GPCR-G Protein-b-Arrestin Super-Complex Mediates Sustained G Protein Signaling

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1

Supplemental Information

Extended Experimental Procedure

Constructs

Constructs expressing FLAG-®$_2$V$_2$R (Shukla et al., 2014), mStrawberry-βarr2 (Burczyk et al., 2015), RlucII-67-Gαs (Carr et al., 2014), RlucII-Gγ2 (Dupre et al., 2006), GFP10-Gγ1 (Gales et al., 2005) and GFP10-®arr2 (Carr et al., 2014) have previously been functionally verified. Functionality of constructs expressing SNAP-®$_2$AR, SNAP-®$_2$V$_2$R, SNAP-V$_2$R were confirmed by measuring their ability to stimulate cAMP production and ERK1/2 phosphorylation upon agonist challenge (Figure S1A-C). Functionality of the constructs expressing mEmerald-67-Gαs and RlucII-67-Gαs were confirmed by determining their ability to stimulate adenylyl cyclase upon receptor stimulation (Figure S1D). Both the GFP10-βarr1 and GFP10-βarr2 constructs were recruited to receptor upon agonist stimulation demonstrating their functionality (Figure S1E). The RlucII-117-Gαs/GFP10-Gγ1 BRET pair was specifically developed for the Gs dissociation experiments and has a >10-fold higher dynamic response window compared to the previously used RlucII-67-Gαs/GFP10-Gγ1 BRET pair (Figure S1F). Addition of cholera toxin, which directly activates Gs, by causing ADP-ribosylation of Gαs subunit resulting in inhibition of its intrinsic GTPase activity (De Haan and Hirst, 2004), promotes a substantial decrease in BRET response of the RlucII-117-Gαs/GFP10-Gγ1 BRET pair confirming that this BRET decrease using this BRET pair reflects true Gs activation/dissociation (Figure S1G). However, the placement of the RlucII-tag in RlucII-117-Gαs impairs its ability to stimulate adenylyl cyclase (Figure S1D).

Real-time Measurement of cAMP Production

Stable HEK293-ICUE2 cell lines previously generated (Violin et al., 2008) were transiently transfected with ®$_2$AR, ®$_2$V$_2$R (Oakley et al., 1999), V$_2$R, SNAP-®$_2$AR, SNAP-®$_2$V$_2$R, SNAP-V$_2$R, ®$_2$V$_2$R–®arr1 (βarr1 directly fused to C-terminal of ®$_2$V$_2$R), or ®$_2$V$_2$R–®arr2 (βarr2 directly fused to C-terminal of ®$_2$V$_2$R)
and plated in poly-D-lysine coated 35-mm glass bottom dishes (MatTek). Forty-eight hours after transfection, the cells were washed once in Dulbecco's phosphate-buffered saline (DPBS) followed by addition of imaging buffer (10 mM HEPES, 125 mM NaCl, 5 mM KCl, 1.5 mM MgCl2, 1.5 mM CaCl2, 10 mM glucose, 0.2% bovine serum albumin (BSA), pH 7.4). Cells were imaged in the dark, on a 37 °C temperature-controlled stage, for the entire experiment using a DeltaVision Deconvolution microscope (GE
Healthcare) with a Coolsnap HQ2 CCD camera (Photometrics) controlled by SoftWoRx 6.1 (GE Healthcare). Dual emission ratio imaging used a CFP/YFP dichroic mirror, and 470 ± 24 nm and 535 ± 25 nm emission filters for CFP and YFP, respectively. Exposure times were 200 ms for CFP and 100 ms for YFP, and images were taken every 15 s. Experiments were initiated by addition of agonist and ICUE2 FRET was followed for 30 min. In experiments investigating the effect of cell membrane permeable and impermeable antagonist, 10 μM of antagonist was added once the cells had initially been stimulated for 10
min with agonist. For the analysis of whole cell cAMP responses, responses were averaged from a field of cells (20-60 transfected cells) and these responses were averaged across experimental replicates. Analysis,
herein, ignores subcellular gradients of cAMP and instead reports the average concentration of cAMP experienced by diffusible proteins, such as ICUE2. Background corrections of fluorescent images were carried out by subtracting the intensity of the background from the emission intensities of fluorescent cells expressing the reporters. All responses represent a decrease in FRET ratio ($FRET_{CFP/YFP}$/CFP) and were normalized to cAMP responses stimulated by addition of 50 μM forskolin + 200 μM IBMX. Baseline experiments were conducted to define "no response" and to correct for bleaching of the fluorophores.

