An inactive receptor-G protein complex maintains the dynamic range of agonist-induced signaling

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Agonist binding promotes activation of G protein-coupled receptors (GPCRs) and association of active receptors with G protein heterotrimers. The resulting active-state ternary complex is the basis for conventional stimulus-response coupling. Although GPCRs can also associate with G proteins before agonist binding, the impact of such preassociated complexes on agonist-induced signaling is poorly understood. Here we show that preassociation of 5-HT7 serotonin receptors with Gs heterotrimers is necessary for agonist-induced signaling. 5-HT7 receptors in their inactive state associate with Gs as these complexes are stabilized by inverse agonists and receptor mutations that favor the inactive state. Inactive-state 5-HT7–Gs complexes dissociate in response to agonists, allowing the formation of conventional agonist–5-HT7–Gs ternary complexes and subsequent Gs activation. Inactive-state 5-HT7–Gs complexes are required for the full dynamic range of agonist-induced signaling, as 5-HT7 receptors spontaneously activate Gs variants that cannot form inactive-state complexes. Therefore, agonist-induced signaling in this system involves two distinct receptor-G protein complexes, a conventional ternary complex that activates G proteins and an inverse-coupled binary complex that maintains the inactive state when agonist is not present.

Results

Agonist Activation Leads to Net Dissociation of Preassociated 5-HT7–Gs Complexes. 5-HT7 serotonin receptors activate Gs heterotrimers to stimulate adenyl cyclase (AC) (9, 10), and previous work has shown that these receptors form complexes with Gs before agonist binding (11–13). We set out to determine the impact of 5-HT7–Gs preassociation on agonist-induced activation of Gs and signaling. Consistent with previous fluorescence studies (13), stimulation with serotonin (5-HT; Fig. L4) decreased bioluminescence resonance energy transfer (BRET) between labeled 5-HT7 receptors and Gs heterotrimers. This is unusual, as energy transfer between GPCRs and G proteins usually increases in response to agonist activation (14) owing to formation of active-state receptor-G protein complexes (e.g., β2 adrenergic receptors [β2AR]) (Fig. L4).

To determine whether 5-HT prompted dissociation of 5-HT7–Gs complexes or a change in complex conformation, we took a luciferase complementation approach (15) that reports protein association and dissociation more directly than energy transfer. We fused a small fragment of luciferase (SmBit) to the C terminus of each receptor and a large fragment of luciferase (LgBit) to the N terminus of Gγ2 and expressed these proteins with Gαs and Gγ1. Luciferase activity decreased on stimulation of 5-HT–SmBit but increased on stimulation of β2AR-SmBit (Fig. L1B), consistent with net dissociation and association of receptor-Gs complexes, respectively. Changes in lucinescence occurred more slowly than corresponding changes in BRET, presumably due to the slow kinetics of luciferase fragment association and dissociation (15). In these experiments, Gβγ was labeled instead of the Gαs subunit so

Significance

G protein-coupled receptors (GPCRs) are targeted by a large fraction of approved drugs and regulate many important cellular processes. Conventional signaling by GPCRs is triggered when agonist-activated receptors associate with heterotrimeric G proteins. We found that serotonin 5-HT7 receptors couple to Gs proteins in an unconventional manner, in which agonist binding instead promotes dissociation of preexisting inactive 5-HT7–Gs complexes. Therefore, agonists can initiate signaling via two distinct mechanisms, by promoting the association of active receptors and G proteins and by promoting dissociation of inactive receptors and G proteins.

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as to minimize interference with normal G protein function and receptor-G protein interactions. This left open the possibility that Go6 subunits remained associated with 5-HT7 receptors after agonist activation. To address this, we used a competition strategy in which luciferase complementation between β2AR receptors and Gs heterotrimers was monitored in the presence and absence of unlabeled 5-HT7 receptors. Expression of 5-HT7 receptors inhibited agonist-induced association of β2AR-SmBit and Goβγ-LgBit, consistent with sequestration of Gs by 5-HT7 (12). This inhibition was relieved by stimulation with 5-HT (Fig. 1C), indicating that agonist activation of 5-HT7 made more Gs heterotrimers available to other GPCRs.

