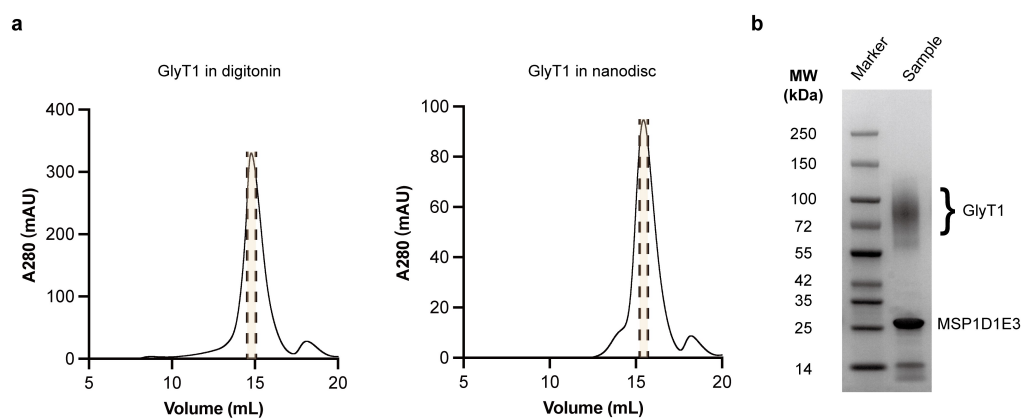


Supplementary Information for

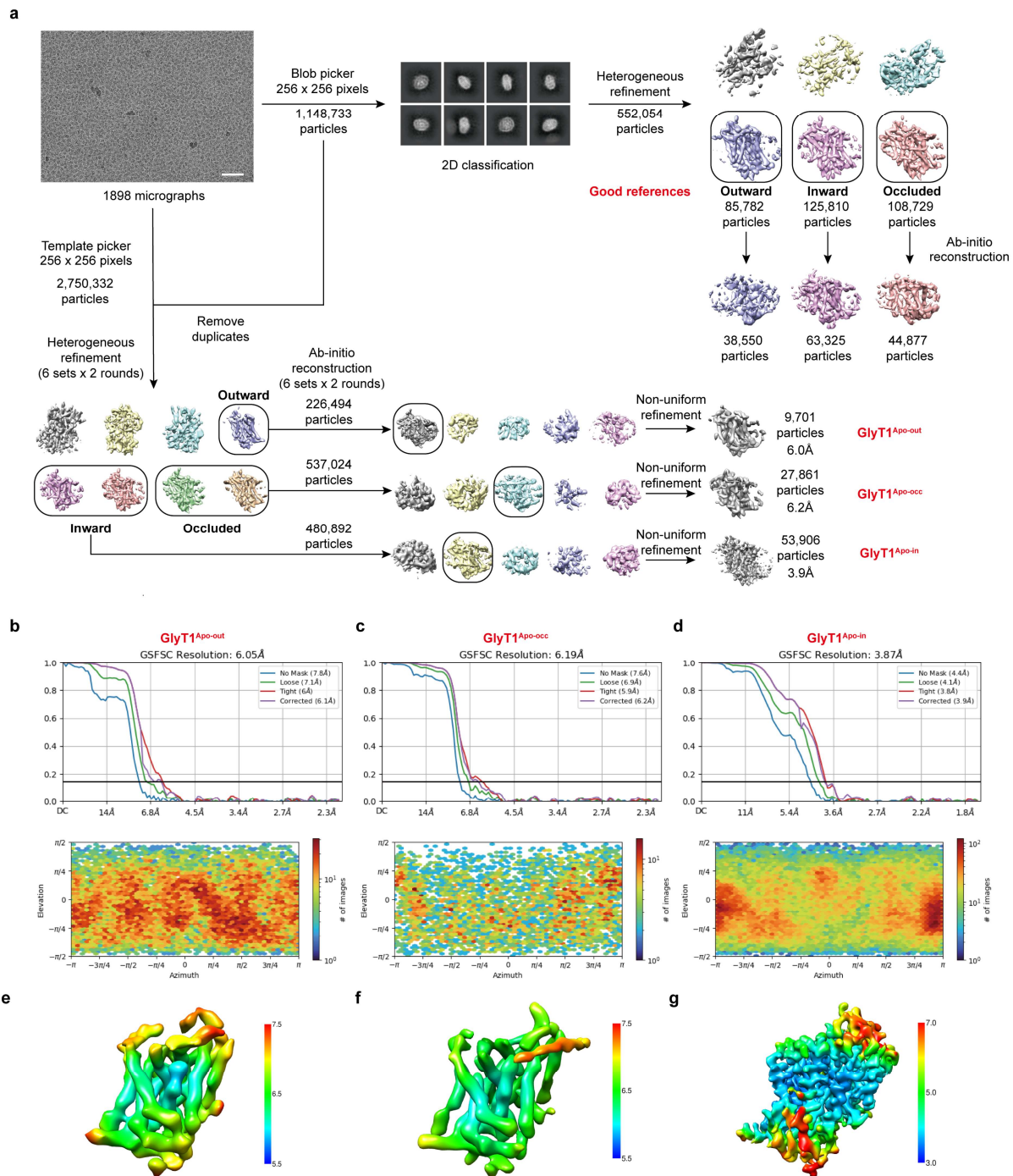
Modulation of the human GlyT1 by clinical drugs and cholesterol

