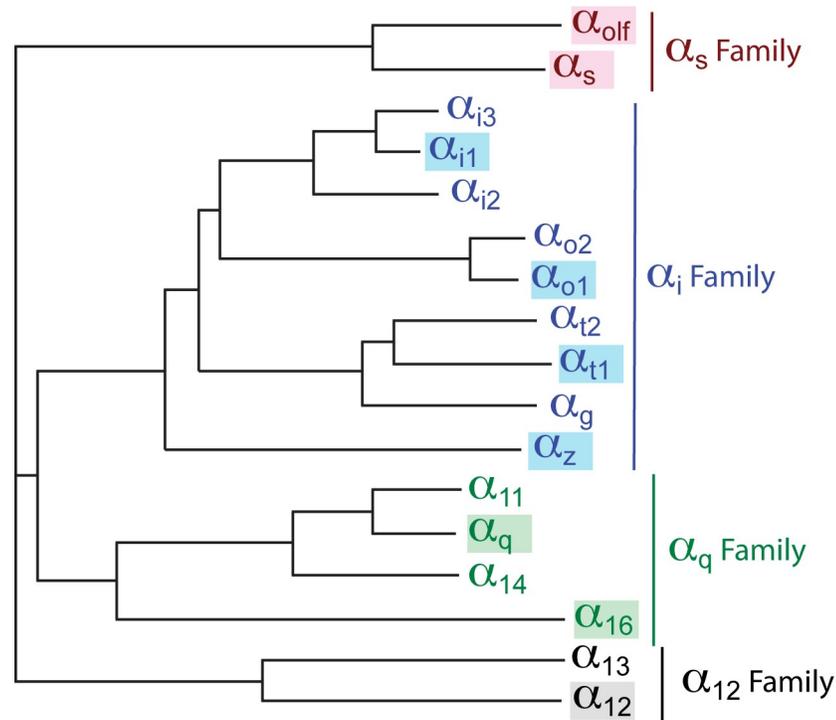


Fig 1. Phylogenetic relationship of human Gα subunits. All the Gα subunits that have been highlighted in the family-specific colours were attempted to be converted into mini-G proteins. The phylogenetic relationships were determined using TreeDyn.

<https://doi.org/10.1371/journal.pone.0175642.g001>

GPCR constructs, expression and purification

Human adenosine A_{2A} receptor (A_{2A}R). Two different A_{2A}R constructs were used during this work. For SEC experiments using purified receptor, an A_{2A}R construct was used that contained an N-terminal thioredoxin fusion protein to increase the molecular weight of the receptor. Without this fusion protein, A_{2A}R and the mini-G protein had identical mobility on SDS-PAGE, thus making it difficult to visualise the separate components when analyzing a complex. The thioredoxin-A_{2A}R fusion protein consisted of an N-terminal cleavable leader sequence (gp67), His10 tag and TEV protease cleavage site, followed by thioredoxin, which was connected to wild-type human A_{2A}R (residues 6–316) through an EAAAKA linker. A_{2A}R contained the N154A mutation to remove a potential N-linked glycosylation site. For all other experiments, a C-terminally truncated human A_{2A}R construct was used (residues 1–317), which contained a C-terminal His10 tag and TEV protease cleavage site and the N154A mutation to remove the potential N-linked glycosylation site. Both constructs were expressed using the baculovirus expression system as described previously [20, 26]. Cells were harvested by centrifugation 72 hours post infection, resuspended in hypotonic buffer (20 mM HEPES pH7.5, 1 mM EDTA, 1 mM PMSF), flash-frozen in liquid nitrogen and stored at –80°C until use. Purification of the receptor was performed in DDM using Ni²⁺-affinity chromatography followed by SEC essentially as described previously [20, 26].

Turkey β₁-adrenergic receptor (β₁AR). A truncated version of wild type turkey β₁AR (construct βAR6; [27]) contained truncations at the N-terminus and the C-terminus and a C-terminal His6 tag for purification [27], and was expressed using the baculovirus expression system at 27°C as described previously [28]. Cells were harvested by centrifugation 48 hours

post infection, resuspended in hypotonic buffer (20 mM Tris-HCl pH8, 1 mM EDTA, 1 mM PMSF), flash-frozen in liquid nitrogen and stored at -80°C until use.

Human angiotensin type II receptor 1 (AT₁R). Wild type AT₁R (residues 1–359) had a C-terminal factor X cleavage site followed by GFP and a His10 tag for purification, and was expressed using the tetracycline-inducible mammalian expression system as a stable cell line in HEK293 cells [29]. Cells were grown in DMEM containing 5% tetracycline-free FBS until they were 80% confluent and then tetracycline was added to a final concentration of 1 $\mu\text{g}/\text{ml}$. Cells were grown for 24 hours and then harvested, and resuspended in PBS, flash frozen in liquid nitrogen and stored at -80°C until use.

Rat neurotensin receptor (NTSR1). NTSR1 was expressed as described previously [13]. The baculovirus construct NTSR1 consisted of the hemagglutinin signal peptide and the Flag tag, followed by the wild-type rat NTSR1 (residues 43–396) and a C-terminal His10 tag. Recombinant baculovirus was generated using a modified pFastBac1 transfer plasmid (Invitrogen). *Trichoplusia ni* cells were infected with recombinant virus, and the temperature was lowered from 27°C to 21°C . Cells were harvested by centrifugation 48 hours post infection, resuspended in hypotonic buffer (10 mM HEPES pH 7.5, 10 mM MgCl_2 , 20 mM KCl), flash-frozen in liquid nitrogen and stored at -80°C until use.

Human serotonin 5HT_{1B} receptor (5HT_{1B}R). Wild-type 5HT_{1B}R (residues 34–390) was modified to contain a C-terminal TEV cleavage site and a His10 tag, cloned into plasmid pBac-PAK8 and recombinant baculoviruses were prepared using the FlashBAC ULTRA system (Oxford Expression Technologies). *Trichoplusia ni* cells were grown in ESF921 media (Expression Systems) to a density of 3×10^6 cells/ml, infected with 5HT_{1B}R baculovirus and incubated for 48 h at 27°C for expression. Purification of the receptor was performed in either DDM or LMNG using Ni^{2+} -affinity chromatography followed by SEC.

Expression, purification and stability of G protein subunits

For constructs see Fig 2 and S1–S3 Figs. Expression, purification and stability measurements by differential scanning fluorimetry (DSF) of the mini-G proteins as well as the non-lipidated $\text{G}\beta_1\gamma_2$ dimer, were performed following the protocols described in [24, 30]. The stability of mini-G proteins was also determined in detergent using native DSF (NanoTemper Prometheus). Mini-G proteins (2 mg/ml) in 50 mM HEPES pH 7.5 (KOH), 20 mM MgCl_2 , 50 mM NaCl, 1 μM GDP were mixed with either no detergent (control), 0.1% LMNG or 0.1% DDM. Samples were incubated on ice (minimum 30 min) prior to heating on the Prometheus (20% excitation, 15°C – 85°C , rate of $2.0^{\circ}\text{C}/\text{min}$) and the onset of scattering determined. The conditions used for the native DSF measurements mimic those used in the formation of a mini-G protein–GPCR complex (contains 1 μM GDP and detergent), which differ from the conditions used for good stability of the GDP-bound state of mini-G proteins (contains 1 mM GDP and no detergent).

SEC

(1) A_{2A}R–mini-G_s complex. The thioredoxin fusion construct of A_{2A}R was purified in DDM essentially as described previously [19]. A_{2A}R and mini-G protein were mixed in a 1:1.2 molar ratio, apyrase (0.1 U/ml final concentration) was added and the sample was incubated overnight on ice before loading onto a Superdex S200 10/300 size exclusion column (10 mM HEPES pH 7.5, 100 mM NaCl, 1 mM MgCl_2 , 100 μM NECA, 0.02% DDM; 4°C , 0.5 ml/min). Peak fractions were analysed by SDS-PAGE.

(2) 5HT_{1B}R–mini-G_{o1} complex. Donitriptan-bound 5HT_{1B}R purified in DDM was mixed with mini-G_{o1} in a 1:1.2 molar ratio. Apyrase (0.1 U/ml final concentration) was added

Fig 2. Alignment of G α GTPase domain protein sequences. The amino acid sequences aligned are of the wild type GTPase domains of the G α subunits used in this study to create the initial mini-G proteins. The G α H domain (not shown) was deleted and replaced by a linker (GGGGGGGG or GGSGGSGG in red). To construct mini-G proteins, the residues highlighted in grey were deleted and residues highlighted in magenta were mutated to the following (G α_s residue number and the CGN in superscript): D49^{S1H1.3}, N50^{S1H1.4}, D249^{S4.7}, D252^{S4H3.3}, D272^{H3.8}, A372^{H5.4}, I375^{H5.7}. The glycine mutation (G217D, highlighted in cyan) was incorporated into G α_{i1} only, to improve expression (see [Results & Discussion](#)). Numbering above the sequences is for G α_s and the CGN system below the sequence is used for reference [6].

<https://doi.org/10.1371/journal.pone.0175642.g002>

and the sample was incubated 4 h on ice before loading onto a Superdex S200 10/300 size exclusion column (20 mM HEPES pH 7.5, 100 mM NaCl, 1 mM MgCl₂, 1 μ M donitriptan, 0.03% DDM; 4°C, 0.5 ml/min). Peak fractions were analysed by SDS-PAGE.

FSEC assays

(1) **A_{2A}R.** Insect cell membranes containing a total of 20 μ g (560 pmol) wild-type A_{2A}R (20 \times 10⁶ cells) were solubilized for 30 min on ice in 40 mM HEPES pH7.5, 500 mM NaCl, 2 mM MgCl₂, 2 U/mL apyrase (Sigma-Aldrich), and 0.5% (v/v) DDM (final volume of 2 ml, concentration of A_{2A}R 280 nM). Insoluble material was removed by ultracentrifugation (30 min, 4°C, 135,000 xg). The supernatant was divided into aliquots for the subsequent assay. To 900 μ l of the supernatant was added either the agonist NECA or the inverse agonist ZM241385 (negative control), both at a final concentration of 60 μ M. GFP-mini-G_s (6 μ g; 110 pmol, final concentration 122 nM) was then added and allowed to bind for 90 min on ice before loading 200 μ l onto a Superdex S200 10/300 size exclusion column (buffer 20 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 μ M NECA or ZM241385, 0.03% DDM, 4°C, flow rate 0.45 ml/min). The control sample contained 6 μ g GFP-mini-G_s only in 500 μ l assay buffer. GFP fluorescence was detected by a Hitachi fluorometer (mV) set to an excitation of 488 nm and an emission of 525 nm.

