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Covalent agonists for studying G protein-coupled receptor activation

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Structural studies on G protein-coupled receptors (GPCRs) provide important insights into the architecture and function of these important drug targets. However, the crystallization of GPCRs in active states is particularly challenging, requiring the formation of stable and conformationally homogeneous ligand-receptor complexes. Native hormones, neurotransmitters, and synthetic agonists that bind with low affinity are ineffective at stabilizing an active state for crystallogenesis. To promote structural studies on the pharmacologically highly relevant class of aminergic GPCRs, we here present the development of covalently binding molecular tools activating G_s-, G_i-, and G_q-coupled receptors. The covalent agonists are derived from the monoamine neurotransmitters noradrenaline, dopamine, serotonin, and histamine, and they were accessed using a general and versatile synthetic strategy. We demonstrate that the tool compounds presented herein display an efficient covalent binding mode and that the respective covalent ligand-receptor complexes activate G proteins comparable to the natural neurotransmitters. A crystal structure of the β_2-adrenoreceptor in complex with a covalent noradrenaline analog and a conformationally selective antibody (nanobody) verified that these agonists can be used to facilitate crystallogenesis.

chemical probes | structural biology | chemical biology

One of the major obstacles to the investigation of the structural basis of G protein-coupled receptor (GPCR) activation is the flexibility of their seven-transmembrane core, particularly in the active state (1), and the resulting biochemical instability of the solubilized protein (2, 3). Protein crystallography, the most powerful tool for the study of GPCR structure, requires the formation of stable and conformationally homogeneous ligand-receptor complexes (4). High-affinity agonists with dissociation constants in the low to subnanomolar range and low off-rates facilitate stabilization of the protein throughout the process of expression, purification, and crystallogenesis (2); however, endogenous neurotransmitters usually show poor binding affinity. Low binding affinity with rapid association and dissociation rates leads to conformational heterogeneity that prevents the formation of diffraction-quality crystals. The rapid dissociation rate of agonists also makes it difficult to generate active-state stabilizing proteins, such as the camelid antibodies (nanobodies) that have been used to obtain active-state structures of the β_2-adrenergic receptor (β_2AR) (5) and M2 muscarinic receptor (6).

To prevent ligand dissociation, irreversible ligation of electrophilic moieties like halomethylketones, isothiocyanates, Michael acceptors, or aziridinium groups of small-molecule ligands with a suitably positioned nucleophilic residue in the receptor has been used (7–16). However, irreversible ligands often suffer from incomplete cross-linking (15) and reduced receptor activation when covalent binding leads to loss of agonist efficacy (10, 16). Furthermore, their highly electrophilic nature and the abundance of nucleophilic groups in biological systems may lead to a low coupling selectivity (7, 8).

Disulfide-based cross-linking approaches (17, 18) offer the advantage that the covalent binding of disulfide-containing compounds is chemoselective for cysteine and enforced by the affinity of the ligand-pharmacophore rather than by the electrophilicity of the cross-linking function (19). We refer to the described ligands as covalent rather than irreversible agonists because cleavage may be promoted by reducing agents and the disulfide transfer process is a reversible chemical reaction in general.

Structural information on the target protein facilitates the development of covalent ligand-receptor pairs. Mutation of $H93^{2.64}$ in the β_2AR to cysteine introduced an anchor for the disulfide-based covalent agonist FAUC50, which does not perturb ligand binding or the activation of the receptor, and thus enabled, to our knowledge, the first agonist-bound GPCR structure (20). Taking advantage of the high structural homology among aminergic GPCRs, we reasoned that the introduction of cysteine into position $X^{2.64}$ should also result in a covalently binding receptor mutant for other aminergic GPCRs.

We here report a methodology to generate disulfide-based covalent ligand-receptor pairs to promote structural and functional studies on GPCRs. We demonstrate that even the low-affinity endogenous agonists noradrenaline, dopamine, and serotonin can be converted into efficient covalently binding

Significance

Protein crystallography has greatly contributed to our understanding of the structure and function of G protein-coupled receptors (GPCRs). Recent success in the structural investigation of active GPCR conformations was guided by the application of high-affinity agonists and G proteins or G protein mimetic nanobodies. However, poor affinities of agonists prevent the formation of diffraction-quality crystals and hamper the generation of state-specific nanobodies. To overcome this limitation, we present a general approach to covalently binding molecular tools for the construction of stable ligand-receptor complexes capable of G protein activation. Besides the promotion of structural studies, tethered agonist-GPCR complexes may find application in biochemical and biophysical experiments that require reliable labeling of distinct receptor populations, underlining the versatility of covalent agonists for studying GPCR activation.