Measurement of cAMP Production for Characterization of G⟨s Constructs

To characterize the ability of mEmerald-67-Gαs, RlucII-67-G⟨s and RlucII-117-G⟨s to stimulate adenylyl cyclase, Flag-V_2R was transiently co-transfected with G⟨s constructs in HEK293 cells. Transfected cells were plated in poly-D-lysine-pretreated 96-well transparent plates (100ul/well; Corning) and maintained in culture for 48 hours before performing the experiments. Forty-eight hours later, cells were washed with DPBS and 90ul/well of IBMX 750 ⌠M in Tyrode's buffer (NaCl 137mM, KCl 0.9M, $MgCl_2$ 1mM, $NaHCO_3$ 11.9mM, NaH_2PO_4 3.6 mM, HEPES 25mM, glucose 5.5mM, $CaCl_2$ 1mM, pH 7.4) was added. Cells were stimulated with 10 ⌠l/well of AVP, incubated at 37°C during 10 min and frozen for 90 min at -
2
80°C. Frozen cells were lysed by 30 min incubation at 37°C followed by 2 min shaking on a Heidolph Titramax 100 platform shaker. 5 ⌠l of cell lysis was combined with 5 ⌠l of 750 ⌠M IBMX in Tyrode's buffer, 5 ⌠l of cAMP-d2 conjugate and 5 ⌠l of anti-cAMP cryptate conjugate according to the cAMP dynamic 2 kit manufacturer (CisBio assays) in a 384-well white flat bottom plate (Corning). The plate was incubated 1 hour at room temperature and FRET between cAMP-d2 and anti-cAMP cryptate was measured
(665 nm/620 nm) using a HTRF Artemis plate reader.

Bioluminescence Resonance Energy Transfer (BRET) Assay

To measure separation between G⟨s and G© (i.e. to monitor G⟨s activation) in HEK293 cells, G®1 was cotransfected
with receptor, G⟨s tagged with RlucII at position 117 (RlucII-117-G⟨s), and G©1 tagged with GFP10 at the N-terminal (GFP10-G©1) (Gales et al., 2005). To measure the interaction between ®arr1/2 and
G⟨s or CD8 in HEK293 cells, ®arr1/2 tagged with GFP10 at its C-terminus (GFP10-®arr1/2) was cotransfected
with G⟨s tagged with RlucII at position 67 (RlucII-67-G⟨s), or CD8 tagged with RlucII at its C-terminus with both receptor, G®1 and G©2. To measure the interaction between ®arr1/2 and G© in HEK293 cells, GFP10-®arr1/2 was co-transfected with receptor, G®1 and G©2 tagged with RlucII at its Nterminus,
and T8-βARKct (Crespo et al., 1995) (when indicated). Transfected cells were plated in poly-Dlysine-pretreated 96-well white microplates (100ul/well; Greiner) for BRET assays and in poly-D-lysinepretreated 24-well plates (500ul/well) for enzyme-linked immunosorbent assay (ELISA). Cells were maintained in culture for 48 h before performing assays. Transfected HEK293 cells were washed with DPBS followed by addition of Tyrode's buffer (NaCl 137mM, KCl 0.9M, $MgCl_2$ 1mM, $NaHCO_3$ 11.9mM, NaH_2PO_4 3.6 mM, Hepes 25mM, glucose 5.5mM, $CaCl_2$ 1mM, pH 7.4). Expression of the energy acceptor (GFP10-tagged biosensor) was monitored by reading fluorescence at 515 nm, while exciting at 400 nm using a FlexStationII microplate reader (Molecular Devices). After addition of agonist, or of vehicle, cells were incubated at 37°C for the specified stimulation time and luciferase substrate coelenterazine 400a (2.5
uM; NanoLight Technology) was added 5 min prior to reading BRET in a Synergy Neo microplate reader (BioTek) equipped with an acceptor filter (515 ± 30 nm) and donor filter (410 ± 80 nm). For agonist washout, Tyrode's buffer containing agonist was removed and cells were washed twice with freshly prepared 37°C Tyrode's buffer. The BRET signal was determined as the ratio of the light emitted by GFP10-tagged biosensors (energy acceptors) and the light emitted by RlucII-tagged biosensors (energy donors). The net BRET signal was defined as the difference between the BRET signal and the one obtained
with luciferase alone (RlucII-tagged biosensor). The agonist-promoted BRET signal (⊗BRET) refers to the difference in BRET recorded from cells treated with agonist and cells treated with vehicle. When BRET is expressed as a percent of control condition, the percent is calculated from the ⊗BRET value obtained from
a given condition divided by the ⊗BRET obtained from the control condition. BRET titration curves were performed by co-transfecting a constant amount of the RlucII-tagged biosensor with increasing amounts of
the GFP10-tagged biosensor. Net BRET values were expressed as a function of the total expression level of