We next tested the hypothesis that preassociated 5-HT7–Gs complexes dissociate in response to agonist because Gs binds GTP and becomes activated. Accordingly, we repeated the above experiments in permeabilized cells in the absence of GTP. To eliminate the possibility that residual GTP was present, we used apyrase to hydrolyze endogenous nucleotides and replaced them with either the hydrolysis-resistant analog GDPβS or Goβγ-LgBit, consistent with sequestration of Gs by 5-HT7 (12). Similar to Fig. 1C, similar to A, (F) to BRET between 5-HT7 receptors in their inactive state associate with Gs. Several 5-HT7 inverse agonists produced small but significant increases in BRET between 5-HT7 receptors and Gs heterotrimers (Fig. 2A). Similarly, in pull-down assays, we also found that detergent-solubilized 5-HT7 receptors retained Gs more efficiently in the presence of an inverse agonist (methiothepin [MT]) than in the presence of an agonist (5-HT) if GDP was present, whereas this was not the case for solubilized β2AR (SI Appendix, Fig. S1). These results with inverse agonists suggest that 5-HT7 receptors in their inactive state associate with Gs.

To further test this idea, we introduced mutations to produce constitutively inactive (CIM) and active (CAM) 5-HT7 receptors. For CIM receptors, residues F334G and N380I were mutated individually to positively charged residues, in both cases to promote interactions with DI275S-GDP that stabilize the inactive state. To produce a CAM receptor residue, L1733F was mutated to alanine to weaken hydrophobic interactions with residues in transmembrane helix 6 and promote activation. We have previously shown that both of these CIM receptors fail to support the activation of Gs and AC.

**Fig. 1.** 5-HT7–Gs complexes dissociate in response to agonist stimulation. (A) In intact cells, BRET between 5-HT7-Rluc8 and Goβγ-Venus decreases in response to agonist [5-hydroxytryptamine; 10 μM; n = 20], whereas BRET between β2AR-Rluc8 and Goβγ-Venus increases in response to agonist (isoproterenol; 10 μM; n = 20). (B) Luciferase complementation between β2AR-SmBit and Goβγ-LgBit decreases in response to agonist (5-HT; n = 20), whereas luciferase complementation between β2AR-SmBit and Goβγ-LgBit increases in response to agonist (iso; n = 16). (C) Iso-induced luciferase complementation between β2AR-SmBit and Goβγ-LgBit is blunted when 5-HT7 receptors are coexpressed, and this is alleviated by stimulation with 5-HT (n = 14). (D and E) Similar to A, (F) to BRET between 5-HT7 receptors in their inactive state associate with Gs. Several 5-HT7 inverse agonists produced small but significant increases in BRET between 5-HT7 receptors and Gs heterotrimers (Fig. 2A). Similarly, in pull-down assays, we also found that detergent-solubilized 5-HT7 receptors retained Gs more efficiently in the presence of an inverse agonist (methiothepin [MT]) than in the presence of an agonist (5-HT) if GDP was present, whereas this was not the case for solubilized β2AR (SI Appendix, Fig. S1). These results with inverse agonists suggest that 5-HT7 receptors in their inactive state associate with Gs.
BRET between the CAM 5-HT7 receptor and Gs was significantly ligand-induced changes in BRET were abolished. In contrast, basal by inactive 5-HT7 receptors.

binds directly to AC, but its actions are highly synergistic with G

tors (18), although the underlying mechanism is unclear. Forskolin decreased compared with WT 5-HT7, and the agonist-induced decrease was occluded (Fig. 2A). We then assessed the relative stability of inactive- and active-state 5-HT7 receptors with mini Gs (mGs) proteins, as these

association of 5-HT7 and mGs was modestly enhanced by 5-HT and inhibited by MT (Fig. 3C). Similar results were obtained in