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The authors declare no conflict of interest.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 4QKX).

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molecular tools for the β_2AR, the dopamine D_2 receptor (D_2R), and the 5-hydroxytryptamine 2A (5-HT_{2A}) serotonergic subtype representing G_s-, G_i-, and G_q-coupled GPCRs, respectively. Analogous studies were conducted starting from histamine and the receptor subtype H_1. We applied this strategy to obtain an active-state crystal structure of the β_2AR^{H93C} and a covalent (nor)adrenaline analog.

Results

Design and Synthesis of the Covalent Agonists. The designed covalent ligands feature the recognition element of neurotransmitters connected to a disulfide moiety via a flexible linker, based on the parent compound FAUC50 (Fig. 1). To develop a general approach to covalent ligands, we planned to exploit the naturally installed nucleophilicity of the neurotransmitters in a chemoselective *N*-alkylation reaction. Starting from a substituted hydroxyphenylethanol, the required electrophilic building blocks were synthesized (Fig. 1 and *SI Appendix*, Fig. S3). The chemoselective *N*-alkylation reaction of the biogenic amines was initially hampered by the instability of the catechol and the 5-hydroxyindole moiety of the endogenous ligands and the disulfide-functionalized derivatives. Catechol groups, for instance, are susceptible to oxidizing conditions, irradiation, and heat, leading to a complex mixture of oxidization products (21). Thus, adjustment of solvent, base, temperature, and handling under an inert atmosphere was necessary to guarantee a good balance between consumption of the starting material and low degradation of the products. Subsequent disulfide exchange and immediate purification by preparative HPLC efficiently yielded the corresponding covalent (nor)adrenaline (**1** and **2**), dopamine (**3**), and histamine (**5**) derivatives (*SI Appendix*, Fig. S1). Because the serotonergic analog (**4**) showed substantial impurities, intermediate silyl-protection of the phenolic moiety was necessary to access the pure product (*SI Appendix*, Fig. S2). Despite their immanent sensitivity to oxygen, irradiation, and heat, all derivatives could be obtained in high quality, as determined by NMR.

Characterization of the Covalent Ligand-Receptor Pairs. To construct covalent GPCR-neurotransmitter complexes, our initial investigations were directed to the linkage of the β_2AR^{H93C} and the disulfide-functionalized (nor)adrenaline analogs **1** and **2**, slightly differing in their spacer length. The efficiency of these compounds in the formation of the covalent bond was evaluated in a time-dependent radioligand depletion assay. Therefore, we used WT β_2AR with a single H93C mutation that was purified and reconstituted into HDL particles (22). Both ligands showed considerable covalent binding after incubation with the mutated receptor that could not be reversed by a large excess of radioligand (Fig. 2*A* and *SI Appendix*, Fig. S5). Compound **2** was equally effective as FAUC50 in blocking radioligand binding, whereas analog **1** displayed a lower propensity for covalent complex formation, indicating that a shorter linker results in a higher degree of covalent linkage (*SI Appendix*, Figs. S4 and S5). To determine whether the tethered ligand-receptor complex is capable of activating heterotrimeric G_s protein, reconstituted WT and β_2AR^{H93C} mutant receptors were incubated with ligand **2** alone or with ligand **2** followed by an excess of the high-affinity inverse agonist ICI-118,551. After addition of G protein, activation was observed by measuring [^{35}S]GTPγS binding to the $G\alpha_s$ subunit. Fig. 2*D* shows that an excess of the inverse agonist ICI-118,551 is unable to reverse compound **2**-induced G protein activation in the case of β_2AR^{H93C} but prevented agonist-induced nucleotide exchange by the WT receptor. Thus, the disulfide-functionalized (nor)adrenaline **2** forms a stable complex with the β_2AR^{H93C} that promotes G protein activation.

