Abstract

5-hydroxytryptamine receptor 5A (5-HT₅A) belongs to the 5-HT receptor family and signals through the Gᵢ/o protein. It is involved in nervous system regulation and an attractive target for the treatment of psychosis, depression, schizophrenia, and neuropathic pain. 5-HT₅A is the only Gᵢ/o-coupled 5-HT receptor subtype lacking a high-resolution structure, which hampers the mechanistic understanding of ligand binding and Gᵢ/o coupling for 5-HT₅A. Here we report a cryo-electron microscopy structure of the 5-HT₅A-Gᵢ complex bound to 5-Carboxamidotryptamine (5-CT). Combined with functional analysis, this structure reveals the 5-CT recognition mechanism and identifies the receptor residue at 6.55 as a determinant of the 5-CT selectivity for Gᵢ/o-coupled 5-HT receptors. In addition, 5-HT₅A shows an overall conserved Gᵢ protein coupling mode compared with other Gᵢ/o-coupled 5-HT receptors. These findings provide comprehensive insights into the ligand binding and Gᵢ protein coupling of Gᵢ/o-coupled 5-HT receptors and offer a template for the design of 5-HT₅A-selective drugs.

Introduction

5-hydroxytryptamine (5-HT) receptors are widely expressed in the central and peripheral nervous systems and are involved in a variety of psychiatric disorders. They are one of the most promising drug targets for the treatment of nervous system diseases. There are seven distinct types (5-HT₁–5-HT₇), comprised of 14 subtypes in the 5-HT receptor family, of which 13 are G protein-coupled receptors (GPCRs) (Fig. 1a). So far, 26 structures of 5-HT receptors have been reported, including crystal structures of 5-HT₁B, 5-HT₂B, 5-HT₂C, and 5-HT₂C, as well as cryo-electron microscopy (cryo-EM) structures of all members of 5-HT₁, including 5-HT₁A, 5-HT₁B, 5-HT₁C, 5-HT₁D, 5-HT₁E, and 5-HT₁F, in complex with Gᵢ/o protein. These structures provide a basis for understanding ligand recognition and functional regulation of these 5-HT receptors. Besides 5-HT₁, 5-HT₅ is another type of Gᵢ/o-coupled 5-HT receptor and also remains the last type of Gᵢ/o-coupled 5-HT receptor without a reported structure.

The 5-HT₅ subfamily consists of two members, designated as 5-HT₅A and 5-HT₅B, which share 69% sequence identity with each other and have 23%–34% homology with other 5-HT receptors. Of note, 5-HT₅B is the first example of a brain-specific receptor that is absent in humans, of which the coding sequence is interrupted by stop codons. Thus, 5-HT₅A stands out as the only 5-HT₅ subtype expressed in human brain regions, including the cerebral cortex, hippocampus, and raphe nuclei. 5-HT₅A shows an antinociceptive role and is involved in the regulation of memory, learning, and food intake. Its specific ligands have shown potential in the treatment of psychosis, depression, schizophrenia, and neuropathic pain. Thus, the development of 5-HT₅A-selective drugs will offer a new opportunity for the treatment of these nervous system diseases.
However, the selective ligands for 5-HT5A are still lacking. 5-Carboxamidotryptamine (5-CT) is a synthetic agonist for 5-HT5A and also activates other Gi/o-coupled 5-HT receptors with distinct affinities. It shows a moderate affinity for 5-HT5A with a $pK_i$ value of 7.7 and displays high affinities for 5-HT1A, 5-HT1B, and 5-HT1D ($pK_i$ = 8.9–9.0)\textsuperscript{21–25}. In contrast, 5-CT displays negligible affinities for 5-HT1E ($pK_i$ = 5.4) and 5-HT1F ($pK_i$ = 6.1)\textsuperscript{26,27}. SB699551 and ASP5736 stand out as two selective antagonists, which have been widely used for functional studies of 5-HT5A\textsuperscript{20,28}. The availability of the structure of ligand-bound 5-HT5A may accelerate the design of 5-HT5A-targeting drugs by providing an accurate structure template.