(2) **β_1 AR.** Insect cell membranes containing a total of 8 μ g (178 pmol) wild-type β_1 AR (30 \times 10⁶ cells) were solubilized for 30 min on ice in 20 mM Tris-HCl pH8, 500 mM NaCl, 5 mM MgCl₂, 2 U/mL apyrase and 0.5% (v/v) DDM (final volume 2 ml, β_1 AR concentration 90 nM). Insoluble material was removed by ultracentrifugation (30 min, 4°C, 135,000 xg). The supernatant was divided into aliquots for the subsequent assay. Isoprenaline (100 μ M final concentration) or ICI118551 (10 μ M final concentration) were added to 500 μ l of the supernatant. GFP-mini-G_s (6 μ g, 110 pmol) was then added to give a final concentration of 122 nM and allowed to bind for 90 min on ice before loading 200 μ l onto a Superdex S200 10/300 size exclusion column (buffer 20 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 μ M isoprenaline or ICI118551, 0.03% DDM, 4°C, flow rate 0.45 ml/min). The control sample contained 6 μ g GFP-mini-G_s only in 500 μ l assay buffer.

(3) **5HT_{1B}R.** When detergent-solubilized unpurified receptor was used, insect cells expressing 610 pmol 5HT_{1B}R (40 \times 10⁶ cells) were resuspended in 20 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 2 U/ml apyrase to a final cell density of 20 \times 10⁶ cells/ml and solubilized with 0.5% DDM (45 min, 4°C, final volume 2 ml, 5HT_{1B}R concentration 305 nM). Insoluble material was removed by ultracentrifugation (30 min, 4°C, 135,000 xg). The supernatant was divided into 900 μ l aliquots for the subsequent assay. GFP-mini-G_{o1} (5 μ g, 100 pmol) was added (final concentration 111 nM) with either donitriptan or SB224289, each to a final concentration of 100 μ M, and allowed to bind for 90 min on ice before loading 500 μ l onto a Superdex S200 10/300 size exclusion column. The control sample contained 5 μ g GFP-mini-G_{o1} in 500 μ l assay buffer.

In some FSEC experiments, purified 5HT_{1B}R was used. Donitriptan-bound, purified receptor (120 μ g; 3 nmol) in either LMNG or DDM was incubated for 90 min on ice with 4 μ g (60–80 pmol) either of GFP-mini-G_{i1}, GFP-mini-G_{o1} or GFP-mini-G_s (negative control) in a final

volume of 450 μl . Samples (200 μl) were then loaded onto Superdex S200 10/300 size exclusion column (buffer 20 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl_2 , 1 μM donitriptan, 0.03% DDM or 0.001% LMNG buffer, 4°C, flow rate 0.45 ml/min). The final concentration of purified 5HT_{1B}R was 6.7 μM and the final concentrations of the mini-G proteins were as follows: GFP-mini-G_{s/i1}, 133 nM; GFP-mini-G_{i1}, 167 nM; GFP-mini-G_{o1}, 170 nM; GFP-mini-G_s, 167 nM; GFP-mini-G_{i1} $\beta_1\gamma_2$, 90 nM.

Fluorescent saturation binding assay (FSBA)

(1) β_1 AR. Membranes prepared from insect cells expressing β_1 AR (50 x 10⁶ cells) were solubilized in 20 mM Tris-HCl pH8, 500 mM NaCl, 3 mM imidazole, 2 U/ml apyrase 0.5% DDM (1 hour, 4°C, final volume 8 ml, β_1 AR concentration 37.5 nM). Insoluble material was removed by ultracentrifugation (30 min, 4°C, 135,000 xg) and the supernatant was divided into two aliquots. The agonist isoprenaline was added to one sample (final concentration 10 μM) and the inverse agonist ICI118551 was added to the other (final concentration 1 μM). Samples were then aliquoted 200 μl per well into a black Ni²⁺-coated 96-well plate (Pierce; Thermo Fisher). The receptor was allowed to bind *via* its His tag for 1 h on ice. The supernatant was then aspirated and 200 μl GFP-mini-G_s at varying concentrations (0 to 2.8 μM) were added and incubated for a further 90 min on ice. The supernatant was then removed by aspiration and each well washed 4 times with buffer A (10 μM isoprenaline (agonist), 20 mM Tris-HCl pH8, 100 mM NaCl, 1 mM MgCl_2 , 1 mg/mL BSA, 30 mM imidazole, 0.03% DDM,) or buffer B (1 μM ICI118551 (inverse agonist), 20 mM Tris-HCl pH8, 100 mM NaCl, 1 mM MgCl_2 , 1 mg/mL BSA, 30 mM imidazole, 0.03% DDM). Elution of the receptor–GFP-mini-G_s complex from the sides of the well to make a homogeneous solution was performed with 200 μl of the respective wash buffers that contained 300 mM imidazole. Fluorescence was then measured using a Pherastar plate reader (BMG Labtech, Inc.) with excitation at 485 nm and emission at 520 nm. ΔF data (fluorescence agonist condition minus fluorescence antagonist condition) corresponding to specific binding were analysed by non-linear regression using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA) and apparent K_D values derived from one site-specific binding analysis.

(2) A_{2A}R. The assay was performed essentially as described above for β_1 AR, but the buffer conditions were different. Solubilisation of insect cell membranes (40 x 10⁶ cells) was performed in 10 ml of 20 mM Tris-HCl pH8, 500 mM NaCl, 10 mM imidazole, 2 U/ml apyrase and 0.5% DDM (1 h, 4°C, final volume 10 ml, A_{2A}R concentration 112 nM). After ultracentrifugation, the agonist NECA (10 μM final concentration) was added to one supernatant sample and the inverse agonist ZM241385 (10 μM final concentration) to the other. Washing buffers for A_{2A}R were buffer C (10 μM NECA, 20 mM Tris-HCl pH8, 100 mM NaCl, 1 mM MgCl_2 , 1 mg/mL BSA, 50 mM imidazole, 0.03% DDM) or buffer D (10 μM ZM241385, 20 mM Tris-HCl pH8, 100 mM NaCl, 1 mM MgCl_2 , 1 mg/mL BSA, 50 mM imidazole, 0.03% DDM).

(3) 5HT_{1B}R. Insect cells expressing 5HT_{1B}R (50 x 10⁶ cells) were solubilized with buffer containing 10 μM Donitriptan, 20 mM Tris-HCl pH8; 500 mM NaCl; 10 mM imidazole, 2 U/ml apyrase and 0.5% DDM (1 h, 4°C, final volume 6 ml, 5HT_{1B}R concentration 127 nM). Insoluble material was removed by ultracentrifugation (30 min, 4°C, 135,000 xg) and 200 μl of supernatant was then aliquoted per well into a black Ni²⁺-coated 96-well plate. The receptor was allowed to bind *via* its His tag for 1 h on ice. The supernatant was then aspirated and 200 μl either of GFP-mini-G_{o1}, GFP-mini-G_{s/i1} or GFP-mini-G_s (negative control) at varying concentrations (from 0 to 5 μM) were added and incubated for a further 90 min on ice. The supernatant was then removed by aspiration and each well washed 4 times with buffer E (1 μM Donitriptan, 20 mM Tris-HCl pH8, 100 mM NaCl, 1 mM MgCl_2 , 1 mg/mL BSA, 50 mM imidazole, 0.03% DDM).

Elution was carried out with 200 μ L of buffer E containing 300 mM imidazole. ΔF data (fluorescence G_{o1} condition minus fluorescence G_s condition) corresponding to specific binding were analysed by non-linear regression using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA) and apparent K_D values derived from one site-specific binding analysis.

Competition binding assay

Insect cells expressing 5HT_{1B}R were resuspended in 1 ml of assay buffer (20 mM HEPES pH7.5, 100 mM NaCl, 1 mM MgCl₂, 1 mM ascorbate, 20 μ M pargyline) at a final concentration of 2×10^6 cells/ml. Cells were sheared by 10 passages through a bent 26G needle. The supernatant was diluted 10-fold in assay buffer and aliquots (900 μ l) taken for each sample. Mini-G protein (100 μ l, 25 μ M final concentration) or buffer (negative control) was added. The mixture was aliquoted into a 0.2 ml PCR plate, 96 μ l per well. Sumatriptan (12 μ l), prepared in assay buffer also containing 2 U/ml apyrase, was added to each well (final concentrations in the range of 100 pM to 1 mM). Non-specific binding was determined in the presence of 100 μ M donitriptan. Samples were mixed and incubated at 4°C for 2 h. [³H]-GR125743 (12 μ l) was added at its apparent K_D (10 nM) concentration. Samples were mixed and incubated at 4°C for 2 h before filtering through 96-well glass fibre GF/B filter plate (Merck Millipore) and washing with ice-cold assay buffer. Filters were dried, punched into scintillation vials and 4 ml Ultima Gold scintillant (Perkin Elmer) were added. Radioactivity was quantified by scintillation counting (1 min per sample) using a Tri-Carb counter (Perkin Elmer), and K_i values were determined using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA)

Thermostability assay

(1) **A_{2A}R.** Membranes from *Trichoplusia ni* cells expressing wild-type human A_{2A}R were resuspended in T_m buffer (25 mM HEPES pH 7.5, 100 mM NaCl, 1 mM MgCl₂) and homogenized by ten passages through a 26G needle. Mini-G protein was added at a final concentration of 25 μ M. ³H-NECA and unlabeled NECA, prepared in assay buffer containing 2 U/ml apyrase, were mixed in a molar ratio of 1:5 and added to the membranes to give a final concentration of 1 μ M (approximately ten-fold above the apparent K_D). The samples were incubated at room temperature for 1 h, then chilled on ice for 30 min. Decylmaltoside (DM) was added to a final concentration of 0.13%, and samples were incubated on ice for 1 h. Cell debris and insoluble material were removed by centrifugation (5 min, 20,000 xg , 4°C) and the supernatant was aliquoted (120 μ l) into PCR strips. Samples were heated to the desired temperature for exactly 30 min, then quenched on ice for 30 min. Samples (50 μ l) were loaded onto gel-filtration resin (Toyopearl HW-40F) packed into a 96-well filter plate (Millipore), which was centrifuged to separate receptor-bound from free radioligand [31]. Nonspecific binding was determined in the presence of 200 μ M unlabelled NECA. Radioactivity was quantified by liquid scintillation counting using a MicroBeta TriLux scintillation counter (PerkinElmer). Data were analysed by nonlinear regression using GraphPad Prism software. Apparent T_m values were derived from sigmoidal dose-response analysis performed by non-linear regression. Results represent the mean \pm SEM of two independent experiments, performed in duplicate.

