Supporting Information

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SI Materials and Methods

Molecular Biology. For construction of pcDNA-Zeo-tetO, an NdeI-XhoI fragment from pACMV-tetO (1), which contained tet operator sequences inserted into the CMV promoter, was ligated into the NdeI/XhoI-digested pcDNA3.1-Zeo backbone. All β_2-adrenergic receptor (β_2AR) constructs were of human origin and contained an N-terminal FLAG-tag and a C-terminal 6xHis-tag. For β_2AR constructs used in sortase ligations, the sortase consensus site (LPETGHH) was inserted after amino acid 365 (β_2AR-LPETGHH). The minimal cysteine β_2AR (β_2ARΔ4) used in bimane fluorescence experiments and the minimal ICL3 mutant (β_2ARΔ238–267) were designed as previously described (2, 3). All β_2AR constructs expressed in Sf9 insect cells contained the N187E glycosylation mutation. Human muscarinic-2 receptor (M_2R) and Mu (μ)-opioid receptor (MOR) were cloned into pcDNA-Zeo-tetO with an N-terminal FLAG-tag and C-terminal sortase consensus site followed by a 6xHis-tag. To enhance stability and expression, a minimal cysteine (C59A, C125S, C140I, C150V, C242V, C251V, and C269S) and truncated (after amino acid 393) variant of rat βarr1 (βarr1–MC-393) in pGEX4T was generated. The Δ62–77 finger loop deletion and the V70C mutation, corresponding to V74C of visual arrestin (4), were introduced in pGEX4T–βarr1–MC-393.

β_2AR Expression and Purification. With the exception of β_2ARΔ238–267, all β_2AR constructs were expressed in Sf9 insect cells using the BestBac Baculovirus Expression System (Expression Systems). Cells were infected at a density of 3×10^6 cells per milliliter and harvested 60 h thereafter. Receptor was solubilized in n-dodecyl-β-D-maltopyranoside (DDM) (Anatrace) and purified using FLAG-M1 and alprenolol-affinity chromatography as previously described (5). β_2ARΔ238–267 in pcDNA-Zeo-tetO was transfected into Expi293F cells (Invitrogen) stably integrated with the plasmid pcDNA/TR (Invitrogen) to express the tetracycline repressor (Expi293F-TR). Cells were transfected using expifectamine (Invitrogen) as described in the manufacturer's protocol with receptor expression being induced 48 h posttransfection with 4 μg/mL doxycycline, 5 mM sodium butyrate, and 1 μM of the β_2AR antagonist alprenolol. Cultures were harvested 30–36 h thereafter, and all purification steps conducted at 4 °C with protease inhibitors (benzamidine and leupeptin) unless stated otherwise. Cell pellets were resuspended (10 mL/g wet cell pellet mass) in room temperature lysis buffer [10 mM Tris, pH 7.4, 2 mM EDTA, 10 mM $MgCl_2$, 5 units/mL benzonase (Sigma), and 2 mg/mL iodoacetamide] with 1 μM alprenolol for 20 min. Membrane was pelleted at 30,000 × *g* for 20 min and resuspended in 10 mL/g original cell pellet mass of solubilization buffer [20 mM Hepes, pH 7.4, 100 mM NaCl, 1% DDM, 0.05% cholesterol hemisuccinate (CHS), 10 mM $MgCl_2$, 5 units/mL benzonase, and 2 mg/mL iodoacetamide] with 1 μM alprenolol. After extensive dounce homogenization, solubilizing membrane was sequentially stirred at room temperature and then 4 °C for 1 h each. Insoluble material was removed by centrifugation at 30,000 × *g* for 30 min, supernatant loaded onto M1-FLAG resin with 2 mM $CaCl_2$ at 1–3 mL/min, and resin washed with 20 column volumes of wash buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% DDM, 0.01% CHS, and 2 mM $CaCl_2$). Receptor was eluted in elution buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% DDM, 0.01% CHS, 0.2 mg/mL FLAG-peptide, and 5 mM EDTA), and monomeric receptor was collected by size exclusion chromatography on a Superdex 200 Increase Column (GE Healthcare Life Sciences).