To investigate covalent receptor binding and activation of the disulfide-functionalized dopamine analog **3**, the D_2R was chosen as a representative model for a G_i-coupled GPCR and the mutation $L94C^{2.64}$ was introduced into WT D_2R. A radioligand depletion assay, using membranes containing the D_2R^{L94C} mutant, revealed extensive blockade of [^{3}H]spiperone binding at the mutated receptor, suggesting a highly efficient covalent binding mode for the dopamine analog **3** (Fig. 2*B*). The ability of dopamine analog **3** to activate WT D_2R, as well as the ability of the covalent **3**-D_2R^{L94C} complex to induce G protein-promoted signaling, was assessed in an inositol phosphate (IP) formation assay using HEK cells transiently transfected with a chimeric $G\alpha_{qi5}$ protein and the WT and mutant receptor, respectively (6, 23) (Fig. 2*D* and *SI Appendix*, Fig. S6). In contrast to the reversibility of the control noncovalent agonist quinpirole, agonist-induced IP accumulation by the dopamine analog **3** could not be diminished by an excess of antagonist (Fig. 2*D*), demonstrating that the dopamine analog **3** is an efficacious covalent agonist for the D_2R.

To extend our strategy to a G_q protein-coupling subtype, the serotonin receptor mutant 5-HT_{2A}^{T134C} was generated from WT receptor to evaluate the disulfide-functionalized serotonin surrogate **4**. Interestingly, we observed only minor blockade of this receptor mutant in the radioligand depletion assay (*SI Appendix*, Fig. S7). This result prompted us to evaluate the role of serine residue 131 in transmembrane helix 2 (TM2), positioned below the cysteine anchor, in sterically hindering efficient covalent binding. Consequently, $S131^{2.61}$ was mutated to glycine. Investigation of the double-mutant 5-$HT_{2A}^{S131G\text{-}T134C}$ revealed that the amount of covalently modified receptor increased in the radioligand depletion assay (Fig. 2*C*), and analogous IP

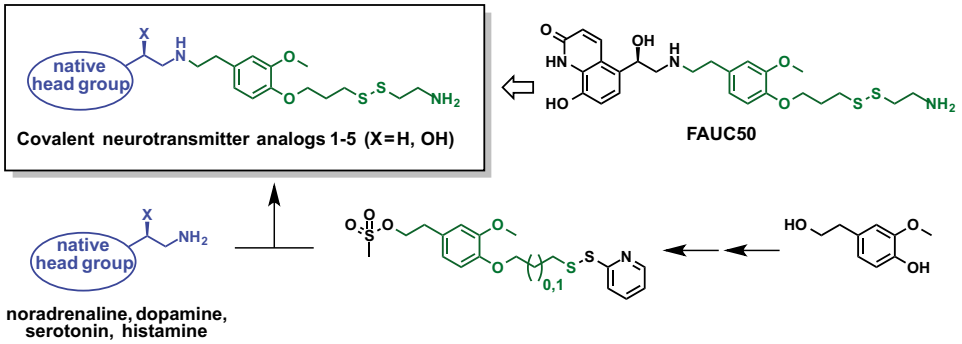

Fig. 1. Covalent β_2AR agonist FAUC50 served as a template for the covalent neurotransmitter-derived agonists. Starting from the corresponding hydroxyphenylethanol, regioselective *O*-alkylation, nucleophilic substitution with potassium thioacetate, methanolysis, treatment with 2,2′-dithiodipyridine, and *O*-activation gave the required electrophilic building blocks that were used in chemoselective *N*-alkylation of the biogenic amines. Reagents and conditions are shown in *SI Appendix*, Figs. S1–S3.