the GFP10-tagged biosensor (recorded using FlexStationII) over the total expression level of the RlucIItagged
biosensor (detected by the Synergy Neo microplate reader) for each transfection condition.

Measurement of Cell Surface Expression by ELISA

For all BRET experiments equal surface expression in HEK293 cells for the different receptors were determined by ELISA. Culture medium was removed and cells were fixed with DPBS containing 3.7% formaldehyde for 5 min. Cells were washed 3 times in washing solution (0.2% BSA in DPBS) and nonspecific
binding sites were blocked by incubating cells for 45 min in blocking solution (1% BSA in DPBS). Cells were washed 3 times and incubated for 45 min with anti-Flag M2 monoclonal antibody (Sigma-Aldrich, 1:10000 in blocking solution). After 3 washes, blocking solution was added for 15 min. Cells were then incubated 45 min with anti-mouse HRP conjugated antibody (1:1000 in blocking solution; GE Healthcare). After 3 washes with washing buffer, followed by 4 washes with DPBS, HRP activity was detected by incubating cells with *o*-phenylenediamine dihydrochloride (Sigma-Aldrich). Adding 0.6M HCl stopped the reaction and absorbance was read at 492 nm using a Tecan GENios multifunction microplate reader (MTX Lab Systems, Inc).

Surface Expression Measured by Radioligand Whole Cell Binding

Equal surface expression of the different receptors was confirmed for all experiments, except for BRET, by

whole cell binding assays using cell membrane impermeable radioligands. HEK293 cells were grown until confluent in 6-well plates. Cells were washed once in cold DPBS followed by cell detachment using 0.02%
EDTA. Cells were re-suspended in Minimal Eagle's Medium (MEM)/0.1% BSA/10 mM HEPES, pH 7.4 and centrifuged at 4000 rpm for 5 min. The cell pellet was re-suspended in MEM/0.1% BSA/10 mM HEPES and samples were transferred to a 96-square-well plate (Labnet International Inc.). For β_2AR- and β_2V_2R-expressing cells, 30 nM radioactive [$_3$H]-CGP 12177 (Perkin Elmer) was added to each sample. For
V_2R-expressing cells, 4 nM radioactive [$_3$H]-(Arg$_8$)-vasopressin (Perkin Elmer) was added to the sample. Non-specific binding was determined by addition of 10 µM propranolol (Sigma-Aldrich) to β_2AR- and β_2V_2R-expressing cells. Non-specific binding was determined by addition of 30 µM AVP to V_2Rexpressing cells. Cells were incubated on ice for 3 h followed by transfer to Whatman GF-B filter paper, and washed 4 times with cold wash buffer (50 mM Tris, pH 7.4, 12.5 mM $MgCl_2$, 2 mM EDTA). Filter paper containing sample was mixed with scintillation fluid (Research Products International Corp.) and counted on a β-counter for 3 min (Perkin Elmer). Protein concentration was determined by BCA assay (Pierce).

