Cryo-EM structure of 5-HT$_{3A}$ receptor in its resting conformation

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Serotonin receptors (5-HT$_{3A}$R) directly regulate gut movement, and drugs that inhibit 5-HT$_{3A}$R function are used to control emetic reflexes associated with gastrointestinal pathologies and cancer therapies. The 5-HT$_{3A}$R function involves a finely tuned orchestration of three domain movements that include the ligand-binding domain, the pore domain, and the intracellular domain. Here, we present the structure from the full-length 5-HT$_{3A}$R channel in the apo-state determined by single-particle cryo-electron microscopy at a nominal resolution of 4.3 Å. In this conformation, the ligand-binding domain adopts a conformation reminiscent of the unliganded state with the pore domain captured in a closed conformation. In comparison to the 5-HT$_{3A}$R crystal structure, the full-length channel in the apo-conformation adopts a more expanded conformation of all the three domains with a characteristic twist that is implicated in gating.
The ion channel class of serotonin receptors (5-HT$_3$AR) are cation selective and belong to the super family of pentameric ligand-gated ion channels (pLGICs)\textsuperscript{12}. The 5-HT$_3$AR pentameric complex is ~250 kDa in molecular weight and is made up of five homologous subunits A (5-HT$_3$A) or a heterologous combination of subunit A with other subunits (B–E) arranged around a pseudo five-fold symmetric axis\textsuperscript{3}. These channels, located in the dorsal vagal complex of the brainstem and in the gastrointestinal (GI) tract, form a circuit that controls gut motility, visceral perception, secretion, and the emesis reflex\textsuperscript{4,5}. 5-HT$_3$AR are implicated in a number of psychiatric and GI disorder conditions including anxiety, depression, bipolar disorder, and irritable bowel syndrome\textsuperscript{6,7}. Currently, serotonin receptor (5-HT$_3$R) antagonists are in clinical use to alleviate nausea and vomiting caused by chemotherapy and radiotherapy, and for the management of post-infection diarrhea and irritable bowel syndrome\textsuperscript{8,9}. However, in several cases, adverse side effects have led to restrictions in use of these drugs\textsuperscript{10}. A better understanding of the structural correlates of 5-HT$_3$AR function will therefore facilitate ongoing drug design efforts for safer therapeutics.

At the functional level, the 5-HT$_3$R gating cycle involves transitions between at least three distinct conformational states, namely: the resting state, a non-conductive conformation with low agonist-affinity; the open state, a conductive conformation with higher agonist-affinity; and the desensitized state, a non-conductive conformation, with the highest agonist-affinity among the three states. In the absence of the agonist (serotonin), the channel resides predominantly in the resting or closed conformation, while in the presence of the agonist, the channel transiently opens and eventually transitions to the desensitized conformation. Several therapeutically interesting compounds act as orthosteric or allosteric ligands and modulate 5-HT$_3$AR channel activity by shifting the equilibrium between these pre-formed functional states. At the structural level, 5-HT$_3$AR is a dynamic allosteric protein where binding of the neurotransmitter serotonin in the N-terminal extracellular domain (ECD) elicits a conformational change leading to pore opening within the transmembrane domain (TMD). In addition to these two domains, the channel has a large intracellular domain (ICD) formed by the region between the transmembrane M3 and M4 helices. Although high resolution structures of several prokaryotic and eukaryotic pLGICs are now available\textsuperscript{11–19}, the key questions regarding the conformational coupling between the ECD, TMD, and the ICD still remain unclear. The ICD is implicated to play a role in receptor trafficking and clustering at the synapse plasma membrane, gating, post-translational modification, and intracellular regulation of channel function\textsuperscript{20}. Additionally, in 5-HT$_3$AR, the relatively large ICD also modulates single-channel conductance, rectification, and desensitization kinetics\textsuperscript{21,22}. In the pLGIC structures solved so far, the ICD is intrinsically missing, or has been genetically removed or considerably digested by trypsin\textsuperscript{13–17}. In the trypsin-digested 5-HT$_3$AR crystal structure, the ICD was partially resolved as two split α-helices; a short horizontal MX helix extending from the post-M3 loop and the MA helix extending from the cytoplasmic side toward M4\textsuperscript{16}. Moreover, the 5-HT$_3$AR was crystallized in the presence of single-chain antibodies (referred to as nanobodies) that bound to the channel in the vicinity of the serotonin binding site. Functional analysis showed that in the nanobody-bound form, the channel is not activated by serotonin, and hence the channel conformation likely corresponds to that of an inhibited, non-conducting channel\textsuperscript{16}. Although the inhibited state (at least in the case of competitive antagonism) could be expected to resemble the resting (closed) conformation, experimental evidences from voltage-clamp fluorometry, X-ray crystallography, and theoretical predictions in pLGIC homologs suggest that the inhibitors elicit conformational changes of their own even though the channel is electrically silent\textsuperscript{23–25}. Interestingly, the antagonist-induced structural changes at some positions are similar while others distinct to those evoked by the agonist suggesting that the antagonist stabilizes the receptor in a different non-conductive conformation\textsuperscript{23,25}. Thus, to determine the basic tenets of the resting conformation, we sought to determine the structure of the full-length 5-HT$_3$AR channel in the apo-conformation by single-particle cryo-electron microscopy (cryo-EM). The cryo-EM structure of apo-5-HT$_3$AR at 4.3 Å resolution is similar to the 5-HT$_3$R crystal structure but is captured in a distinct state with differences in the ECD, TMD, and the ICD conformations.