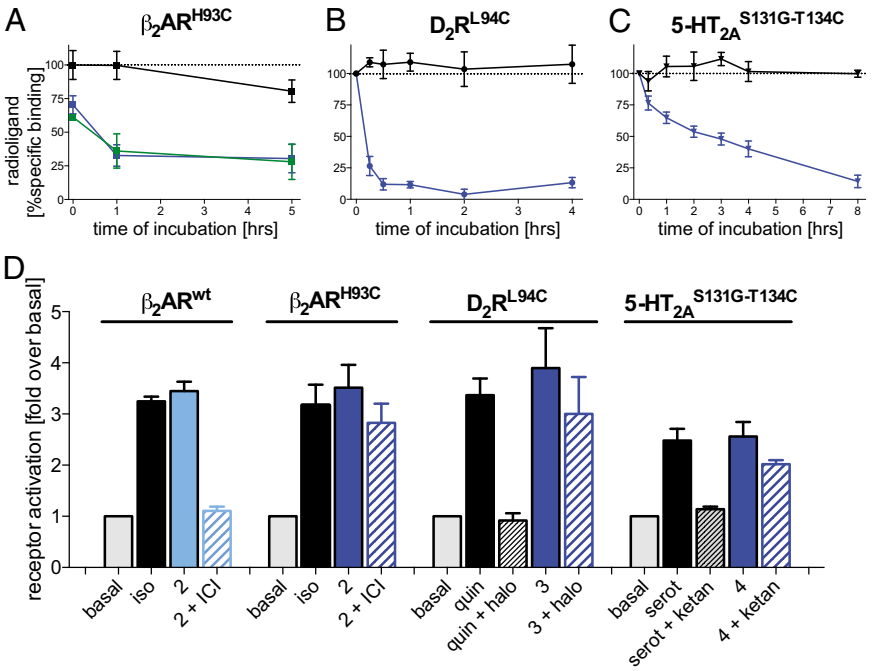

Fig. 2. (*A*) Radioligand depletion assay of the covalent ligands **2** (blue) and FAUC50 (green) incubated with purified β_2AR^{H93C} reconstituted into HDL particles yields equally high fractions of covalent ligand-receptor complex compared with the reversible reference alprenolol (black) after dilution with [^{3}H]dihydroalprenolol. (*B*) Membrane-bound D_2R^{L94C} was preincubated with dopamine analog **3** (blue) and the reversible agonist quinpirole (black). After washing the membranes, radioligand displacement with [^{3}H]spiperone indicates nearly complete covalent binding of compound **3** to D_2R^{L94C} compared with quinpirole. (*C*) Preincubation and a radioligand binding assay using membrane-bound 5-$HT_{2A}^{S131C-T134C}$ and compound **4** (blue) show a considerable fraction of nonreversible ligand-receptor complex, whereas membranes treated with the reversible ligand serotonin (black) are fully bound by [^{3}H]ketanserin. (*D*) Receptor activation of WT β_2AR and β_2AR^{H93C} was determined by a [^{35}S]GTPγS binding assay using purified and reconstituted receptors and purified heterotrimeric G_s protein. Although compound **2** activates both receptors similar to isoproterenol (iso), the activation at β_2AR^{wt} is reduced to basal activity by adding an excess of inverse agonist ICI-118,551 (ICI), whereas the compound **2**-β_2AR^{H93C} complex displays significant nucleotide binding even in presence of ICI-118,551. An IP accumulation assay was conducted to measure activation of compound **3** at D_2R^{L94C} in comparison to the reference quinpirole (quin). Although the effect of quinpirole is fully blocked by an excess of the antagonist haloperidol (halo), the compound **3**-D_2R^{L94C} complex induces stable and nonreversible IP formation. Similarly, IP accumulation of compound **4** reveals substantial activation of 5-$HT_{2A}^{S131C-T134C}$, which is equal to the effect of serotonin (serot), but cannot be inhibited by the antagonist ketanserin (ketan). Data represent the mean of three experiments ± SEM, each performed in triplicate.

accumulation assays indicated substantial nonreversible G_q protein activation by the covalent neurotransmitter-receptor complex (Fig. 2*D* and *SI Appendix*, Fig. S8).

Investigation of the covalent histamine analog **5** directed us to the construction of the histamine H_1 receptor mutant H_1R^{Y87C} as an additional example of a G_q-coupled GPCR. In a corresponding IP accumulation assay, histamine and histamine analog **5** show comparable activation of WT and mutant receptors (*SI Appendix*, Figs. S9 and S10). Although the response of histamine can be substantially reduced by an excess of the antagonist diphenhydramine, histamine analog **5** still displays significant $G\alpha_q$ activation, thus demonstrating considerable formation of a covalent ligand-receptor complex (*SI Appendix*, Fig. S10).

Structure of β_2AR^{H93C} in Complex with a Covalent (Nor)adrenaline Analog. To test the applicability of our methodology in structural studies, we conducted crystallography trials for β_2AR^{H93C} using compound **2** and the engineered nanobody 6B9 (Nb6B9), which is a high-affinity version of a single-domain antibody fragment obtained from llamas that was further evolved to display optimized properties for crystallography (24). Nb6B9 mimics the G protein by binding at the intracellular surface of the β_2AR, and thus stabilizes the active state of the receptor (Fig. 3*A*). Crystals of the compound **2**-bound β_2AR^{H93C}-Nb6B9 complex, grown in lipidic mesophase (25), displayed excellent size and stability at 20 °C, in contrast to the crystals obtained for the adrenaline-bound complex of β_2AR, stabilized by Nb6B9 (24), which were inherently unstable and had to be harvested shortly after their appearance.