whereas the CAM receptor activates Gα and AC spontaneously (17). Basal BRET between both CIM 5-HT7 receptors and Gα was significantly increased compared with wild-type (WT) 5-HT7, and ligand-induced changes in BRET were abolished. In contrast, basal BRET between the CAM 5-HT7 receptor and Gα was significantly decreased compared with WT 5-HT7, and the agonist-induced decrease was occluded (Fig. 2B and C). These results suggested that CIM 5-HT7 receptors should efficiently sequester Gα heterotrimer, whereas CAM 5-HT7 should activate Gα. As expected, CIM 5-HT7 receptors completely prevented β2AR-mediated activation of AC, whereas CAM 5-HT7 constitutively activated Gα. (SI Appendix, Table S1), consistent with a transient association of inactive 5-HT7 and Gα under these conditions. However, in the presence of 5-HT, β2AR activation caused an even more rapid decrease in BRET between 5-HT7 and Gα (kfast = −0.5 s−1; kslow = −0.03 s−1; 83% fast). This rapid decrease started from a lower baseline due to 5-HT–induced dissociation of inactive-state complexes, but nonetheless demonstrates the existence of active-state 5-HT7–Gα complexes in the presence of 5-HT. Similar kinetic results were obtained with CIM and CAM 5-HT7 mutants (SI Appendix, Table S1). These results suggest that even in the absence of nucleotides, inactive-state 5-HT7–Gα complexes are more stable than active-state 5-HT7–Gα complexes in cell membranes, and are consistent with agonist-induced net dissociation under the same conditions (Fig. 1E).

5-HT7 Readily Adopts the Active State. We next examined the interaction of 5-HT7 receptors with mini Gα (mGs) proteins, as these engineered Gα subunits were designed to stabilize the active state of Gα-coupled GPCRs (23). We found that unliganded 5-HT7 receptors spontaneously recruited mGs proteins to the plasma membrane, as assessed by both confocal imaging (Fig. 3A and B) and BRET assays (Fig. 3C). Moreover, 5-HT7 interactions with mGs were only weakly sensitive to agonists or inverse agonists but in a manner opposite to that observed with Gα heterotrimers; the association of 5-HT7 and mGs was modestly enhanced by 5-HT and inhibited by MT (Fig. 3C).
pull-down assays with detergent-solubilized 5-HT_7 receptors and mGs (SI Appendix, Fig. S1). Consistent with these observations, CIM 5-HT_7 receptors lost the ability to interact with mGs, whereas the CAM 5-HT_7 receptors retained this ability (SI Appendix, Fig. S3). These results suggest that mGs is unable to form complexes with inactive 5-HT_7 that are analogous to inactive-state 5-HT_7–G_s complexes, whereas active 5-HT_7 can form complexes with mGs. Furthermore, spontaneous association with mGs implies that 5-HT_7 receptors readily adopt an active state in the absence of an agonist. This was not the case for β_2AR, which required agonist activation for robust association with mGs under similar conditions (Fig. 3A–C).

Most GPCRs intrinsically favor inactive conformations (2), and high-affinity agonist binding is usually not evident unless a nucleotide-free G protein (or a G protein surrogate) is present to stabilize the active state. An unusual characteristic of 5-HT_7 receptors is a high-affinity agonist binding that persists even in the presence of guanine nucleotides (24–26). This could reflect either stabilization of active 5-HT_7 by nucleotide-bound G_s (25) or, alternatively, an intrinsic tendency of the receptor to adopt active states even when G_s is not present. To test these alternatives, we performed [3H]SB269970 competitive binding assays using membranes prepared from gene-edited cells that do not express Gα_s family subunits, with and without the expression of exogenous Gα_s. We found that high-affinity agonist binding was maintained even in the complete absence of G_s (Fig. 4A) and was unaffected by addition of guanine nucleotides (Fig. 4B). As has been described previously (25, 27), we also observed a small population of low-affinity agonist-binding sites, and the fraction of low-affinity sites was modestly larger when G_s was present (~30%) than when G_s was absent (~20%) (SI Appendix, Table S2). The affinity of the inverse agonist [3H]SB269970 was slightly higher when G_s was present (Fig. 4C). These results are consistent with the suggestion that 5-HT_7 receptors readily adopt active states that bind agonist with high affinity even in the absence of G_s, and further suggest that G_s may stabilize an inactive state that binds agonists with low affinity.

