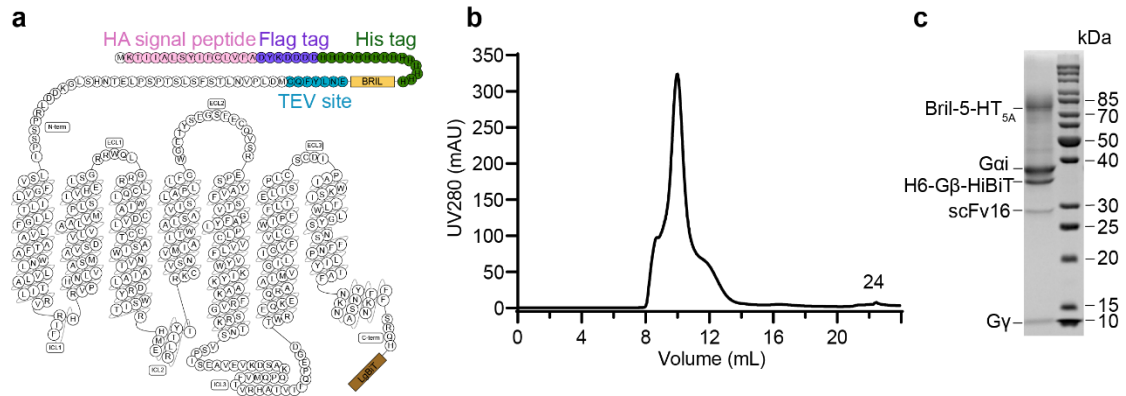**Supplementary Information**

**Fig. S1 Characterization and purification of the 5-CT–5-HT$_{5A}$–G$_i$ complex. a** Schematic

illustration of the HA–Flag–His15–BRIL–5-HT$_{5A}$–TEV site–LgBiT construct used in the study.

The HA signal peptide (pink), Flag (purple), His15 (green), BRIL (yellow), TEV (blue) and

LgBiT (brown) are indicated. **b** Analytical size-exclusion chromatography of the purified

complex on Superdex 200 Increase 10/300 column. **c** SDS-PAGE analysis of the purified