**Results**

**Function and the cryo-EM structure of 5-HT$_3$AR.** The full-length mouse 5-HT$_3$R was cloned into an oocyte expression vector for functional measurements. In response to application of serotonin, robust concentration-dependent inward macroscopic inward currents were elicited that desensitize in the continued presence of the activating ligand (Supplementary Fig. 1A). A plot of peak response as a function of serotonin concentration yielded an EC$_{50}$ of 2.7 ± 0.09 μM which is in agreement with the previous studies\textsuperscript{28} (Supplementary Fig. 1A). For structural studies, the full-length 5-HT$_3$AR gene was codon-optimized, cloned into pFast-Bac1 plasmid, and expressed in Spodoptera frugiperda (Sf9) cells. Milligram quantities of 5-HT$_3$R were purified to homogeneity. The purified protein was deglycosylated with PNGase treatment and gel-filtration fractions corresponding to pentamers (Supplementary Fig. 1B) were used for single-particle cryo-EM analysis (Supplementary Fig. 1C). Three dimensional reconstructions of the detergent-solubilized, full-length 5-HT$_3$AR channel led to a density map with a nominal resolution of 4.3 Å with imposed Cs symmetry (Supplementary Figs. 1D, 2A, and 2B). The final reconstruction was built from 108,277 particles (Supplementary Fig. 3) and had a local resolution in most regions between 3.5 and 4.5 Å (Supplementary Fig. 1D). The map contained density for the entire ECD, TMD, and a large region of the ICD, and was used for model building and refinement (Supplementary Fig. 4). The final refined apo-5-HT$_3$R model contains residues Asp6-Gln338 and L397-Ser461 (Table 1).

When viewed from the plane of the bilayer, perpendicular to the pore axis, the apo-5-HT$_3$R pentamer is 165 Å in length and has a diameter of 80 Å (Fig. 1a, b). The ECD reveals protruding non-protein densities corresponding to N-glycans. The mouse 5-HT$_3$AR has three N-glycosylation sites at positions Asn82, Asn148, and Asn164, and strong densities for glycans were observed at each of the asparagine sidechains. Site-directed mutagenesis shows that prevention of glycosylation at sites Asn82, Asn148, and Asn164, and strong densities for glycans were observed at each of the asparagine sidechains. Site-directed mutagenesis shows that prevention of glycosylation at sites Asn82, Asn148, and Asn164 abolishes the expression of functional receptors on the membranes\textsuperscript{29}. Densities of the glycans were also resolved in several previous pLGIC structures including 5-HT$_3$AR, GABA-β3, nAChR, GlyR, and GluCl\textsuperscript{13,16,17,30}. The overall topology of apo-5-HT$_3$AR is as previously observed in other members of the pLGIC family with each subunit comprised of a twisted β-sandwich ECD and a four-helical TMD. In addition, in apo-5-HT$_3$AR, the density for the ICD is only partially resolved with post-M3 loop, MX, and MA helices visible.