(2) **NTSR1.** Cell pellets from 10 ml of insect cell cultures were resuspended in 1.8 ml buffer containing DDM to give a final buffer composition of 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 1% (w/v) DDM. The samples were placed on a rotating mixer at 4°C for 1 hour. Cell debris and non-solubilized material were removed by ultracentrifugation (152,800 xg , 4°C, 30 min), and the supernatant containing detergent-solubilized NTSR1 was used to test for thermal stability in the presence of NTS and mini-G proteins. For thermal denaturation

curves, the supernatants were diluted 6.67-fold into assay buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM MgCl₂) containing 22.5 μM mini-G protein and 10 nM ³H-NTS and incubated for 1 hour on ice. After addition of apyrase (0.25 units/ml, NEB), the sample was placed on ice for an additional 30 min. Samples (120 μl aliquots) were exposed to different temperatures between 0°C and 60°C for 30 min and placed on ice. Separation of receptor–ligand–mini-G protein complex from free ³H-NTS (100 μl) was achieved by centrifugation-assisted gel filtration (spin assay) using Bio-Spin 30 Tris columns (BioRad), equilibrated with RDB buffer [50 mM Tris-HCl pH7.4, 1 mM EDTA, 0.1% (w/v) DDM, 0.2% (w/v) CHAPS, 0.04% (w/v) CHS], essentially as described previously [32]. Control reactions on ice were recorded at the start and at the end of each denaturation experiment. The percentage of activity remaining after heat exposure was determined with respect to the unheated control. Data were analyzed by nonlinear regression using a Boltzmann sigmoidal equation in the Prism software (GraphPad).

(3) AT₁R. HEK 293 cells expressing wild type AT₁R were resuspended in a radioligand binding assay buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% BSA, 40 μg/ml bacitracin) and homogenized by sonication (4 sec pulse). Mini-G protein and apyrase were added at a final concentration of 25 μM and 0.1 U/ml, respectively. ¹²⁵I-Ang II and unlabeled Ang II were added at a concentration of 0.5 nM and 25 nM respectively (approximately 50 times the apparent K_D value). The sample was incubated at room temperature (20°C) for an hour, chilled on ice for 10 minutes and then digitonin was added to a final concentration of 1% and incubated on ice for an hour. Insoluble material was removed by centrifugation (2 min, 20,000 xg, 4°C). The reaction mix was split into a number of 115 μl aliquots and each was incubated at various temperatures for exactly 30 minutes. The reactions were then quenched on ice for 5 minutes. ¹²⁵I-Ang II bound to AT₁R was separated from unbound ¹²⁵I-Ang II using centrifugation-assisted gel filtration column, essentially as described previously [29]. Non-specific binding was determined using a 500-fold excess of cold ligand. Radioactivity was measured using liquid scintillation counting. Data was analysed by non-linear regression using GraphPad prism software and apparent T_m values were derived by non-linear regression of the sigmoidal dose-response curve.

Results and discussion

Initial development of new mini-G proteins

The recently designed minimal G protein, mini-G_s [24], comprises only the GαGTPase domain from G_s and 3 deletions and 7 mutations to thermostabilise it (Fig 2). Mini-G_s coupled to both the β₁-adrenergic receptor (β₁AR) and the adenosine A_{2A} receptor (A_{2A}R), and resulted in the same increase in agonist affinity as observed for heterotrimeric G_s coupling [20, 24]. However, there are 4 families of Gα subunits (Fig 1) and GPCRs couple to distinct G proteins depending upon their physiological function [25]. Therefore, to provide tools for the structure determination of GPCRs in their fully active state, it was necessary to develop versions of mini-G proteins for at least one member from each of the other families. All of the mutations and deletions used to create mini-G_s are located within conserved regions of the Gα subunit (Fig 2). Therefore, in theory, these mutations were potentially transferable to the other Gα families, allowing the production of a panel of mini-G proteins capable of coupling to any GPCR.

Archetypal members from each Gα family were selected and include the following: G_{olf} from the G_s family, G_{i1}, G_{o1}, G_z and G_t from the G_i family, G_q and G₁₆ from the G_{q/11} family, and G₁₂ from the G_{12/13} family. The mutations required to convert Gα_s into mini-G_s were transferred *en bloc* to the selected Gα proteins to produce a mini-G protein version of each (Fig 2). These mutations were the following: (i) deletion of all amino acid residues N-terminal of Ile/

Leu^{HN43}; (ii) deletion of the α -helical domain between residues H^{H1S2.12} and the Thr, three residues N-terminal to Ile^{S2.1}, and replacement with an 8 amino acid residue linker; (iii) deletion of 10 amino acid residues of switch III between Tyr^{S4H3.4} and Asn/Ser^{S4H3.15}; (iv) mutating 7 residues to D49^{S1H1.3}, N50^{S1H1.4}, D249^{S4.7}, D252^{S4H3.3}, D272^{H3.8}, A372^{H5.4}, I375^{H5.7}. Residue numbers are for G α_s and superscripts refer to the CGN system for comparing residues in G proteins [6]. Initial characterization of each mini-G protein was performed by assessing expression in *Escherichia coli* and purification by Ni²⁺-affinity chromatography and size exclusion chromatography (SEC). Four out of the eight engineered mini-G proteins (mini-G_{ol6}, mini-G_{i11}, mini-G_{o1} and mini-G₁₂) fulfilled these initial criteria *i.e.* they were all stable enough in their basal conformation to allow high-yield expression and purification. The yield of purified mini-G protein per litre of culture and their stability as measured by differential scanning fluorimetry (in parentheses) are as follows: mini-G_s, 100 mg/L (65°C); mini-G_{ol6}, 80 mg/L (65°C); mini-G_{o1}, 100 mg/L (64°C); mini-G₁₂, 25 mg/L (73°C). The worst expressed of the four new mini-G proteins was mini-G_{i11}, so an additional mutation G217D was incorporated and the truncation at the N-terminus shortened, which increased the yield of pure protein to 12 mg/L, although the stability was only 48°C. Thus, mini-G_{ol6}, mini-G_{i11}, mini-G_{o1} and mini-G₁₂ were all of sufficient stability to be used to test their ability to couple to relevant GPCRs. The amino acid sequences of the mini-G proteins are given in [S1 Fig](#).

Four mini-G proteins were not expressed in *E. coli*, namely mini-G_z, mini-G_t, mini-G₁₆ and mini-G_q (amino acid sequences are given in [S2 Fig](#)). The failure of the *en bloc* transfer of the deletions and mutations from mini-G_s, despite the high conservation of G protein structures, highlights our lack of understanding of the folding of these proteins. Indeed, it is well known that an accessory factor, Ric8, is required for the efficient folding of G_q in mammalian cells [33], and other unknown factors may also be required. For the purpose of this study, we therefore did not perform any further development of mini-G_{i11} and mini-G_z, given that two other members of the G_i family, mini-G_{i11} and mini-G_{o1}, already gave stable mini-G proteins. In contrast, as neither member of the G_q family tested produced a stable mini-G protein, we decided to develop alternative strategies to make a usable version of mini-G_q, whilst further work on mini-G₁₆ was terminated. The successful engineering of a version of mini-G_q chimera will be discussed later.

Assay development and validation using the mini-G_s system

The ultimate goal of developing mini-G proteins is the structure determination of GPCRs in the fully active state bound to an agonist and a mini-G protein. In the simplest format, this necessitates the purification of the GPCR in detergents and forming the G protein–GPCR complex from the purified components *in vitro*. It was therefore essential to devise some simple assays that could assess whether a mini-G protein had coupled to a GPCR in detergent solution. This turned out to be not as straightforward as originally anticipated due to the potential instability of either the GPCR and/or mini-G protein in either their inactive and/or active conformations. These issues were not obvious when the original work on the development of mini-G_s was performed, because mini-G_s is one of the most stable mini-G proteins developed and also the thermostabilised β_1 -adrenergic receptor (β_1 AR) and the wild type adenosine A_{2A} receptor (A_{2A}R) were both much more stable than other GPCRs. We therefore developed five separate assays for assessing whether a mini-G protein coupled to a GPCR and/or formed a stable complex in detergent. These were all first tested using mini-G_s coupling to β_1 AR and A_{2A}R. Each assay has its own limitations, which are often apparent in the subsequent sections where they were used on less stable receptors and the newly developed mini-G proteins, and these are discussed below. The five different assays that were used are the following:

(i) agonist affinity shift assay; (ii) thermostability assay (TSA); (iii) fluorescence-based saturation binding analysis (FSBA) of GFP-mini-G protein binding; (iv) fluorescence-detection size exclusion chromatography (FSEC); (v) size exclusion chromatography (SEC) of purified complex. A brief rationale for the use of each assay with their advantages and disadvantages are given below.

(i) Agonist affinity shift assay. The development of mini-G_s relied on the agonist affinity shift assay to identify those mutants that coupled to β_1 AR [24]. It is generally considered that the defining feature of G protein coupling is an increase in the affinity of an agonist for the G protein–GPCR complex compared to the GPCR alone. For example, wild type β_2 AR binds an agonist 100-fold more tightly when coupled to a G protein than the receptor alone [34]. However, the shift in agonist affinity in other receptors is often considerably smaller than that observed for β_2 AR, such as the 10-fold shift in agonist affinity observed in β_1 AR [34]. However, the advantage of this assay is that it can be performed using standard pharmacological procedures in high-throughput, using receptors in either membrane preparations or solubilized in detergent. Assays may use either a radiolabelled agonist in saturation binding experiments or, more usually, a radiolabelled antagonist in competition binding experiments [20, 24] (and see experiments below on the serotonin 5HT_{1B} receptor). The advantage of this assay is that it is very sensitive and can be performed on membrane-bound receptors *i.e.* in a format where the receptor is most stable in all conformations. The disadvantage of this assay is that it requires suitable radioligands for the receptor under study and these are not readily available for all GPCRs.

(ii) Thermostability assay. The thermostability of a detergent-solubilised GPCR depends upon the type of detergent used and whether the receptor is either ligand-free, agonist-bound or antagonist-bound [35, 36]. In addition, the receptor stability tends to be increased by an increase in affinity and/or decrease in the off-rate of the ligand [37]. Often, the agonist bound state is one of the least stable conformations of a receptor, presumably because agonists increase the probability of transitions to a fully active state. In the inactive state there is close packing of the intracellular surface of the transmembrane α -helices. Upon activation, the outward movement of helices 5 and 6 disrupts this close packed structure and creates a crevice where the C-terminus of the G protein binds, thus allowing G protein coupling [38]. The structures of non-rhodopsin GPCRs in the fully active state have been determined only when they have been stabilized through binding of a heterotrimeric G protein [2], a conformation-specific nanobody [15, 18, 19] or a mini-G protein [20]. The interface between a GPCR and a G protein is over 1000 Å² [2, 20], and is therefore predicted to increase the thermostability of the agonist-bound GPCR–G protein complex compared to the agonist-bound GPCR. This was observed for both β_1 AR and A_{2A}AR, which were consistently more stable in the agonist-bound state when coupled to mini-G_s in a variety of different detergents compared to when mini-G_s was absent [20, 24].

A typical thermostability assay measures how much of a radiolabelled agonist remains bound to a detergent-solubilised receptor after heating at different temperatures for 30 minutes [36]. The advantage of this assay is that it is fast and high-throughput and can be performed in any detergent of choice. Another advantage is that the agonist–GPCR–mini-G protein complex can be pre-formed in membranes, which may stabilise the receptor upon detergent solubilisation. If there is a shift in thermostability in the presence of a mini-G protein, then this is strongly suggestive of binding or coupling.