complex with Coomassie blue staining.
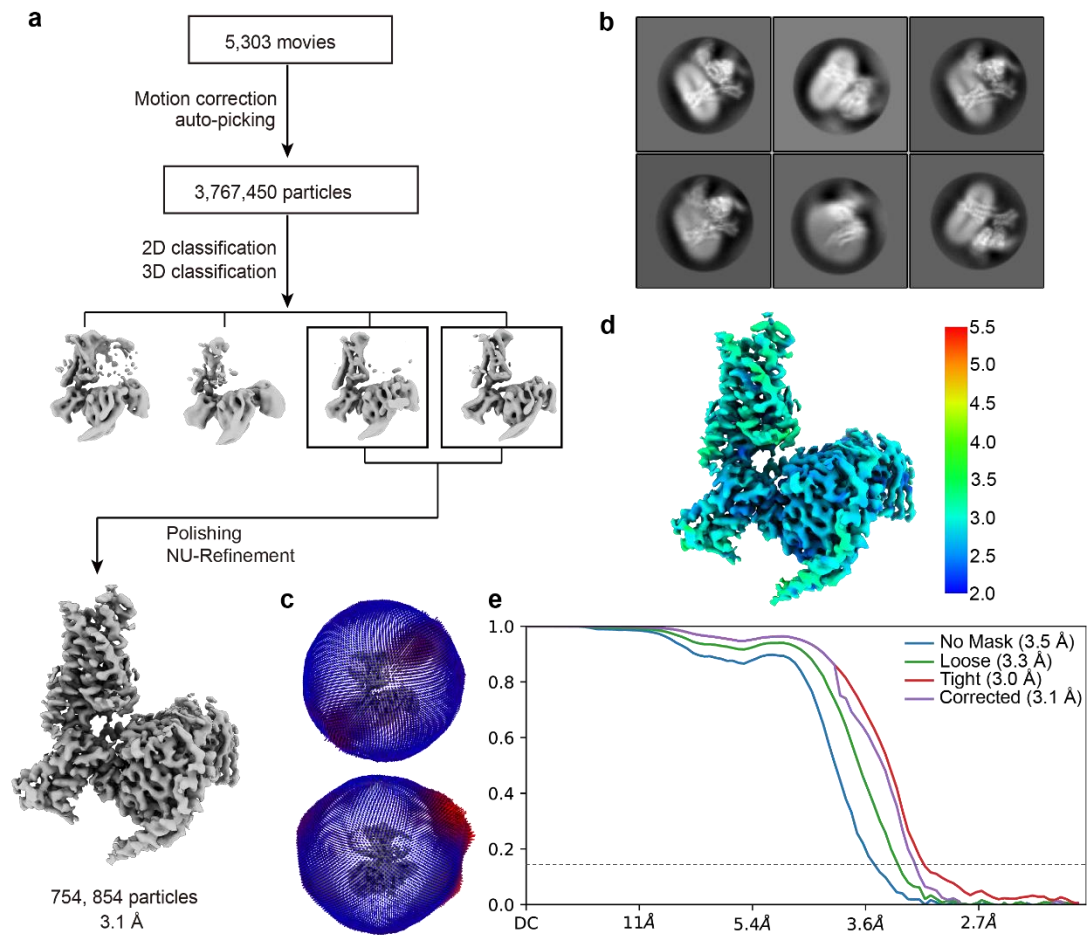
Fig. S2 The cryo-EM data processing and density map of the 5-CT–5-HT$_{5A}$–G$_i$ complex.
a Flow chart of cryo-EM data processing. **b** Representative cryo-EM micrograph from 5,303
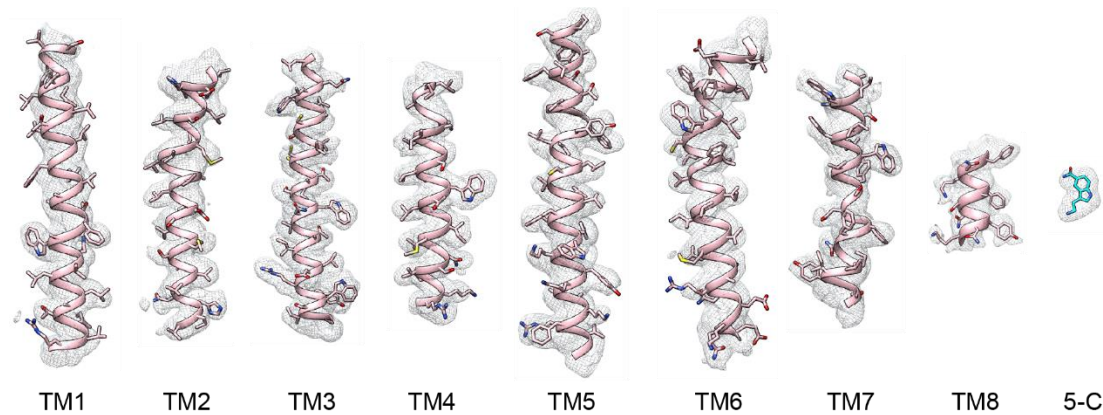movies and representative two-dimensional class averages determined using approximately 3.8
million particles of the 5-CT–5-HT$_{5A}$–G$_i$ complex were shown. **c** Angle distribution maps. **d**
Cryo-EM map of the 5-CT–5-HT$_{5A}$–G$_i$ complex, colored by local resolution (Å) calculated
using Resmap package. **e** 'Gold-standard' Fourier shell correlation curves of the 5-CT–5-
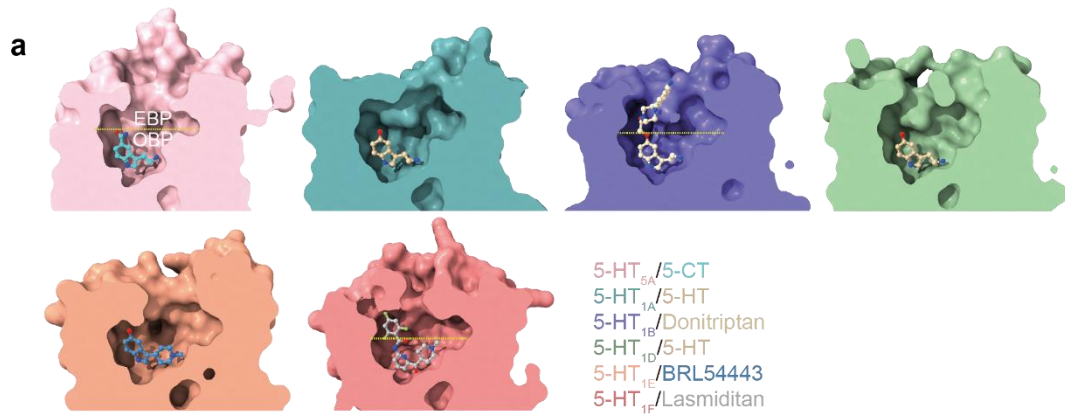HT$_{5A}$–G$_i$ complex.

TM1 TM2 TM3 TM4 TM5 TM6 TM7 TM8 5-CT

Fig. S3 Representative cryo-EM density map of the 5-CT–5-HT$_{5A}$–G$_i$ complex. Cryo-EM density of transmembrane helices, helix 8 and 5-CT.

Fig. S4 Comparison of the binding pose of agonists and sequence alignment of residues in the binding pockets of G$_{i/o}$-coupled 5-HT receptors. a Comparison of the agonist-binding poses in G$_{i/o}$-coupled 5-HT$_{5A}$ (light pink), 5-HT$_{1A}$ (cyan, PDB code: 7E2Y), 5-HT$_{1B}$ (purple, PDB code: 6G79), 5-HT$_{1D}$ (green, PDB code: 7E32), 5-HT$_{1E}$ (wheat, PDB code: 7E33), and 5-HT$_{1F}$ (coral, PBD code: 7EXD). **b** Sequence alignment of residues in the ligand-binding pocket of G$_{i/o}$-coupled 5-HT receptors. The conserved residues are highlighted in solid dark green circles.