This document contains Supplementary Figure 1–9 and Supplementary Table 1-2.



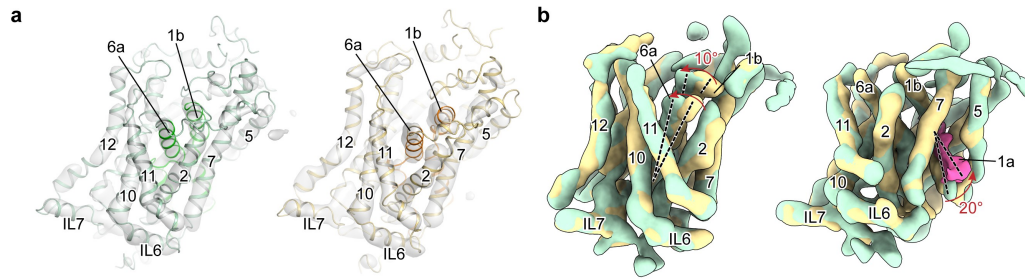
Supplementary Figure 1. Purification and biochemical characterization of GlyT1.

a, SEC purification of GlyT1 in the digitonin or reconstituted nanodisc. Peak fractions used for cryo-EM sample preparation are highlighted. **b**, SDS-PAGE of sample utilized for cryo-EM grids preparation, visualized by Coomassie blue staining.



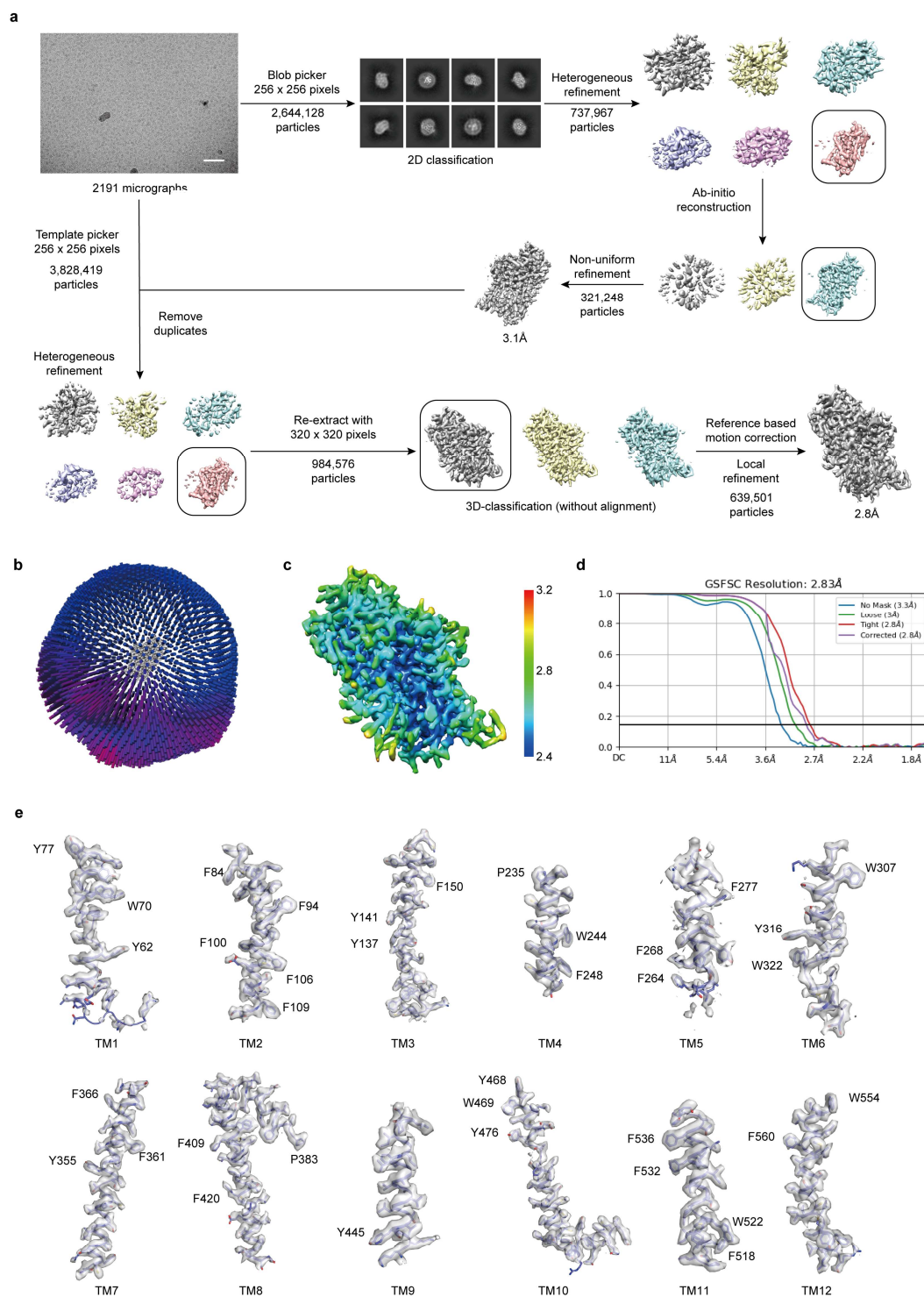
Supplementary Figure 2. Cryo-EM data processing of GlyT1 in the apo state.