(iii) Fluorescence-detection size exclusion chromatography (FSEC). FSEC is a rapid methodology for assessing whether a membrane protein fused to GFP is stable in detergent by performing SEC on an unpurified detergent solubilise and monitoring GFP fluorescence in the eluate [39]. A membrane protein stable in detergent gives a symmetrical peak at a size

consistent with the molecular weight of the membrane protein plus the mass of specifically bound detergent and lipid. By fusing GFP to the N-terminus of mini-G proteins (S3 Fig), it was possible to use FSEC to monitor whether a stable complex was formed between the mini-G protein and a GPCR. The GFP-mini-G_s fusion protein has a molecular weight of 54 kDa and migrated with a retention volume of 15.1 ml on FSEC. When this was mixed with either unpurified DDM-solubilised β_1 AR or A_{2A}R in the presence of an agonist, then an additional peak was observed at 12.1–12.5 ml (Fig 3A and 3C), which was consistent with the molecular weight of the detergent-solubilised receptor bound to GFP-mini-G_s (~180 kDa). This additional peak was not observed if the receptors were bound to an inverse agonist. An additional peak was sometimes observed at a retention volume of 8 ml, which corresponds to the void volume of the SEC column and was due presumably to aggregates of GFP-mini-G_s.

The advantage of this assay is that it is a quick assessment of whether a GPCR forms a complex with a mini-G protein, because the receptor does not need to be purified and the SEC experiment takes under an hour. However, a major limitation is that only small amounts of GFP-mini-G_s can be used per experiment to avoid saturation of the detector and producing a very broad peak that would obscure the presence of the complex between the GPCR and GFP-mini-G protein. An additional limitation is the amount of membranes that can be solubilized efficiently using 0.5% DDM (to ensure maximal stability of mini-G_s; see below) and maintaining the small volume (500 μ l) required for loading on the FSEC column (24 ml). Thus in the data shown (Fig 3A and Fig 3C) the amount of receptor is limiting and only a proportion of the GFP-mini-G_s is bound to receptor and is shifted in apparent molecular weight. In addition, the receptor–mini-G protein complex must be detergent-stable for a peak to be observed. Many GPCR–G protein complexes are too unstable to be observed in DDM and therefore it is essential to assess milder detergents such as LMNG (see section on the serotonin 5HT_{1B} receptor).

(iv) Fluorescence-based saturation binding analysis of mini-G protein binding. To determine the affinity of mini-G protein binding to a receptor, the fluorescence-based saturation binding assay (FSBA) was developed. In this assay, the amount of the GFP-mini-G protein specifically bound to an immobilized receptor was determined using a fluorescent plate reader. As proof of principle, DDM-solubilized β_1 AR or A_{2A}R were immobilized onto Ni²⁺-coated wells of a 96-well plate *via* their C-terminal poly-histidine tag, in the presence of either an agonist or inverse agonist. GFP-mini-G_s was then added at increasing concentrations. After washing to remove any non-specifically bound GFP-mini-G_s, the amount of GFP-mini-G_s fluorescence was measured (Fig 3B and 3D). GFP-mini-G_s showed a specific saturated binding to the receptor with apparent K_D values of 200 ± 1 nM ($n = 2$) and 430 ± 24 nM ($n = 2$) for GFP-mini-G_s binding to β_1 AR and A_{2A}R, respectively.

The FSBA is a simple assay for determining the affinity of mini-G protein binding to a receptor *in vitro*. However, it must be appreciated that the apparent affinity determined may be specific only for the conditions in the assay. In particular, the type of detergent used may have a profound effect on the affinity, especially if it slightly destabilizes the active state of the receptor. The agonist may also affect the apparent affinity of the mini-G protein, depending on how effective the agonist is in stabilizing the active state of the receptor. However, the FSBA remains a useful tool for biophysical analyses of mini-G protein binding to a receptor.

(v) Size exclusion chromatography (SEC). The ultimate biochemical assay for observing coupling of mini-G proteins to a receptor is combining the purified components *in vitro* and then observing the co-elution of the relevant proteins on SEC [24]. Purified A_{2A}R and purified mini-G_s were mixed at a molar ratio of 1:1.2 in the presence of the agonist NECA, the complex allowed to form and then separation was performed by SEC. The A_{2A}R–mini-G_s complex resolved as a predominant peak with an apparent molecular weight of 153 kDa compared with

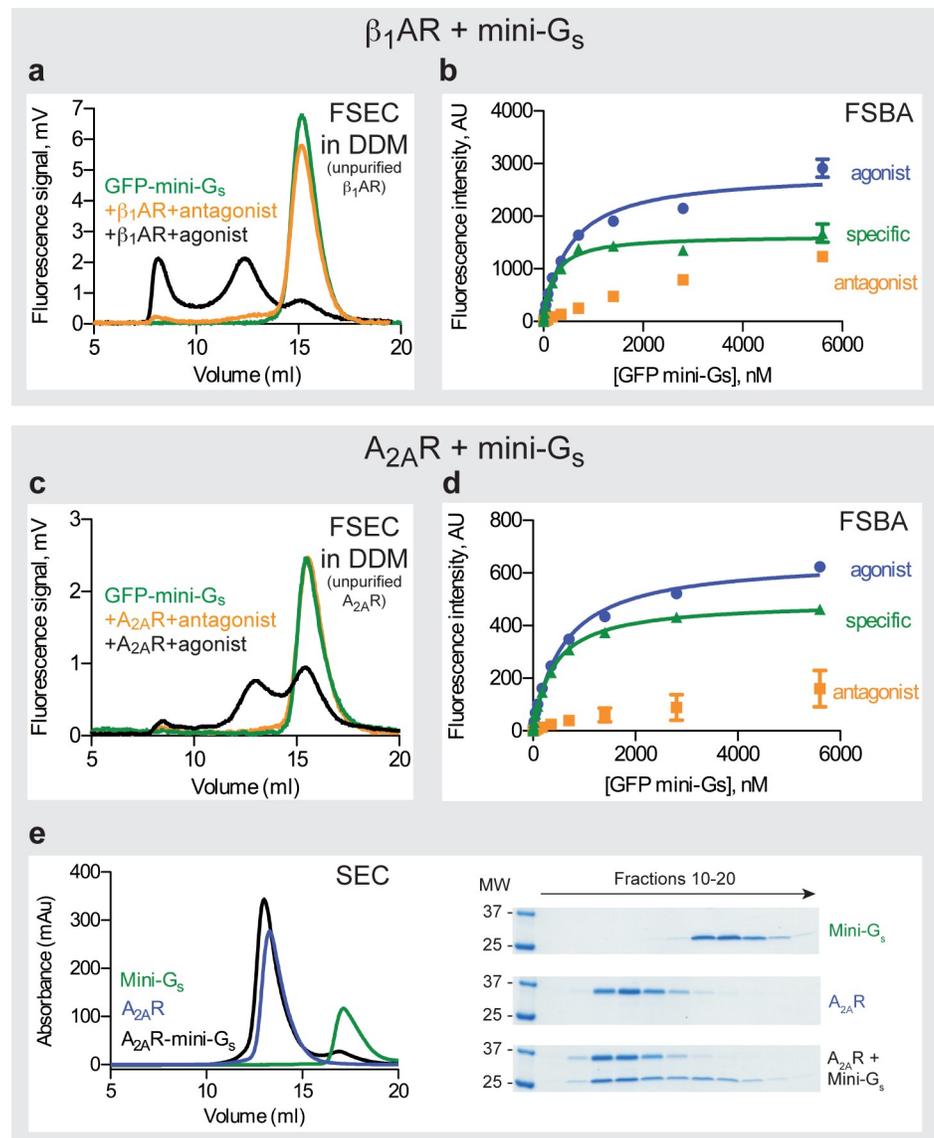


Fig 3. The β₁AR–mini-G_s and A_{2A}R–mini-G_s complexes. (a) FSEC traces of GFP-mini-G_s with β₁AR (retention volumes are given in parentheses): green, GFP-mini-G_s (15.1 ml); orange, GFP-mini-G_s with β₁AR bound to the inverse agonist ICI118551 (15.1 ml); black, GFP-mini-G_s with β₁AR bound to the agonist isoprenaline (8 ml, 12.1 ml and 15.1 ml). Representative chromatograms from at least two independent experiments are shown. (b) Measurement of GFP-mini-G_s affinity to DDM-solubilized β₁AR using a fluorescent saturation binding assay (FSBA); blue circles, β₁AR bound to the agonist isoprenaline (total binding); orange squares, β₁AR bound to the inverse agonist ICI118551 (non-specific binding); green triangles, specific binding, with an apparent *K_D* of 200 ± 1 nM (mean ± SEM, *n* = 2). Curves shown are from a representative experiment. (c) FSEC traces of GFP-mini-G_s with DDM-solubilised A_{2A}R (retention volumes are given in parentheses): green, GFP-mini-G_s (15.1 ml); orange, GFP-mini-G_s with A_{2A}R bound to the inverse agonist ZM241385 (15.1 ml); black, GFP-mini-G_s with A_{2A}R bound to the agonist NECA (12.5 ml and 15.1 ml). Representative chromatograms from at least two independent experiments are shown. (d) Measurement of mini-G_s affinity to DDM-solubilized A_{2A}R using FSBA: blue circles, A_{2A}R bound to the agonist NECA (total binding); orange squares, A_{2A}R bound to the inverse agonist ZM241385 (non-specific binding); green triangles, specific binding, with an apparent *K_D* of 430 ± 24 nM (mean ± SEM, *n* = 2). (e) Analytical size exclusion chromatography (SEC) of mini-G_s bound to purified A_{2A}R (retention volumes are given in parentheses): black, A_{2A}R–mini-G_s complex, 153 kDa (13 ml); blue, A_{2A}R, 133 kDa (13.3 ml); green, mini-G_s, 22 kDa (17.2 ml). Three panels to the right of the SEC traces are coomassie blue-stained SDS-PAGE gels of fractions from 3 separate SEC experiments: top panel, mini-G_s; middle panel, A_{2A}R; bottom panel, mini-G_s mixed with NECA-bound A_{2A}R (1.2:1 molar ratio).

<https://doi.org/10.1371/journal.pone.0175642.g003>

133 kDa for the receptor alone and 22 kDa for mini-G_s alone. SDS-PAGE analysis confirmed the presence of both A_{2A}R and mini-G_s in fractions from the 153 kDa complex (Fig 3E).

The advantage of using purified components and SEC for analyzing complex formation is that complex formation is observed unambiguously. The conditions for complex formation can be refined and the stability of the complex can be assessed readily after a period of days by repeating the SEC. These data are essential for successful determination of the structure of a GPCR–mini-G protein complex. The disadvantage of this assay is that sufficient quantities of purified receptor are required and this may be limiting in the initial stages of a project. The limitation of the amount of purified receptor required for SEC may be partially overcome by analyzing complex formation using FSEC (see section on 5HT_{1B}R).

