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Noncanonical scaffolding of $G_{\alpha i}$ and β -arrestin by G protein-coupled receptors

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Heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) are common drug targets and canonically couple to specific G_{α} protein subtypes and β -arrestin adaptor proteins. G protein- and β -arrestin-mediated signaling have been considered separable. We show GPCRs promote a direct interaction between $G_{\alpha i}$ protein subtype family members and β -arrestins, regardless of their canonical $G_{\alpha i}$ protein subtype coupling. $G_{\alpha i}$ - β -arrestin complexes bound extracellular signal-regulated kinase (ERK) and their disruption impaired both ERK activation and cell migration, consistent with β -arrestins requiring a functional interaction with $G_{\alpha i}$ for certain signaling events. These results introduce a GPCR signaling mechanism distinct from canonical G protein activation in which GPCRs cause the formation of $G_{\alpha i}$ - β -arrestin signaling complexes.

Heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) couple to specific G_{α} protein subtypes and activate G proteins by catalyzing guanine nucleotide exchange (1). These distinct G_{α} protein subtypes then amplify specific second messenger signaling systems. β -arrestins inhibit, or 'arrest,' canonical G protein-mediated signaling, and promote other forms of GPCR signaling, such as phosphorylation of extracellular signal-regulated kinase (ERK) (2, 3). G proteins and β -arrestins may coordinate their signaling as they can be found in signaling complexes consisting of a GPCR bound to both G protein and β -arrestin (4, 5).

We developed a bioluminescent resonance energy transfer (BRET) method to identify tripartite interactions between GPCRs, G_{α} proteins, and β -arrestins. Agonist treatment of the $G_{\alpha s}$ -coupled vasopressin type 2 receptor (V_2R) expressed in human embryonic kidney 293T (HEK 293T) cells catalyzed the formation of $G_{\alpha i}$ - β -arrestin scaffolds that were not observed between β -arrestin and other G_{α} subtypes. A similar pattern of $G_{\alpha i}$ - β -arrestin complex formation was also observed after activation of each GPCR that we tested, which

included receptors canonically coupled to $G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha q}$. Notably the $G_{\alpha i}$ - β -arrestin scaffolds formed complexes with GPCRs and also bound ERK. Disrupting $G_{\alpha i}$ and β -arrestin interactions attenuated V_2R -mediated activation of ERK phosphorylation. Disrupting $G_{\alpha i}$ and β -arrestin interactions eliminated GPCR-mediated migration toward a β -arrestin-biased agonist that does not stimulate canonical $G_{\alpha i}$ signaling. These results reveal the formation of $G_{\alpha i}$ - β -arrestin signaling scaffolds as a GPCR signaling mechanism.

Results

β -arrestin, $G_{\alpha i}$, and receptors form complexes

GPCRs differentially associate with β -arrestins in cells exposed to agonists (6). Activation of V_2R results in a long-lived receptor association with β -arrestin (7). This contrasts with other receptors, such as the β_2AR , that form transient interactions with β -arrestin dissociation occurring at or near the plasma membrane (8). $G_{\alpha s}$, β -arrestin, and a GPCR can be detected in a complex (4). We used BRET to examine such complexes (Fig. 1A) (9, 10). We used a split luciferase system, nanoluciferase binary technology (NanoBiT), relying on a

small 11-amino acid complementing peptide (smBiT) fused to one protein and a near-full length nanoluciferase (LgBiT) fused to another protein (fig. S1A) (11). The complementation of the split luciferase components is driven by the interaction of their fusion partners (e.g., β -arrestin and G_α proteins) as smBiT and LgBiT have low affinity for each other (12). When in close proximity, the split luciferase can then transfer energy to a third protein tagged with the fluorescent protein acceptor, monomeric Kusabira Orange (mKO), generating a BRET response (fig. S1, B and C) (13). This technology enables real-time quantification of interactions between three proteins in living cells, and we used it to confirm simultaneous interactions between $G_{\alpha s}$, β -arrestin, and V_2R expressed in HEK 293T cells following agonist treatment (Fig. 1, B and C). The canonically $G_{\alpha s}$ -coupled V_2R also formed a complex with $G_{\alpha i}$ and β -arrestin in HEK 293T cells treated with the V_2R agonist arginine vasopressin (AVP) (Fig. 1, D and E). We also swapped the location of the dipole donor and acceptor components (Fig. 1F). Rearranging the BRET components increased the observed signal and confirmed that $G_{\alpha i}$: β -arrestin complexes can associate with the V_2R (Fig. 1, G and H, and fig. S2A). Agonist treatment of the $G_{\alpha s}$ -coupled β_2AR also induced formation of $G_{\alpha i}$: β -arrestin: β_2AR complexes (Fig. 1, I and J, and fig. S2B). We validated the specificity of $G_{\alpha i}$: β -arrestin:GPCR complex formation by co-transfecting both untagged and mKO-tagged β_2AR and V_2R . $G_{\alpha i}$: β -arrestin:GPCR complexes were only detected in cells treated with the cognate agonist for the mKO-tagged receptor (fig. S2, C to H), indicating a specific interaction of $G_{\alpha i}$: β -arrestin with activated receptors.

$G_{\alpha i}$: β -arrestin: V_2R complexes at the plasma membrane

We visualized localization of $G_{\alpha i}$, β -arrestin, and V_2R by confocal microscopy (Fig. 2). We validated the imaging parameters using single-color controls to ensure accurate quantification of each component channel (fig. S3). Colocalization of $G_{\alpha i}$, β -arrestin, and V_2R occurred after agonist treatment and was most prominent at the plasma membrane (Fig. 2, A and B). Line scan analyses demonstrated plasma membrane-localized puncta containing $G_{\alpha i}$, β -arrestin, and V_2R components after 5 min of agonist treatment (Fig. 2C). Thirty minutes after agonist treatment, clear endosomal localization of β -arrestin and V_2R was observed that largely lacked $G_{\alpha i}$ (Fig. 2D). These observations are consistent with colocalization of $G_{\alpha i}$, β -arrestin, and V_2R that occurs after agonist treatment and is most prominent at the plasma membrane.