In this study, we report the structure of G\textsubscript{i} coupled 5-HT\textsubscript{5A} complex bound to 5-CT at a resolution of 3.1 Å. This structure clarified the feature of 5-CT recognition by 5-HT\textsubscript{5A} and identified a determinant for 5-CT affinities against G\textsubscript{i/o}-coupled 5-HT receptors, thus providing a rationale for designing drugs targeting 5-HT\textsubscript{5A}. Structural comparison of the 5-HT\textsubscript{5A}–G\textsubscript{i} with other G\textsubscript{i/o}-coupled 5-HT receptor complexes deepens our understanding of the mechanism underlying ligand recognition and G\textsubscript{i/o} coupling.

### Results

**Cryo-EM structure of the 5-CT–5-HT\textsubscript{5A}–G\textsubscript{i}–scFv16 complex**

We used the full-length human 5-HT5A for structural studies. A BRIL was fused to the N-terminus of 5-HT5A to improve expression. The NanoBiT tethering strategy was applied to stabilize the 5-HT5A–Gi complex, which had been widely used in the structure determination of several GPCR–G protein complexes\textsuperscript{29–31} (Supplementary Fig. S1). The C-terminus of the receptor and the G\textsubscript{\beta1} subunit were connected to the LgBiT and HiBiT, respectively. A dominant-negative form of the human G\textsubscript{\alpha1} mutant containing four mutations (S47N, G203A, E345A, and A326S), referred to as G\textsubscript{\alpha1} (4DN), was applied\textsuperscript{32}. The 5-HT\textsubscript{5A}–G\textsubscript{i} complex was assembled by co-expressing the engineered receptor with G\textsubscript{\alpha1} (4DN), G\textsubscript{\beta1}, G\textsubscript{\gamma2} subunits, and scFv16 in High Five (Hi5) cells in the presence of 5-CT.

The structure of the 5-CT–5-HT\textsubscript{5A}–G\textsubscript{i}–scFv16 complex was determined with an overall resolution of 3.1 Å (Fig. 1b, c; Supplementary Fig. S2 and Table S1). The high-quality density maps are clear for modeling 5-HT5A from residue 31 to residue 353, with the exception of residues 237–275 in the intracellular loop 3 (ICL3). The
majority of the residue side chains in the seven-transmembrane helical domain (TMD), three extracellular loops (ECL1–ECL3), and two ICLs (ICL1 and ICL2) of 5-HT5A were well-defined. 5-CT, scFv16, and the three subunits of Gi protein are also well-fitted in the EM map. The entire model provides detailed structural information on the 5-CT-binding pocket and 5-HT5A–Gi interaction interface (Fig. 1c, d; Supplementary Fig. S3).

The recognition of G_{i/o}-coupled 5-HT receptors by agonists

The binding pocket in 5-HT5A is largely overlapped with that in other G_{i/o}-coupled 5-HT receptors, sharing 11 of 16 identical residues. 5-CT is embedded deep into the pocket constituted by TM3, ECL2, and TM5–TM7 of 5-HT5A (Fig. 2a). Compared with other ligands bound to G_{i/o}-coupled 5-HT receptors, 5-CT adopts a similar binding pose in 5-HT5A (Supplementary Fig. S4a). Its indole scaffold is anchored through a salt bridge between its positively charged nitrogen at the 3-aminoethyl group and the carboxylate of D121^{3.32} (Fig. 2a, b). This salt bridge is highly conserved across ligand-bound 5-HT receptors with known structures (Supplementary Fig. S4b). Mutating D121^{3.32} to alanine abolished the 5-CT-induced 5-HT5A activation, highlighting its importance to 5-CT activity (Fig. 2c). In addition, the side chain of D121^{3.32} is further stabilized by an intramolecular hydrogen bond between D121^{3.32} and Y328^{7.43}, which is supported by the alanine mutagenesis data. On the other side, the nitrogen at the 5-carboxamide of 5-CT forms a hydrogen bond with the side chain of E305^{6.55}. Besides polar interactions, the indole scaffold of 5-CT tightly packs against a hydrophobic cleft comprising side chains of V122^{3.33}, F301^{6.51}, F302^{6.52}, and L324^{7.39}. These hydrophobic residues substantially contribute to 5-CT-induced 5-HT5A activation (Fig. 2c; Supplementary Table S2).