Because most GPCRs intrinsically favor inactive conformations, the pharmacologic properties of receptors in the absence of nucleotide-free G proteins or surrogates are thought to reflect primarily the inactive state. Accordingly, agonist-binding affinity under these conditions is relatively low and is only modestly decreased by mutations that inhibit constitutive receptor activity (17), but is significantly increased by mutations that activate constitutive activity (28). However, we found that the CIM 5-HT_7 F336R displayed >10,000-fold lower agonist-binding affinity than WT 5-HT_7 receptors (Fig. 4D). In contrast, the CAM 5-HT_7 L173A displayed agonist binding similar to the high-affinity binding component of WT 5-HT_7 receptors (Fig. 4D and SI Appendix, Table S3). As expected, inverse-agonist–binding affinity was higher for CIM 5-HT_7 receptors than for CAM 5-HT_7 receptors (Fig. 4E). Therefore, inactive mutant 5-HT_7 receptors that bind G_s tightly bind 5-HT with low affinity, whereas active mutant 5-HT_7 receptors that bind G_s weakly bind 5-HT with high affinity. These results are consistent with a negative allosteric interaction between agonist and G_s binding to WT 5-HT_7 receptors and a net dissociation of 5-HT–G_s complexes on agonist binding.

Inactive-State 5-HT_7–G_s Complexes Prevent Constitutive Signaling.

The foregoing results suggested that inactive- and active-state 5-HT_7 receptors form distinct complexes with G_s heterotrimer. Because the C terminus of the Gα_s subunit is required for active-state GPCR–G protein complexes (29), we guessed that by altering this region, it might be possible to prevent formation of active-state complexes without impairing inactive-state complexes. However, removing a single amino acid from the distal C terminus of Gα_s (Gα_s Δ1) decreased the basal BRET between 5-HT_7 and G_s, which partially occluded the agonist-induced decrease and enhanced the inverse agonist-induced increase (Fig. 5A and B and SI Appendix, Fig. S4 A and B). Removing two amino acids (Gα_s Δ2) reduced the basal BRET to background levels and converted the agonist-induced decrease observed in the presence of apyrase into an increase, implying net receptor-G protein association. Therefore, truncation of the Gα_s C terminus was in fact more effective at disrupting inactive-state 5-HT_7–G_s complexes and left active-state complexes at least partially intact. By comparison, the same truncations had no effect on the basal BRET between β_2AR and G_s (Fig. 5C) and progressively inhibited agonist-induced coupling of β_2AR to G_s (Fig. 5D and SI Appendix, Fig. S4 C and
that 5-HT7 should constitutively activate truncated mutants. Indeed, in cells expressing 5-HT7, basal cAMP levels increased when Goα was truncated, peaking at Goα Δ2 and declining back to baseline by Goα Δ4 (Fig. 5G). Stimulation with 5-HT produced only modest further increases in cAMP when Goα was truncated, even though the AC activator forskolin could produce large further increases (Fig. 5G and SI Appendix, Fig. S7). These trends were not due to changes in spontaneous nucleotide release or hydrolysis, as truncation of Goα progressively inhibited basal and agonist-stimulated cAMP accumulation mediated by β2AR receptors (Fig. 5H), mirroring the progressive impairment seen in direct coupling assays. 5-HT7 (but not β2AR) also constitutively activated heterotrimers with polar residues in position 394 of Goα, and agonist-induced activation was occluded (SI Appendix, Fig. S5). Therefore, 5-HT7 receptors constitutively activated Go heterotrimer with which they were unable to form inactive-state complexes, again consistent with a tendency of these receptors to adopt active conformations even when not bound by agonist.