M_2R and MOR Expression and Purification. The M_2R and the MOR were expressed in and purified from Expi293F-TR cells as described above with the following modifications. The antagonists atropine (1 μM) and naloxone (1 μM) were included during expression and purification for the M_2R and the MOR, respectively. For M_2R solubilization, 10% glycerol was added, and NaCl was increased to 750 mM based on previous studies (6). Additionally, M1-FLAG resin was washed with five column volumes of high (750 mM) and low (100 mM) NaCl-containing wash buffer at ratios of 4:0, 3:1, 2:2, 1:3, and 0:4, respectively. In addition to DDM, MOR solubilization buffer contained 0.3% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (7).

G Protein, βarr1, and Nb80 Purification. Heterotrimeric G protein was purified as previously described (8). In brief, *Trichoplusia ni* HighFive insect cells were infected with two viruses made from BestBac baculovirus system, the first expressing both human $G\beta_1$-His6 and $G\gamma_2$, and the second $G\alpha_i$ or the short $G\alpha_s$ splice variant. Cells were harvested 48 h postinfection. Heterotrimeric Gs or Gi was purified from solubilized cell membranes using Ni-NTA chromatography and HiTrap Q Sepharose anion exchange (GE Healthcare Life Sciences). Purification of βarr1 was conducted as previously described (9). In short, GST–βarr1–MC-393 was expressed in BL21(DE3) bacteria, lysed using a microfluidizer, and captured using glutathione sepharose. βArr1 was removed from GST by thrombin digestion and further purified using HiTrap Q sepharose anion exchange. Nb80 was purified as previously described (10).

High-Density Lipoprotein Reconstitution. Receptor reconstitution into high-density lipoprotein (HDL) particles was conducted as described elsewhere (10). In short, DDM-solubilized receptor (2 μM) was incubated for 1 h at 4 °C with 80 μM apolipoprotein A1 (MSP1D1) and a 3:2 molar ratio of 8 mM phosphatidylcholine (POPC) with phosphatidylglycerol (POPG). Bio-beads (Bio-Rad) were added (0.5 mg/μL reconstitution volume) thereafter and rotated overnight at 4 °C. HDL receptor was isolated from nonreceptor-containing HDL particles using M1-FLAG chromatography.

Sortase Ligation Reactions. All sortase reactions were conducted in buffer containing 20 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% DDM, 0.01% CHS, and 5 mM $CaCl_2$. Detergent-solubilized receptor (10 μM) was incubated with GGG-V_2Rpp (GGG-ARGRpTPPpSLGPQDEpSCpTpTApSpSpSLAKDTSS) (50 μM) or nonphosphorylated GGG-V_2R, and 2 μM sortaseA containing five mutations that increase ligation efficiency, as described previously (11). Ligations were incubated overnight at 4 °C, and unligated receptor (containing C-terminal 6xHis-tag) was removed using Talon resin (Invitrogen). Size exclusion chromatography was utilized to specifically isolate monomeric ligated receptor.

Bimane Fluorescence. For bimane labeling of proteins, alprenolol-pure β_2AR, β_2AR-LPETGGH, or ion-exchange-pure βarr1–MC-393 V70C was incubated with 100 μM TCEP at 4 °C for 15 min, then with a threefold excess of monobromobimane (Sigma) at 4 °C overnight. An additional threefold molar excess of monobromobimane was added the next day, and the reaction was allowed to continue for 1 h at room temperature before quenching with excess L-cysteine. Excess label was removed by size exclusion chromatography. Bimane-labeled βarr1 was concentrated and flash frozen with 15% glycerol. For bimane-labeled receptors, phosphopeptide ligation (β_2AR-LPETGGH-bimane) and HDL

reconstitution (β_2AR-bimane and β_2ARpp-bimane) were carried out as described above.

