Supplementary information

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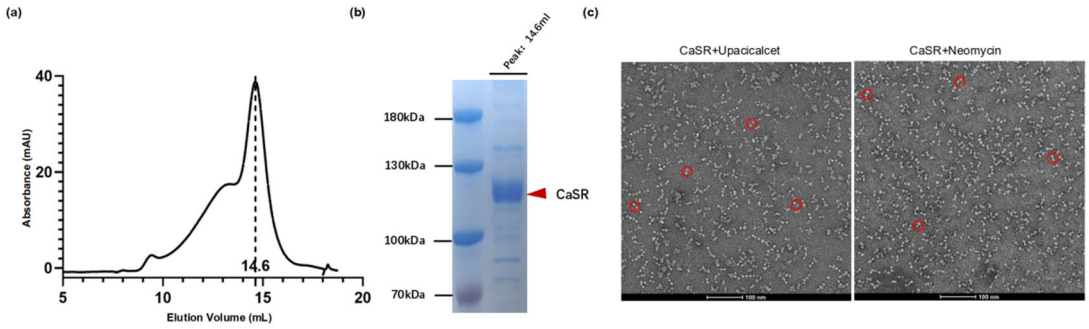


Fig. S1 Purification and negative-stain characterization of CaSR-ligand complexes. (a) Size-exclusion chromatography (SEC) trace of the CaSR complexes. Fractions around the main peak at ~14.6 mL (dashed line) were pooled for downstream analyses. (b) Coomassie-stained SDS-PAGE of the peak fraction showing a predominant band corresponding to CaSR (arrowhead). (c) Representative negative-stain electron micrographs of CaSR bound to upacicalcet (left) and to neomycin (right), where red circles highlight individual receptor particles. Scale bars, 100 nm.