The structure of the compound **2**-bound β_2AR-Nb6B9 complex was solved at a resolution of 3.3 Å with clear electron density for the covalent ligand (*SI Appendix*, Fig. S11). It reveals a remarkable similarity to the 3.2 Å adrenaline-bound structure. Fig. 3*B* shows a superimposition of the binding pocket of both complexes. The adrenaline-pharmacophores form identical ionic and hydrophilic interactions. A unique feature of catechol-bound structures is the tilt of TM6 toward the catechol moiety to form a hydrogen bond between the *meta*-hydroxyl group and $Asn293^{6.55}$ (24) (*SI Appendix*, Fig. S12). In the adrenaline-bound structure, a water molecule further mediates an extended hydrogen bond network from $Asn293^{6.55}$ to $His296^{6.58}$ and up to Asn301 in extracellular loop 3 (ECL3) (Fig. 3*B*). The electron density of the covalent complex structure is suggestive of the existence of a corresponding water molecule but is insufficient to confirm its presence definitively. $His296^{6.58}$ and the Asn301 in ECL3 appear to adopt different positions in the covalent complex structure; however, the electron density maps for this region are relatively weak, which may reflect the inherent flexibility of ECL3.

The linker of the covalent ligand is positioned in an extended pocket formed by TM3, TM7, and ECL2 (*SI Appendix*, Fig. S13). A comparison of the orthosteric and extended pocket of the adrenaline- and compound **2**-bound structures illustrates that the covalent ligand perfectly fills additional space without altering the overall shape of the adrenaline pocket (Fig. 3*C*). Despite

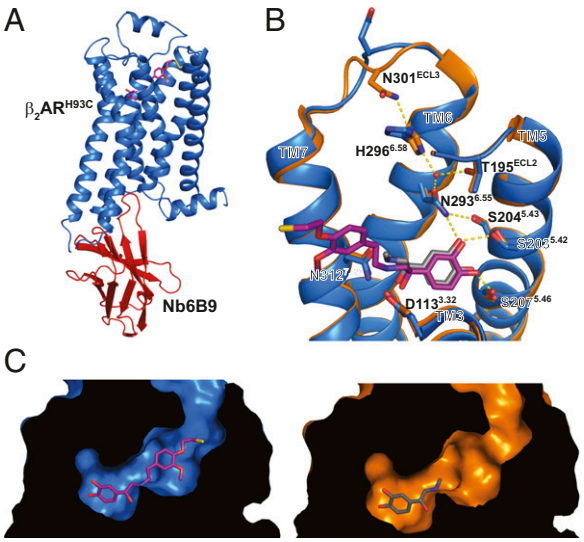

Fig. 3. (*A*) Overall structure of the β_2AR^{H93C} (blue) forming a covalent complex with compound **2** (magenta) and Nb6B9 (red) that binds at the intracellular G protein interface and stabilizes the active state. (*B*) Superimposition of the bound compound **2**-β_2AR^{H93C} complex (blue, ligand in magenta) and the adrenaline-bound active-state structure of the β_2AR (orange, ligand in gray). The water molecule and the extended hydrogen-bond network are resolved in the adrenaline-bound structure. Compound **2** forms highly similar polar interactions. It is likely that minor differences may result from low resolution in this part of the compound **2**-bound structure in which the water molecule is not clearly resolved and the side chain of H296$^{6.58}$ and the ECL3 adopt different conformations. (*C*) Surface view on the binding pocket of both complexes reveals overall identical contraction and shape. The covalent ligand is positioned in an extended binding pocket and does not interfere with ligand binding or activation of the receptor.

minor differences in ECL3 of the β_2AR, our approach yielded a structure that is virtually identical to the adrenaline-β_2AR complex.