Distinction of $G_{\alpha i}$ complexes with β -arrestin from $G_{\alpha s}$: β -arrestin:receptor complexes

The difference in magnitude of signal observed in BRET between $G_{\alpha i}$ and $G_{\alpha s}$ in G protein: β -arrestin: V_2R complexes may reflect distinct interaction orientations. Sustained G protein

signaling can exist at receptors after β -arrestin-dependent internalization (14) and β -arrestins can be catalytically activated by an agonist-occupied receptor (15). We therefore tested if a direct functional interaction between G proteins and β -arrestins could be catalyzed by agonist treatment of the V_2R . We confirmed that the V_2R canonically signaled via $G_{\alpha s}$ (Fig. 3A), increased intracellular concentration of cyclic adenosine monophosphate (cAMP) (Fig. 3B), and recruited β -arrestin (Fig. 3C). V_2R did not canonically signal through $G_{\alpha i}$ in a TGF- α shedding assay (16) that works by coupling G_α protein activation to alkaline phosphatase activity to generate an absorbance signal (Fig. 3A).

We evaluated the four primary G_α families, $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 12}$, in the split luciferase (nanoBiT) system (Fig. 3D). In contrast to canonical V_2R - $G_{\alpha s}$ protein signaling, only $G_{\alpha i}$, but not $G_{\alpha s}$, $G_{\alpha q}$, or $G_{\alpha 12}$, were detected in complexes with β -arrestin when V_2R was treated with AVP (Fig. 3, E and F).

Given that the V_2R is not known to signal through $G_{\alpha i}$, and the absence of canonical $G_{\alpha i}$ signaling in our assays, we then varied the amounts of G_α subunits transfected by up to 10-fold in our G_α : β -arrestin complex formation assay. We did not observe an interaction between $G_{\alpha s}$, $G_{\alpha q}$, or $G_{\alpha 12}$ and β -arrestin with agonist activated V_2R or β_2AR . However we observed an interaction between $G_{\alpha i}$ and β -arrestin under these conditions (fig. S4, A to H). Transient overexpression of increased amounts of $G_{\alpha i}$ directly correlated with increased basal association between $G_{\alpha i}$ and β -arrestin (fig. S5). Taken together with the decreased agonist-induced signal, these data are supportive of a specific association between $G_{\alpha i}$ and β -arrestin that can be promoted by GPCRs. As controls, we verified that the various G_α protein subtypes were expressed in similar amounts (fig. S6). Furthermore, $G_{\alpha i}$ isoforms 2 and 3, as well as the very similar $G_{\alpha o}$, also interacted with β -arrestin after agonist activation of V_2R (fig. S7). A similar $G_{\alpha i}$ -family interaction with β -arrestin-1 was also observed (fig. S8, A and B).

We then tested whether $G_{\alpha i}$ and β -arrestin could interact in multiple orthogonal assays. We performed nanoBRET between G_α -NanoLuc and β -arrestin-2-mKO to confirm that this complex formed only with $G_{\alpha i}$ and not $G_{\alpha s}$ (fig. S9A). Purified $G_{\alpha i}$ and β -arrestin demonstrated a physical interaction in an immunoprecipitation assay (Fig. 3G). A thermal shift assay was used that measures the thermodynamic effects of protein-protein interactions through the binding of a hydrophobic dye (17). The presence of non-hydrolysable GTP with purified $G_{\alpha i}$ and β -arrestin-2-394X (which induces an active conformation of β -arrestin (18)) shifted the transition temperatures relative to either $G_{\alpha i}$ plus non-hydrolysable GTP or β -arrestin alone, indicating an interaction (fig. S9, B to E). These biochemical experiments are consistent with $G_{\alpha i}$ and β -arrestin directly interacting without the need for other partners. In a cellular context, overexpressed and tagged β -

arrestin co-immunoprecipitated with similarly overexpressed and tagged V₂R and G_{ai} in HEK 293T cells (fig. S10).

The interaction between G_{ai} and β -arrestin was partially sensitive to pertussis toxin (Fig. 3H), which promotes enzymatic ADP ribosylation of cysteine 352 in helix 5 of G_{ai} (19). Mutation of cysteine 352 to isoleucine relieved sensitivity to pertussis toxin (Fig. 3I). Pertussis toxin pretreatment of HEK 293T cell expressing either the V₂R or β_2 AR did not affect β -arrestin recruitment (fig. S11, A and B). This pertussis toxin treatment did not appear to reduce G_{ai}: β -arrestin complex formation by interfering with β -arrestin recruitment to the receptor (15, 20).

We investigated if the V₂R could recruit G_{ai} and if β -arrestin was required. G_{ai} was recruited to activated V₂R in both wild-type HEK 293T cells and cells depleted of β -arrestin1 and β -arrestin2 (fig. S12, A and B). G_{as} was also recruited to the V₂R in cells treated with agonist (fig. S12, C and D). These results are consistent with prior findings that canonically G_{as}-coupled receptors can also recruit G_{ai} (21).

We investigated if disrupting β -arrestin recruitment would impair G_{ai}: β -arrestin complex formation. GPCR kinases (GRKs) phosphorylate intracellular serine and threonine residues of the receptors, often within the C-terminal tail, to promote binding of β -arrestin (22, 23). β -arrestin interacts with both a phosphorylated GPCR tail, as well as the receptor core (24). To probe the contribution of the phosphorylated tail we used the canonically G_{ai}-coupled chemokine receptor CXCR3 that has a functional C-terminal truncation mutant (CXCR3 L344X, lacking phosphorylatable tail residues) (25). Both the L344X mutation and a mutation of four C-terminal serine and threonine residues to alanine (CXCR3 4xA) resulted in CXCR3 receptors that were equivalently expressed on the surface of HEK 293T cells as WT CXCR3 (fig. S13, A and B). Canonical G_{ai} signaling downstream of CXCR3 L344X showed a greater response (left shifted EC₅₀ and higher E_{max}) than either WT CXCR3 or CXCR3 4xA (fig. S13C). For β -arrestin-2 recruitment, both CXCR3 L344X and CXCR3 4xA had an attenuated response (lower E_{max}) relative to that of WT CXCR3 (fig. S13D). Similarly, the mutant CXCR3s displayed reduced G_{ai}: β -arrestin complex formation (fig. S13E) but with similar kinetics (fig. S13F) to those of WT CXCR3. This β -arrestin recruitment to the receptor appears necessary for G_{ai}: β -arrestin complex formation, (4, 26)

We used V₂R antagonists to assess the reversibility of G_{ai}: β -arrestin complex formation. Treatment of HEK 293T cells overexpressing V₂R with either a membrane-permeant V₂R antagonist (SR121463) or a membrane-impermeant V₂R antagonist (H3192) prevented formation of G_{ai}: β -arrestin complexes (Fig. 3J). We assessed the ability of these antagonists with different cell permeability properties to disrupt β -arrestin association either with V₂R or G_{ai}. Interactions

between the V₂R and β -arrestin occur both at the plasma membrane and within intracellular endosomes (6). The membrane-permeant antagonist was more efficient at reversing V₂R: β -arrestin association than the membrane-impermeant antagonist (Fig. 3K). In contrast, both antagonists equivalently disrupted G_{ai}: β -arrestin complexes (Fig. 3L) indicating that G_{ai}: β -arrestin complexes are present primarily at the plasma membrane, consistent with the confocal findings. The antagonist experiments also demonstrate the reversible nature of this complex as well as confirm that the underlying interaction between G_{ai} and β -arrestin generated the signal in the nanoBiT system. After antagonist addition the half-life of the G_{ai}: β -arrestin complexes was longer than that of the V₂R: β -arrestin complexes (table S1). This G_{ai}: β -arrestin complex appears to be distinct from the previously described G_{as}: β -arrestin:GPCR complex (4).