Bimane-labeled β_2AR or β_2ARpp HDLs were incubated with or without isoproterenol for 15 min at room temperature before the addition of excess transducer. Final concentrations were 250 nM HDL, 10 μM isoproterenol, 500 nM Gs (+5 mM $MgCl_2$), 1 μM βarr1-393 minimal cysteine or βarr1-393 minimal cysteine Δ62–77 in buffer composed of 20 mM Hepes, pH 7.4, 100 mM NaCl. The reactions were equilibrated for at least 30 min in black, solid-bottom 96-well microplates before fluorescence emission spectra were collected on a CLARIOstar plate reader (BMG Labtech) in top-read mode, with excitation at 370 nm (16 nm bandpass) and emission scanning from 400 nm to 600 nm (10 nm bandpass) in 1-nm increments. Reactions were set up in duplicate in each experiment, and wells for background subtraction contained all components except the bimane-labeled HDLs. Experiments were repeated at least three times.

For experiments with βarr1-bimane, HDL receptors were incubated with ligands (and for β_2ARpp, Nb80) for 15 min at room temperature. βArr1-bimane was added to each well, and the reactions were equilibrated for at least 30 min before being read as described above. Final concentrations were 375 nM HDLs, 10 μM ligand, 500 nM Nb80, and 250 nM βarr1-bimane. Reactions were set up in duplicate in each experiment, and wells for background subtraction contained all components except βarr1-bimane. To normalize data, the area under each averaged, background-subtracted curve between 425 nm and 600 nm was calculated (GraphPad Prism), and areas were normalized to the maximum value (M_2Rpp plus iperoxo) in each experiment. Experiments were repeated at least three times.

Receptor–Transducer Coimmunoprecipitation. Since β_2AR and βarr1 are of similar molecular weight, we used a β_2AR construct with T4-lysozyme (T4L) fused to the receptor's N terminus to obtain separation by SDS/PAGE (12). Assay and wash buffer consisted of 20 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% DDM, and 2 mM $CaCl_2$. Unligated or phosphopeptide-ligated FLAG-T4L-β_2AR in DDM were mixed with 10 μM isoproterenol and stoichiometric amounts of heterotrimeric G protein or βarr1–MC-393. The antibody fragment Fab30 was added to βarr1 to stabilize the interaction with the phosphorylated receptor C terminus (13). After reactions were incubated at room temperature for 1 h, FLAG-T4L-β_2AR was immunoprecipitated using FLAG-M1 resin, and transducers were eluted by the addition of 1 mg/mL FLAG peptide and 10 mM EDTA.

Radioligand Binding. All equilibrium competition radioligand binding studies were conducted in a final volume of 200 μL containing HDL receptor, radioligand, a titration of unlabeled competitor, and the presence or absence of the indicated transducer protein. All components were diluted in assay buffer containing 20 mM Hepes, pH 7.4, 100 mM NaCl, and 1 mg/mL BSA. Radioligands used in β_2AR, M_2R, and MOR competition binding experiments were [^{125}I]-cyanopindolol (CYP; 60 pM), [^{3}H]-*N*-methyl-scopolamine (NMS; 1 nM), and [^{3}H]-naloxone (2.5 nM), respectively. G protein or βarr1 were used at a final concentration of 100 nM or 1 μM, respectively, unless specified otherwise. Binding reactions proceeded at room temperature and were harvested onto glass-fiber filters (GF/B) with 0.3% polyethyleneimine (PEI) using a 96-well Brandel harvester. Binding data were analyzed in GraphPad Prism using a sigmoidal dose–response curve fit, and differences in log IC_{50} values were analyzed by one-way ANOVA.

G Protein GTPase Assay. The GTPase activity of $G\alpha_s$ or $G\alpha_i$ was measured in vitro using the GTPase Glo Assay (Promega) with the following modifications. Final reaction buffer consisted of 20 mM Hepes, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mg/mL BSA. HDL-β_2AR (4 nM), -M_2R (100 nM), or -MOR (100 nM) was incubated in the absence or presence of the indicated agonist (5 μM) and βarr1 (1 μM) for 15 min at room temperature. The antibody fragment Fab30 (1 μM) was included with βarr1 to stabilize its interaction with the ligated receptor C terminus (13). G protein (500 nM) and GTP (2.5 μM) were subsequently added, and reactions proceeded for 1 h at room temperature before addition of GTPase Glo reagent and ADP, as described in the manufacturer's protocol.