a, Representative data processing workflow for the GlyT1^{Apo} dataset (scale bar = 50nm). **b–d**, Final resolutions are reported at 6.0 Å, 6.2 Å, and 3.9 Å for GlyT1^{Apo-out}, GlyT1^{Apo-occ} and GlyT1^{Apo-in}, respectively, according to the gold-standard Fourier shell correlation (GSFSC) criterion. Angular distribution for each reconstruction is shown below. **e–g**, Local resolution distributions of GlyT1^{Apo-out} (e), GlyT1^{Apo-occ} (f), and GlyT1^{Apo-in} (g).



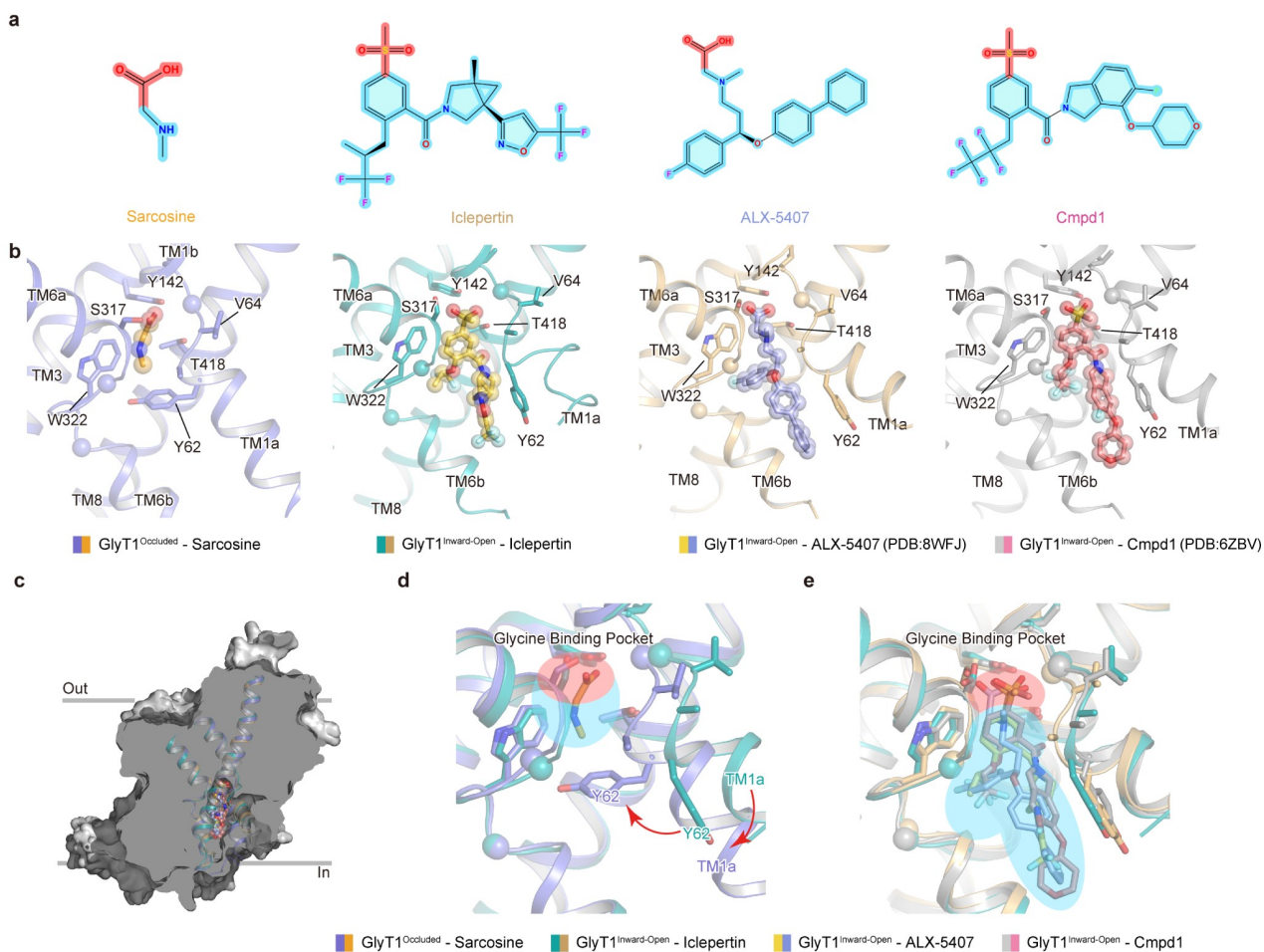
Supplementary Figure 3. Apo GlyT1 in the occluded state and outward-facing state.