Multiple GPCRs promote the formation of G_{ai}: β -arrestin complexes

Given the paradoxical results of the G_{as}-coupled V₂R catalyzing a G_{ai}: β -arrestin complex, we investigated if this phenomenon was generalizable to other GPCRs. We selected five well-studied GPCRs (β_2 AR, CXCR3, neurotensin 1 receptor (NTS₁R), and dopamine receptors D1 (D₁R) and D2 (D₂R)) to compare formation of G_{ai}: β -arrestin complexes with that of β -arrestin and their canonically coupled G_α subtype (or G_{as} in the case of canonically G_{ai}-coupled GPCRs) when overexpressed in HEK 293T cells. We confirmed the canonical G protein coupling of these GPCRs (Fig. 4, A to E). Only CXCR3 and D₂R canonically signaled through G_{ai}, with β_2 AR and D₁R primarily coupling to G_{as} and NTS₁R primarily to G_{aq}. All five of these GPCRs formed G_{ai}: β -arrestin complexes in response to agonist treatment with no discernable complexes between the other G_α subunits and β -arrestin (Fig. 4, F to J). Thus, formation of G_{ai}: β -arrestin complexes is a common mechanism across GPCRs.

G_{ai}: β -arrestin complexes facilitate ERK scaffolding and signaling

We tested potential functional consequences of G_{ai}: β -arrestin complexes by assessing whether they could scaffold ERK1 and ERK2, which are kinases that function in regulation of cell proliferation, cell survival, and apoptotic signaling (27, 28). GPCRs are thought to regulate ERK activation through phosphorylation by separate G protein and β -arrestin signaling pathways (29). β -arrestins regulate ERK signaling, however, CRISPR-Cas9 genome editing approaches have demonstrated a lack of ERK signaling in the collective absence of functional G proteins, leading some to conclude that β -arrestin signaling might be dispensable for ERK activation (30, 31). However, other studies using CRISPR-Cas9 demonstrate an essential role for β -arrestin in some pathways regulating ERK

activation (32). For G_{ai} -coupled receptors, β -arrestin-mediated ERK activation is pertussis toxin sensitive (33–35). However, how the G_{ai} and β -arrestin transducers cooperate in this pathway is unclear.

To investigate if G_{ai} : β -arrestin complexes could bind ERK in response to activated V_2R , we tagged ERK at either its N- or C terminus with the dipole acceptor mKO (Fig. 5A). Data were normalized to an untagged (cytosolic) mKO to account for changes in protein localization. Agonist treatment of V_2R catalyzed the formation of a G_{ai} : β -arrestin:ERK complex (Fig. 5B). The magnitude of the adjusted complex BRET ratio was dependent on the location of the mKO tag on ERK (ERK-mKO compared to mKO-ERK), consistent with orientation and distance dependence of resonance energy transfer between the luciferase donor and mKO dipole acceptor. To selectively evaluate the contributions of G_{ai} signaling on ERK phosphorylation, we used ‘ $\Delta 3G$ ’ HEK 293T cells depleted by CRISPR-Cas9 technology of three G_α protein subfamilies, $G_{as}/G_{aol}/G_{aq/11}$, and $G_{a12/13}$ (only G_{ai} members present) (fig. S14) (30). Agonist treatment of these ‘ $\Delta 3G$ ’ HEK 293T cells overexpressing V_2R increased phosphorylation of ERK (Fig. 5, C and D). Pretreatment with pertussis toxin decreased, but did not eliminate, ERK phosphorylation in ‘ $\Delta 3G$ ’ cells or cells lacking the 3 G_α proteins (Fig. 5, C and D). In these cells, pertussis toxin pretreatment in combination with β -arrestin1 and β -arrestin2 knockdown had a slightly greater effect on ERK phosphorylation (Fig. 5, C and D), consistent with possible functional coordination of G_{ai} and β -arrestin. Similar effects of pertussis toxin pretreatment on ERK phosphorylation were observed in ‘ $\Delta 3G$ ’ cells transiently overexpressing the β_2AR , D_1R , or NTS_1R (fig. S15). To support these findings, we used HEK 293T cells lacking all G_α proteins and restored G_{ai} expression, increased expression of β -arrestin, or both. Increasing expression of both, but not of either protein alone, significantly increased ERK phosphorylation after AVP treatment in these cells in which all G_α proteins had been suppressed (Fig. 5, E and F). These results are consistent with a role of G_{ai} : β -arrestin complexes in promoting ERK phosphorylation, although they do not preclude other G_α - or β -arrestin-mediated mechanisms for ERK activation.

A β -arrestin-biased AT_1R agonist promotes formation of the G_{ai} : β -arrestin complex and promotes pertussis toxin-sensitive cell migration

To assess whether G_{ai} : β -arrestin complexes also occur in the absence of canonical G protein activation, we used the angiotensin type 1 receptor (AT_1R) β -arrestin-biased agonist, TRV120023, which does not promote canonical G protein signaling but does cause recruitment of β -arrestin (36, 37) (Fig. 6A). The endogenous ligand of AT_1R , Angiotensin II (AngII), signals through both G_{aq} and G_{ai} (Fig. 6B) and recruits β -arrestin (Fig. 6A). We verified that TRV120023 is a β -arrestin-

biased agonist in our assays and that it had no little or no activity at promoting canonical G protein signaling through any of the four G_α -family proteins tested (Fig. 6C) but did stimulate β -arrestin recruitment to the receptor (Fig. 6A). Because TRV120023 does not activate canonical G protein signaling, it would be predicted not to induce cell migration, which is thought to require canonical G protein signaling (38). However, not only did TRV120023 promote migration, this migration was pertussis toxin sensitive, as pretreatment of cells with pertussis toxin reduced TRV120023-mediated migration by ~50%. Furthermore, inhibition of both G_{ai} and β -arrestin through pertussis toxin pretreatment and siRNA mediated depletion of β -arrestin1 and β -arrestin2 eliminated migration in HEK 293T cells stably expressing AT_1R (Fig. 6, D and E). Migration of primary human pulmonary arterial smooth muscle cells (PASMCs) toward TRV120023 was also pertussis toxin sensitive, indicating that this observation was not unique to immortalized model cell lines and this mechanism can occur with physiological amounts of receptor and effector expression (fig. S16, A to D). Since treatment of the AT_1R with either the endogenous agonist AngII or the β -arrestin-biased agonist TRV120023 induced G_{ai} : β -arrestin complex formation when overexpressed in HEK 293T cells (Fig. 6F), the migration findings are consistent with the possibility that G_{ai} : β -arrestin complexes can promote functional responses after GPCR activation.