Confocal Microscopy

For co-localization studies, HEK293 cells were transfected with N-terminal SNAP-tagged receptors (New England Biolabs), mStrawberry-βarr2 (Burczyk et al., 2015), mEmerald-67-Gαs (mEmerald was tagged at position 67), Gβ_1, and Gγ_2, 48 h prior to the experiments. For localization studies of the fusions, HEK293 cells were transfected with either SNAP-tagged ®$_2V_2$R or ®$_2V_2$R–®arr1/2 48 h prior to the experiment. The
day before the experiment, cells were plated on poly-D-lysine coated 35-mm glass bottom dishes (MatTek)
and were starved for at least 2 h in serum-free medium prior to the experiment. For the co-localization studies, SNAP-tagged receptors were labeled with SNAP-Surface 649 (New England Biolabs) for 10-15 min, and then washed with FluoroBrite DMEM (Life Technologies). For the localization studies, SNAP-®$_2V_2$R or SNAP-tagged fusions were labeled with SNAP-Surface 549 (New England Biolabs) for 10-15 min, followed by washing with FluoroBrite DMEM (Life Technologies). All experiments were conducted at 37°C in FluoroBrite DMEM (Life Technologies). Prior to, or at different time points, during stimulation of the receptors (or the fusions), cells were fixed with ice cold 6% formaldehyde (Sigma-Aldrich) diluted in DPBS. Confocal images were obtained on a Zeiss LSM510 laser-scanning microscope using single line (568 nm) or multi-track sequential excitation (488, 568, 633 nm) and emission (515–540 nm, mEmerald-67-Gαs; 585–615 nm, mStrawberry-βarr2 or SNAP-Surface 549; 650 nm, SNAP-Surface 649) filter sets.

HEK293 Membrane Purification and Radioligand Binding Assays

HEK293 cells transfected with either β_2V_2R, β_2V_2R–βarr1, or β_2V_2R–βarr2, and GRK2-CAAX were harvested in cold wash buffer (20 mM Tris-HCl and 150 mM NaCl, pH 7.4) and dounce-homogenized (100
strokes) in cold homogenization buffer (20 mM Tris-HCl, EDTA-free protease inhibitor mixture, 5 nM microcystin LF, 10 nM calyculin A, 10 mM sodium fluoride, 1 mM tetrasodium pyrophosphate, and 1 mM β-glycerophosphate, pH 7.4). Following differential centrifugation, the P2 microsomal membrane fraction was resuspended in cold buffer (50 mM Tris-HCl, protease inhibitor mixture, 5 nM microcystin LF, 10 nM calyculin A, 10 mM sodium fluoride, 1 mM tetrasodium pyrophosphate, 1 mM β-glycerophosphate, 150 mM NaCl, 12.5 mM $MgCl_2$, 0.2% BSA, and 10% glycerol, pH 7.4), aliquoted, and stored at −80°C. Competition radioligand binding assays using β_2V_2R, β_2V_2R–βarr1, or β_2V_2R–βarr2 expressing HEK293 membranes were conducted in parallel. Competition binding assays measuring βarr1/2 coupling were performed in assay buffer (50 mM Tris-HCl, pH 7.4, 50 mM potassium acetate, 150 mM NaCl, 5 mM $MgCl_2$, and 0.2% BSA) containing 60 pM ^{125}I-cyanopindolol (Perkin Elmer), β_2V_2R + GRK2-CAAX, β_2V_2R–βarr1 + GRK2-CAAX, or β_2V_2R–βarr2 + GRK2-CAAX membranes, and a serial dilution of test ligand. Nonspecific binding was determined in the presence of 10 μM propranolol (Sigma-Aldrich), whereas total binding was determined in the absence of a competitor. After a 1.5 h incubation at 25°C, bound radioactivity was collected on 0.3% polyethyleneimine-treated GF/C filters using wash buffer (50 mM Tris-HCl, pH 7.4, and 50 mM potassium acetate). Bound radioactivity was quantified on a Packard Cobra gamma counter (GMI). ISO was fitted to a two-site binding model by sharing the low affinity value between unfused and βarr-fused receptors, and ICI-118551 was fitted to a one-site binding model in Prism
(GraphPad Software, Inc.). The log Ki values were fitted directly by setting [^{125}I]-CYP concentration to 60pM and [^{125}I]-CYP affinity to 60pM.