It has been thought that the ligand-binding pocket of each G_{i/o}-coupled 5-HT receptor is comprised of two
subpockets, the orthosteric binding pocket (OBP) and the extended binding pocket (EBP) \(^2\). OBP of \(G_{\alpha_i}\)-coupled 5-HT receptors locates deep into the core of the TMD pocket, whereas the EBP approaches the extracellular surface of the entire binding pocket (Supplementary Fig. S4a). The 11 conserved residues in the binding pocket across \(G_{\alpha_i}\)-coupled 5-HT receptors are located in the OBP, including D3.32, C3.36, T3.37, I4.56, S4.42, T5.43, A5.46, \(W_6.48\), F6.51, F6.52, and Y7.43 (Supplementary Fig. S4b), of which D3.32 is thought critical to ligand binding for 5-HT and other monoamine receptors by forming a conserved salt bridge with the basic cyclic amine of ligands \(^2\). The featured benzene ring of the ligand is surrounded by conserved hydrophobic residues in \(G_{\alpha_i}\)-coupled 5-HT receptors, including \(W_6.48\), F6.51, F6.52, and Y7.43. This hydrophobic environment is crucial for ligand-induced receptor activation. Inspection of the binding poses of ligands in \(G_{\alpha_i}\)-coupled 5-HT receptors reveals that 5-HT, 5-CT, and BRL54443 only occupy the OBP. In contrast, Donitriptan and Lasmiditan, two anti-migraine drugs selectively targeting 5-HT1B/1D and 5-HT1F, respectively, are relatively bulky and occupy both OBP and EBP of specific receptors (Supplementary Fig. S4a). These structural observations are consistent with the contention that OBP is critical to the binding potency of ligands, whereas the EBP plays a predominant role in determining ligand selectivity \(^2\). Together, these findings provide insights into the 5-CT recognition for 5-HT5A and deepen our understanding of ligand selectivity for 5-HT receptors.

**Role of the residue at 6.55 in the determination of 5-CT selectivity for \(G_{\alpha_i}\)-coupled 5-HT receptors**

5-CT shows different selectivity for \(G_{\alpha_i}\)-coupled 5-HT receptors. It exhibits high affinities for 5-HT1A, 5-HT1B, and 5-HT1D \((pK_i = 7.9–8.1)\) and relatively weak affinities for 5-HT1E, 5-HT1F, and 5-HT5A \((pK_i = 5.4–7.0)\) (Fig. 3b; Supplementary Table S3). Sequence comparison of residues in the EBP of \(G_{\alpha_i}\)-coupled 5-HT receptors reveals a low sequence identity at position 6.55 (Supplementary Fig. S4b). The residue at 6.55 is alanine in 5-HT1A and serine in 5-HT1B and 5-HT1D. In contrast, the cognate residue in 5-HT1E, 5-HT1F, and 5-HT5A is glutamic acid. The difference in residue composition raises a hypothesis that the residue at position 6.55 is involved in 5-CT selectivity for \(G_{\alpha_i}\)-coupled 5-HT receptors.

To prove this hypothesis, we introduced swap mutations to residues at 6.55 across \(G_{\alpha_i}\)-coupled 5-HT receptors. Our \(G_i\) protein recruitment data support two facets of roles of the residue at 6.55 in 5-CT-induced receptor activation. One is the steric hindrance arising from the side chain of the residue at position 6.55 (Fig. 3c). For 5-HT1A, A365F.E and A365S.S mutations, which increase the size of the side chain, reduced 5-CT-induced receptor activation relative to the WT receptor. S334E mutation of 5-HT1B decreased 5-CT activity, while substituting the serine with a smaller side chain residue alanine dramatically increased 5-CT potency. Similarly, mutating glutamic acid of 5-HT1E and 5-HT5A to alanine or serine, two residues with a relatively small side chain, notably promoted receptor activation. These findings corroborate the idea that the bulkier side chain of glutamic acid relative to alanine and serine may prevent the binding of 5-CT and receptor activation through steric hindrance. On the other hand, 5-CT may form hydrogen bonds with the glutamic acid at 6.55 in \(G_{\alpha_i}\)-coupled 5-HT receptors, which may dominate the ligand–receptor interaction over the hindrance effects of the side chain (Fig. 3c). This point is supported by functional analysis of 5-HT1D and 5-HT1F. S321E mutation of the 5-HT1D enhanced 5-CT activity, despite the increased side-chain size. Consistently, E313S.S and E313A.A mutations in 5-HT1F almost abolished 5-CT activity. Thus, residues at position 6.55 modulate the activity of \(G_{\alpha_i}\)-coupled 5-HT receptors through two aspects of roles: the steric hindrance effects for 5-HT1A, 5-HT1B, 5-HT1E and 5-HT5A and the hydrogen bond-forming capacity as an acceptor for 5-HT1D and 5-HT1F. Together, our findings provide further evidence for the previous speculation that the residue at 6.55 is responsible for the ligand-recognition specificity of 5-HT receptors and offer a new opportunity for the design of drugs selectively targeting 5-HT receptors \(^1\).