We then compared the apo-5-HT$_3$R conformation with that of 5-HT$_3$AR crystal structure\textsuperscript{16}. An alignment of the two structures reveals an expansion of all the three domains in apo-5-HT$_3$AR resulting from a radial outward twist of the ECD outer β-sheet and an iris-like movement of the TM helices and the ICD (Fig. 2a). The ECD undergoes a clockwise twist with respect to the
crystal structure, which changes the relative positions of the interfacial loops at the ECD–ECD and ECD–TMD interfaces (Fig. 2b). Particularly, the movement in the outer β-strands is more pronounced than the inner β-strands. In the TMD, the M3 and M4 helices show an outward displacement with respect to an axis through the intrasubunit helical bundle composed of M1–M4 helices, while there is very little change in the position of the M2 helix (Fig. 2b). Changes in M3 and M4 conformations relative to M2 lead to a widening of the intrasubunit cavity lined by M1–M4 helices (indicated by dotted lines, Fig. 2b). These structural differences are also reflected as an increase in total surface area (protein-only region) of the apo-5-HT3AR pentamer (102,850 Å²) in comparison to the crystal structure (85,000 Å²). The increase in total surface area is accompanied by an increase in solvent accessible areas for apo-5-HT3AR pentamer (73,210 Å²) in comparison to the crystal structure (54,530 Å²). The analysis of accessible areas for apo-5-HT3AR pentamer (73,210 Å²) in total surface area is accompanied by an increase in solvent inter-subunit interfaces reveals that the buried areas between crystal structure, which changes the relative positions of the pLGIC putative closed conformations such that it lies further along the pathway that defines the global motion going from closed to open structures. In apo-5-HT3AR, the ECD twist is clockwise with respect to the crystal structure, and in the direction toward the closed conformation.

The ion permeation pathway and the TMD. Viewing from the extracellular end (Fig. 3a, b), the ion permeation pathway in apo-5-HT3AR is wide open with a radius greater than 3.5 Å throughout the ECD followed by a narrower pathway in the TMD and the ICD with multiple constriction sites (Supplementary Fig. 5). A slight constriction is seen at the center of the ECD lined by Lys108 in the β4–β5 loop, although the extent of constriction is less than that seen in the crystal structure (Fig. 3b). A negative charge in the vicinity of Lys108 (at position Asp105) has been shown to regulate single-channel conductance in pLGICs. The Asp105 position is conserved among most cation-selective pLGICs, whereas this position is a lysine in anion-selective members of the family.