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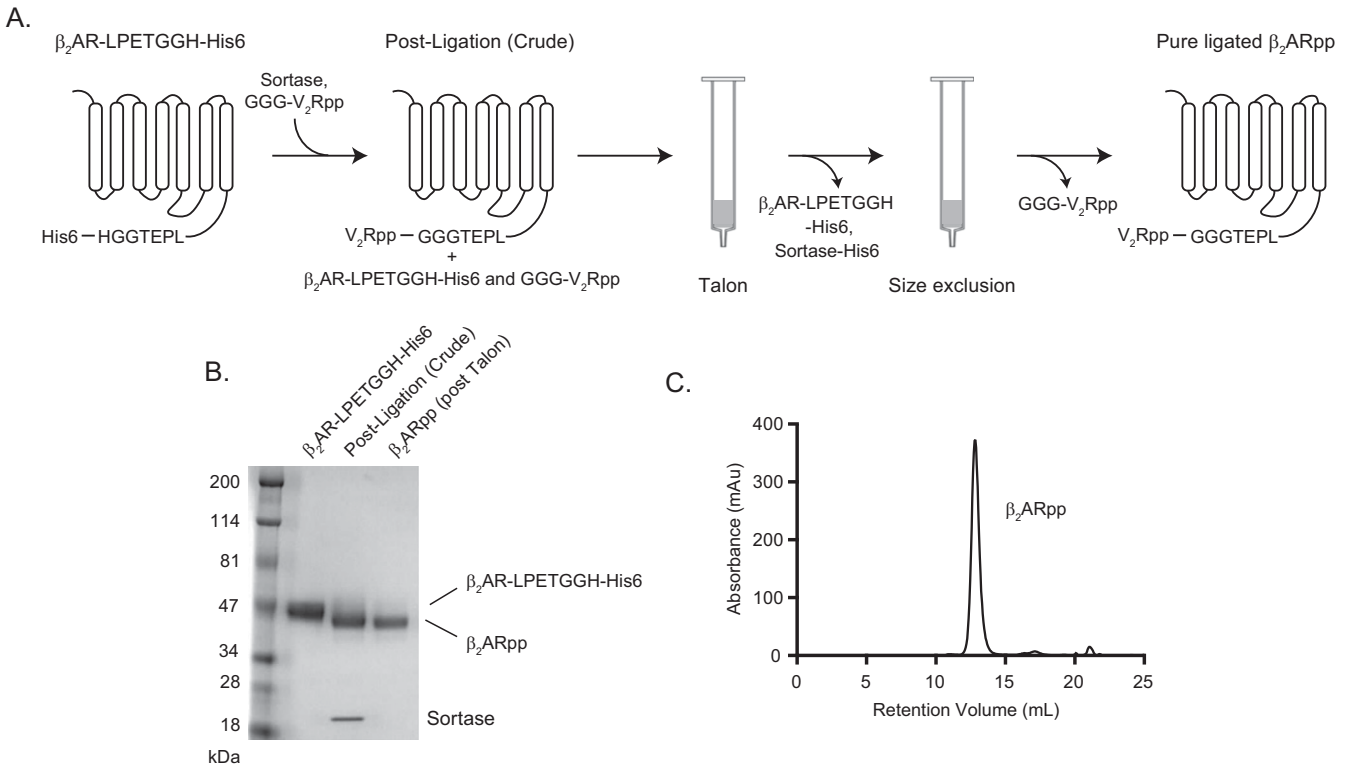

Fig. S1. Generation and purification of phosphopeptide (pp)-ligated β_2AR (β_2ARpp). (*A*) Purified FLAG-β_2AR in detergent containing the sortase consensus site LPETGHH after amino acid 365 and a C-terminal 6xHis tag (β_2AR-LPETGHH-His6) was modified using sortase-His6 and GGG-V_2Rpp. Ligated β_2ARpp was subsequently purified from unligated receptor (β_2AR-LPETGGH-His6), sortase-His6, and GGG-V_2Rpp by Talon metal affinity resin and size exclusion chromatography, as shown in the schematic. (*B*) Protein (Coomassie) staining of β_2ARpp purification. (*C*) Size exclusion chromatographic analysis of β_2ARpp.