**Discussion**

Taken together, our results support a model wherein agonist binding to 5-HT7 receptors is linked to Go activation in a manner distinct from conventional GPCR-G protein coupling (Fig. 6A). We propose a model wherein 5-HT7 receptors in their basal state (RnG, reversibly form encounter complexes (RnG) with Go, heterotrimer. RnG encounter complexes can transition to conventional active-state complexes (RnG) but are more likely to transition to inactive-state complexes (RnG), a process we term “inverse coupling.” Constitutive Go activation occurs through the RnG coupling pathway but is kept in check by accumulation of RnG. Agonist binding does not change the rates governing the formation of RnG encounter complexes or RnG active-state complexes, but does decrease the accumulation of RnG complexes. This decreases the net 5-HT7-G protein association and allows for increased formation of RnG and Go activation. Our data suggest that the conformational transitions between RnG and RnG are sensitive to agonist binding to the receptor but less sensitive to nucleotide binding to Go, whereas the conformational transitions between RnG and RnG are sensitive to nucleotide binding to Go, but less sensitive to agonist binding to the receptor. If the RnG-to-RnG pathway is blocked (e.g., by truncations or mutations of RnG), RnG complexes form spontaneously even in the absence of agonist, because the basal state of 5-HT7 intrinsically favors active conformations (RnG ~ RnG). In contrast, conventional GPCRs in their basal state intrinsically favor inactive conformations (RnG ~ RnG), but RnG complexes do not form or accumulate (Fig. 6A). Conventional RnG encounter complexes either dissociate or progress to RnG, and conformational transitions between RnG and RnG are sensitive to both agonist binding to the receptor and nucleotide binding to the G protein.

Based on these general principles, we defined a set of ordinary differential equations to construct deterministic models of conventional and inverse coupling (SI Appendix, Table S5). Simulations based on these models recapitulated the essential features of receptor-G protein association, dissociation, and activation that we observed for β2AR and 5-HT7 receptors. Specifically, agonist binding led to a net association of β2AR and Go, and a net dissociation of 5-HT7 and Go, either the presence or absence of guanine nucleotides, but increased formation of RnG (and thus Goα-GTP) in intact cells (Fig. 6B). Notably, our inverse coupling model also predicts that increasing 5-HT7 receptor density will not lead to higher potency signaling: that is, a receptor reserve will not be apparent (Fig. 6C). The absence of a receptor reserve has been observed experimentally for 5-HT7 (11), and several studies have reported lower agonist potency than expected based on agonist-binding affinity (9, 11, 27, 30).

Our model suggests that this anomalous property of 5-HT7 receptors reflects sequestration of Go heterotrimer in RnG.

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**Fig. 4.** High-affinity agonist binding to 5-HT7 does not require Goα. (A) Competitive binding assays between the inverse agonist ([3H]SB269970 and 5-HT using membranes prepared from cells lacking endogenous Goα subunits, with or without coexpression of exogenous Goα, in the presence of 100μM GDP. Data are mean ± SD; n = 6 to 9. Least squares fits to one- and two-site binding models are superimposed. (B) As in A but with coexpression of Goα, and in the presence of no added nucleotide or 100μM GTPγS. Data are mean ± SD; n = 6 to 9. (C) Homologous competitive binding with unlabelled SB269970 with or without expression of Goα. Data are mean ± SEM; n = 3. (D) Agonist binding to the activated mutant 5-HT7 (L173A) is similar to high-affinity agonist binding to WT 5-HT7, whereas agonist binding to the inactive mutant 5-HT7 (F336R) is severely impaired. Data are mean ± SEM; n = 3. (E) Inverse agonist binding to the inactive mutant 5-HT7 (F336R) is higher affinity than for the active L173A mutant. Data are mean ± SEM; n = 3. Grouped data from all radioligand-binding experiments are provided in SI Appendix, Tables S2–S5.
complexes when agonist concentrations are below the level at which receptors are saturated.

Our model predicts that Go heterotrimers should decrease agonist-binding affinity at 5-HT7 receptors by stabilizing the inactive receptor state. Although we and others have observed a small population of low-affinity agonist-binding sites (25, 27), this fraction was only modestly increased when Go was present (Fig. 4A). It is possible that negative allosterism between agonist and Go binding is difficult to observe in equilibrium-binding experiments due to the transient nature of inactive-state 5-HT7-Go complexes (Fig. 2E), as well as possible loss of Go from membrane preparations. A similar problem exists for some active-state GPCR-G protein complexes, as high-affinity agonist binding can be difficult to detect for some receptors in some expression systems (31). Strategies that have been successful in stabilizing active-state complexes for ligand-binding experiments (32) may eventually be able to reveal more robust Go-mediated inhibition of agonist binding to 5-HT7 receptors.