a, Representative cryo-EM density maps and models for the GlyT1 in the occluded state (GlyT1^{Apo-occ}, left, colored green) and outward-facing state (GlyT1^{Apo-out}, right, colored yellow). Helices are shown as cartoon and labelled. **b**, Superposition of the cryo-EM density maps of the GlyT1^{Apo-occ} (green) and GlyT1^{Apo-out} (yellow). The movements of TM1b and TM6a from outward-facing state to the occluded state are indicated with red arrows and represented by the angle (about 10 degrees) between the dashed lines (left). The movement of TM1a from outward-facing state (or occluded state) to the inward-facing state (colored pink) are indicated with red arrows and represented by the angle (about 20 degrees) between the dashed lines (right).



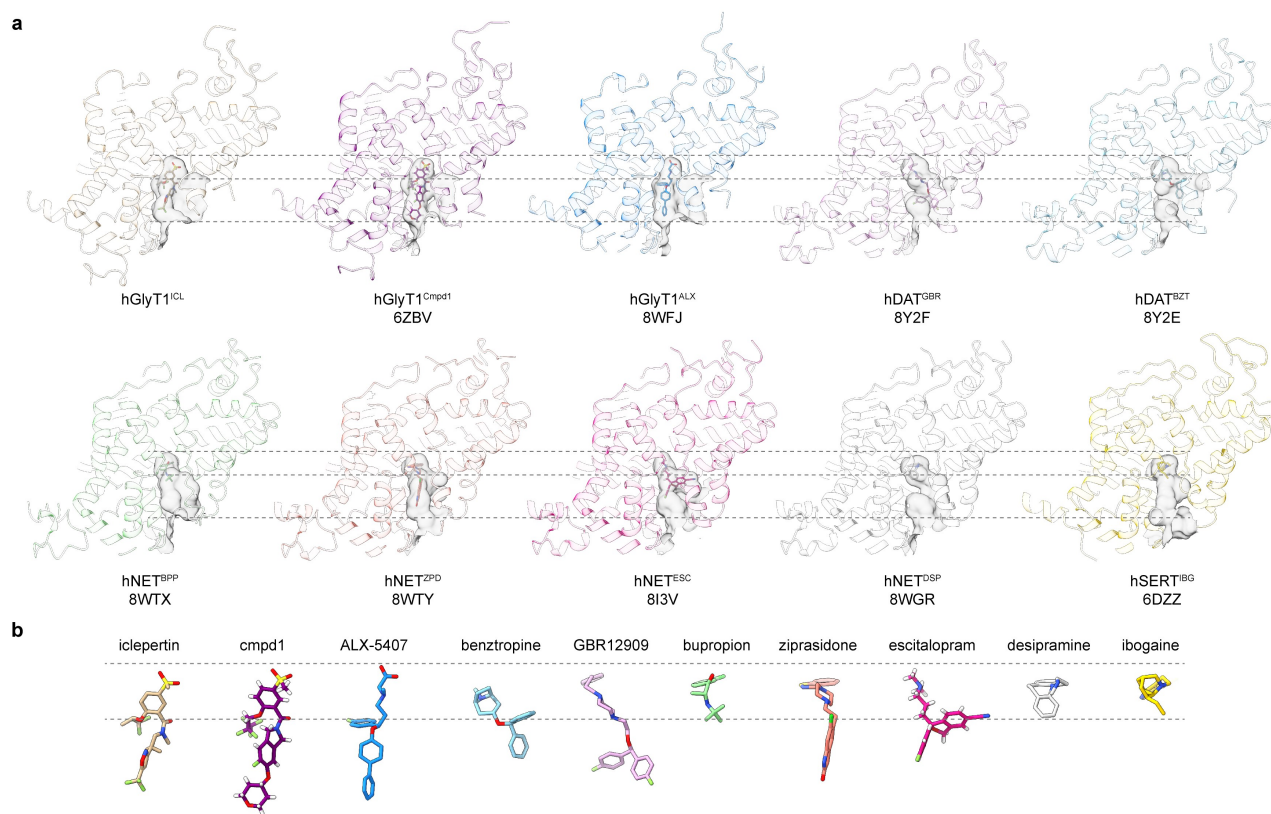
Supplementary Figure 4. Cryo-EM data processing of GlyT1^{Sar}.