Discussion

Our results reveal a GPCR signaling mechanism in which GPCRs can promote formation of G_{ai} : β -arrestin complexes. These G_{ai} : β -arrestin complexes were observed downstream of all receptors tested, even with receptors that do not canonically signal through G_{ai} . Various GPCR ligands drove formation of G_{ai} : β -arrestin complexes, even the β -arrestin-biased ligand TRV120023, which promotes little or no classical G protein-mediated signaling. A major determinant of β -arrestin association with G_{ai} appears to be GPCR-mediated recruitment of β -arrestin to the plasma membrane. G_{ai} : β -arrestin scaffolds can include a GPCR, or a signaling effector (ERK), or possibly both, and may form functional signaling complexes. Such signaling complexes were associated with V_2R -mediated ERK activation, even though the stimulatory GPCR ligand (AVP) does not activate canonical G_{ai} signaling. It remains to be addressed if G_{as} is also a component of these G_{ai} : β -arrestin complexes formed in response to agonist stimulation of G_{as} -coupled receptors. Using HEK 293T cells depleted of the $G_{as}/q/12$ proteins and overexpressing the V_2R , we demonstrated that AVP-induced ERK phosphorylation is decreased after G_{ai} inhibition with pertussis toxin and β -arrestin depletion with siRNA. Furthermore, expression of G_{ai} in cells lacking all functional G proteins combined with overexpression of β -arrestin, but not either β -arrestin

overexpression or G_{ai} rescue alone, significantly increased AVP-induced ERK phosphorylation. Pertussis toxin impaired migration of both primary human PSMCs and immortalized cells treated with a β -arrestin-biased AT_1R ligand, TRV120023. It remains unclear exactly how G_{ai} : β -arrestin complexes participate in the process of ERK activation and cell migration (Fig. 6G).

β -arrestin signaling is necessary for full efficacy responses in many cellular and physiological activities (22). However signaling functions attributed to β -arrestin are absent when functional G proteins were eliminated (30). This study bridges these seemingly contradictory results concerning the interplay of G protein and β -arrestin signaling (31, 32) by delineating a G_{ai} : β -arrestin scaffolding complex. Furthermore, this study provides further validity to computational simulations and other experimental observations suggesting that the activated state of β -arrestin persists even when it is not bound to a receptor (39). This work also offers plausible mechanistic insight into our initially paradoxical observations that G_{ai} can drive ERK phosphorylation downstream of the canonically G_{as} -coupled V_2R and that pertussis toxin inhibits cell migration in response to a β -arrestin-biased agonist.

A number of caveats must be considered when interpreting our results. Many experiments rely on the overexpression of components which have been genetically modified by insertion of various fluorescent reporter probes. Probe architecture can impact the intensity of the signal generated. Because of overexpression, interactions may be detected which would not be seen at physiologically relevant concentrations of these molecules. However, our control experiments, and our observation that the lowest concentrations of expression vector provide the highest signal-to-noise for G_{ai} : β -arrestin complex formation (fig. S4), and our findings in primary human PSMCs, support our interpretation that this complex formation is neither an artifact of probe orientation nor dependent on overexpressed conditions. The presence of G_{ai} : β -arrestin association in orthogonal assays (TSA, immunoprecipitation in both purified and non-purified conditions, and nanoBRET) provides further support for the existence of G_{ai} : β -arrestin complexes.

In summary, our findings demonstrate that GPCRs promote formation of G_{ai} : β -arrestin complexes, and that these G_{ai} : β -arrestin complexes appear necessary for certain aspects of β -arrestin signaling. A better understanding of their physiological roles may provide strategies for therapeutic targeting of GPCRs.

Materials and Methods

Cell culture and transfection

Human embryonic kidney cells (HEK 293, HEK 293T, Rockman β arrestin-1/2 HEK 293 knockout, triple G_a subfamily

knockout (' $\Delta 3G$ ') HEK 293T cells, and quadruple G_a subfamily knockout (' $\Delta 4 G$ ') HEK 293T cells) were maintained in minimum essential medium supplemented with 1% antibiotic-antimycotic (GIBCO) and 10% fetal bovine serum. Rockman β arrestin-1/2 HEK 293 knockout were supplied by Dr. H. Rockman and validated as previously described (32). Cells were grown at 37°C with humidified atmosphere of 5% CO_2 . For BRET and luminescence studies, HEK 293T cells were transiently transfected via an optimized calcium phosphate protocol as previously described (20). For immunoblot studies utilizing siRNA, ' $\Delta 3G$ ' or ' $\Delta 4G$ ' HEK 293T cells were transiently transfected with Lipofectamine 3000 (Thermo Fisher) or RNAiMAX (Thermo Fisher) according to manufacturer specifications. For TGF alpha shedding assay studies, ' $\Delta 3G$ ' HEK 293T cells were transfected using Fugene 6 (Promega) or Lipofectamine 2000 (Thermo Fisher) according to manufacturer specifications.

Generation of constructs

Construct cloning was performed using conventional techniques such as restriction enzyme/ligation methods. Linkers between the fluorescent proteins or luciferases and the cDNAs for receptors, transducers, kinases, or adaptor proteins were flexible (GGGGS) and ranged between 15-18 amino acids. CXCR3 C terminus phosphomutant constructs were generated using a Quikchange mutagenesis kit (Agilent, Santa Clara, CA). See table S2 for a list of constructs used in this manuscript.

Drugs

VUF10661, arginine vasopressin (AVP), dopamine, angiotensin II, neurotensin and isoproterenol were purchased from Sigma-Aldrich (St. Louis, MO). VUF10661 and isoproterenol were dissolved in dimethyl sulfoxide (DMSO) and stored in a desiccator cabinet. Stock solutions of AVP, angiotensin II, neurotensin, (Sigma-Aldrich) were prepared according to manufacturer specifications. TRV120023 was synthesized by Genscript (Piscataway, NJ). Stock solutions of neurotensin were made in 0.1% BSA in PBS. Dopamine was prepared fresh in BRET media supplemented with 0.03% ascorbic acid (Sigma-Aldrich). H3192 and SR121463 were kindly provided by the laboratory of R. J. Lefkowitz. All drug dilutions were performed with BRET media or cell culture media. PTX was obtained from List Biological Laboratories (Campbell, CA). All compound stocks were stored at $-20^\circ C$ until use.