The pore of apo-5-HT3AR is lined by M2 which appear as straight helices lying parallel to the pore axis (Fig. 3c). At the mouth of the extracellular end, the pore is lined by Asp20′, followed by three rings of hydrophobic residues at Ile17′, Val13′, and Leu9′. Below Leu9′, the pore is polar lined by Thr6′, Ser2′, and negatively charged at the intracellular end with Glu-1′. The Asp-4′ is positioned further away from the pore axis. The radius of the pore at Asp20′ is 3.8 Å and decreases to 2.1 Å at the level of Leu9′ position. Strong density (7–6 σ) and weaker density (5–4 σ) are present within the permeation pathway in the TMD and the ICD (Fig. 3c). The weaker densities are arranged as two sets of five-membered rings: one as a loose arrangement, in-plane with Asp20′ sidechain with a mean distance of 3.94 Å and the other as a tight pentagon ring beneath it with a mean distance of 2.4 Å. These values are close to those estimated for water polygons in high-resolution protein crystals. Based on these distances, the pore radii at this location (>hydrated Na⁺ ion radius), and the B-factors for the modeled ions and waters (Table 1), we suggest that these densities likely correspond to ions and water. A similar arrangement of ions and water pentagons was also observed in the intracellular end of M2 in the GLIC open structure. Further down, the pore at Leu9′ is constricted to ~2.3 Å which is too small to allow a hydrated Na⁺ to pass through and in addition the hydrophobicity of this region prevents ion dehydration. There are two additional constrictions below the Leu9′ position, at Thr6′ and Ser2′ with a radius of 2.7 Å and 2.1 Å, respectively. Although the pore radii at these locations are smaller than a hydrated Na⁺, the hydroxyl sidechain at these positions may allow coordination of partially dehydrated ions. The intracellular end is lined by two rings of negative charges at positions Glu-1′ and Asp-4′ which are involved in governing charge selectivity and constitute the selectivity filter. This suggests that the channel in this conformation is non-conducting and the Leu9′ position acts as a gate impeding ion permeation. This is not surprising considering that the channel was imaged in the absence of any activating ligands. The finding that the activation gate is located at the center of M2 is also in agreement with SCAM studies in 5-HT3AR which showed that extracellularly positioned Cys in M2, particularly the 17′ position, were accessible even in the closed state. In contrast, residues below the 13′ are accessible in the open but not in the closed state. In the strychnine-bound GlyR structure, the channel is constricted primarily at the extracellular end with constrictions to below 3 Å radii at 20′, 13′, and 9′ positions. The “closed” GLIC conformation is also much narrower in the extracellular end with positions 16′ and 17′ constricted to 2 Å. On the other hand, the 5-HT3AR structures are similar to the apo-GluCl structure with a relatively wider pore at the external end (Supplementary Fig. 6).
At the extracellular end, M2 extends into the M2–M3 linker, which in apo-5-HT₃AR is bent and tilted upward (above the level of the cys loop), such that it is in close proximity with the pre-M1 region as well as the β₈–β₉ loop of the neighboring subunit (Supplementary Fig. 7A). Residue Ala277 in the M2–M3 linker makes a H-bond contact with Arg219 in the pre-M1 region, while Thr276 interacts with the backbone carbonyl of Gly185 in the β₈–β₉ loop. The M2–M3 linker was only partially resolved in the 5-HT₃AR crystal structure. The M2–M3 linker is seen in multiple orientations in different pLGIC structures with this region adopting a distinct extended conformation in the apo-5-HT₃AR (Supplementary Fig. 7B). The M2–M3 linker in the agonist-bound GlyR structure is retracted from the inter-subunit interface in comparison to the antagonist-bound structure. The M2–M3 linker and the pre-M1 region are implicated to be a part of the signal transduction machinery that communicates ligand-binding events in the ECD to the channel gate in the M2 bundle. Consequently, mutations in these regions are shown to impact channel function.

The M4 segment has a well-defined density and the tip of M4, which in apo-5-HT₃AR is bent and tilted upward (above the level of the cys loop), is α-helical extending above the putative membrane limits (Supplementary Fig. 8). The tip of M4 is in close proximity to the β₆–β₇ (Cys) loop with Trp459 in M4 oriented toward the conserved Phe144 in the Cys loop in a potential stacking interaction. The tip of M4 was not well-resolved in the 5-HT₃AR crystal structure and Trp459 was built only in two subunits. Of the two, one of the Trp sidechains was in a similar stacking orientation. In nAChR, the interaction between the post-M4 and cys loop is predicted to modulate gating.