Characterisation of mini-G proteins

Mini-G_{off} couples and stabilizes A_{2A}R. The GTPase domains of G_{off} and G_s share 87% sequence identity (80% for the full length α subunits) and both G proteins couple to A_{2A}R [40]. Of the 17 amino acid residues in mini-G_s that make direct contact to residues in A_{2A}R in the crystal structure of the A_{2A}R–mini-G_s complex [20], all of these residues are identical except that two Arg residues in G_s are replaced with two Lys residues in G_{off}. Despite the high degree of sequence homology between these two isoforms, G α_{off} is far more difficult to overexpress than G α_s , in fact, the only method reported to produce functional G α_{off} is co-expression with the molecular chaperone RIC8B in insect cells [41]. Therefore, we constructed mini-G_{off} to investigate whether the mini-G protein version would be better expressed than native α subunit. Mini-G_{off} was constructed by transferring the 7 point mutations and 3 deletions from mini-G_s (Fig 2) and mini-G_{off} was highly expressed in *E. coli* and as stable as mini-G_s. The coupling of mini-G_{off} to A_{2A}R was assessed by SEC of the complex assembled *in vitro* from purified proteins and a thermostability assay [20, 24]. Purified NECA-bound A_{2A}R was mixed with mini-G_{off} and analysed by SEC and SDS-PAGE (Fig 4A). The apparent molecular weight of mini-G_{off} was 23 kDa (17.1 ml); theoretical molecular weight 26 kDa) and the apparent molecular weight of purified A_{2A}R in DM was 133 kDa (13.3 ml). The complex A_{2A}R–mini-G_{off} resolved as a predominant peak with an apparent molecular weight of 153 kDa (13 ml) and

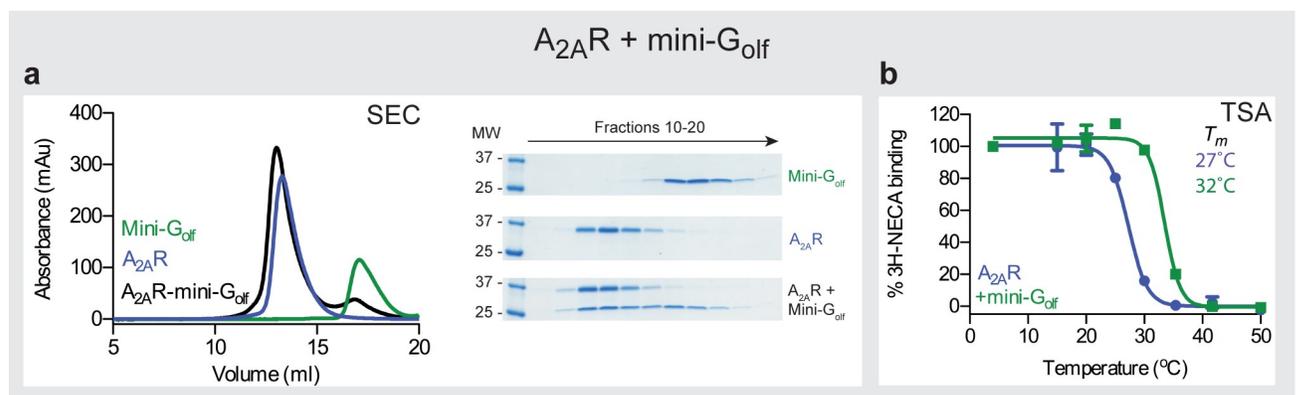


Fig 4. The A_{2A}R–mini-G_{off} complex. (a) Analytical SEC of mini-G_{off} bound to purified A_{2A}R (retention volumes are given in parentheses): black, A_{2A}R–mini-G_{off} complex, 153 kDa (13 ml); blue, A_{2A}R, 133 kDa (13.3 ml); green, mini-G_{off}, 23 kDa (17.1 ml). Three panels to the right of the SEC traces are coomassie blue-stained SDS-PAGE gels of fractions from 3 separate SEC experiments: top panel, mini-G_{off}; middle panel, A_{2A}R; bottom panel, mini-G_{off} mixed with NECA-bound A_{2A}R (1.2:1 molar ratio). (b) Thermostability assay (TSA) of unpurified DM-solubilized, ³H-NECA-bound A_{2A}R. Data were analysed by nonlinear regression and apparent *T_m* values were determined from analysis of the sigmoidal dose-response curves fitted. *T_m* values represent mean±SEM of two independent experiments, each performed in duplicate: blue circles, no mini-G_{off} (27 ± 0.3 °C); green squares, mini-G_{off} (33 ± 1 °C). Curves shown are from a representative experiment.

<https://doi.org/10.1371/journal.pone.0175642.g004>

contained both A_{2A}R and mini-G_{olf}. Mini-G_{olf} also stabilized agonist-bound DM-solubilised A_{2A}R, with mini-G_{olf}-coupled A_{2A}R showing an apparent T_m of $32 \pm 1^\circ\text{C}$ in comparison with $27 \pm 0.3^\circ\text{C}$ for the receptor alone (Fig B). This stability was similar to that obtained with mini-G_s (33°C) under the same conditions [20].

The results with mini-G_{olf} were very encouraging in terms of both the transferability of the mutations, the expression and stability of the mini-G_{olf} and the stability of the A_{2A}R–mini-G_{olf} complex. Thus where there is a high degree of homology between G proteins, then there is good transferability of the mutations, as was previously observed for the transfer of thermostabilising mutations between GPCRs [42]. These data also suggested that even if the native α subunit is poorly expressed the mini-G protein version may be highly expressed and very stable.

Development of chimeric mini-G_{s/q} to study G_q-coupled receptors. The expression of mini-G_q in *E. coli* was unsuccessful. One possibility to explain this is that efficient folding of G_q *in vivo* is dependent on the molecular chaperone Ric8 [33] and that mini-G_q had a similar requirement. Indeed, co-expression of Ric8 with mini-G_q in the baculovirus expression system led to the overproduction of mini-G_q. However, upon purification of mini-G_q it was not possible to dissociate Ric8 (results not shown), suggesting that the mini-G_q was perhaps not correctly folded and/or was very unstable. Given the lack of success in transferring the mini-G protein mutations from G_s to G_q, another strategy was developed.

The second strategy used to try and develop mini-G_q was to transfer the specificity determinants of G_q onto mini-G_s. It is well established that the C-terminal region of a G α subunit forms the main receptor binding site [43] and is one of the main determinants of coupling specificity [44, 45]. Mutating as few as 3–5 amino acids at the C-terminus of the G alpha subunit has been shown to switch the specificity of coupling to some GPCRs [44, 45]. However, the two GPCR–G protein structures published to date [2, 20] revealed an extensive interface between the receptors and G α , suggesting that other regions of the G protein may also play a role in specificity. Recent *in vivo* FRET studies suggest that residues within the $\alpha 5$ helix, but distal to the five C-terminal residues, strongly influence specificity [46].

Mini-G_s did not couple to any of the G_q-coupled receptors tested (results not shown). We then evaluated a number of mini-G_{s/q} chimeras (S4 Fig) for both gain of binding to G_q-coupled receptors (Fig 5A and 5B) and loss of binding to the cognate G_s-coupled receptor A_{2A}R, predominantly using thermostability assays (Fig 5C) and SEC (S5 Fig). First, the chimera mini-G_{s/q}57 was constructed in which the five C-terminal amino acids of mini-G_s (Q^{H5.22}YELL^{H5.26}) were changed to those found in G α_q , which required three mutations (Q390E^{H5.22}, E392N^{H5.24} and L394V^{H5.26}). There was no increase in thermostability of agonist-bound AT₁R or NTSR1 in the presence of mini-G_{s/q}57 that might suggest coupling (Fig 5A and 5B). In fact, there was a decrease in thermostability of AT₁R in the presence of mini-G_{s/q}57, suggesting that binding had occurred, but that residues of G_s were clashing with the receptor and causing its destabilisation. Furthermore, a complex between mini-G_{s/q}57 and A_{2A}R was still observed (Fig 5C and S5 Fig), suggesting that the mutations were insufficient to change the specificity of G_s to G_q. Therefore, the chimera mini-G_{s/q}58 was constructed in which the final 19 amino acid residues in the $\alpha 5$ helix of mini-G_s (Phe376^{H5.8}—Leu394^{H5.26}) were changed to those in G α_q ; this required 13 mutations (N377A^{H5.9}, D378A^{H5.10}, C379V^{H5.11}, R380K^{H5.12}, I382T^{H5.14}, Q384L^{H5.16}, R385Q^{H5.17}, M386L^{H5.18}, H387N^{H5.19}, R389K^{H5.21}, Q390E^{H5.22}, E392N^{H5.24} and L394V^{H5.26}). Mini-G_{s/q}58 did not couple to A_{2A}R (Fig 5C and S5 Fig), demonstrating that residues in the $\alpha 5$ helix beyond the C-terminal 5 amino acids are important in G protein specificity. However, there was no significant shift in the thermostability of the G_q-coupled receptor NTSR1 in the presence of mini-G_{s/q}58 (Fig 5B). We reasoned that this may be because the stability of mini-G_{s/q}58 was impaired, because mutating the last 19 amino acid residues in mini-G_s would have also changed residues buried in