a, Representative data processing workflow for the GlyT1^{Sar} dataset (scale bar = 50nm). **b**, Angular distribution is calculated and shown. **c**, Final cryo-EM density map is colored by local resolution. **d**, Final resolution is reported at 2.8 Å according to the gold-standard Fourier shell correlation (GSFSC) criterion. **e**, Cryo-EM density maps and atomic models of TM1–TM12 of GlyT1^{Sar}.



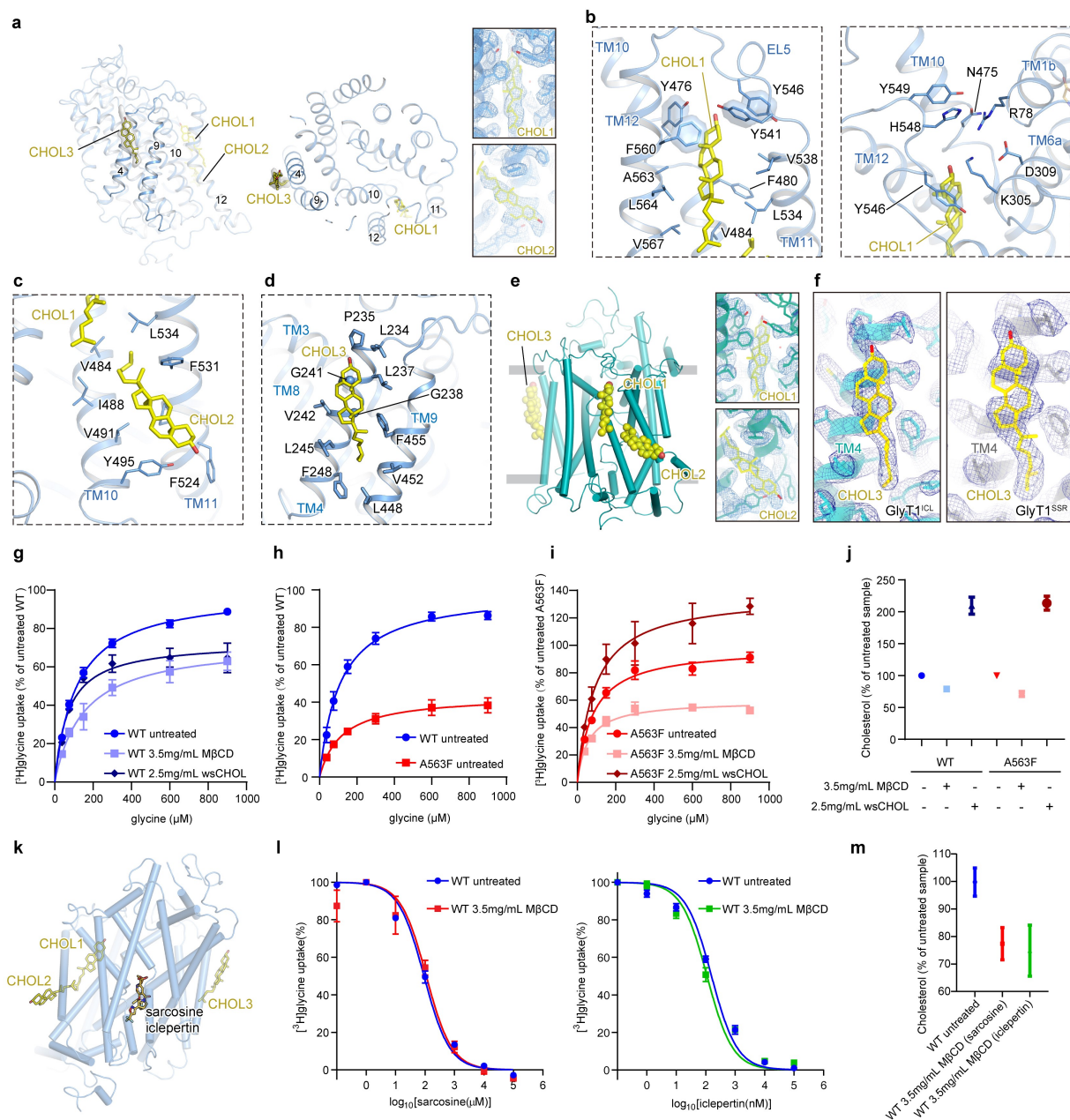
Supplementary Figure 6. Comparison of the binding pocket of GlyT1 in the occluded state and inward-facing state.