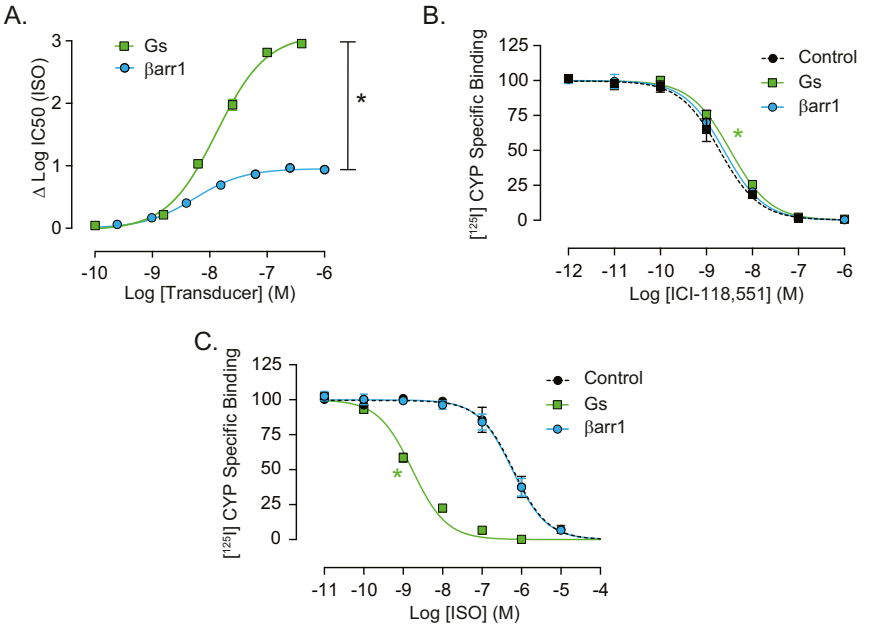

Fig. S2. The increase in agonist affinity to the β_2ARpp induced by β-arrestin1 is 100-fold less than that induced by G protein (*A*) and requires receptor phosphorylation (*B*). (*A*) Plot of changes in the log IC_{50} values of isoproterenol (ISO) derived from competition radioligand binding experiments using [^{125}I]-cyanopindolol (CYP) and HDL-β_2ARpp in the absence or indicated concentration of transducer [Gs and β-arrestin1 (βarr1)]. (*B* and *C*) Competition binding assays using HDL-β_2ARpp, [^{125}I]-CYP, and the indicated concentration of ICI-118,551 (*B*) or ISO (*C*) in the presence or absence of Gs (100 nM) or βarr1 (1 μM). In *C*, HDL-β_2ARpp was treated with calf-intestinal alkaline phosphatase (CIP) before assay setup. Error bars represent SE from at least three independent experiments. The asterisk (*) in *A* indicates that curve fit maxima are significantly different ($P < 0.05$, *t* test); the asterisks in *B* and *C* indicate log IC_{50} values significantly different from the control curves ($P < 0.05$, one-way ANOVA).