**General features of the activation and \(G\) protein coupling of \(G_{\alpha_i}\)-coupled 5-HT receptors**

Similar to agonists bound to other \(G_{\alpha_i}\)-coupled 5-HT receptors, 5-CT directly contacts the toggle switch residue \(W_6.48\) of 5-HT5A and triggers its rotameric switch. The change of \(W_6.48\) initiates the rotation and outward movement of the TM6 cytoplasmic end of 5-HT5A relative to inverse agonist-bound 5-HT1B (PDB: 5V54), the hallmark of class A GPCR activation (Fig. 4a, b).

Structural comparison of the \(G_i\)-coupled 5-HT5A with other \(G_{\alpha_i}\)-coupled 5-HT receptors whose structures had been solved revealed an almost overlapped receptor activation conformation. However, the a5 helices of \(G_{\alpha_i}\) subunits in these 5-HT receptor complexes showed slight tilts to different extents, which cause rotation of the entire \(G_{\alpha_i}\) proteins, leading to the most noticeable translational movement of the aN helices (Fig. 4a). The global coupling interface profile analysis showed that the majority of the \(G_{\alpha_i}\) subunit-interacting residues in TM3, ICL2, TM5, TM6, TM7, and helix 8 are conserved across \(G_{\alpha_i}\)-coupled 5-HT receptors, including R\(^i\).30, I\(^i\).34, I\(^i\).61, A\(^i\).67, R\(^i\).6.29, R/K\(^i\).6.32, and N\(^i\).4.7. Differently, no substantial interactions were seen between ICL3 of \(G_{\alpha_i}\)-coupled 5-HT receptors and \(G_i\) protein, with an exception of 5-HT1D, which shows an additional EM density of ICL3–\(G_i\) interaction \(^1\) (Fig. 4c).
Two major interfaces exist between 5-HT<sub>5A</sub> and G<sub>i</sub> protein. The cytoplasmic receptor cavity constituted by TM3, TM5, TM6, and the TM7–helix 8 junction accommodates the distal C-terminal end of the α5 helix of the G<i>α</i> subunit, forming a primary interface (Fig. 4d). The residues of α5 helix hydrophobically contact the receptor cavity. L348, C351, L353, and F354 in the α5 helix of the G<i>α</i> subunit contact a hydrophobic patch comprised of residues in TM3 (I143.54), TM5 (I223.56, A227.65, and V231.69), and TM6 (A283.63 and V287.67). In addition, residues R230.58 and S233.57 of TM5 form well-defined hydrogen bonds with D341 and K345 from the α5 helix, respectively (Fig. 4d). The ICL2 also interacts with G<i>α</i>, constituting the other major interface. M147.80 of ICL2 inserts into the groove constituted by hydrophobic residues in the α5 helix, the β1 and β3 strands, and αN of the G<i>α</i> subunit. A hydrogen bond present between R152.43 and E28 may further stabilize the ICL2–G<i>α</i> interface (Fig. 4e). Together, these findings clarify the activation and G<i>α</i> coupling features of 5-HT<sub>5A</sub> and provide a comprehensive understanding of the G protein coupling mechanism of G<i>α</i>/<i>β</i>-coupled 5-HT receptors.