a, Molecular structures of sarcosine, iclepertin, ALX-5407, and Cmpd1. The electron-rich group of each compound is highlighted in red, and the rest portion is highlighted in blue. **b**, The binding pockets of sarcosine (colored blue and orange), iclepertin (colored cyan and wheat), ALX-5407 (colored light yellow and light blue, PDB: 8WFJ) and Cmpd1 (colored grey and pink, PDB: 6ZBV) in GlyT1. Residues involved in the interactions are shown as sticks and labelled. Sarcosine, iclepertin, ALX-5407 and Cmpd1 are shown as sticks and spheres. Sodium and chloride ions are shown as purple and green spheres, respectively. **c**, Superposition of the binding pockets of sarcosine, iclepertin, ALX-5407 and Cmpd1 in their cut-open views. Overall structures are shown as grey surfaces. TM1, TM6, and TM7 are shown as cartoon. Sarcosine, iclepertin, ALX-5407 and Cmpd1 are shown as spheres. **d**, Conformational changes between the GlyT1 in the occluded state and the GlyT1 in the inward-facing state. The movements of TM1a and residue Y62 are indicated with red arrows. The binding pocket of glycine is highlighted in red. **e**, Superposition of the binding pockets of iclepertin, ALX-5407 and Cmpd1. The binding pocket of glycine and its electron-rich group is highlighted in red. The rest portion of each compound is highlighted in blue.



Supplementary Figure 7. Structural comparison of the binding pocket of neurotransmitter transporter bound to inhibitor in the inward-facing state.

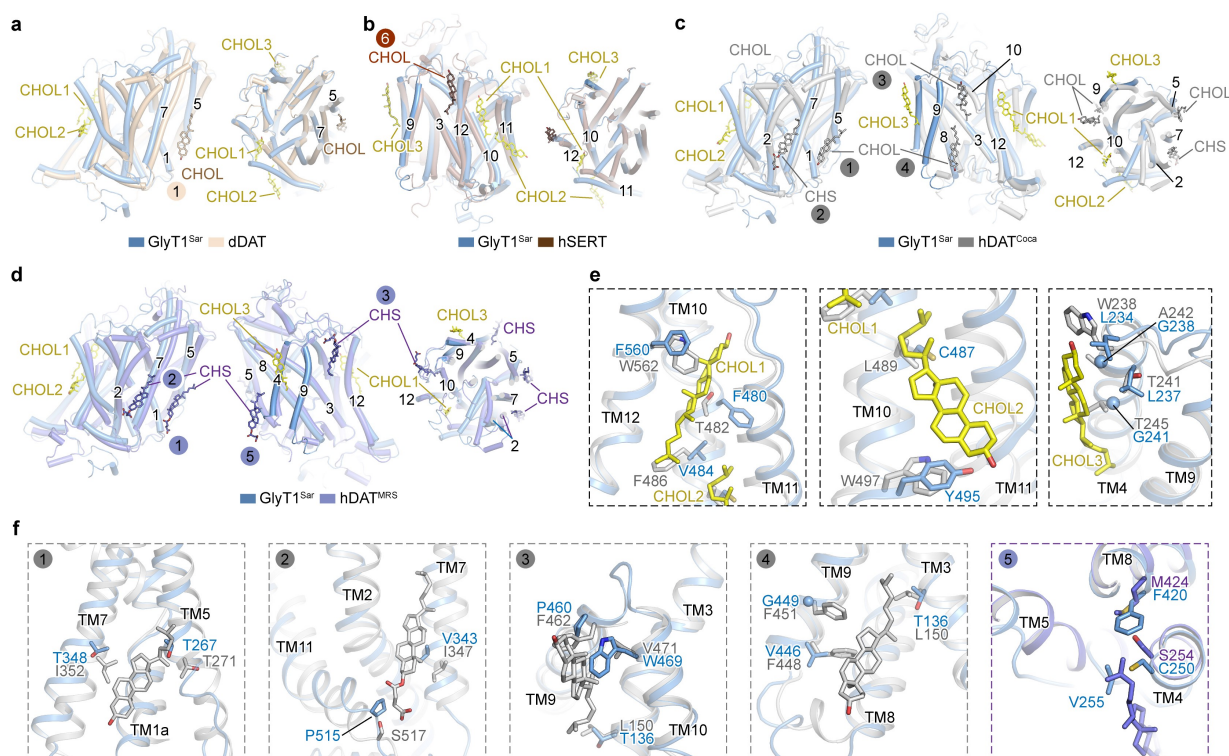
a, Overall structure of neurotransmitter transporters bound to inhibitors in the inward-facing state. Models are shown as cartoon representations and the inhibitors are shown as sticks. The regions of binding pockets are shown as gray surfaces. **b**, Molecular structures of the inhibitors are aligned on the same level depicted with dashed lines.