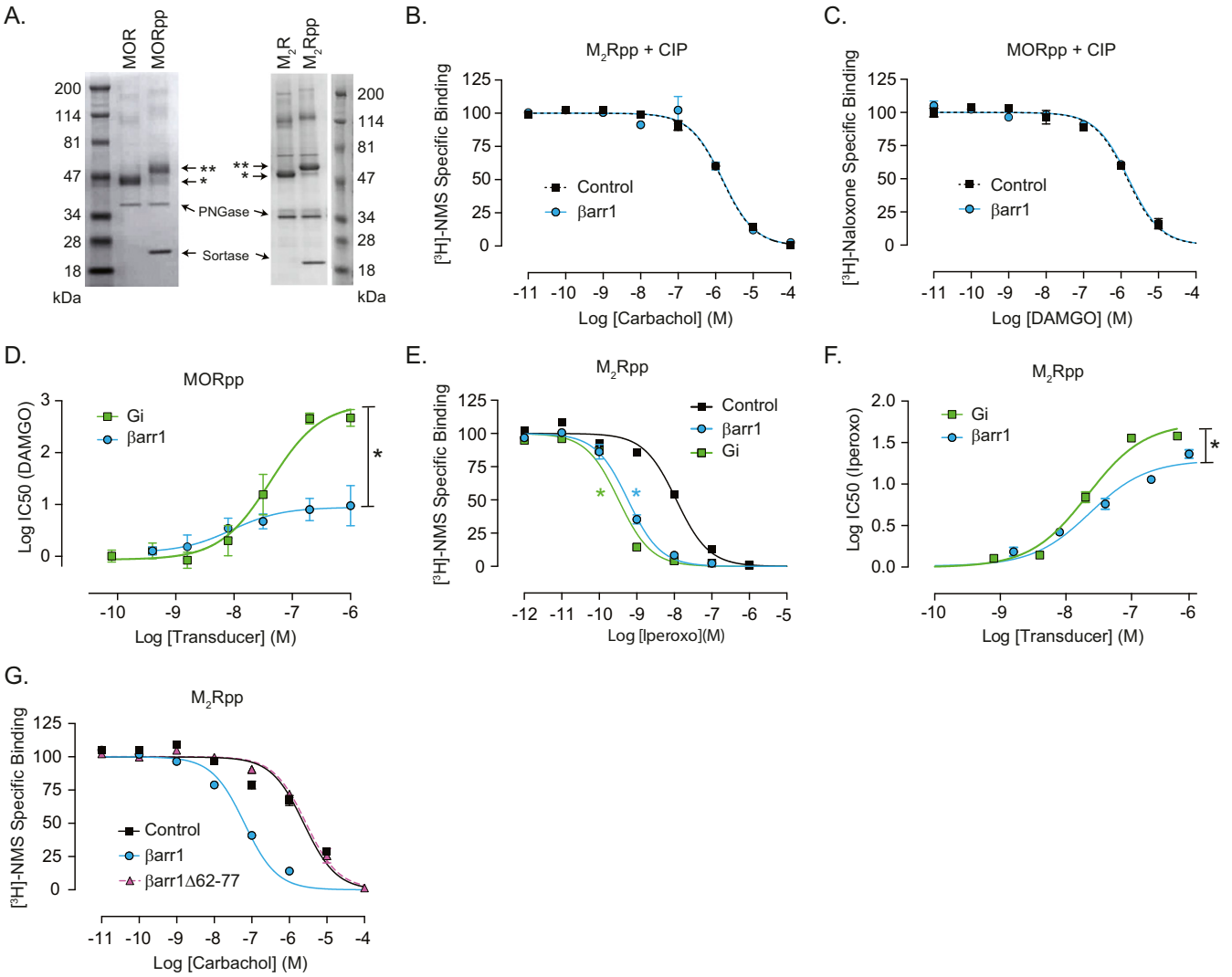

Fig. S3. Allosteric regulation of MOR and M_2R by Gi and β-arrestin1. (*A*) Phosphopeptide-ligated (**) MOR (MORpp) and M_2R (M_2Rpp) were generated by incubating receptors containing a C-terminal sortase recognition site (*) with GGG-V_2Rpp and sortase. To determine ligation efficiency, a small fraction of the reaction was deglycosylated with PNGase to visualize changes in receptor molecular weight. Coomassie-stained protein gels are shown. (*B* and *C*) Competition binding assays using HDL-M_2Rpp (*B*) or -MORpp (*C*) performed as in Fig. 5 *A* and *B*, respectively, except that HDLs were treated with calf-intestinal alkaline phosphatase (CIP) before assay setup. (*D*) Graph of changes in the log IC_{50} values for DAMGO determined from competition radioligand binding experiments using [^{3}H]-naloxone and HDL-MORpp in the absence or indicated concentration of transducer [Gi and β-arrestin1 (βarr1)]. (*E*) Competition binding assays using HDL-M_2Rpp performed as in Fig. 5*A* using iperoxo as the competitor ligand in the presence or absence of Gi (100 nM) or βarr1 (1 μM). (*F*) Plot of changes in the log IC_{50} values of iperoxo obtained from competition binding experiments using [^{3}H]-NMS and HDL-M_2Rpp in the absence or indicated concentration of transducer (Gi and βarr1). (*G*) Competition binding assays using HDL-M_2Rpp performed as in Fig. 5*A* using a finger loop deletion mutant of βarr1 (Δ62–77) (1 μM). Error bars represent SE from at least three independent experiments. Asterisks (*) in *E* and *G* indicate log IC_{50} values significantly different from the control curve ($P < 0.05$, one-way ANOVA); asterisks in *D* and *F* indicate that the curve fit maxima are significantly different ($P < 0.05$, *t* test).