In summary, our present results explain several unusual biophysical and pharmacologic properties of 5-HT7 receptors. We propose that this receptor intrinsically favors active conformations but avoids unstrained activation of Go heterotrimers by forming inactive-state 5-HT7-Go complexes. Agonist binding acts primarily to prevent the formation of unproductive 5-HT7-Go complexes, which indirectly promotes the formation of productive complexes. Thus, a negative allosteric interaction between agonist binding and Go association is necessary for agonist-induced 5-HT7 signaling. Recent studies have shown that the allosteric range of GPCRs is broader than previously anticipated (33). Engineered antibodies can stabilize both active and inactive receptor conformations (33–35), and the basal state (Rn in our model) represents a time-weighted average of conformational sampling. Our results suggest that Go proteins can also act to stabilize both active and inactive receptor conformations and cooperate with agonist binding in both a positive and a negative manner. Although our results indicate that the distal C terminus of Go, required for inactive-state 5-HT7-Go complexes, further studies are needed to establish the structural mechanism through which Go stabilizes the inactive state of the receptor. It will be interesting to determine whether Go acts in a manner similar to the way in which negative allosteric antibodies stabilize inactive GPCRs (33–35). Several other GPCRs are thought to interact with Go proteins before agonist binding (4–8); therefore, it seems possible that inverse coupling will prove to be a conserved mechanism for regulating the sensitivity and dynamic range of cell signaling.

Materials and Methods

Materials. Trypsin, DPBS, PBS, FBS, MEM, DMEM, penicillin/streptomycin, L-glutamine were obtained from Thermo Fisher Scientific. Receptor ligands (5-HT, isoproterenol, ICI-118,551, and MT) and forskolin were purchased from Cayman Chemical or MilliporeSigma. Detergents (n-dodecyl-β-o-maltoside [DDM] and cholesteryl hemisuccinate [CHS]) were obtained from AvantiPak, Digitonin, apyrase, GDPβS, and GDP were purchased from MilliporeSigma. Detergents (n-dodecyl-β-o-maltoside [DDM] and cholesteryl hemisuccinate [CHS]) were obtained from Avantor, Digitonin, apyrase, GDPβS, and GDP were purchased from MilliporeSigma or BioBasic. [3H]SB269970 was obtained from PerkinElmer, and polyethyleneimine (PEI) MAX was purchased from Polysciences.

Plasmid DNA Constructs. 5-HT7-Rluc8 was made by amplifying the human 5-HT7 coding sequence (splice variant d) using the PCR results for 5-HT7–Tango (36) (Roth Lab PRESTO-Tango Kit; Addgene) and ligating into pRluc8-Δ2 (37). Numerous oligonucleotides were used to amplify and ligate into pRluc8-Δ2 with HindIII and KpnI. Inactivating and activating mutations were introduced into 5-HT7–Rluc8 using the QuickChange Mutagenesis Kit (Agilent Technologies) and gblock fragments (Integrated DNA Technologies) as primers. Plasmids encoding unlabeled human 5-HT7, βAR, Goα-long, and Gβγ2 were purchased from the cDNA Resource Center. Truncated and mutated Goα subunits were derived from WT Goα-long by amplifying the coding sequence with reverse primers incorporating the desired mutation and ligating the resulting fragment into pCDNA3.1(+) using KpnI and XhoI. A plasmid encoding βAR-SmBit was derived from unlabeled βAR using the QuickChange Mutagenesis Kit and a gblock primer. A plasmid encoding 5-HT7–SmBit was derived from unlabeled 5-HT7 by standard subcloning into a SmBit vector. A plasmid encoding LgBit-Gγ2 was kindly provided by Stephen.
Parameters and conditions are provided in each well of a six-well plate. Experiments 24 to 48 h later. Up to 3.0 μg of plasmid DNA was transfected in growth medium using linear PEI MAX (made in China, authenticated and propagated as described previously (39). Cells were a generous gift from Asuka Inoue, Tohoku University, and were propagated in 24-well plates. Plasmids encoding the Nluc-EPAC-VV cAMP sensor was kindly provided by Kirill Martemyanov, The Scripps Research Institute. Plasmids encoding the β2AR (C-terminal truncated) were verified by Sanger sequencing.