Split luciferase and BRET assays

HEK 293T cells seeded in 6-well plates were co-transfected with 500 ng of smBiT tagged β -arrestin-2, and either 250 ng of LgBiT tagged receptor or 2000 ng of untagged receptor and varying concentrations of LgBiT G_a protein expression vector (most experiments were conducted between 50-200 ng of G_a

plasmid) or 2000ng of mKO tagged β -arrestin-2 and 500 ng of smBiT tagged V₂R using a previously described calcium phosphate protocol (40). Twenty-four hours post-transfection, cells were plated onto clear bottom, white-walled 96-well plates at 50,000-100,000 cells/well in “BRET media” - clear minimum essential medium (GIBCO) supplemented with 2% FBS, 10 mM HEPES, 1x GlutaMax, and 1x Antibiotic-Antimycotic (GIBCO). Select cells were then treated overnight with pertussis toxin at a final concentration of 200 ng/mL. The following day, media were removed, and cells were incubated at room temperature with 80 μ L of Hanks’ balanced salt solution (GIBCO) supplemented with 20mM HEPES and 3 μ M coelenterazine-h (Goldbio or Promega) for ~15 min. For luminescence split luciferase studies, plates were read with a BioTek Synergy Neo2 plate reader set at 37°C with a 485 nm emission filter. Cells were stimulated with either vehicle (Hank’s Balanced Salt Solution with 20 mM HEPES) or indicated concentration of agonist. For split luciferase luminescence experiments, plates were read both before and after ligand treatment to calculate Δ net change in luminescence and subsequently normalized to vehicle treatment. For BRET experiments, plates were read on a Berthold Mithras LB940 using pre-warmed media and instrument at 37°C using a standard Rluc emissions filter (480 nm) with a custom mKO 542 nm long-pass emission filter (Chroma Technology Co., Bellows Falls, VT). Readings were performed using a kinetic protocol. The BRET ratio was calculated by dividing the mKO signal by the luciferase signal. For some experiments, a net BRET ratio was calculated by subtracting the vehicle BRET ratio from the ligand stimulated BRET ratio, or an adjusted BRET ratio was calculated by subtracting the ligand treated cytosolic mKO signal from the ligand treated effector mKO signal.

Immunoblotting

Experiments were conducted as previously described (40). Briefly, cells were serum starved for at least four hours, stimulated with the indicated ligand, subsequently washed 1x with ice-cold PBS, lysed in ice-cold RIPA buffer containing phosphatase and protease inhibitors (Phos-STOP (Roche), cOmplete EDTA free (Sigma)) and rotated for forty-five minutes, and cleared of insoluble debris by centrifugation at $>12,000 \times g$ (4°C, 15 min), after which the supernatant was collected. Protein was resolved on SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with the indicated primary antibody overnight (4°C). phospho-ERK (Cell Signaling Technology, #9106) and total ERK (Millipore #06-182) were used to assess ERK activation. AI-CT antibody that recognizes both isoforms of β -arrestin was utilized (32), with protein loading assessed by alpha-tubulin (Sigma #T6074). Galpha i-1 (13533, Santa Cruz Biotechnology), Galpha q/11/14 (365906, Santa Cruz Biotechnology),

Galpha 12 (515445, Santa Cruz Biotechnology), Galpha 13 (293424, Santa Cruz Biotechnology), Galpha s/olf (55545, Santa Cruz Biotechnology) antibodies were used to verify ‘ Δ 3G’ HEK 293 cells.

Horseshoe peroxidase-conjugated polyclonal mouse anti-rabbit-IgG or anti-mouse-IgG were used as secondary antibodies. Immune complexes on nitrocellulose membrane were imaged by SuperSignal enhanced chemiluminescent substrate (Thermo Fisher). Following detection of phospho signal, nitrocellulose membranes were stripped and reblotted for total kinase signal. For quantification, phospho-protein signal was normalized to total protein signal using ImageLab (Bio-Rad) within the same immunoblot.

siRNA knockdown

‘ Δ 3G’ HEK 293T cells were transiently transfected with either Lipofectamine 3000 (Thermo Fisher) or RNAiMAX (Thermo Fisher) per manufacturer specifications in a six-well tissue culture sterile plate with 1 μ g of receptor and either 7 μ g of either control siRNA or 3.5 μ g siRNA directed to β -arrestin-1 and β -arrestin-2 sequences (Lipofectamine 3000 conditions) or 1000 ng receptor and 400 ng β -arrestin-1 and β -arrestin-2 siRNA or 800ng of control siRNA (RNAiMAX conditions) similar to that previously described (32). 24 hours later, the cells were transiently transfected with 1000 ng of receptor using Lipofectamine 3000 (Thermo Fisher) and left to incubate for an additional 48 hours.

Wound-healing migration assay – immortalized cells

HEK 293T cells stably expressing the AT₁R were utilized (41). Briefly, 70 μ L of cell suspension at a concentration of 5×10^5 cells per mL was applied into each well of silicone inserts (Ibidi, Martinsried, Germany) on 24 well plate, and after 24hrs incubation, the inserts were removed to create a wound field. The cells were incubated additionally for 12 hrs with 1 μ M of TRV120023 and visualized with a Zeiss Axio Observer microscope (Carl Zeiss, Thornwood, NY). Wound healing was then analyzed using ImageJ (NIH, Bethesda, MD) wound healing tool macros.

Wound-healing migration assay – primary human cells

Human pulmonary arterial smooth muscle cells (PASMCs) were isolated from donor lungs as previously described (42) (gift of Suzy Comhair and Serpil Erzurum, Cleveland Clinic Foundation). Human PASMCs were seeded into 24-well plates at a density of 1.5×10^4 cells per well in Human Smooth Muscle Cell Basal Medium supplemented with Human SMC Growth Supplement (Cell Application, Inc.). At 30% confluence, cells were transfected with 50 ng of control or β -arrestin-1 and β -arrestin-2 ON-TARGETplus siRNA pools (Dharmacon, Inc.). When the cells reached 100% confluency,

they were serum starved for 4 hours with 2 hours of PTX (200 ng/mL) treatment. Scratches were made using small pipette tips. Cells were washed using DPBS and stimulated with basal smooth muscle cell medium containing 1 μ M Angiotensin-II (Millipore Sigma) or 10 μ M TRV120023 (Genscript USA Inc.). The wound closure was monitored using live-cell station Zeiss Axio Observer microscope (Carl Zeiss, Thornwood, NY). The images were captured in real-time at 0 hour and for every hour until 16 hours. The initial edges of the scratch at 0 hour time point were marked and migrated distance 12-16 hours afterwards was measured using MetaMorph Premier software (Molecular Devices, San Jose, CA) at the Duke Light Microscopy Core Facility (Durham, NC).