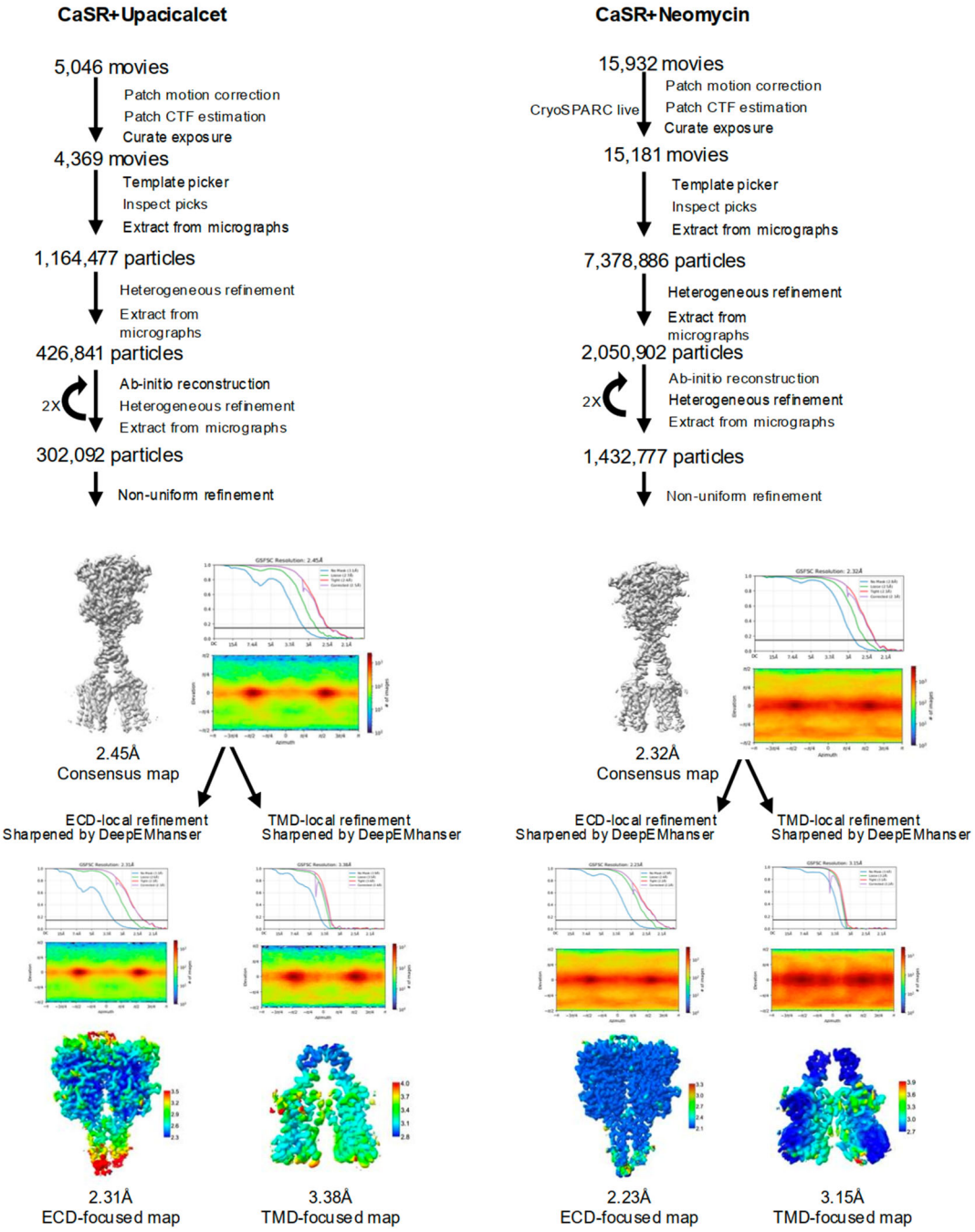


Fig. S2 Cryo-EM data processing workflow.