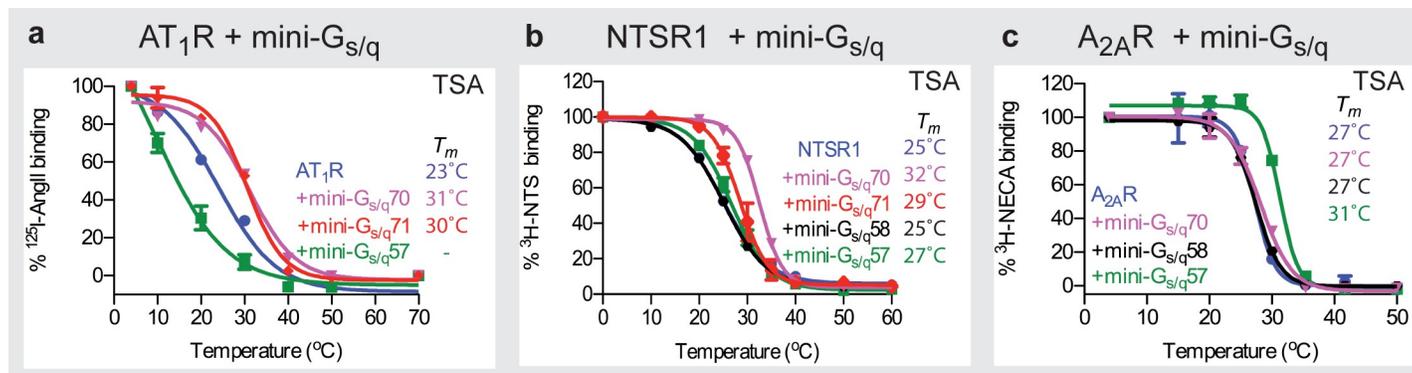


Fig 5. Thermostability assays of various complexes between mini-G_{s/q} chimeras and GPCRs. (a) Thermostability of unpurified digitonin-solubilized, ¹²⁵I-AngII-bound AT₁R (*T_m* values in parentheses): blue circles, no mini-G_{s/q} (23 ± 0.4°C); green squares, mini-G_{s/q}57 (*T_m* not determined); magenta inverted triangles, mini-G_{s/q}70 (31 ± 1°C); red triangles, mini-G_{s/q}71 (30 ± 0.8°C). (b) Thermostability of unpurified DDM-solubilized, ³H-NTS-bound NTSR1: blue, no mini-G_{s/q} (25 ± 0.4°C); green squares, mini-G_{s/q}57 (27 ± 0.7°C); black circles, mini-G_{s/q}58 (25 ± 0.4°C); magenta inverted triangles, mini-G_{s/q}70 (32 ± 0.3°C); red diamonds, mini-G_{s/q}71 (29 ± 1.1°C). (c) Thermostability of unpurified DM-solubilized, ³H-NECA-bound A₂AR: blue circles, no mini-G_{s/q} (27 ± 0.3°C); green squares, mini-G_{s/q}57 (31 ± 0.3°C); black circles, mini-G_{s/q}58 (27 ± 0.5°C); magenta inverted triangles, mini-G_{s/q}70 (27 ± 0.2°C). In all panels, data (n = 3) were analysed by nonlinear regression and apparent *T_m* values were determined from analysis of the sigmoidal dose-response curves fitted with values shown as mean ± SEM. Curves shown are from a representative experiment.

<https://doi.org/10.1371/journal.pone.0175642.g005>

the core of the G protein, thus affecting the stability of the mini-G_s backbone. Therefore, a refined version of this chimera, mini-G_{s/q}70, was constructed in which residues in the α5 helix whose side chains formed direct contacts (3.9 Å cut-off) with either β₂AR [2] or A₂AR [20] in the G protein-bound structures were mutated to match those in Gα_q (R380K^{H5.12}, Q384L^{H5.16}, R385Q^{H5.17}, H387N^{H5.19}, E392N^{H5.24} and L394V^{H5.26}; S4 Fig). In addition, the mutation Q390E^{H5.22} was included, despite only making contact to A₂AR via its backbone, as it is buried in the receptor-G protein interface and may be important for binding to G_q-coupled receptors. Mini-G_{s/q}70 gave better binding to both G_q-coupled receptors tested, NTSR1 and AT₁R, and showed no binding to A₂AR (Fig 5 and S5 Fig).

Two other chimeras were also constructed to try and improve on mini-G_{s/q}70. Mini-G_{s/q}72 contained the additional mutation C379V^{H5.11} compared to mini-G_{s/q}70 and, although the C379^{H5.11} side chain does not form direct contacts with either A₂AR or β₂AR, its mutation to Val is predicted to introduce a direct interaction between the Val γ2 carbon and Leu110 from A₂AR. However, the AT₁R-mini-G_{s/q}72 complex did not have a higher thermostability than AT₁R-mini-G_{s/q}70 (results not shown). Finally, the chimera mini-G_{s/q}71 was constructed in which residues from other regions of Gα that form direct contacts with either β₂AR [2] or A₂AR [20] were mutated to match those in Gα_q. This included the seven mutations in mini-G_{s/q}70 (R380K^{H5.12}, Q384L^{H5.16}, R385Q^{H5.17}, H387N^{H5.19}, Q390E^{H5.22}, E392N^{H5.24} and L394V^{H5.26}) and six additional mutations (A39R^{HNS1.3}, H41L^{S1.2}, D343K^{H4.23}, L346V^{H4.26}, R347D^{H4.27} and Y358I^{H4S6.11}). D343^{H4.23} was the only amino acid residue whose side chain did not interact with either A₂AR or β₂AR, but the mutation to Lys was included because the longer side chain could potentially interact with a receptor and the charge reversal may be important for specificity. Conversely, Thr350^{H4S6.3} was *not* mutated to Pro in mini-G_{s/q}71 even though its side chain forms direct contacts with β₂AR. Alignment of Gα_s with two independently solved structures of Gα_q [47, 48] showed that this region of the G proteins differ significantly and thus, in Gα_q, this residue is unlikely to interact with the receptor. However, after all these considerations to make an improved version of mini-G_{s/q}70, mini-G_{s/q}71 did not improve the thermostability of agonist-bound G_q-coupled receptors compared to mini-G_{s/q}70 (Fig 5A and 5B).

Mini-G_{i1}: Tackling stability issues. Transfer of the 7 point mutations and 3 deletions from mini-G_s into Gα_{i1} to make mini-G_{i1} was not successful, as the resultant protein was very

poorly expressed and had low stability (results not shown). Whilst the work on developing chimeras of mini- $G_{s/q}$ was underway, we decided to first study the reasons why mini- G_{i1} appeared to be so unstable. Therefore, to improve expression, stability and to allow binding of the mini- G_{i1} to the $\beta\gamma$ subunits, the N-terminus (residues 4–18) was re-inserted, Asp249^{H3.8} was mutated back to Leu, and the G217D^{H2S4.3} mutation introduced based on a sequence comparison between G_{i1} (poorly expressed) and G_s/G_o (highly expressed) (Fig 2 and S1 Fig). The resultant mini- G_{i1} (construct 46) yielded only 12 mg of purified protein per litre of culture and was 17°C less stable than mini- G_s , but was suitable for initial studies in GPCR coupling.

The serotonin 5HT_{1B} receptor (5HT_{1BR}) was used as a model G_i -coupled receptor for developing mini- G_{i1} because it could be expressed and purified in DDM using the baculovirus expression system and its structure determined in the inactive state. Initially, GFP-mini- G_{i1} was tested using FSEC for binding to purified 5HT_{1BR} (in DDM) and bound to the agonist donitriptan. However, the GFP-mini- G_{i1} (S3 Fig) migrated at 13.5 ml in the absence of receptor or in the presence of donitriptan-bound 5HT_{1BR}, indicating that no coupling occurred (Fig 6C). However, when the LMNG-purified 5HT_{1BR} was used, the FSEC showed two peaks, one corresponding to free GFP-mini- G_{i1} with a retention volume of 14.3 ml and the other corresponding to GFP-mini- G_{i1} bound to donitriptan-activated 5HT_{1BR}, with a retention volume of 12.2 ml (Fig 6D). The presence of two peaks, suggested that mini- G_{i1} was not stable in detergent when in a complex with 5HT_{1BR}. The detergent instability of the complex could also have been due to the receptor, but because agonist-bound 5HT_{1BR} had been crystallised previously [10], we felt this was unlikely. In order to improve the stability of mini- G_{i1} , the heterotrimer was formed between GFP-mini- $G_{i1}46$ (S3 Fig) and $\beta_1\gamma_2$ and tested by FSEC. The GFP-minotrimer in complex with the LMNG-purified 5HT_{1BR} resolved as a single peak with a retention volume of 11.8 ml compared to 14.3 ml for the free GFP-mini- $G_{i1}\beta_1\gamma_2$ trimer (Fig 6F). Thus the $\beta_1\gamma_2$ subunits restored the stability of mini- G_{i1} .

Although the mini- $G_{i1}\beta_1\gamma_2$ trimer coupled successfully to LMNG-solubilised 5HT_{1BR}, this is not as desirable for crystallography as a mini-G protein coupled receptor due to the large size of the heterotrimeric G protein. Therefore, following the successful strategy of changing the coupling of mini- G_s to that of G_q by making a mini- $G_{s/q}$ chimera, the same strategy was applied to engineer a mini- $G_{s/i1}$ chimera (S1 Fig and S6 Fig). Therefore 9 mutations (C379V^{H5.11}, R380T^{H5.12}, Q384I^{H5.16}, R385K^{H5.17}, H387N^{H5.19}, Q390D^{H5.22}, Y391C^{H5.23}, E392G^{H5.24} and L394F^{H5.26}) were introduced into the $\alpha 5$ helix of mini- G_s to change its coupling specificity to that of G_{i1} . A complex between GFP-mini- $G_{s/i1}43$ (S3 Fig) with donitriptan-bound DDM-purified 5HT_{1BR} resolved as a single peak with a retention volume of 13.2 ml compared to 15.1 ml for the free GFP-mini- $G_{s/i1}$ (Fig 6E). Thus mini- $G_{s/i1}$ was indeed more stable than mini- G_{i1} . The specificity of mini- G_s compared to mini- $G_{s/i1}$ for donitriptan-bound, DDM-solubilised 5HT_{1BR} was confirmed using FSBA (Fig 6B). No specific coupling of GFP-mini- G_s to 5HT_{1BR} was observed, although specific coupling to GFP-mini- $G_{s/i1}$ (apparent K_D 390 nM; Fig 6B) was confirmed.

In order to compare all of the mini- G_{i1} constructs and the role of $\beta_1\gamma_2$, agonist affinity shift assays were performed on 5HT_{1BR}. The uncoupled receptor showed a K_i for the agonist sumatriptan in this assay of 280 ± 10 nM, which was shifted by mini- $G_{i1}46$ and mini- $G_{s/i1}43$ to 80 ± 13 nM and 36 ± 2 nM, respectively (Fig 6A). However, addition of $\beta_1\gamma_2$ to the mini-G proteins resulted in a further increase in agonist affinity to 15 ± 1 nM and 7.2 ± 0.8 nM for mini- $G_{i1}46\text{-}\beta_1\gamma_2$ and mini- $G_{s/i1}43\text{-}\beta_1\gamma_2$, respectively. Thus despite the successful generation of both mini- G_{i1} and mini- $G_{s/i1}$, their stability is still not perfect as binding of $\beta_1\gamma_2$ stabilises the mini-G proteins and elicits a greater increase in agonist affinity upon coupling of the mini-trimers.

Coupling of mini- G_{o1} to 5HT_{1BR}. The GTPase domain of G_{o1} and G_{i1} are highly conserved (80% identity), but the mini-G proteins derived from them behaved very differently.