Conformation of the ECD. The neurotransmitter binding site has been extensively studied and a wealth of structural data is available from the acetylcholine binding protein (AChBP) and from the 5-HT₃A-AChBP chimera bound to a battery of pLGIC agonists and antagonists. The serotonin binding site is located at the subunit interface lined by residues from Loops A, B, and C on the (+) subunit and Loops D, E, and F from the (−) subunits. There are clear densities for aromatic residues from each of these loops lining the binding pocket, particularly Trp156-Loop B, Phe199 and Tyr207-Loop C, Trp63-Loop D, Tyr126-Loop E. Densities for Trp168, Arg169, and D177 in Loop F are also shown and are implicated in ligand binding. In comparison to the crystal structure, the β-sheets forming the ECD undergo a clockwise twist (viewed from the extracellular end) leading to a widening of the ligand-binding pocket with rearrangement of the binding site residues. A partly "closed" conformation of Loop C in the crystal structure is likely from its interaction with the nanobody bound in the vicinity. The orientation of Trp156 in Loop B, lining the back wall of the neurotransmitter binding pocket, in apo-5-HT₃AR is flipped relative to its position in the agonist-bound state.
5-HT3AR crystal structure. This position is highly conserved as a Trp in 5-HT3AR α-subunits of nAChRs, and AChBP, while it is a Tyr in the anionic members of pLGIC family. The indole ring of Trp is involved in a cation-π interaction with the quaternary ammonium ligands binding to the neurotransmitter binding pocket. An alignment with AChBP and nAChR shows that the Trp156 orientation in apo-5-HT3AR is similar to the conformation seen in these structures (Supplementary Fig. 9). Further studies are necessary to understand the potential role of Trp156 re-orientation in channel 5-HT3AR inhibition.

Conformation of the ICD. The ICD sequence is the least conserved region among the pLGIC family, and in human isoforms the length is always greater than 70 residues and varies up to 150 residues. In apo-5-HT3AR, the post-M3 region forms a loop that extends away from the pore axis and terminates in an α-helix referred to as MX helix. MX is a short helix which forms a belt around the MA helical bundle, lying parallel to the plane of the membrane, and is seen to bend downward toward the cytoplasmic region (Fig. 5a). Although we see densities for the entire MX and MA helices and for a short region of the unstructured portion from MX, the density for a stretch of 59 residues is missing until beginning of the MA helix, presumably due to the unstructured and flexible nature of this loop. Not surprisingly, this is also the region that is chipped away during trypsin digestion. The apo-5-HT3AR structure suggests the presence of
**Fig. 3** Profile of ion permeation pathway. **a** The pore profile generated by the HOLE program depicts an ion permeation pathway of ~165 Å encompassing the ECD, TMD, and the ICD. Only two subunits are shown for clarity. Sidechains of residues that line the constricted areas are shown as sticks. **b** A comparison of the pore radii along the pore axis for the 5-HT₃AR cryo-EM structure (salmon red) with that of the crystal structure (pale green). The dashed line indicates an approximate radius of a hydrated Na⁺ ion. The pore is constricted below 3 Å radius at three sites: L260 and E250 along M2 and R416 in the ICD. **c** Non-protein densities along the pore axis were modeled as water (red), Na⁺ (magenta), and Cl⁻ (green). The map around the ions is shown as a mesh representation calculated at various σ values (outer water ring: 4 σ; inner water ring: 5 σ; Na⁺ ion: 6 σ; Cl⁻ ion: 7 σ).

**Fig. 4** The neurotransmitter binding site. **a** The map around the aromatic residues at the subunit interface that constitutes the neurotransmitter binding site (top). The map for the residues in Loop F that are involved in ligand binding (bottom). **b** Alignment of 5-HT₃AR apo (salmon red) and crystal (pale green) structures reveals a twist and an expansion at the region lined Loop C, Loop B, and Loop F. The arrows indicate the direction of movement. **c** A comparison of the orientations of the residues that are involved in neurotransmitter binding.
multiple intrasubunit interactions between MX, unstructured loop, and MA. The post-M3 loop is held in its position through potential polar interactions with M3 (Arg306) and M4 helix (Trp426) within the same subunit (Fig. 5b). Although in comparison to the 5-HT3AR crystal structure the post-M3 loop is positioned away from the pore axis (with minimal interaction with the neighboring subunit) (Fig. 5a), it still partially blocks the inter-subunit cavity or lateral portals which have been implicated to serve as conduits for ion permeation.