TGF- α shedding assay

GPCR G_{α} activity was assessed by the transforming growth factor- α (TGF- α) shedding assay as previously described (16). Briefly, HEK 293 cells lacking $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha s/olf}$, and $G_{\alpha 12/13}$ were transiently transfected with receptor, modified TGF- α -containing alkaline phosphatase (AP-TGF- α), and the indicated G_{α} subunit. Cells were reseeded twenty-four hours later in Hanks' Balanced Salt Solution (HBSS) (Gibco, Gaithersburg, MD) supplemented with 5mM HEPES in a Costar 96-well plate (Corning Inc., Corning, NY). Cells were then stimulated with the indicated concentration of ligand for one hour. Conditioned media (CM) containing the shed AP-TGF- α was transferred to a new 96-well plate. Both the cell and CM plates were treated with para-nitrophenylphosphate (p-NPP, 100mM) (Sigma-Aldrich, St. Louis, MO) substrate for one hour, which is converted to para-nitrophenol (p-NP) by AP-TGF- α . This activity was measured at OD₄₀₅ in a Synergy Neo2 Hybrid Multi-Mode (BioTek, Winooski, VT) plate reader immediately after p-NPP addition and then after a one-hour incubation. G_{α} activity was calculated by first determining p-NP amounts by absorbance through the following equation:

$$100 * \left(\frac{\Delta OD_{405, CM}}{\Delta OD_{405, CM} + \Delta OD_{405, cell}} \right)$$

where $\Delta OD_{405} = OD_{405, 1hr} - OD_{405, 0 \text{ hours}}$ and $\Delta OD_{405, cell}$ and $\Delta OD_{405, CM}$ represent the changes in absorbance after one hour in the cell and CM plates, respectively. Data were normalized to a single well that produced the maximal signal.

Thermal shift assay

Protein thermal melting shift experiments were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems). Proteins were buffered in 20 mM HEPES pH 7.5, 100 mM NaCl, 4 mM MgCl₂, β -arrestin-2, $G_{\alpha i \beta \gamma}$, V₂Rpp, Fab30, and GTP γ S (nonhydrolyzable GTP analog) were added at a final concentration of 5 μ M, 10 μ M, 30 μ M, and 120 μ M, respectively. All reactions were set up in a 96-well plate at final

volumes of 20 μ L and SYPRO Orange (Thermo Fisher Scientific) was added as a probe at a dilution of 1:1000. Excitation and emission filters for the SYPRO-Orange dye were set to 475 nm and 580 nm, respectively. The temperature was raised with a step of 0.5°C per 30 s from 25°C to 99°C and fluorescence readings were taken at each interval. All measurements were carried out three times. Data were analyzed using Applied Biosystems® Protein Thermal Shift Software. Expression and purification of heterotrimeric G protein was conducted as previously described (17). In brief, Hive Five insect cells were infected with two viruses made from BestBac baculovirus system, one expressing human $G_{\beta 1}$ -His6 and $G_{\gamma 2}$ and the other $G_{\alpha i}$. Approximately forty-eight hours after infection the cells were harvested, solubilized, and heterotrimeric $G_{\alpha i}$ purified using Ni-NTA chromatography and HiTrap Q sepharose anion exchange (GE Healthcare Life Sciences).

Purified component pull-down

Expression and purification of β -arrestin-2 and heterotrimeric $G_{\alpha i}$ were purified similarly to previously described (43). Briefly, purified glutathione S-transferase tagged β -arrestin-2 and purified heterotrimeric G protein were added together at a final concentration of 4 μ M and 1 μ M, respectively, and placed on a tube revolver at room temperature for 30 min. Once complete, the solution was added to 40 μ L of washed glutathione agarose beads and placed on a tube rotator at room temperature for 60 min. After removal of the supernatant, samples were washed three times with 300 μ L of 20mM HEPES pH 7.5, 100mM NaCl. The sample was then eluted with 200 μ L of 2x SDS-sample buffer (8% SDS, 5% β -mercaptoethanol, 10% glycerol and 25 mM Tris pH 6.8). Protein was resolved on SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with antibodies directed to either 400 ng β -arrestin-1/2 (A1-CT, from laboratory of RJ Lefkowitz) or $G_{\alpha i 1}$ (13533, Santa Cruz Biotechnology).

Immunoprecipitation

Immunoprecipitation was conducted as previously described (44). Briefly, 4 μ g of HA-V₂R, 4 μ g of $G_{\alpha i}$ -GFP and 4 μ g of pcDNA-ARRB1-Flag and/or pcDNA were transfected into HEK 293T cells seeded in 6 cm plates. Forty-eight hours post-transfection, after approximately 4 hours of serum starvation, cells were stimulated with AVP for 5 and 10 min at 37°C. Cells were then lysed on ice for 10 min in FLAG lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA) supplemented with protease inhibitor cocktail tablet (Roche). Cell lysates were incubated with anti-FLAG M2 affinity gel (A2220, Sigma) overnight and immunoprecipitated ARRB1-FLAG were eluted with Flag peptides (F3290, Sigma). For primary antibody incubation, GFP polyclonal antibody (A6455, Invitrogen), HA-Tag (3724S, cell signaling biotechnology), and ANTI-FLAG M2 antibody (F3165, Sigma) were utilized.

Confocal microscopy

HEK293T cells plated in fibronectin-coated 35 mm glass bottomed dishes (MatTek Corp. P35G-0-14-C) were transiently transfected via the calcium-phosphate method with DNA encoding G_{ai}-mVenus (125 ng), β arr2-mKO (125 ng), and/or Mars1-V₂R (500 ng). Mars1 binds a membrane impermeant fluorogen (SCi1) and induces its fluorescence in the near-infrared spectrum (45). Cells were pulse labeled with SCi1 (diluted 1:5000 from 0.5 mg/mL stock) for 15 min before treatment with or without 100 nM AVP. Cells were then fixed at basal, 5 min, and 30 min after treatment with 4% paraformaldehyde. Samples were then imaged with a Plan-Apochromat 63x/1.4 Oil lens on a Zeiss LSM880 using corresponding laser lines to excite mVenus, mKO, or Mars1 (488 nm, 561 nm, 633 nm respectively). Spectral gating via a 34 spectral array detector was performed using single color transfection controls.

