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Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐ A description of all covariates tested
☒	☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated
☐	☒ Clearly defined error bars *State explicitly what error bars represent (e.g. SD, SE, CI)*

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection	Latitude S (Gatan Co.); GROMACS version 5.1 (www.gromacs.org) was used for performing MD simulations.
Data analysis	1. Drift correction: MotionCor version 2.1.0.5 2. CTF estimation: GCTF version 1.06 3. 2D and 3D Reconstruction, 3D refinement, post-processing: Relion Version 2.1 4. Local resolution estimation: Resmap version 1.1.4 5. Pore profile calculation: HOLE version 3.0 6. Model visualization: PyMOL version 2.0.4 7. 3D volume visualization: Chimera version 1.11.2 8. MRC to MTZ conversion: CCP4I version 7.0.057 9. Manual model building: Coot 0.8.9.1 10. Structure refinement: Phenix version 1.13-2998 11. Find tunnels: Caver version 3.0.1 12. Find ligand binding residues: Ligplot+ version 1.4.5 13. Electrostatic potential calculation: APBS version 1.5 14. MD simulations: CHAP version 0.9 (www.channotation.org; Trick JL, Chelvaniththilan S, Klesse G, Aryal P, Wallace EJ, Tucker SJ, Sansom MSP (2016) Structure 24:2207-2216; Rao S, Klesse G, Stansfeld PJ, Tucker SJ, Sansom MSP (2017) Channels 11:347-353.), with trajectories visualized using VMD version 1.9.1 15. Various CRYO-EM data conversion: EMAN version 2.2,

16. Figure generation: CorelDraw version 20.1.0.708
17. Electrophysiology data analysis: Clampfit version 10.2
18. Electrophysiology plot generation: Origin Version b9.5.0.193

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The coordinates of the 5-HT3AR structure and the Cryo-EM map has been deposited under PDB ID: 6DG7 (State 1) and 6DG8 (State 2); EMD ID: EMD-7882 (State 1) and EMD-7883 (State 2) with the wwPDB and EMDB. All relevant data are available from the authors.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	For electrophysiological recordings, the wt and mutants were recorded from independently injected oocytes. The n values refers to each oocyte and is mentioned in the figure legend.
Data exclusions	No data exclusion
Replication	Simulation runs were repeated thrice. The replicates were initiated from separately assembled protein-membrane systems.
Randomization	N/A
Blinding	N/A

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
☒	☐ Unique biological materials
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	We used 1D4 antibody for affinity purification of 5-HT3AR protein. The antibody was produced using hybridoma cell line in CWRU core facility.
Validation	*Describe the validation of each primary antibody for the species and application, noting any validation statements on the manufacturer's website, relevant citations, antibody profiles in online databases, or data provided in the manuscript.*