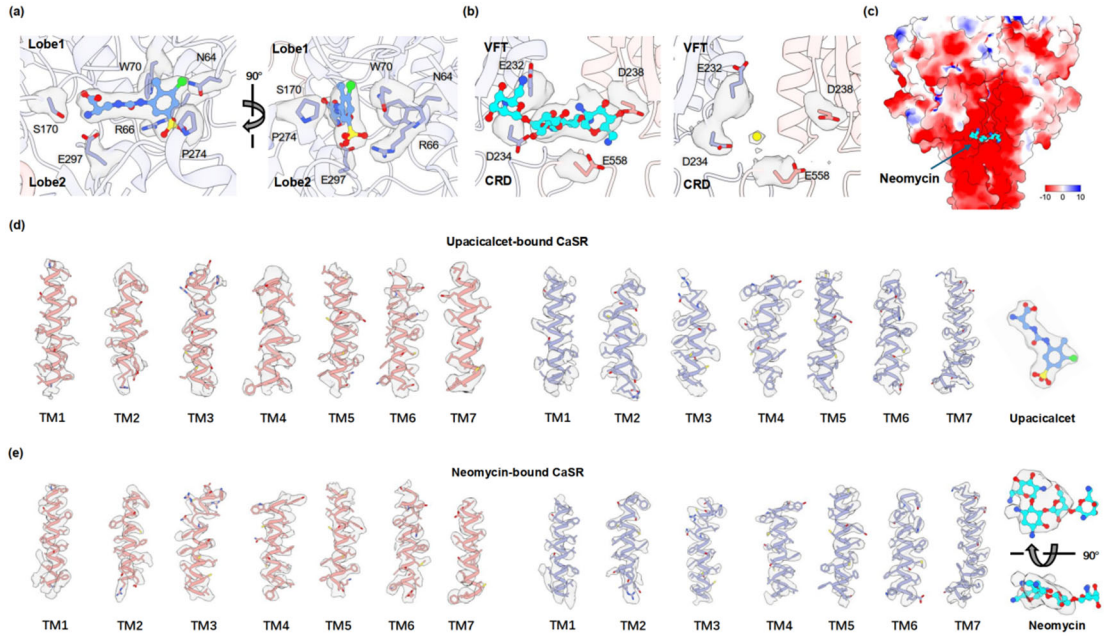


Fig. S3 Cryo-EM density fitting and ligand-binding features of CaSR complexes. (a) Close-up views of the upacicalcet-binding site in the VFT Lobe 1-Lobe 2 cleft, showing upacicalcet and surrounding residues fitted into the cryo-EM density (two orthogonal views). (b) Close-up views of the neomycin-binding site at the inter-protomer VFT-CRD interface, showing neomycin and surrounding residues fitted into the cryo-EM density (left). For comparison, the corresponding site in the upacicalcet-bound structure shows a clear much smaller density of the coordinated Ca^{2+} ion (right). (c) Electrostatic surface potential of the CaSR ECD illustrating that neomycin binds in a negatively charged pocket formed between the two protomers at the VFT (red, negative; blue, positive). (d) Upacicalcet-bound complex: transmembrane helices (TM1-TM7) and the ligand are shown with the corresponding cryo-EM densities, demonstrating the quality of fit. (e) Neomycin-bound complex: TM1-TM7 and the ligand modeled into the cryo-EM densities, confirming the consistency between models and maps. For density panels, maps are shown as semi-transparent meshes at representative contour levels.

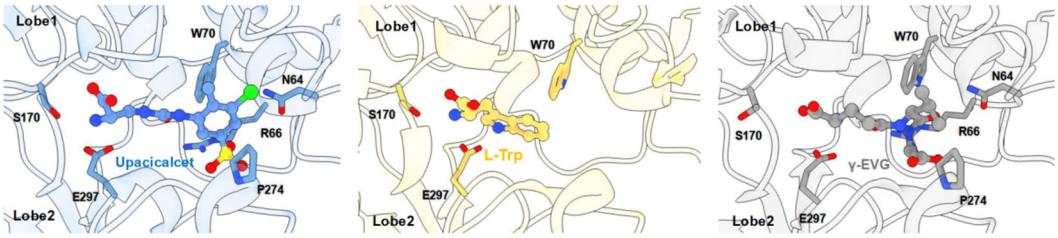


Fig. S4 Comparison of ligand-pocket interaction networks at the CaSR amino-acid binding site. Representative views of the VFT amino-acid binding pocket in the upacicalcet-CaSR structure (this study; left), the L-Trp-bound CaSR structure (PDB: 7M3G; middle), and the γ-EVG-bound CaSR structure (PDB: 9J7I; right). Upacicalcet is shown as blue sticks, L-Trp as yellow sticks, and γ-EVG as gray sticks. The key residues are shown as sticks and are labeled.

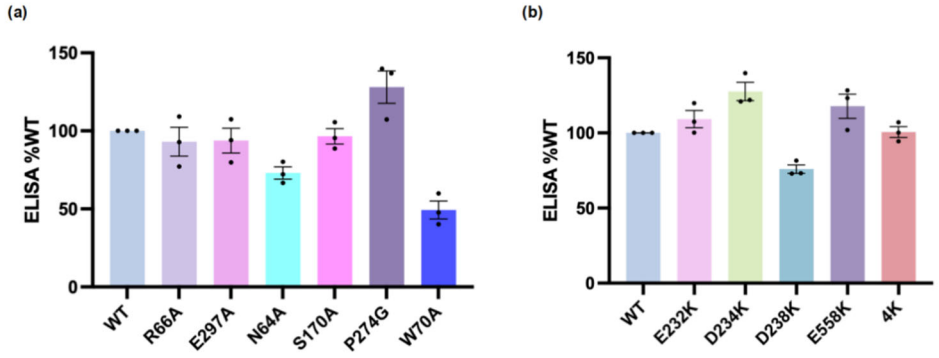


Fig. S5 Analysis of relative expression levels of wild-type and mutant CaSR. (a-b) ELISA assays measuring cell surface expression levels of wild-type and mutant CaSR constructs used in BRET experiments. These constructs were employed to assess the functional impact of upacicalcet (a) and neomycin (b) on CaSR activation through their interactions with the receptor. 4K indicates a quadruple mutation in which E558, D238, D234, and E232 were all substituted with lysine (K).