Image analysis

Confocal images were arranged and analyzed using ImageJ (NIH, Bethesda, MD). All image adjustments were identical and consistent across all samples and were based on the single-color control samples. The line scan analysis function was used to measure the intensity of each channel.

Graphing and statistical analyses

Data were analyzed in Microsoft Excel (Microsoft, Redmond, WA) and graphed in Prism 8.0 (GraphPad, San Diego, CA). Dose-response curves were fitted to a log agonist versus stimulus with three parameters (span, baseline, and EC₅₀) with the minimum baseline corrected to zero using Prism 8.0. Statistical analysis was also performed in Prism 8.0. For comparing ligands in concentration-response assays or time-response assays, a two-way ANOVA of ligand and concentration or ligand and time, respectively, was conducted. For sample size determination, most experiments were conducted three times, with some additional replicates acting as positive or negative controls for other experiments. Experiments were not randomized, and investigators were not blinded to treatment conditions. Critical plate-based experiments were independently replicated by at least two different investigators when feasible. For some analyses, a post hoc comparison was also made, with details included within figure legends. Replicates in figure legends refer to distinct cellular conditions (biological replicates); certain experiments also include technical replicates to show intra-replicate variation. Unless otherwise noted, statistical tests were two-sided and Bonferroni analyses were corrected for multiple comparisons. Further details of statistical analysis and replicates are included in the figure captions.

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and analyzed confocal experiments. J.S.S., T.F.P., K.Z., I.C., and S.R. analyzed all other data. J.S.S., T.F.P., M.G.C., and S.R. wrote the paper. All authors discussed the results and commented on the manuscript. **Competing interests:** A patent application for the BRET technique described in this manuscript has been filed on behalf of J.S.S., T.F.P., M.G.C., and S.R. by Duke University. All other authors declare no competing interests. **Data and materials availability:** All data are available in the manuscript or the supplementary materials. Data and materials will be fully available upon reasonable request. CXCR3, AT1R-RLucII, V2R-LgBiT, V2R-smBiT, β 2AR-LgBiT, smBiT- β arr2, smBiT- β arr1, V2R-mKO, β 2AR-mKO, β arr2-mKO, Erk2-mKO, mKO-ERK2, and Gna11-mVenus constructs (see table S2) are available from Sudarshan Rajagopal under a material agreement with Duke University. The G-protein-KO HEK293 cells and the NanoBiT-G-protein constructs are available from Asuka Inoue under a material agreement with Tohoku University.

SUPPLEMENTARY MATERIALS

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Figs. S1 to S16

Tables S1 and S2

MDAR Reproducibility Checklist

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Fig. 1. G protein:β-arrestin:GPCR complex formation. (A) Arrangement of luciferase fragments and mKO acceptor fluorophore on G protein (LgBiT), β-arrestin-2 (mKO), and V₂R (smBiT). HEK 293T cells were transiently transfected and stimulated with agonist or vehicle. (B) BRET ratio of G_{αs}-LgBiT:β-arrestin-mKO:V₂R-smBiT following AVP (500 nM) treatment. After AVP treatment, an increase in the BRET ratio was observed in cells expressing β-arrestin-mKO, but not in cells expressing cytosolic (untagged) mKO. (C) Quantification of G_{αs}-LgBiT:β-arrestin-mKO:V₂R-smBiT complex formation in cells treated with either vehicle or AVP at a single five-minute timepoint. (D) Similar experiment to panel (B), except testing the ability of G_{αi} to form a complex. BRET ratio of G_{αi}-LgBiT:β-arrestin-mKO:V₂R-smBiT following treatment with AVP. After AVP treatment, an increase in the BRET ratio was observed in cells expressing β-arrestin-mKO, but not in cells expressing cytosolic mKO (similar to panel (B)). (E) G_{αi}-LgBiT:β-arrestin-mKO:V₂R-smBiT formation at a single five-minute timepoint. (F) Rearrangement of BRET components; G_{αi} protein (LgBiT), β-arrestin (smBiT), and V₂R (mKO). (G) BRET ratio of G_{αi}-LgBiT:β-arrestin-smBiT:V₂R-mKO following AVP treatment. Rearrangement of BRET tags increased the observed signal when compared to panel (D). (H) Five-minute quantification of G_{αi}-LgBiT:β-arrestin-smBiT:V₂R-mKO complexes. (I) Similar experiment to panel (G), except testing the ability of G_{αi}-LgBiT:β-arrestin-smBiT to form a complex with the β₂AR-mKO as opposed to the V₂R-mKO. After isoproterenol (10 μM) treatment, an increase in the BRET ratio was observed in cells expressing β₂AR-mKO, but not in cells expressing cytosolic mKO. (J) Five-minute quantification of G_{αi}-LgBiT:β-arrestin-smBiT:β₂AR-mKO complexes. For kinetic experiments, **P*<0.05 by two-way ANOVA, Fischer's post hoc analysis with a significant difference between treatments; for five-minute quantification, **P*<0.05 by student's two-tailed *t* test; Panels (B) through (E), *n*=3 per condition; panels (G) through (J), *n*=4 per condition. Graphs show mean ± s.e.m. Cyto, cytoplasmic.

Fig. 2. Confocal microscopy of $G_{\alpha i}$: β -arrestin: V_2R complexes. Confocal microscopy analysis of AVP-induced complexes of $G_{\alpha i}$: β -arrestin: V_2R in HEK 293T cells transfected with mVenus-tagged $G_{\alpha i}$, mKO-tagged β -arrestin-2 and Mars1-tagged V_2R . (A) Single cells preceding treatment (basal), at 5 min, or at 30 min. Localization of $G_{\alpha i}$ -mVenus: β -arrestin-mKO: V_2R -Mars1 was observed at 5 min, with less at 30 min. Scale bars, 5 mm. (B) inset of images in (A), scale bars, 1 mm. (C) line scan analysis of 5-min time point, demonstrating colocalization of fluorophores following AVP (100 nM) treatment. (D) line-scan analysis of 30-min time point. Data are representative of ten (basal), twenty (5 min) or fifteen (30 min) fields of view from three independent experiments.