**Discussion**

5-HT<sub>5A</sub> is a G<i>α</i>/<i>β</i>-coupled 5-HT receptor subtype and is involved in nervous system disorders, thus serving as an important drug target. It is the only G<i>α</i>/<i>β</i>-coupled 5-HT receptor subtype lacking a high-resolution structure to date. In this paper, we report a 3.1 Å-resolution cryo-EM structure of the 5-HT<sub>5A</sub>–G<i>α</i> complex bound to a synthetic agonist, 5-CT, which is also the first 5-CT-bound 5-HT receptor structure. Our structure reveals the recognition mechanism of 5-HT<sub>5A</sub> by 5-CT and adds to the pool of the structures for deepening our understanding of the ligand-binding mode of 5-HT receptors. Furthermore, structural comparison and functional analysis of the ligand-binding pockets reveal that the residue at 6.55 serves as a determinant for the 5-CT specificity for G<i>α</i>/<i>β</i>-coupled 5-HT receptors. This ligand specificity is partly attributed to the steric hindrance arising from the
side chain of the residue at 6.55 or its potential polar interaction with ligands. In addition, our structure reveals a similar activation mechanism and an overall conserved G\textsubscript{i/o} coupling mode for 5-HT\textsubscript{5A} compared with other G\textsubscript{i/o}-coupled 5-HT receptors. These findings broaden our understanding of ligand recognition in the 5-HT system.

Although 5-HT\textsubscript{5A} has been cloned for ~3 years, it is still one of the less well-characterized receptors in the 5-HT receptor family. The lack of selective ligands has delayed the functional studies on 5-HT\textsubscript{5A} until the discovery of the selective antagonists SB699551 and ASP5736, which have improved our understanding of the localization of 5-HT in the brain and its function. However, it should be noted that we are still far from fully understanding the pharmacological characteristics of 5-HT\textsubscript{5A}. Meanwhile, no drugs targeting 5-HT\textsubscript{5A} have been registered for clinical trials or approved. Recently, virtual screening based on a homology model identified UCSF678, a 42 nM new chemical probe with partial agonism activity for 5-HT\textsubscript{5A}. UCSF678 exhibits enhanced selectivity for 5-HT\textsubscript{5A} and a more restricted off-target profile than the existing 5-HT\textsubscript{5A} antagonist SB699551. Unlike the promiscuous ligand 5-HT, molecular docking reveals that

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**Fig. 4 Structural features of the activation and G\textsubscript{i/o} coupling of 5-HT\textsubscript{5A} and other G\textsubscript{i/o}-coupled 5-HT receptors.**

a The activation of 5-HT\textsubscript{5A} and other G\textsubscript{i/o}-coupled 5-HT receptors. The outward movement of TM6 of agonist-bound G\textsubscript{i/o}-coupled 5-HT receptors compared with methiothepin-bound 5-HT\textsubscript{1B} in the inactive state is shown as a black arrow. The movements of the \(\alpha\)N and \(\alpha\)5 helix of G\textsubscript{\alpha} protein in the 5-HT\textsubscript{5A}–G\textsubscript{\alpha} complex relative to that in other G\textsubscript{i/o}-coupled 5-HT receptors are indicated as black arrows. The structures of the active 5-HT\textsubscript{5A} (light pink), 5-HT\textsubscript{1A} (cyan, PDB: 7E2Y), 5-HT\textsubscript{1B} (purple, PDB: 6G79), 5-HT\textsubscript{1D} (green, PDB: 7E32), 5-HT\textsubscript{1E} (wheat, PDB: 7E33), 5-HT\textsubscript{1F} (coral, PDB: 7EXD) and inactive-state 5-HT\textsubscript{1B} (gray, PDB: 4IAQ) complexes were superimposed based on TM2, TM3 and TM4.

b Structural comparison of ligands and the W\textsubscript{6.48} residues of active 5-HT receptors with that of inactive 5-HT\textsubscript{1B}. Ligands directly contact W\textsubscript{6.48}. Compared with methiothepin, the inverse agonist of 5-HT\textsubscript{1B}, agonists trigger the rotameric switch of W\textsubscript{6.48}, which leads to the outward movement of TM6.

c The sequence alignment of receptor residues at receptor–G\textsubscript{\alpha} protein interfaces. The conserved residues are highlighted in solid green circles.

d Detailed interactions between TM5, TM6, and the TM7–helix 8 junction of 5-HT\textsubscript{5A} and the \(\alpha\)5 helix of the G\textsubscript{\alpha} subunit (d). Detailed interactions between ICL2 of 5-HT\textsubscript{5A} and the \(\alpha\)5 and \(\alpha\)N helices, \(\beta\)1 and \(\beta\)3 strands of the G\textsubscript{\alpha} subunit (e).
USCF678 extends into the upper region of the binding pocket, known as EBP. W1173.28 in EBP of 5-HT5A is further proved to be responsible for the high-affinity binding of USCF638, and the tryptophan at position 3.28 may contribute to the off-target binding of USCF638 analogs. These findings are consistent with the two pockets (OBP and EBP) binding model and highlight the importance of EBP to the discovery of selective ligands. Consequently, the 5-HT5A structure provides an accurate template for the rational design of drugs targeting 5-HT5A and may offer a new opportunity for the treatment of nervous system diseases, including psychosis, depression, schizophrenia, and neuropathic pain.