The ICD reveals a notable constriction to 2.1 Å at the level of Arg416 with a strong density (σ = +15 kT e⁻¹) at the center of the pore axis, which we modeled as a Cl⁻ (Fig. 3c and Supplementary Fig. 5). Interestingly, previous studies have suggested that intracellular phosphates interact with MA arginine residues near the cytoplasmic mouth of the channel facilitating pore block and inhibition of ion conduction. Besides the constriction at Arg416, the MA helix offers a continuous conduit of radius over 3 Å. Serotonin-mediated activation, therefore, likely evokes a conformational change within the ICD to allow permeation. 5-HT3AR has an unusually low conductance which is well below the charged cluster further up.

The electrostatic potential map along the ion permeation pathway shows that in contrast to the ECD and the TMD, where the surface charge is electronegative (lined by Asp52, Glu53 (β1-β2 loop), and Glu186 (β8-β9 loop) in the ECD and Glu-2' and Asp-4' (M2) in the TMD), the ICD has a cluster of positively charged residues (highlighted by the blue region in Fig. 5c). This is a unique feature of 5-HT3AR among the cation-selective pLGICs. At the edge of the TMD–ICD interface, there is a density present in the cavity lined by M3 of one subunit and M1 and M4 helices of the neighboring subunit. This density was partially modeled as a phospholipid based on putative polar contacts with neighboring Arg sidechains (Arg306 in M3 and Arg435 in M4). Interestingly, a recently solved structure of the GABA (β3–α5) chimeric channel reveals a neurosteroid binding site that aligns with the lipid site suggesting that this lipid pocket may be involved in allosteric modulation of pLGICs.

Discussion

The cryo-EM structure of apo-5-HT3AR determined in the solution state, in the absence of inhibitors, Fab fragments, truncation, or constraints from crystal lattice, bears the hallmarks of the resting conformation. In comparison to the 5-HT3AR crystal structure, the full-length channel adopts an expanded

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Methods

induced conformational changes leading to open and desensitized structure serves as a starting conformation to determine ligand–mechanisms underlying channel modulation. The apo-5-HT3AR structure serves as a starting conformation to determine ligand-induced conformational changes leading to open and desensitized states.

Methods

Cloning and transfection. Codon-optimized mouse 5-HT3AR gene (NCBI Reference Sequence: NM_001099644.1) was purchased from GenScript USA Inc. Subsequently, it was subcloned into pFastbac1 vector containing four Strep-tags (Anatrace) in a buffer containing 500 mM NaCl, 50 mM Tris pH 7.4, 10% glycerol, and 0.5% protease inhibitor by rotating for 2 h at 4 °C. Non-solubilized material (Anatrace) in a buffer containing 500 mM NaCl, 50 mM Tris pH 7.4, 10% glycerol, and 0.01% C12E9 (Buffer A). The protein was then eluted with Buffer A supplemented with 3 mg/ml 1D4 peptide (NH2-TETSQVAPA-CO2H). Eluted protein was then concentrated and deglycosylated with PNGase F (NEB) by incubating 5 units of the enzyme per 1 µg of the protein for 2 h at 37 °C under gentle agitation. Eluted protein was then applied to a Superose 6 column (GE healthcare) equilibrated with Buffer A. The peak fractions around 13.9 ml were pooled and concentrated to 2–3 mg/ml using 50 kDa MWCO Millipore filters (Amicon) and used subsequently for cryo-EM studies.

Sample preparation and cryo-EM data acquisition. The 5-HT3AR protein (−2.5 mg/ml) was filtered and incubated with 3 mM Fluorinated Fos-choline 8 (Ana- trace) to improve particle distribution48. The sample was double blotted (3.5 µl per blot) onto Cu 300 mesh Quantifoil 1.2/1.3 grids (Quantifoil Micro Tools), and immediately after the second blot, the grid was plunge frozen using a Vitrobot (FEI). The grids were imaged on a Titan Krios microscope (FEI), operating at 300 kV, and equipped with a K2 Summit direct detector camera (Gatan). 40-frame movies were collected at 130,000× magnification (set on microscope) in super-resolution mode with a physical pixel size of 0.532 Å/pixel. The dose rate was 4 electrons/pixel/second, with a total exposure time of 12 s. The defocus values ranged from ~0.75 µm to ~2.5 µm (input range setting for data collection) as per the automated imaging software42.