Immunoprecipitation and Western Blot of Fusion Proteins

4

Either $®_2V_2R$ or $®_2V_2R$–®arr1/2 were isolated by IP from transfected HEK293 cell lines using standard procedures. Briefly, cells were solubilized in cold lysis buffer (5 mM HEPES, pH 7.5, 250 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 2 mM EDTA, 0.01% n-dodecyl-β-D-maltoside, and EDTA-free protease inhibitor mixture). After centrifugation, the cleared lysate was incubated overnight at 4°C with anti-M2 FLAG-agarose beads (Sigma-Aldrich). Receptors bound to beads were washed extensively with cold lysis
buffer, deglycosylated with peptide N-glycosidase F, and eluted with lysis buffer containing 1 mg/ml FLAG peptide (Sigma-Aldrich). Immunoprecipitated receptors were resolved on 4–20% SDS-PAGE gels and western blotted using standard procedures. Immunoreactive bands representing the $®_2V_2R$ (polyclonal
α-FLAG, Sigma), $®_2V_2R$–®arr1 fusion protein (polyclonal α-βarr1, A1CT(Attramadal et al., 1992)), and $®_2V_2R$–®arr2 fusion protein (polyclonal α-βarr2, A2CT (Attramadal et al., 1992)) were visualized using Bio
Imaging system (Syngene).

ERK1/2 Phosphorylation Assays

To test for ERK activation, HEK293 cells expressing similar surface levels of $®_2AR$, $®_2V_2R$, V_2R, SNAP-$®_2AR$, SNAP-$®_2V_2R$, SNAP-V_2R, $®_2V_2R$–®arr1, or $®_2V_2R$–®arr2 were starved for 18 h in serum-free medium in 6-well plate prior to stimulation. At different time points following initial stimulation cells were solubilized by directly adding 2×SDS sample buffer followed by sonication. Equal amounts of cellular extracts were separated on 4–20% SDS-PAGE gels and transferred to nitrocellulose membranes for western
blotting. Phosphorylated ERK1/2 and total ERK1/2 were detected by western blotting with rabbit polyclonal anti-phospho-ERK (Cell Signaling, 1:2000), anti-ERK (Millipore, 1:10000) antibodies, respectively. Chemiluminescent detection was performed using the SuperSignal West Pico reagent (Pierce),
and all western blots were visualized and quantified by the Bio Imaging system (Syngene). The levels of ERK phosphorylation were normalized to a loading control (total ERK).

Protein Purification

Expression and purification of Fab30 was performed as previously described (Shukla et al., 2013). In short,