**Materials and methods**

**Constructs**

The human full-length 5-HT5A was cloned into the pfastBac with an N-terminal haemagglutinin (HA) sequence followed by a Flag-tag, 15× His-tag, BRIL-tag, and an LgBiT sequence at the C-terminus to facilitate the protein expression and purification. The human Ga3, with four dominant-negative mutations, S47N, G203A, E245A, and A326S was applied. Human Gβ1, human Gγ2, and scFv16 were cloned into pFastBac vector using homologous recombination (ClonExpress One Step Cloning Kit, Vazyme).

**Expression and complex purification**

5-HT5A-LgBiT, DN_Ga3, Gβ1-SmBiT, Gγ2, and scFv16 were co-expressed in Hi5 insect cells (Invitrogen) using the Bac-to-Bac baculovirus expression system (ThermoFisher). Cell cultures were grown in ESF 921 medium (Expression Systems) to a density of 3 × 10⁶ cells/ml, and then infected with five separate baculoviruses, respectively, at the ratio of 1:1:1:1:1.5. The culture was harvested by centrifugation 48 h post-infection and stored at −80 °C for further usage.

Cell pellets were lysed in 20 mM HEPES, pH 7.4, 20 mM KCl, 10 mM MgCl2, 5 mM CaCl2, and 10% glycerol supplemented with Protease Inhibitor Cocktail (ThermoFisher). The 5-HT5A–Gαi complex was formed on the membrane for 1.5 h at room temperature by addition of 10 μM 5-CT and 25 μM apyrase (Sigma), and then solubilized from the membrane by using 0.5% (w/v) n-dodecyl-β-D-maltoside (DDM) (Anatrace) and 0.1% (w/v) cholesteryl hemisuccinate (CHS) (Anatrace) for 2 h at 4 °C, followed by centrifugation at 85,000 × g for 30 min to extract the solubilized complex. The supernatant was subsequently incubated by nickel affinity chromatography (Ni Smart Beads 6F, SMART Lifesciences) at 4 °C for 3 h. The resin was washed with 20 column volumes of the buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 25 mM imidazole, 0.01% (w/v) LMNG (Anatrace), 0.005% GDN (Anatrace), 0.004% (w/v) CHS (Anatrace), and 5μM 5-CT. The complex was then eluted with six column volumes of the same buffer containing 300 mM imidazole. The protein was concentrated and subjected onto a Superdex 200 Increase 10/300 column (GE Healthcare) in the buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.00075% (w/v) LMNG (Anatrace), 0.00025% (w/v) GDN (Anatrace), 0.0002% (w/v) CHS (Anatrace) and 10 mM 5-CT. The purified complex fractions were collected and concentrated for cryo-EM experiments.

**Cryo-EM grid preparation and data collection**

For the cryo-EM grid preparation, 3 μL of the purified 5-CT–5-HT5A–Gαi complex at a final concentration of 25 mg/mL was applied to glow-discharged holey carbon grids (Quantifoil R1.2/1.3, 300 mesh), and vitrified using a Vitrobot Mark IV (ThermoFisher Scientific) subsequently. Grids were plunge-frozen in liquid ethane using Vitrobot Mark IV (Thermo Fischer Scientific). Frozen grids were transferred to liquid nitrogen and stored for data acquisition. Cryo-EM images were collected by an FEI Titan Krios at 300 kV accelerating voltage equipped with a Gatan K3 Summit direct electron detector at the Center of Cryo-Electron Microscopy Research Center, Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China). A total of 5303 movies were automatically acquired using SerialEM10 in super-resolution counting mode at a pixel size of 1.071 Å. The images were recorded at a dose rate of about 26.7 e/Å²/s with a defocus ranging from −1.2 to −2.2 μm. The total exposure time was 3 s, and intermediate frames were recorded in 0.083-s intervals, resulting in a total of 36 frames per micrograph.