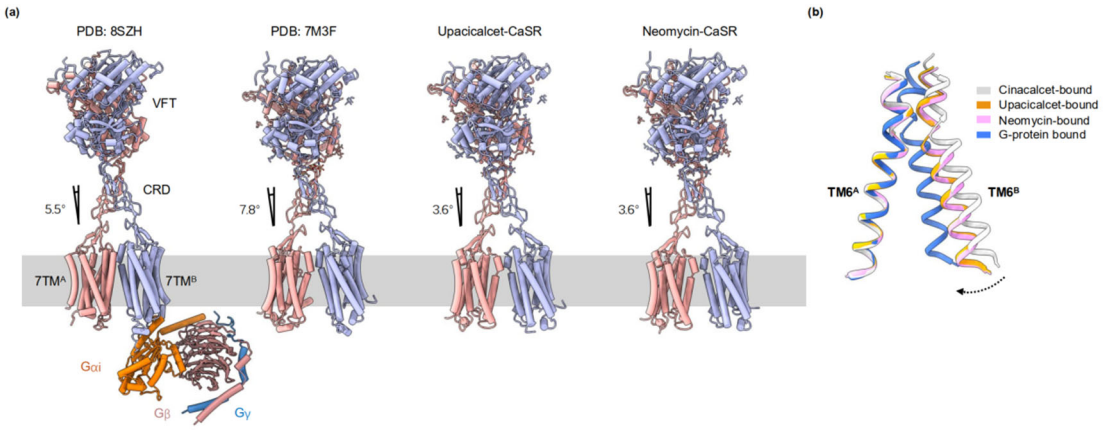


Fig. S6 Comparison of active-state CaSR assemblies relative to G-protein and cinacalcet references. (a) Structures of the CaSR-Gi3 complex (PDB: 8SZH), cinacalcet-bound CaSR (PDB: 7M3F), and the upacicalcet- or neomycin-bound CaSR. A membrane slab is shown in grey, while protomers are colored pink and light blue. The numbers indicate the angle between the extracellular-domain (ECD) principal axis and the membrane normal. (b) Comparison of TM6 from protomer A across the three ligand-bound structures with TM6 from the Gi-bound complex. The dashed arrows indicate that, relative to the cinacalcet-bound complex, TM6 in the upacicalcet- or neomycin-bound complex shifts toward the TM6 position observed in the G-protein-bound complex. Colors: cinacalcet-bound, grey; upacicalcet-bound, orange; neomycin-bound, pink; G-protein bound, blue.

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

	CaSR-upacicalcet	CaSR-neomycin
Data collection and processing		
Magnification	130,000	130,000
Voltage (kV)	300	300
Electron exposure (e-/Å^2)	52	50
Defocus range (μm)	-0.7~-1.5	-0.7~-1.5
Pixel size (Å)	0.93	0.93
Symmetry imposed	C1	C1
Initial particle images (no.)	3,133,373	7,289,498
Final particle images (no.)	302,092	1,432,777
Map resolution (Å)	2.31 (ECD)	2.23 (ECD)
	3.38 (TMD)	3.15 (TMD)
FSC threshold	0.143	0.143
Refinement		
Initial model used (PDB code)	7M3F	7M3F
Model composition		
Non-hydrogen atoms	12,660	12,739
Protein residues	1579	1579
Ligands	21	18
B factors (Å^2)		
Protein	64.82	58.63
Ligand	66.79	50.65
R.m.s. deviations		
Bond lengths (Å)	0.006	0.005
Bond angles (°)	1.024	0.950
Validation		
MolProbity score	1.51	1.40
Clash score	8.70	4.53
Poor rotamers (%)	0.00	0.00
CaBLAM outliers (%)	1.16	1.55
Ramachandran plot		
Favored (%)	97.82	97.05
Allowed (%)	2.18	2.95
Disallowed (%)	0.00	0.00
PDB	9XTM	9XTP
EMDB	EMD-67239	EMD-67244