Fab30 was expressed in the periplasm of *E. coli* strain M55244, extracted, and purified on Protein A/G agarose (Pierce). Fab30 was eluted with 10 mM sodium acetate pH 3.0 with 40 mM NaCl and neutralized with 0.1 M sodium acetate, pH 5.0. Fab30 was subsequently purified on a pre-packed Resource S column
followed by dialysis in 20 mM HEPES, pH 7.4, 100 mM NaCl. Expression and purification of the Fab30 complex has been described previously (Shukla et al., 2014). Briefly, T4L-β_2V_2R was co-expressed with untagged bovine βarr1 (1-393) and untagged GRK2-CAAX in *Sf*9 cells, and complex formation was initiated by addition of 100 nM BI. After harvesting and lysis of cells, Fab30 was added to stabilize the T4L-β_2V_2R-βarr1 complex and was enriched from detergent-solubilized membranes using immobilized M1 anti-FLAG affinity chromatography. Finally, the Fab30 complex was further purified by size-exclusion chromatography (SEC). Gs heterotrimer was expressed in *Trichoplusia ni* (HighFive™; Invitrogen) insect cells and purified as previously described (Rasmussen et al., 2011). Briefly, after harvesting and lysis of cells, Gs heterotrimer was enriched from detergent-solubilized membranes using immobilized metal ion affinity chromatography (IMAC) and treated with lambda phosphatase. The Gs protein then underwent further purification by ion-exchange chromatography (IEC) and SEC. Expression and purification of Nb35 was achieved as previously described (Rasmussen et al., 2011). Briefly, Nb35 was expressed in the periplasm of *E. coli* strain WK6, extracted, and purified by IMAC, followed by IEC. Selected Nb35 fractions were dialyzed against buffer (10 mM HEPES, pH 7.5, 100 mM NaCl). To form stable T4L-β_2V_2R megaplexes, purified Fab30 complex was incubated with purified heterotrimeric Gs for 1 h at room temperature, in 1:1.5 molar ratio, in buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 100 nM BI, 0.01% Lauryl Maltose Neopentyl Glycol (LMNG), and 100 µM TCEP. Additionally, 3 molar excess of Nb35 was added to enhance stability of the complex. Next, the megaplex was treated with 25 mU/ml of apyrase (New England Biolabs) for 1 h at room temperature and the $CaCl_2$ concentration was adjusted to 4
mM. Finally, the megaplex was loaded on to a preparative size exclusion column (Superdex 200, 16/600, GE Healthcare). The SEC column was run at 0.3 ml/min and the column running buffer consists of 20 mM HEPES pH 7.4, 150 mM NaCl, 100n M BI, 0.01% LMNG, 100 µM TCEP. SEC elution fractions corresponding to the complex peak were either pooled or used individually for further studies. Expression of GST-βarr1 was done in *E. Coli* BL21 (DE3) cells and then extracted and purified on glutathione sepharose beads (GE Healthcare). GST-βarr1 was eluted by 75 mM HEPES, 150 mM NaCl, 5 mM
5
dithiothreitol (DTT), 5 mg/ml reduced glutathione, pH 7.0, followed by dialysis in 20 mM HEPES, pH 7.4, 100 mM NaCl.

Co-immunoprecipitation of In Vitro Complexes

FLAG-tagged Fab30 complex, BI-occupied β_2V_2R, or Cz-occupied β_2V_2R were mixed with purified heterotrimeric Gs in a molar ratio of 1:1.5 in an assay buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 0.01% LMNG, 100 nM BI or Cz, respectively. For experiments investigating the effect of GTPγS, the assay buffer was further supplemented with 4 mM $MgCl_2$, 20 µM GDP + 4 mM $MgCl_2$, or 20 µM GTPγS + 4 mM $MgCl_2$. Next, FLAG-β_2V_2R was immobilized on M1 anti-FLAG agarose-beads and $CaCl_2$ concentration adjusted to 2 mM, followed by 5 washes with washing buffer (assay buffer + 2 mM $CaCl_2$). Finally, FLAG-β_2V_2R and all proteins associated with FLAG-β_2V_2R were eluted by elution buffer containing 1 mg/ml FLAG peptide (Sigma-Aldrich), 20mM HEPES, pH 7.4, 150mM NaCl, 0.01% LMNG, 100 nM BI, 5 mM EDTA. A similar protocol was used for the pull-down assays immobilizing Fab30 using protein A/G agarose beads (Pierce) and 2×SDS sample buffer for elution of Fab30 and Fab30-associated proteins. Eluted proteins were separated on 4–20% Tris glycine polyacrylamide gels and stained with coomassie blue. For quantification of Gs binding, separated proteins were further transferred onto nitrocellulose membranes for western blotting. Gαs subunit was detected using a primary mouse monoclonal anti-Gαs antibody (Millipore, 1:2000). Chemiluminescent detection was performed using the SuperSignal West Pico reagent (Pierce), and all western blots were visualized and quantified by the Bio Imaging system (Syngene).