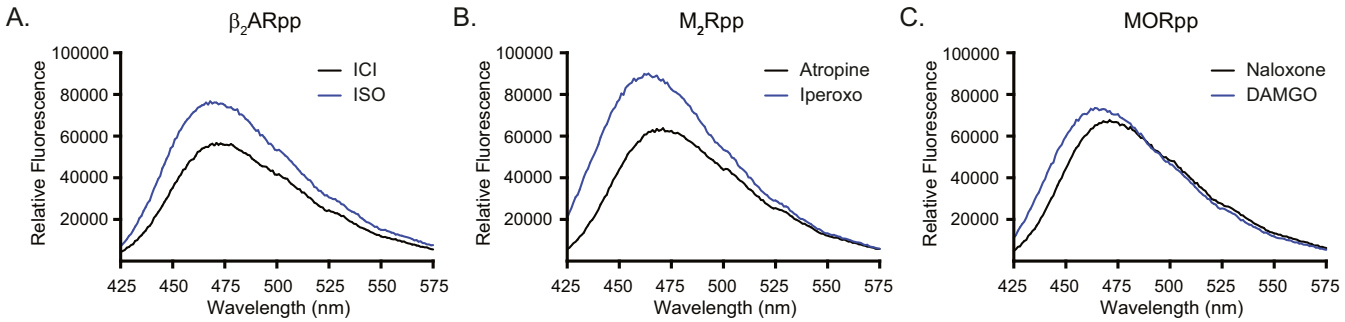

Fig. S4. Analysis of β-arrestin1 coupling to the transmembrane core of β_2ARpp, MORpp, and M_2Rpp HDLs by bimane fluorescence. Fluorescence spectra of β-arrestin1 (βarr1) labeled with monobromobimane at residue 70 in the presence of β_2ARpp (*A*), M_2Rpp (*B*), and MORpp (*C*) HDLs and the indicated antagonist (black line) or agonist (blue line). Data are representative of three independent experiments.

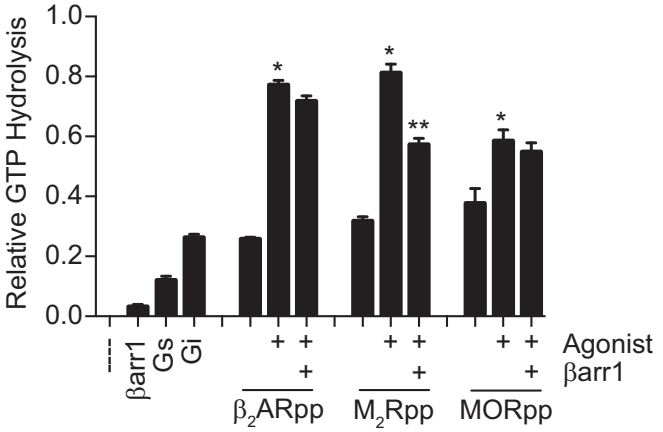

Fig. S5. The inhibition of G protein activation by β-arrestin1 correlates with its coupling strength to the receptor transmembrane core. The GTPase activity (GTP hydrolysis) of purified G protein (Gs or Gi) was measured in vitro in the presence and absence of β_2ARpp (Gs), M_2Rpp (Gi), and MORpp (Gi) HDLs. Treatment with agonists isoproterenol (ISO) (β_2ARpp), iperoxo (M_2Rpp), and DAMGO (MORpp) significantly increases G protein activation (*$P < 0.05$, one-way ANOVA). The presence of β-arrestin1 (βarr1) significantly blocks activation M_2Rpp (**$P < 0.05$, one-way ANOVA) but not for β_2ARpp or MORpp. Error bars represent SE from three independent experiments.