Image processing. Movies were motion-corrected to compensate for the beam-induced motion using MotionCor259 with a B-factor of 150 pixels6. Super-resolution counting images were 2 × 2 binned in Fourier space with a pixel size of 1.064 Å. All subsequent data processing was performed using RELION 2.037. The density map of the motion-corrected micrographs was estimated using Gnome software62. Approximately, 2000 particles were manually picked from the 3550 micrographs and sorted into two-dimensional (2D) classes. The best of these classes were then used as templates for auto-picking. A loose auto-picking threshold was selected to ensure no good particles were missed. This resulted in ~327,000 auto-picked particles that were subjected to multiple rounds of 2D classification to remove suboptimal particles. An initial three-dimensional (3D) model was generated from 5-HT3AR crystal structure (PDB code: 4PR) and low-pass filtered to 60 Å using EMAN260. The best ~117,000 particles were then subjected to 3D auto-refinement, followed by 3D classification into three classes. The best 3D class, containing ~108,000 particles, was subjected to a final round of 3D auto-refinement and post-processing to yield a 5-HT3AR map at an overall resolution of 4.3 Å (calculated based on the gold-standard Fourier shell coefficient (FSC) = 0.143 criterion). In the RELION post-processing step, a soft mask was applied to the two half-maps before the FSC was calculated. The post-processing step also included B-factor estimation and map sharpening. ResMap software55 was used for estimation of local resolutions.

5-HT3AR model building. The 5-HT3AR crystal structure (PDB-ID: 4PR)66 was used as an initial model and aligned to the 5-HT3AR cryo-EM map calculated with RELION 2.03. The cryo-EM map was converted to an .mrc format using the mrcformat tool from the PHENIX software67. The mtz map was then used for model building in COOT68. In comparison to the 5-HT3AR crystal structure, all ligands, ions, water molecules, and nanobodies (in the crystal structure) were removed from the PDB files. Additional residues in our model and sidechains were built for seven additional residues. After initial model building, the model was refined against the EM-derived maps using the phenix.real_space_refinement tool from the PHENIX software package67, employing rigid body, local grid, NCS, and gradient minimization. This model was then subjected to additional rounds of manual model-fitting and refinement which resulted in a final model-to-map cross-correlation coefficient of 0.774. Stereochemical properties of the model were evaluated by Molprobity69.

Protein surface area and interfaces were analyzed by using PDB2PISA server (http://www.ebi.ac.uk/pdbj/cgi/pisa/). To compare apo-5-HT3AR and the 5-HT3AR crystal structure, all ligands, ions, water molecules, and nanobodies (in the crystal structure) were removed from the PDB files. Additional residues in the apo-5- HT3AR structure were also removed before analysis so that surface area comparisons were made between identical construct lengths. Electrostatic surface potential calculations were carried out using the APBS tools plug-in PyMOL70. The pore profile was calculated using the HOLE program71.

Data availability. The coordinates of the 5-HT3AR structure and the cryo-EM map have been deposited under PDB ID 6BE1 and EMD-7088 with the wwPDB and EMDDB. Data supporting the findings of this manuscript are available from the corresponding author upon reasonable request.

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**Author contribution**

S.B. and S.C. conceived the project and designed the experimental procedures. S.B. purified the protein and carried out all aspects of cryo-EM sample preparation, screening, data analysis, model building, and refinement. Y.G. performed the two-electrode voltage-clamp recordings. A.S. and V.Y.M.-B. trained S.B. in freezing samples and provided guidance to optimize grid preparation. S.M. trained S.B. and S.C. in the use of Tecnai TF20 microscope for screening grids. M.F. and M.N. trained S.B. in insect cell expression. W.H., T.H., D.J.T., and V.Y.M.-B. provided guidance at various stages of cryo-EM analysis. S.C. supervised the execution of the experiments, data analysis, and interpretation. S.B. and S.C. drafted the manuscript. All authors reviewed the final manuscript.

**Additional information**

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