Fig. 3. The canonically G_{α_s} -coupled V_2R forms G_{α_i} : β -arrestin complexes. (A) Canonical G protein signaling of the V_2R . (B) cAMP generation of the V_2R . (C) Canonical β -arrestin-2-smBiT recruitment following AVP (500 nM) treatment of the V_2R -LgBiT. (D) Arrangement of luciferase fragments on G protein (LgBiT) and β -arrestin (smBiT). (E) Only G_{α_i} -LgBiT formed complexes with β -arrestin-2-smBiT after AVP (500 nM) treatment in cells overexpressing the V_2R . (F) Concentration-response on G_{α_i} -LgBiT: β -arrestin-2-smBiT association in cells overexpressing the V_2R . (G) G_{α_i} -LgBiT: β -arrestin-2-smBiT complex formation is sensitive to pertussis toxin pretreatment. Data are normalized to maximal signal within each replicate. (H) Loss of pertussis toxin sensitivity on G_{α_i} -LgBiT: β -arrestin-2-smBiT by mutation of the ADP-ribosylation site (C352). (I) Pretreatment for 30 min with a membrane permeable V_2R antagonist (SR121463, 10 μ M) or a membrane impermeant V_2R antagonist (H3192, 10 μ M) on G_{α_i} -LgBiT: β -arrestin-2-smBiT complex formation. (J) V_2R -LgBiT association with β -arrestin-2-smBiT after either membrane permeable SR121463 or impermeant H3192 V_2R antagonists. (K) G_{α_i} -LgBiT association with β -arrestin-2-smBiT after membrane permeable (SR121463) or impermeable (H3192) V_2R antagonists. (L) GST- β -arrestin-2 association with purified G_{α_i} . For panel (A), TGF- α shedding assay was conducted in 'Δ3G' HEK 293T cells. All other experiments were conducted in WT HEK 293T cells overexpressing the indicated assay components. For panels (A) and (E), $*P < 0.05$ by two-way ANOVA, with a main effect of G_{α_s} , or $G_{\alpha_{is}}$, respectively, vs other G_{α} subunits. For panels (G) and (H), $*P < 0.05$ by two-way ANOVA, main effect of pertussis toxin treatment. For panel (I), $*P < 0.05$ by two-way ANOVA, main effect of vehicle relative to either antagonist. For panels (J) and (K), $*P < 0.05$ by two-way ANOVA, Bonferroni post hoc of vehicle vs either antagonist. $^{#}P < 0.05$, by two-way ANOVA, Bonferroni post hoc of SR121463 vs H3192. Panels (A) through (C), (E), (F) and (H) through (J), $n = 3$ per condition; (K) $n = 6-9$ per condition, (G) $n = 8$; (L) is representative of three pulldown experiments. Graphs show mean $\pm$ s.e.m.

Fig. 4. GPCRs form $G_{\alpha i}$: β -arrestin complexes irrespective of canonical G protein coupling. Canonical G protein signaling at (A) β_2 AR; (B) CXCR3; (C) D_1 R; (D) D_2 R; (E) NTS_1 R assessed with the TGF- α shedding assay. $G_{\alpha i}$: β -arrestin complex formation at (F) β_2 AR (10 μ M isoproterenol); (G) CXCR3 (1 μ M VUF10661); (H) D_1 R (500 nM dopamine); (I) D_2 R (500 nM dopamine); (J) NTS_1 R (10 nM neurotensin). * P <0.05 by two-way ANOVA, main effect of $G_{\alpha i}$ subtype. For panels (A) through (E), n =4 per condition. For panel (F) n =3-6; for panel (G) n =3-4; for panel (H) n =4, for panel (I) n =3-4; for panel (J) n =3 biological replicates per condition. Graphs show mean + s.e.m. Iso, isoproterenol.

Fig. 5. $G_{\alpha i}$: β -arrestin scaffolds form functional complexes with ERK. (A) Arrangement of split luciferase components and mKO on G_{α} protein (LgBiT), β -arrestin (smBiT), or ERK (mKO). (B) BRET of $G_{\alpha i}$ -LgBiT, β -arrestin-smBiT, and ERK2-mKO in cells overexpressing untagged V_2R treated with AVP (500 nM). (C) Representative immunoblot of phospho and total ERK1/2 in ‘Δ3G’ HEK 293T cells pretreated with PTX (200 ng/mL) and/or β arr1/2 siRNA stimulated with either vehicle or AVP (500 nM). (D) Quantification of ERK immunoblots in ‘Δ3G’ HEK 293T cells. 100% represents the maximal signal of phospho ERK / total ERK in the control, vehicle-treated condition. (E) Representative immunoblot of β -arrestin, $G_{\alpha i}$, phospho-ERK, and total ERK1/2 in ‘Δ4G’ HEK 293T cells (lacking all G proteins) transfected with the indicated amounts of $G_{\alpha i}$ and/or β arr1/2. 100% represents the signal of phospho ERK / total ERK in the control siRNA, absent PTX, AVP stimulated condition (F) Quantification of ERK immunoblots in ‘Δ4G’ HEK 293T cells. * P < 0.05, *** P < 0.001, two-way ANOVA with Bonferroni post hoc to no treatment, control siRNA group. The net BRET ratio of cytosolic mKO control was subtracted from the net BRET ratio of ERK-mKO to yield an adjusted BRET ratio that is the ordinate of panel (B). Immunoblots are representative of four to five separate experiments. For panel (B) n =5; panel (D) n =4; (F) n =5 per condition. NT, no transfection of either $G_{\alpha i}$ or β -arrestin. PTX, pertussis toxin. Graphs show mean $\pm$ s.e.m.

Fig. 6. Cell migration to the β -arrestin-biased ligand TRV120023 requires both $G_{\alpha i}$ and β -arrestins. (A) BRET assay quantifying recruitment of β -arrestin-2-YFP to AT₁R-RlucII following treatment with either angiotensin II or TRV120023. Canonical G protein signaling via TGF- α shedding assay at the Angiotensin II type 1 receptor (AT₁R) following treatment with either (B) the endogenous ligand, angiotensin II or (C) TRV120023. (D) Representative images of the four TRV120023 migration conditions in HEK 293T cells stably expressing AT₁R. (E) Quantification of PTX pretreatment (200 ng/mL) and/or β arr1/2 siRNA on TRV120023-induced migration for the experiment shown in panel (D). (F) Split luciferase assay for monitoring $G_{\alpha i}$ -LgBiT: β -arrestin-smBiT association after treatment of AT₁R with either angiotensin II or TRV120023. (G) Cartoon schematic of three described GPCR transduction effectors. * $P < 0.05$, two-way ANOVA with Bonferroni post hoc to no treatment, control siRNA group. # $P < 0.05$, two-way ANOVA with Bonferroni post hoc compared to control siRNA, PTX pretreated group. For panel (A) $n = 4$ per condition, for panels (B) and (C) $n = 4-5$ per condition, for panels (E) and (F) $n = 4$ per condition. Graphs show mean $\pm$ s.e.m.