**Cell Culture and Transfection.** HEK 293 cells (American Type Culture Collection; CRL-1573) were propagated in plastic flasks and on six-well plates according to the supplier’s protocol. HEK 293 cells with targeted deletion of GNAS and GNASL were a generous gift from Asuka Inoue, Tohoku University, and were derived, authenticated and propagated as described previously (39). Cells were transiently transfected in growth medium using linear PEI MAX (molecular weight 40,000) at a nitrogen/phosphate ratio of 20 and were used for experiments 24 to 48 h later. Up to 3.0 μg of plasmid DNA was transfected in each well of a six-well plate.

**BRET and Luminescence Assays.** Intact cells were washed twice with 1x DPBS, harvested by trituration, and transplanted to opaque black (for BRET) or white (for luminescence) 96-well plates. Permeabilized cells were washed twice with permeabilization buffer (KPS) containing 140 mM KCl, 10 mM NaCl, 1 mM MgCl2, 0.1 mM KEGTA, and 20 mM NaHEPES (pH 7.2); harvested by trituration; permeabilized in KPS buffer containing 10 μg mL−1 high-purity digitonin; and then transferred to 96-well plates. Measurements were made from permeabilized cells supplemented with 100 μM GDP, 2 U mL−1 apyrase, or apyrase with 100 μM GDP/PS. Steady-state BRET and luminescence measurements were performed using a Mithras LB940 photon-counting plate reader (Berthold Technologies). Kinetic BRET and luminescence time course measurements were obtained with a POLARStar Optima plate reader (BMG Labtech). Coelenterazine h (5 μM; Nanolight) or furimazine (NanoGlo; 1:1,000; Promega) were added to all wells immediately before taking measurements with Rluc8 and Nluc, respectively. Raw BRET signals were corrected for the emission intensity at 520 to 545 nm divided by the emission intensity at 475 to 495 nm. Net BRET is the raw BRET ratio minus the ratio measured from cells expressing only the donor.

**Confo Imaging.** Cells grown on 25-mm round coverslips were transferred to an imaging chamber and washed with DPBS. Drug solutions were added directly to the chamber by pipetting. Confocal images were acquired using a Leica SP8 scanning confocal microscope with a 63×, 1.4 NA objective. Venus was excited with a 488-nm diode laser and detected at 500 to 550 nm. BG-649-PEG-biotin was excited with a 633-nm diode laser and detected at 540 to 750 nm.

**Membrane Preparation and Radioligand Binding.** Transfected cells were washed twice with cold PBS/EDTA and resuspended in cold DPBS. After pelleting at 600 × g for 10 min at 4 °C, cells were resuspended in cold homogenization buffer containing 75 mM Tris-HCl pH 7.4, 2 mM EDTA, and protease inhibitor mixture (Roche). Cells were sonicated (three 5-s pulses at 20% amplitude with a 50-s cooldown period between each pulse), debris was pelleted at 500 × g for 10 min at 4 °C, and supernatants were centrifuged at 50,000 × g for 30 min at 4 °C. Pellets were resuspended in assay buffer containing 100 mM NaCl, 10 mM MgCl2, and 20 mM Hepes, pH 7.4, then snap-frozen and stored at −80 °C. Competitive binding assays were performed as described previously (26) by incubating membranes with [3H]SB269970 (1.5 to 2.8 nM) and increasing concentrations of unlabeled SB269970 in a 1:1:2:1 ratio or SNAPf-5HT7 in a 1:1:1:2:1 ratio, and either SNAPf-β2AR or SNAPf-5HT7 in a 1:1:1:2:1 ratio or SNAPf-β2AR or SNAPf-5HT7 in a 1:1:1:2:1 ratio. membranes were prepared as above, with the addition of 10 μM GDP or 1 U mL−1 apyrase, and receptor ligands (10 μM 5-HT, MT, ERP, or apyrase with 100 μM GDP/PS). Membrane preparations were made from permeabilized cells supplemented with 100 μM GDP and receptor ligands (10 μM 5-HT, MT, isoproterenol, or ici-118,551) and GDP or 2 U mL−1 apyrase and receptor ligands as above for 3 h at 4 °C with gentle rotation. Solubilized membranes were incubated with 250 μg of streptavidin (SAV) beads (Dynabeads MyOne SAV C1; Thermo Fisher Scientific) that had been washed with buffer wash 200 μM MgCl2 (pH 7.8, 150 mM NaCl, 2 mM MgCl2, 20% [vol/vol] glycerol, 1% [vol/vol] DDM, 0.2% [vol/vol] CHS, and protease inhibitor mixture (Roche), 100 μM GDP or 2 U mL−1 apyrase, and receptor ligands as above for 3 h at 4 °C with gentle rotation. Solubilized membranes were incubated with 250 μg of streptavidin (SAV) beads (Dynabeads MyOne SAV C1; Thermo Fisher Scientific) and washed with buffer wash 200 μM HEPES (pH 7.8, 150 mM NaCl, 2 mM MgCl2, 10% [vol/vol] glycerol, 0.1% [vol/vol] DDM, 0.02% [vol/vol] CHS, and protease inhibitor mixture) for 2.5 h at 4 °C with gentle rotation. Beads were washed five times with 1 mL of wash buffer supplemented with either 50 μM GDP or 1 U mL−1 apyrase and