Discussion

Endogenous neurotransmitters typically bind with a low affinity to their respective receptors. Thus, obtaining active structures often requires synthetic high-affinity agonists. However, appropriate agonists may not be available or may not stabilize physiological conformations. This study presents a general and practical approach for conversion of the native neurotransmitters noradrenaline, dopamine, serotonin, and histamine into covalent GPCR agonists. The convergent synthetic strategy facilitates fast and facile access to the respective disulfide-functionalized molecular tools, allowing one to optimize the linker length between the pharmacophore and the cross-linking functionality (*SI Appendix*, Figs. S1, S4, and S5). By using different head groups endowed with a primary or secondary amine, this general synthetic approach provides access to a variety of tailored covalent ligands for aminergic GPCRs.

The formation of covalent ligand-receptor complexes was evaluated in radioligand depletion assays for compounds **2**, **3**, and **4**. In addition to synthetic modification of the compounds, the mutation of amino acid residues in proximity to the disulfide-forming cysteine, perturbing covalent bond formation provides an additional tool with which to optimize the target complex. Functional assays confirmed the covalent binding mode for all described agonists and, most importantly, the ability of the covalent ligand–receptor pairs to activate G proteins.

The applicability of the covalent agonists in structural studies is demonstrated by the active-state crystal structure of β_2AR^{H93C} in complex with a covalent (nor)adrenaline-derived agonist, verifying that the designed ligands can be used to facilitate crystallogenesis. A comparison of the presented structure and the adrenaline-bound structure revealed that compound **2** induces a virtually identical receptor conformation, providing evidence that the covalent agonists can yield structural insights into the binding mode of endogenous agonists.

In addition to their benefit in structural studies, covalent agonists can find application in biophysical and biochemical assays that require stable ligand-receptor complexes. Although the crystallization of GPCR-G protein complexes is still notoriously difficult (4, 26, 27), a recent key strategy to obtain active-state structures comprised the use of conformationally selective nanobodies that serve as G protein mimetics and stabilize active conformations (5, 6, 24). Covalent ligand-receptor complexes can be useful for the generation of G protein-mimicking nanobodies because they prevent ligand dissociation during the immunization of llamas (28).

A mustard-based covalent agonist FAUC123 facilitated the identification of a rare G protein mimetic nanobody for the M2 muscarinic receptor from a yeast display library using fluorescence-activated cell sorting. The covalent agonist enabled simultaneous staining of yeast with both agonist- and inverse agonist-occupied M2 receptor populations, which were distinguishably labeled with separate fluorophores (6). The disulfide-based approach will further extend the scope and complement the conformational selection of nanobodies.

This strategy may also be useful for studying oligomerization of aminergic GPCRs and allosteric interactions between protomers. In a system that coexpresses WT receptor and a corresponding X2.64C mutant, a covalent complex of a disulfide-based agonist and the mutant receptor could be formed selectively. One could then examine the functional properties of the WT receptor in the presence and absence of receptor bound to covalent agonist.

In conclusion, we demonstrated the generation of efficacious disulfide-based covalent agonists for aminergic GPCRs representing examples for G_s-, G_i-, and G_q-coupled receptors. We believe that this general and adaptable strategy provides a framework for the development of novel covalent agonists for this class of receptors. Applications of covalent ligands extend well beyond structural biology, highlighting their versatility and utility in promoting structural and functional studies on GPCRs.

Materials and Methods

Synthesis and Characterization of the Covalent Agonists. Detailed schemes and conditions for the synthesis of covalent agonists **1**–**5** are provided in *SI Appendix*, Figs. S1 and S2. The synthesis of additional intermediates is summarized in *SI Appendix*, Fig. S3. Detailed methods and characterization for all compounds are provided in *SI Appendix, SI Note S1*. ^{1}H and ^{13}C NMR spectra for covalent agonists **1**–**5** are provided in *SI Appendix, SI Note S2*.

β_2AR Expression and Purification. For functional experiments, human β_2AR bearing an amino-terminal FLAG epitope tag truncated after residue 365 and with or without an H93C mutation was expressed in *Sf9* cells using the FastBac baculovirus system (Expression Systems). Receptor was extracted and purified as described previously (24, 29) (details are provided in *SI Appendix, SI Materials and Methods*). For crystallographic experiments, a construct of human β_2AR fused to an N-terminal T4 lysozyme (30) containing an H93C mutation was expressed and purified from *Sf9* insect cells. After purification, the receptor was incubated for 60 min at room temperature with a stoichiometric excess of compound **2**. A 1.3-fold molar excess of Nb6B9 was then added and incubated for an additional 30 min. The sample was then concentrated using a 50-kDa spin concentrator and purified over a Sephadex S200 size-exclusion column (GE Healthcare) (details are provided in *SI Appendix, SI Materials and Methods*).