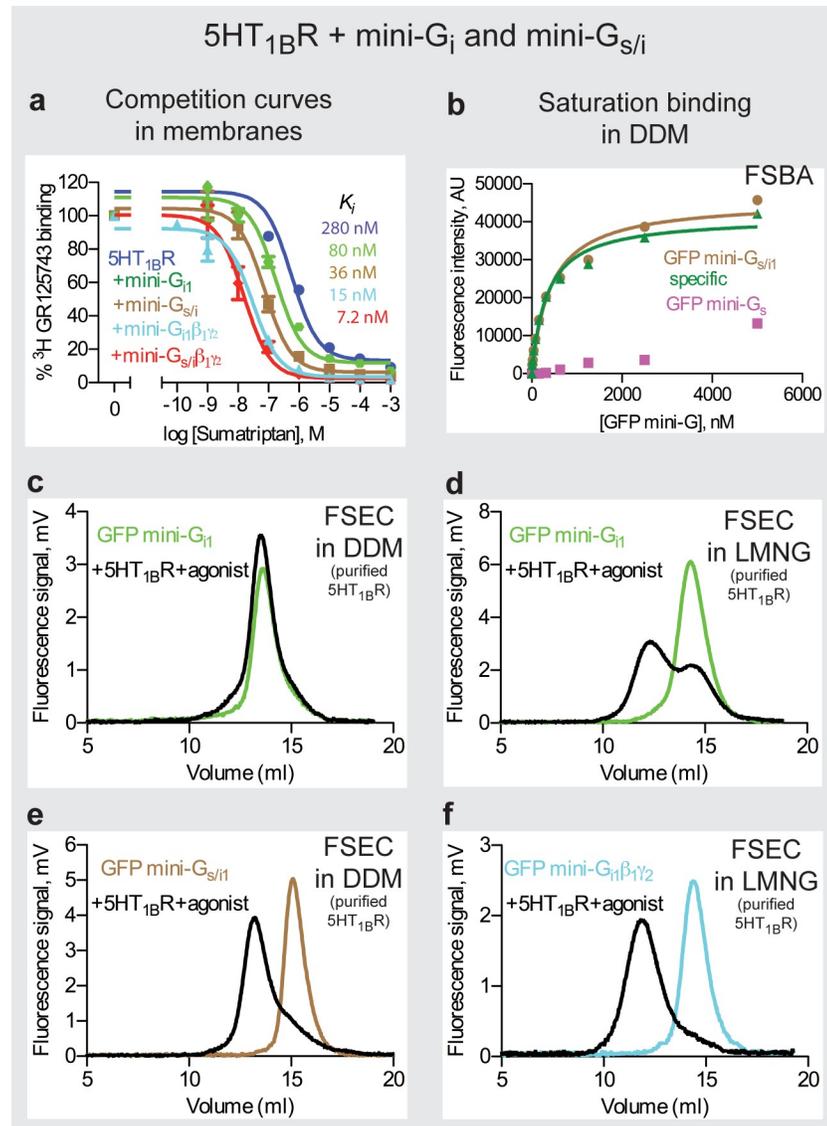


Fig 6. The 5HT_{1B}R-mini-G_{i1} complexes. (a) Mini-G_{i1} coupling increases agonist affinity to 5HT_{1B}R. Competition binding curves were performed in duplicate (n = 2) by measuring the displacement of the antagonist ³H-GR125743 with increasing concentration of the agonist sumatriptan (K_i values representing mean ± SEM in parentheses): blue circles, 5HT_{1B}R (K_i 280 ± 10 nM); green hexagons, 5HT_{1B}R and mini-G_{i1} (K_i 80 ± 13 nM); brown squares, 5HT_{1B}R and mini-G_{s/i1} (K_i 36 ± 2 nM); pale blue triangles, 5HT_{1B}R and mini-G_{i1}β₁γ₂ (K_i 15 ± 1 nM); red diamonds, 5HT_{1B}R and mini-G_{s/i1}β₁γ₂ (K_i 7.2 ± 0.8 nM). Error bars represent the SEM. (b) Measurement of mini-G_{s/i1} chimera affinity to the DDM-solubilized, donitriptan-bound 5HT_{1B}R using FSBA: brown circles, 5HT_{1B}R and GFP-mini-G_{s/i1} (total binding); purple squares, 5HT_{1B}R and GFP-mini-G_s (non-specific binding); green triangles, specific binding. The apparent K_D of 390 ± 47 nM represents the mean ± SEM of two independent experiments. Curves shown are from a representative experiment. (c) FSEC traces of GFP-mini-G_{i1} with 5HT_{1B}R in DDM: black, GFP-mini-G_{i1} and donitriptan-bound 5HT_{1B}R purified in DDM (13.5 ml); green, GFP-mini-G_{i1} (13.5 ml). (d) FSEC traces of GFP-mini-G_{i1} with 5HT_{1B}R in LMNG: black, GFP-mini-G_{i1} and donitriptan-bound 5HT_{1B}R purified in LMNG (12.2 ml and 14.3 ml); green, GFP-mini-G_{i1} (14.3 ml). (e) FSEC traces of GFP-mini-G_{s/i1} with 5HT_{1B}R: black, GFP-mini-G_{s/i1} and donitriptan-bound 5HT_{1B}R purified in DDM (13.2 ml); brown, GFP-mini-G_{s/i1} (15.1 ml). (f) FSEC traces of GFP-mini-G_{i1}β₁γ₂ with 5HT_{1B}R: black, GFP-mini-G_{i1}β₁γ₂ and donitriptan-bound 5HT_{1B}R purified in LMNG (11.8 ml); pale blue, GFP-mini-G_{i1}β₁γ₂ (14.3 ml). In panels c-f, retention volumes are given in parentheses.

<https://doi.org/10.1371/journal.pone.0175642.g006>

Unlike the unstable mini-G₁₁, mini-G_{o1} expressed well (100 mg/L), had high stability comparable to mini-G_s and it was largely insensitive to the presence of mild detergents. Since 5HT_{1B}R couples to both G_o and G_i family members [49], we tested mini-G_{o1} coupling to 5HT_{1B}R and compared the results to coupling with mini-G₁₁ (see above). On FSEC, GFP-mini-G_{o1}12 (S3 Fig) partially coupled to donitriptan-bound, DDM-solubilised 5HT_{1B}R (unpurified), with the higher molecular weight species (retention volume 13 ml) reduced when the receptor was bound to an antagonist (Fig 7C). This was in contrast to the results with mini-G₁₁ under the same conditions where no binding was observed (Fig 6C). The partial coupling probably resulted from the low amount of 5HT_{1B}R used in the assay, because when the experiment was repeated using purified 5HT_{1B}R and GFP-mini-G_{o1}, all of the GFP-mini-G_{o1} bound to the receptor (Fig 7E). This is consistent with what was observed for β₁AR and A_{2A}AR (Fig 3). The 5HT_{1B}R–mini-G_{o1} complex was purified by SEC and SDS-PAGE indicated co-elution of 5HT_{1B}R and mini-G_{o1} in a 1:1 molar ratio (Fig 7D). GFP-mini-G_{o1} bound to DDM-solubilised 5HT_{1B}R in the presence of donitriptan with an apparent K_D of 180 ± 24 nM (Fig 7B). In membranes, mini-G_{o1}12 shifted the agonist affinity for 5HT_{1B}R from 280 ± 10 nM to 32 ± 3 nM (Fig 7A).

The properties of mini-G_{o1} make this an ideal choice for structural studies of G_o/G_i coupled receptors, rather than using mini-G_{s/i}, as it is more highly expressed and more tolerant of detergents. This is exemplified by the stability of the donitriptan-bound 5HT_{1B}R–mini-G_{o1} complex made from purified components (S7 Fig).

Conclusions

The aim of the work presented here was to generate a range of mini-G proteins that could be used as a basis for the structure determination of GPCRs in their fully active state. The original work in developing mini-G proteins was performed on G_s [24], which turned out to be one of the best expressed and most stable of the mini-G proteins. The structures of GPCRs and G proteins are highly conserved [4], and there are thought to be highly conserved networks of side chain interactions within the GPCR [5] and G protein [6] that are essential for receptor activation and G protein activation. It therefore seemed reasonable to use the two G_s-coupled GPCR structures [2, 20] to guide the engineering of G_i, G_o or G_q, given that there are no structures of receptors coupled to these G proteins. Transfer of the relevant mutations from mini-G_s to other G proteins was successful in deriving mini-G_{olf}, mini-G_{o1} and mini-G₁₂. Both mini-G_{olf} and mini-G_{o1} coupled to relevant receptors only in the presence of an agonist and formed stable complexes that could be purified by SEC. Currently, we have not been able to demonstrate binding of mini-G₁₂ to any receptor (results not shown), even though it is highly expressed in *E. coli* and has high thermal stability, suggesting that the protein is in a folded state. In contrast, initial trials to generate mini-G₁₁, mini-G₂, mini-G_q and mini-G₁₆ were unsuccessful due to no expression in *E. coli*; we have not yet tested the chimera strategy on these targets. Mini-G₁₁ expressed very poorly, but was improved upon further mutagenesis, but was still not as stable as mini-G_s and required binding of βγ subunits to attain a full agonist affinity shift in the 5HT_{1B}R.

The second approach to generate mini-G proteins for those that did not work initially was to make chimeras by converting the specificity of mini-G_s to the specificity of the desired G protein. This was developed initially for G_q by mutating in mini-G_s only those residues in the α5 helix whose side chains make contact to either β₂AR or A_{2A}AR in the crystal structures of the relevant complexes [2, 20], to match the equivalent residues in G_q. The final mini-G_{s/q} chimera was stable, overexpressed in *E. coli* and coupled to G_q-coupled receptors, but not to G_s-coupled receptors. The process was also successful in generating a mini-G_{s/i1} and mini-G_{s/o} chimera.