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**Fig. 6.** An inverse coupling model describes the unconventional properties of 5-HT7 receptors. (A) Inverse and conventional coupling models describing the formation of encounter complexes (R(G)), active-state complexes (R(G)), and inactive-state complexes (R(G)). Boxes indicate rates that are influenced by agonist binding to the receptor and nucleotide binding to the G protein. (B) Simulations based on ODE models corresponding to A recapitulating net dissociation of receptor-G protein complexes for 5-HT7, but not for β2AR in response to agonist (Top), but increases in RaG complexes in intact cells for both (Bottom). (C) Simulated curves plotting normalized [Gαi-GTP] vs. [5-HT] across a 200-fold increase in 5-HT7 expression (Left) and plots of simulated pEC50 vs. receptor expression for both 5-HT7, and β2AR (Right). Model parameters are provided in SI Appendix, Table S5.
receptor ligands, diluted in 500 μL of working solution (20 mM Hepes pH 7.8, 100 mM NaCl, 2 mM MgCl₂, 0.1% [wt/vol] DDM, and 0.02% [wt/vol] CHS) and transferred to opaque black 96-well plates. BG-PEG-SNAP-649 fluorescence was determined using a Synergy Neo plate reader (BioTek; excitation, 640 nm; emission, 676 nm). Furimazine (NanoGlo, 1:1,000; Promega) was added, and luminescence was measured without wavelength selection. Recovered Nluc activity (G or mG) was normalized to fluorescence (receptor).

Computational Modeling. Rule-based deterministic models of conventional and inverse coupling based on ordinary differential equations (ODE) were constructed using the Virtual Cell (VCell) modeling platform (40, 41). Initial reactions and parameters followed a previously published analytical model (42), which was modified to include three receptor states, Rₐ complexes (for the inverse coupling model only), and inverse agonist binding. Both models included basal (Rₙ), inactive (Rᵯ), and active (Rₐ) receptor states, each of which could bind reversibly to agonist (L) or inverse agonist (Lᵯ). G proteins could be empty, bound to GDP, or bound to GTP and could bind reversibly to ligand-bound or unbound receptors. Reactions, parameters, and initial conditions are given in Table S5. The VCell, “SHT7_Jang_2020” by user “wojang,” can be accessed within the VCell software (available at https://vcell.org).

Statistical Testing. Hypothesis tests were carried out with the two-tailed pairwise t test, one-sample t test, one-way ANOVA using Dunnett’s test for multiple comparisons against a control, or two-way ANOVA using Sidak’s test for multiple comparisons, as indicated in figure legends. Replicates were separate cultures of transfected cells derived from the two cell lines used. All data were analyzed using GraphPad Prism.

Data Availability. All study data are included in the main text and SI Appendix.

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