Expression and Purification of Nb6B9. Nanobody was expressed in *Escherichia coli* and purified as described previously (5) (details are provided in *SI Appendix, SI Materials and Methods*).

Crystallization, Data Collection, and Refinement of the 2-β_2AR^{H93C}–Nb6B9 Complex. Crystals were grown in lipid mesophase using 35 nL of protein/lipid drops with 600 nL of overlay solution, which consisted of 24–27% PEG 400, 100 mM Tris (pH 7.8–8.4), and 5–10 mM sodium formate. The X-ray diffraction data were collected at the General Medical Sciences and Cancer Institutes Structural Biology Facility at the Advanced Photon Source GM/CA at APS beamline 23ID-B (further details are provided in *SI Appendix, SI Materials and Methods*). Data from 10 crystals have been merged. Diffraction data were processed in HKL-2000 (HKL Research, Inc.) (31), and the structure was solved using molecular replacement with the structures of active β_2AR bound to Nb6B9 (24) (Protein Data Bank ID code 4LDO) as search models in Phaser (32). The resulting structure was iteratively refined in Phenix (33) and manually rebuilt in Coot (34). Final refinement statistics are summarized in *SI Appendix*, Table S1.

Radioligand Depletion Assay and G Protein Activation Assay for β_2AR. Purified WT β_2AR or β_2AR^{H93C} reconstituted into recombinant HDL particles (22) was used as described (20) (details are provided in *SI Appendix, SI Materials and Methods*).

Radioligand Depletion Assay for the D_2 and 5-HT_{2A} Receptor Ligands. Membranes from HEK 293 cells transiently expressing the human D_2R^{L94C} or human 5-$HT_{2A}^{S131G\text{-}T134C}$ were prepared and incubated with the covalent agonists **3** and **4** and the reversible ligands quinpirole and serotonin, respectively (details are provided in *SI Appendix, SI Materials and Methods*). Membranes were then used for radioligand binding experiments with [^{3}H]spiperone (specific activity = 81 Ci/mmol) or [^{3}H]ketanserin (specific activity = 53 Ci/mmol) (both from PerkinElmer) to determine specific binding at the D_2R and the 5-HT_{2A} receptor, respectively, as described (35) (details are provided in *SI Appendix, SI Materials and Methods* and Table S2).

Determination of Covalent D_2, 5-HT_{2A}, and H_1 Receptor Activation via IP Assays. Agonist-induced activation of the human D_2R^{L94C}, 5-$HT_{2A}^{S131G\text{-}T134C}$, and H_1^{Y87C} receptors was studied using IP accumulation assays as described (6, 23). For activation studies with G_i-coupled GPCRs, HEK 293 cells were transiently cotransfected with cDNA encoding for D_2R^{L94C} and the hybrid G protein $G\alpha_{qi5}$ ($G\alpha_q$ protein with the last five amino acids at the C terminus replaced by the corresponding sequence of $G\alpha_i$; a gift from The J. David Gladstone Institutes) (36). Cells were preincubated with *myo*-[^{3}H]inositol (specific activity = 22.5 Ci·mmol^{-1}; PerkinElmer) for 15 h. After incubation with compound **3** for 30 min, the antagonist haloperidol was added to half of the samples (buffer was added to the other half). Incubation was continued for additional 150 min, and total IP was determined (details are provided in *SI Appendix, SI Materials and Methods*). Activation studies with a G_q-coupled GPCR were done with HEK 293 cells, which were transiently transfected with cDNA encoding for the 5-$HT_{2A}^{S131G\text{-}T134C}$ or H_1^{Y87C} receptor, respectively, similar to the described method (details are provided in *SI Appendix, SI Materials and Methods*). After incubation of 5-$HT_{2A}^{S131G\text{-}T134C}$ with compound **4** and serotonin for 90 min, ketanserin was added to half of the samples to inhibit further receptor activation. After further incubation for 150 min, total IP was determined as described. Likewise, the H_1^{Y87C} receptor was incubated with compound **5** and histamine for 90 min, and diphenhydramine was then added to half of the samples (details are provided in *SI Appendix, SI Materials and Methods*).

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