Supplementary Figure 8. Allosteric binding sites of cholesterol in the GlyT1^{Sar} and GlyT1^{ICL}.

a, Side view and top-down views of cholesterol binding sites (CHOL), namely CHOL1, CHOL2, and CHOL3 in the GlyT1. CHOL3 is shown as yellow sticks with grey surface. CHOL1 and CHOL2 are shown as yellow sticks with blue meshes on the right panels. **b–d**, Interactions between GlyT1 and CHOL1 (**b**), CHOL2 (**c**), and CHOL3 (**d**). GlyT1^{Sar} and cholesterol molecules are colored light blue and yellow, respectively. Residues participating in the interactions are shown as sticks and labelled. **e**, Cholesterol binding sites in the GlyT1^{ICL}. GlyT1^{ICL} is shown as cylinder and cholesterol molecules are shown as yellow spheres. **f**, Cryo-EM densities of CHOL3 in the GlyT1^{ICL} and GlyT1^{SSR}. Cholesterol molecules are shown as sticks and embedded in blue meshes. **g and i**, The Michaelis-Menten kinetics of WT GlyT1 and cholesterol-binding site 1 mutant A563F treated with 3.5 mg/mL M β CD or 2.5 mg/mL water-soluble cholesterol (wsCHOL). Compared to untreated cells, there is a significant difference in V_{max} values ($P < 0.05$).

0.0001 for M β CD treated WT and wsCHOL treated WT, $P < 0.0001$ for M β CD treated A563F, and $P = 0.0047$ for wsCHOL treated A563F, respectively), while there is no significant difference in K_m values ($P = 0.1887$ for M β CD treated WT, $P = 0.0721$ for wsCHOL treated WT, $P = 0.4722$ for M β CD treated A563F, and $P = 0.6507$ for wsCHOL treated A563F, respectively). **h**, Plots of [^{3} H]glycine uptake by wild-type GlyT1 (blue) and cholesterol-binding site 1 mutant A563F (red). There is a statistically significant difference in V_{max} value between WT and A563F ($P = 0.0045$), while there is no significant difference in K_m values ($P = 0.6578$). **j**, Cell surface cholesterol analysis post-M β CD or wsCHOL treatment. The concentration of cholesterol was normalized to that of the untreated sample. Treated and untreated groups of WT GlyT1 or A563F mutant are represented by specific symbols and lines defined in the panel. **k**, Relative position of the cholesterol binding sites with respect to the sarcosine and iclepterin binding pockets. Overall structure of GlyT1 is shown as blue cylinders. The iclepterin, sarcosine, and cholesterol are shown as sticks and colored wheat, orange, and yellow, respectively. **l**, Effect of cholesterol depletion on the concentration-inhibition curves of iclepterin (left) and sarcosine (right) of GlyT1 in HEK-293F cells. The IC_{50} values of iclepterin and sarcosine are 161.8 ± 19.6 nM and 96.81 ± 13.67 μ M in wild type (WT) GlyT1, and are 106.9 ± 15.6 nM and 113.4 ± 32.8 μ M in GlyT1 under the M β CD treatment, respectively. There is a statistically significant difference in IC_{50} values and the resulting P-values are 0.1576 and 0.5887, respectively. The [^{3} H]glycine uptake has been normalized to the uptake of cells without the treatment of M β CD. **m**, Determination of cell surface cholesterol content after 3.5 mg/mL M β CD treatment. The concentration of cholesterol was normalized to that of the untreated cells expressing WT GlyT1. Data represent as mean $\pm$ S.E.M. ($n = 3$). P-Values are calculated by unpaired two-tailed t-test with a significance level of 0.05.