Table S1. Cryo-EM data collection, model refinement, and validation statistics

	5-HT-5-HT$_{5A}$–G$_i$ (PDB: 7X5H, EMD-33014)
Data collection and processing	
Magnification	46,685
Voltage (kV)	300
Electron exposure (e$^-$/Å^2)	70
Defocus range (μm)	-1.2 ~ -2.2
Pixel size (Å)	1.071
Symmetry imposed	C1
Initial particle projections (no.)	3,767,450
Final particle projections (no.)	754,854
Map resolution (Å)	3.1
FSC threshold	0.143
Map resolution range (Å)	2.5-5.0
Refinement	
Initial model used	7E2Y
Model resolution (Å)	3.3
FSC threshold	0.5
Map sharpening B factor (Å^2)	159.5
Model composition	
Non-hydrogen atoms	8768
Protein residues	1129
Lipid	0
Water	0
B factors (Å^2)	
Protein	151.3
Ligand	85.6
R.m.s. deviations	
Bond lengths (Å)	0.003
Bond angles (°)	0.882
Validation	
MolProbity score	1.21
Clashscore	4.27
Rotamer outliers (%)	0.00
Ramachandran plot	
Favored (%)	98.38
Allowed (%)	1.62
Disallowed (%)	0

35 **Table S2. Effects of alanine mutations of residues in the 5-HT$_{5A}$ pocket on the 5-CT-**
36 **induced G$_i$ recruitment.** The NanoBiT assay was used to evaluate the G$_i$-coupling activity of
37 wild-type 5-HT$_{5A}$ and its mutants. Data shown are means ± S.E.M. of three independent
38 experiments, conducted in triplicate. All data were analyzed by ordinary one-way ANOVA with
39 Dunnett's test. $^*P<0.05$, $^{**}P<0.01$; $^{***}P<0.001$, *vs.* WT receptor. U.D., undetectable.

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Const.	pEC_{50} ± S.E.M.	Fold over basal (Mean ± S.E.M.)	Cell surface expression(%WT)
WT	7.331 ± 0.047	2.218 ± 0.003	100
D121A	U.D.	1.111 ± 0.012	208.25
V122A	4.952 ± 0.076***	2.426 ± 0.067	187.97
C125A	5.643 ± 0.048***	2.627 ± 0.026	78.08
V194A	6.524 ± 0.057***	2.098 ± 0.021	68.79
S204A	6.097 ± 0.072*	2.090 ± 0.013	79.05
T205A	5.790 ± 0.128***	2.401 ± 0.044	88.33
W298A	7.013 ± 0.104	1.167 ± 0.010	131.16
F301A	6.210 ± 0.104***	2.066 ± 0.019	145.33
F302A	7.066 ± 0.075	1.728 ± 0.041	139.10
E305A	7.838 ± 0.026**	2.399 ± 0.008	102.65
L324A	7.009 ± 0.124	1.537 ± 0.019	79.39
Y328A	4.614 ± 0.053***	1.070 ± 0.009	110.59

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Receptor	Mutants	pEC_{50} ± S.E.M.	Fold over basal (Mean ± S.E.M.)	Cell surface expression (%WT)
5-HT$_{5A}$	WT	7.331 ± 0.063	1.834 ± 0.003	100
	E305A	7.838 ± 0.026**	2.397 ± 0.013	102.65
	E305S	7.686 ± 0.082*	1.812 ± 0.003	114.30
5-HT$_{1A}$	WT	7.787 ± 0.082	2.190 ± 0.025	100
	A365E	7.093 ± 0.032***	2.551 ± 0.024	148.36
	A365S	7.553 ± 0.033*	2.734 ± 0.003	107.20
5-HT$_{1B}$	WT	8.160 ± 0.091	1.580 ± 0.006	100
	S334E	6.951 ± 0.177***	1.574 ± 0.036	75.20
	S334A	8.054 ± 0.014	2.550 ± 0.008	86.33
5-HT$_{1D}$	WT	7.859 ± 0.093	1.386± 0.012	100
	S321E	8.408 ± 0.032*	1.356 ± 0.005	83.39
	S321A	7.823 ± 0.131	1.319± 0.004	114.37
5-HT$_{1E}$	WT	5.660 ± 0.034	1.308 ± 0.031	100
	E311A	7.105 ± 0.180**	1.510 ± 0.026	136.07
	E311S	6.400 ± 0.086**	1.243± 0.003	102.91
5-HT$_{1F}$	WT	5.365 ± 0.056	1.863 ± 0.032	100
	E313A	U.D.	0.823 ± 0.024	94.03
	E313S	U.D.	1.031 ± 0.023	104.70

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