**Image processing and 3D reconstruction**

Image stacks were subjected to beam-induced motion correction using MotionCor234, while contrast transfer function (CTF) parameters were determined by Gctf35. Automated particle selection and data processing were performed using Relion 3.036. Automated particle selection yielded 3,767,450 particles. The particles were subjected to reference-free 2D classification, which produced two good subsets showing clear structural features accounting for 754,854 particles. The total exposure time was 3 s, and intermediate frames were recorded in 0.083-s intervals, resulting in a total of 36 frames per micrograph.

The cryo-EM structure of the 5-CT–5-HT5A–Gαi complex (PDB: 7E2Y) and the Gαi protein model (PDB: 6DDE)
were used to generate the initial model and refinement against the electron microscopy map. The model was docked into the EM density map using UCSF Chimera \(^{38}\), followed by iterative manual adjustment and rebuilding in COOT \(^{39}\) and ISOLDE \(^{40}\) according to side-chain densities. Real-space refinement was performed using Phenix programs \(^{41}\). The model statistics were validated using MolProbity \(^{42}\). Structural figures were prepared in Chimera, Chimerax \(^{43}\), and PyMOL (https://pymol.org/2/). The final refinement statistics are provided in Supplementary Table S1.

**NanoBit G protein recruitment assay**

NanoBit, a NanoLuc luciferase-based method, is used to detect the interaction between receptor and G protein in living cells \(^{44}\). The full-length 5-HT\(_{5A}\) was fused with a LgBiT fragment (17.6 kDa) at its C-terminus via a 15-amino acid flexible linker. SmBiT, a 13-amino acid peptide, was C-terminally fused to the G\(\beta\) subunit using the same linker. The cDNAs of 5-HT\(_{5A}\)-LgBiT, G\(\alpha\)\(_{i}\), G\(\beta\)\(_{1}\)-SmBiT, and G\(\gamma\)_2 were cloned into pFastBac vector (Invitrogen). The baculoviruses were prepared using the bac-to-bac system (Invitrogen). Hi5 cells were cultured in ESF 921 medium (Expression Systems) to a density of 2.5–3 million cells per mL and then infected with four separate baculoviruses at the ratio of 1:1:1:1. After 48 h infection, the culture was collected by centrifugation, and the cell pellet was resuspended with PBS. The cell suspension was seeded onto 384-well microtiter plates (40 \(\mu\)L per well) and loaded with 5 \(\mu\)L of 50 \(\mu\)M coelenterazine (Yeasen) diluted in the assay buffer. 5\(\text{F}^\text{ITC}\) using a BD ACCURI C6.

**Surface expression analysis**

Cell surface expression for each mutant was monitored by detecting the fluorescent intensity of FITC using a BD ACCURI C6.

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**Author contributions**

Y.T. and P.X. optimized the conditions of protein samples for final structure determination; Y.T. and S.H. generated the expression constructs and optimized the 5-HT\(_{5A}\)–G\(_{i}\) protein complex; Y.T. purified the protein samples for final structure determination, participated in cryo-EM grid inspection, data collection, model building, executed the functional assay, and prepared the figures and manuscript draft; P.X. evaluated the specimen by negative-stain EM; screened the cryo-EM conditions; prepared the cryo-EM grids, collected cryo-EM images, and refined the structures; G.Y. participated in the functional assay; F.Z. analyzed the interactions between 5-HT\(_{5A}\) and 5-CT by LigPlot\(^{\text{TM}}\) program; X.H. and H.M. participated in structure analysis and model building. Y.J. participated in the supervision of Y.T., S.H., P.X., X.H., and F.Z., analyzed the structures, prepared the figures, and edited the manuscript. H.E.X. and Y.J. conceived and supervised the project, and wrote the manuscript with inputs from all authors.

**Data availability**

The atomic coordinate and the electron microscopy map for the 5-CT–5-HT\(_{5A}\)–G\(_{i}\)-scFv\(_{16}\) complex have been deposited in the Protein Data Bank (PDB) under accession number 7XSH and Electron Microscopy Data Bank (EMDB) under accession number EMD-33014, respectively.

**Conflict of interest**

The authors declare no competing interests.

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**Supplementary information**

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