Supplementary Figure 9. Allosteric binding sites of cholesterol in GlyT1, dDAT, hSERT, and hDAT.

a, Superposition of GlyT1^{Sar} and dDAT (PDB: 4M48). Cholesterol molecules in GlyT1 and dDAT are colored yellow and incarnadine, respectively, and shown as sticks. Helices involved in forming the cholesterol binding sites are labelled. **b**, Superposition of GlyT1^{Sar} and hSERT (PDB: 5I71). Cholesterol molecules in GlyT1 and hSERT are colored yellow and brown, respectively, and shown as sticks. Helices involved in forming the cholesterol binding sites are labelled. Residues participating in the interactions are shown as sticks and labelled. **c**, Superposition of GlyT1^{Sar} and hDAT^{Coca} (PDB: 9EO4). Cholesterol molecules in GlyT1 and cholesteryl hemisuccinate (CHS) in hDAT^{Coca} are colored yellow and grey, respectively, and shown as sticks. Helices involved in forming the cholesterol binding sites are labelled. **d**, Superposition of GlyT1^{Sar} and hDAT^{MRS} (PDB: 8VBY). Cholesterol molecules in GlyT1 and CHS in hDAT^{MRS} are colored yellow and purple, respectively, and shown as sticks. Helices involved in forming the cholesterol binding sites are labelled. Cholesterol binding sites that are different from those in GlyT1 are labelled with number 1 to 6. **e**, Superposition of binding sites of CHOL1, CHOL2, and CHOL3 in GlyT1^{Sar} with corresponding sites in hDAT^{Coca}. Residues interacting with cholesterol are shown as sticks and labelled. **f**, Superposition of binding sites of cholesterol or CHS in hDAT^{Coca} (1–4) and hDAT^{MRS} (5) with corresponding sites in GlyT1^{Sar}. Residues interacting with cholesterol or CHS are shown as sticks and labelled.

Supplementary Table 1. Cryo-EM data collection, refinement and validation statistics.

	GlyT1 ^{Apo-out} (EMDB-61580)	GlyT1 ^{Apo-occ} (EMDB-61581)	GlyT1 ^{Apo-in} (EMDB-61226) (PDB 9J8B)	GlyT1 ^{Sar} (EMDB-61227) (PDB 9J8C)	GlyT1 ^{ICL} (EMDB-61228) (PDB 9J8D)
Data collection and processing					
Magnification		105,000 ×		105,000 ×	105,000 ×
Voltage (kV)		300		300	300
Electron exposure (e ⁻ /Å ²)		60		60	60
Defocus range (μm)		-1.0 ~ -2.0		-1.0 ~ -2.0	-1.0 ~ -2.0
Pixel size (Å)		0.85		0.85	0.85
Symmetry imposed		C1		C1	C1
Initial particle images (no.)		2,750,332		3,828,419	4,095,098
Final particle images (no.)	9,711	27,861	53,906	639,501	333,562
Map resolution (Å)	6.0	6.2	3.9	2.8	2.9
FSC threshold	0.143	0.143	0.143	0.143	0.143
Refinement					
Initial model used (PDB code)	-	-	8WFJ	8WFI	8WFJ
Model resolution (Å)	-	-	4.0	2.9	3.1
FSC threshold	-	-	0.5	0.5	0.5
Map sharpening <i>B</i> factor (Å ²)			165.7	142.3	150.9
Model composition					
Non-hydrogen atoms			4158	4418	4349
Protein residues			524	543	530
Ligands			0	3	4
<i>B</i> factors (Å ²)					
Protein			81.51	59.29	58.83
Ligand			-	65.22	61.33
R.m.s. deviations					
Bond lengths (Å)			0.003	0.003	0.003
Bond angles (°)			0.642	0.598	0.584
Validation					
MolProbity score			1.91	1.35	1.62
Clash score			8.93	4.59	4.09
Poor rotamers (%)			0.00	0.00	0.00
Ramachandran plot					
Favored (%)			93.44	97.40	93.51
Allowed (%)			6.56	2.60	6.49
Disallowed (%)			0.00	0.00	0.00

Supplementary Table 2. Primers used in this study

Name	Sequence (5' to 3')	Source
GlyT1-F	GGAGCTGGCAGAGGTGTGAAT	Tsingke
GlyT1-R	TCCCCTGGCAGCTGTGCTCATAT	Tsingke
GlyT2-F	ATGGATTGCAGTGCTCCCAA	Tsingke
GlyT2-R	GCACTGAGTGCCCAGTTCCAAA	Tsingke
GlyT1-G319S-F	CTCACTGAGTTGCGCGTGGGGAGGC	Tsingke
GlyT1-G319S-R	CACGCGCAACTCAGTGAGTAGAAGATC	Tsingke
GlyT1-L422T-F	CAGTTCTGCACTCTGGAGACGCTGGTC	Tsingke
GlyT1-L422T-R	TCTCCAGAGTGCAGAACTGAGTGCCCA	Tsingke
GlyT1-A563F-F	TCCTCATGTTTCTGTCCTCCGTCCTCTG	Tsingke
GlyT1-A563F-R	GAGGACAGAAACATGAGGAAGCCAATG	Tsingke
GlyT2-S479G-F	ACTCATCACTCTCGGTTCTTACAACAAGTTTCACAATAACGTC	Tsingke
GlyT2-S479G-R	AACTTGTTGTAAGAACCGAGAGTGATGAGTCCGCCC	Tsingke