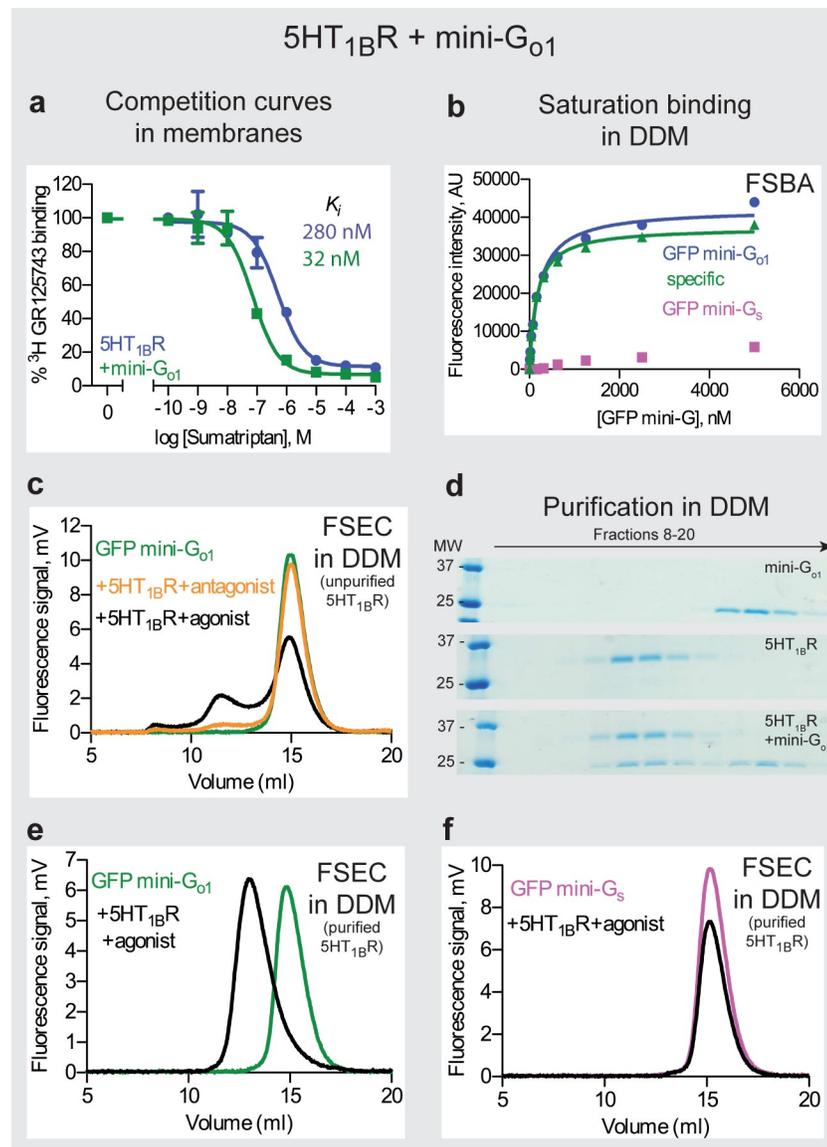


Fig 7. The 5HT_{1B}R-mini-G_{o1} complex. (a) Competition binding curves were performed on membranes in duplicate (n = 2) by measuring the displacement of the antagonist ³H-GR125743 with increasing concentration of the agonist sumatriptan (apparent K_i values representing mean ± SEM are in parentheses): blue circles, 5HT_{1B}R (K_i 280 ± 10 nM); green squares, 5HT_{1B}R and mini-G_{o1} (K_i 32 ± 3 nM). Error bars represent SEM. (b) Measurement of GFP-mini-G_{o1} affinity to DDM-solubilized, donitriptan-bound 5HT_{1B}R using the FSBA: blue circles, 5HT_{1B}R and GFP-mini-G_{o1} (total binding); purple squares, 5HT_{1B}R and GFP-mini-G_s (non-specific binding); green triangles, specific binding. The apparent K_D value (180 ± 24 nM) represents mean ± SEM of two independent experiments. Curves shown are from a representative experiment. (c) FSEC traces of GFP-mini-G_{o1} with DDM-solubilized unpurified 5HT_{1B}R bound to the following (retention volumes are shown in parentheses): orange, the antagonist SB224289 (14.9 ml); black, the agonist donitriptan (11.3 ml and 14.9 ml). Free GFP-mini-G_{o1} (green) resolved as a predominant peak with a retention volume of 14.9 ml (d) Mini-G_{o1} forms a complex with purified 5HT_{1B}R. The three panels are coomassie blue-stained SDS-PAGE gels of fractions from 3 separate SEC experiments: top panel, mini-G_{o1}; middle panel, 5HT_{1B}R; bottom panel, mini-G_{o1} mixed with donitriptan-bound 5HT_{1B}R (1:1 molar ratio). (e) FSEC traces of GFP-mini-G_{o1} with purified 5HT_{1B}R: black, GFP-mini-G_{o1} with 5HT_{1B}R purified in DDM (13 ml); green, GFP-mini-G_{o1} (14.8 ml). (f) FSEC traces of GFP-mini-G_s with purified 5HT_{1B}R: black, GFP-mini-G_s with 5HT_{1B}R purified in DDM (negative control; 15.1 ml); purple, GFP-mini-G_s (15.1 ml). Retention volumes are shown in parentheses.

<https://doi.org/10.1371/journal.pone.0175642.g007>

Table 1. Mini-G proteins and their characteristics.

Mini-G protein	Construct	Yield of pure protein per L of <i>E. coli</i> (mg)	Stability measured by DSF (°C)	Stability in detergent measured by native DSF (°C)		
				No detergent	0.1% LMNG	0.1% DDM
G _s	393	100	65 ± 0.0	48 ± 0.2	45 ± 0.2	39 ± 0.0
G _{o1f}	6	80	65 ± 0.4	44 ± 0.1	42 ± 0.0	37 ± 0.2
G _{s/q}	70	50	67 ± 0.4	47 ± 0.3	44 ± 0.2	36 ± 0.1
G _{s/i1}	43	40	69 ± 0.1	45 ± 0.0	41 ± 0.1	36 ± 0.1
G _{o1}	12	100	64 ± 0.1	44 ± 0.2	41 ± 0.1	33 ± 0.2
G ₁₂	8	25	73 ± 0.3	50 ± 0.1	46 ± 0.1	41 ± 0.2

<https://doi.org/10.1371/journal.pone.0175642.t001>

The α5 helix provides ~70% of the buried surface area between the GTPase domain and the receptor in the two G protein–GPCR complexes crystallised to date. The work here shows that changing these contacts is sufficient to alter the specificity of coupling. However, this is not to say that the remaining 30% of the interface is not important, merely that a range of amino acid residues can be accommodated in this interface and therefore it plays a less important role in defining both specificity and the affinity of G protein binding.

The mini-G proteins and their properties are shown in Table 1 and Table 2. On the whole, the expression levels are satisfactory in *E. coli* and the stability of the mini-G proteins in the absence of detergent is also good. However, their stability decreases in detergent, particularly in high concentrations, with the greatest decrease in stability observed at high detergent concentrations (greater than 0.5% w/v) and with detergents that are regarded as harsh for membrane protein purification [50]. Thus care must be exercised in the initial choice of detergent for forming receptor–mini-G protein complexes. However, GPCRs in the active state are also unstable in detergent and this also needs to be carefully addressed before structural studies are a possibility. This may require the use of very high-affinity agonists that stabilize the receptor and/or the stabilisation of the GPCR–mini-G protein complex by conformational thermostabilisation. It is not trivial to define which component, receptor or mini-G protein, is the least stable within a complex under a given set of conditions. This is why we recommend that initial tests are performed on the receptor solubilized in very mild detergents such as digitonin, GDN or LMNG.

Despite the successes described here, mini-G proteins are not a panacea for all GPCRs. The specificity determinants for G protein coupling are not determined solely by the α5 helix and the subtypes of βγ can also affect the efficiency and specificity of coupling within a cell [51, 52]. As we have not taken any subtype-specific G protein–GPCR interactions into account, further engineering of the mini-G proteins in the light of new structural data of GPCRs coupled to G_i, G_o or G_q may improve their performance further. Additional complications to making stable receptor–G protein complexes are the kinetics of dissociation of the G protein from the

Table 2. Mini-G proteins that bind βγ subunits and their characteristics.

Mini-G protein that bind βγ	Construct	Yield of pure protein per L of <i>E. coli</i> (mg)	Stability measured by DSF (°C)
G _s	399	100	72 ± 0.0
G _{o1f}	9	144	66 ± 0.1
G _{s/q}	76	30	71 ± 0.1
G _{i1}	46	12	48 ± 0.3
G _{s/i1}	48	10	72 ± 0.1
G _{s/o1}	16	15	69 ± 0.1

<https://doi.org/10.1371/journal.pone.0175642.t002>

activated receptor and the stability of the activated receptor itself. There has probably been strong evolutionary pressure for some GPCRs to evolve transient signaling complexes that are inherently unstable to provide tight regulation of the signaling process. Thus it may be necessary to thermostabilise the receptor in the active conformation [36] to generate a complex sufficiently stable for structural studies, although for other GPCR-G protein complexes this may not be necessary.

Supporting information

S1 Fig. Sequence of mini-G proteins used in this study. The poly-histidine tag is highlighted in red, the TEV protease cleavage site is highlighted in grey, and the linker used to replace the G α AH domain is highlighted in turquoise. Mutations are shown in bold type and underlined. (DOCX)

S2 Fig. Sequence of mini-G proteins that were *not* successfully expressed in *E. coli*. The poly-histidine tag is highlighted in red, the TEV cleavage site highlighted in grey and the linker used to replace the G α AH domain is highlighted in turquoise. Mutations are shown in bold type and underlined. The constructs were cloned into plasmid pET15b for *E. coli* expression using *Nco*I (yellow) and *Xho*I (magenta) restriction sites. Start and stop codons are in red. (DOCX)

S3 Fig. Sequence of GFP-mini-G proteins used in this study. GFP (highlighted in green) was fused to the N-terminus of the mini-G proteins with a GGGGS linker (highlighted in yellow). The poly-histidine tag is highlighted in red, the TEV cleavage site highlighted in grey and the linker used to replace the G α AH domain is highlighted in turquoise. (DOCX)

S4 Fig. Sequence alignment of selected mini-G_{s/q} chimeras used in this study. Residues in red are the signature mutations of a mini-G protein. Residues in blue are those found in G_q. Diamonds above the sequences identify the amino acid residues in G α_s where the side chains that make atomic contacts to residues in either β_2 AR (β_2 con) or A_{2A}AR (2A con). Ovals above the sequences identify the amino acid residues in G α_s where only the main chain atoms make contacts to the receptor. (DOCX)

S5 Fig. Analytical SEC and SDS-PAGE analyses of purified A_{2A}AR with mini-G_{s/q} chimeras. Analytical SEC of mini-G_{s/q}57 (a), mini-G_{s/q}58 (b) and mini-G_{s/q}70 (c) bound to purified A_{2A}AR: black, A_{2A}AR-mini-G_{s/q} complex; blue, A_{2A}AR; green, mini-G_{s/q}. Three panels below the SEC traces are coomassie blue-stained SDS-PAGE gels of fractions from 3 separate SEC experiments: top panel, mini-G_{s/q}; middle panel, A_{2A}AR; bottom panel, NECA-bound A_{2A}AR mixed with mini-G_{s/q} (1:1.2 molar ratio). (PDF)

S6 Fig. Sequence alignment of the different mini-G_{i1} and mini-G_{o1} proteins used in this study. Residues in red are the signature mutations of a mini-G protein. Note the additional G217D mutation (highlighted in yellow; residue 114 in the mini-G protein) in mini-G_{i1} to improve expression. Residues highlighted in cyan in mini-G_s were mutated to their equivalent in mini-G_{i1} or mini-G_{o1} (highlighted in magenta and grey respectively) to make the mini-G_{s/i1} or mini-G_{s/o1} chimeras. Note the re-insertion of the N-terminus and the back mutation (D to L) highlighted in green in the constructs that were used to form a heterotrimer with $\beta_1\gamma_2$ (*i.e.* mini-G_{i1_46}; mini-G_{s/i1_43} and mini-G_{s/o1_16}). (PDF)

S7 Fig. Stability of the purified donitriptan-bound 5HT_{1B}R–mini-G_{o1} complex. (a) The complex was assembled from purified components and analysed by SEC immediately after assembly (blue line) or after the sample was stored at 4°C for 48h (red line). Fractions (0.5 ml) were collected and analysed by SDS-PAGE: (b) immediately after assembly; (c) after 48 h at 4°C.
(PDF)

Acknowledgments

We thank F. Marshall for helpful comments in the preparation of the manuscript.

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