In Vitro GTPase Activity

In vitro GTPase activity of purified heterotrimeric Gs modulated by reconstituted and purified BI-occupied β_2V_2R or Fab30 complex was measured using the GTPase assay kit (Innova Biosciences). Briefly, 5 µg of Gs was mixed with a varying amount of receptor/complex. To initiate the GTPase reaction, 50 µl of commercially supplied GTP buffer was added (and incubated) for 1 h at room temperature. Commercially supplied “Gold Mix” was added to the reaction and incubated for another 2 min at room temperature,

followed by the addition of commercially supplied “Stabilizer” and incubated for 15 min at room temperature. Absorbance was read at 595 nm and inorganic phosphate generated was determined based on
an inorganic phosphate standard curve.

GST-βarr1 Pull-down Assay

Purified GST-βarr1 was mixed with V_2R C-terminal phosphopeptide (1:5 molar ratio) (Shukla et al., 2013), Fab30 (1:3 molar ratio), and purified heterotrimeric Gs (1:1.5 molar ratio) in assay buffer (20 mM HEPES, 150 mM NaCl, 0.01% LMNG, pH 7.4) containing either control assay buffer, 20 μM GDP, or 20 μM GDP + 120 μM $AlCl_3$ + 20 mM NaF + 4mM $MgCl_2$. GST-βarr1 and GST-βarr1 associated proteins were immobilized on glutathione sepharose beads (GE Healthcare) and washed 5 times using assay buffer. Finally, the pulled-down proteins were eluted using elution buffer containing 75 mM HEPES, 150 mM NaCl, 5 mM DTT, 5 mg/ml reduced glutathione, pH 7.0. Eluted proteins were separated on 4–20% Tris glycine polyacrylamide gels. For quantification of the Gαs subunit or Gβγ subunits binding, separated proteins were transferred to nitrocellulose membranes for western blotting. The Gαs subunit or $Gβ_1$ subunit
were detected using a primary mouse monoclonal anti-Gαs antibody (Millipore, 1:2000) or a primary rabbit
polyclonal anti-$Gβ_1$ antibody (Santa Cruz, 1:2000), respectively. Chemiluminescent detection was performed using the SuperSignal West Pico reagent (Pierce), and all western blots were visualized and quantified by the Bio Imaging system (Syngene).

Electron Microscopy

In vitro reconstituted megaplex was prepared for EM using a conventional negative staining protocol (Peisley and Skiniotis, 2015). The negative stained sample was imaged at room temperature with a Tecnai
T12 electron microscope operated at 120 kV using low-dose procedures. Images were recorded at a magnification of ×71,138 and a defocus value of ~1.5 μm on a Gatan US4000 CCD camera. All images were binned (2 × 2 pixels) to obtain a pixel size of 4.16 Å on the specimen level. Particles were excised using Boxer (part of the EMAN 2.1 software suite) (Ludtke et al., 1999). Two-dimensional reference-free alignment and classification of particle projections was performed using ISAC (Yang et al., 2012). 6052 0° particle projections of native megaplexes were subjected to Iterative Stable Alignment and Classification (ISAC), producing 52 classes accounting for 4097 particle projections.

Data and Statistical Analysis

All graphs were generated and analyzed using GraphPad Prism 6 (GraphPad Software, Inc.). Data represents the mean ± SEM of three or more independent experiments. Statistical significance for more than two conditions was assessed using a one-way ANOVA with Dunnett's multiple comparisons post hoc test, a one-way ANOVA with Sidak’s multiple comparisons post hoc test, or a two-way ANOVA with Sidak's post hoc test. When values were normalized by another value, a one-sample t test was used.

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