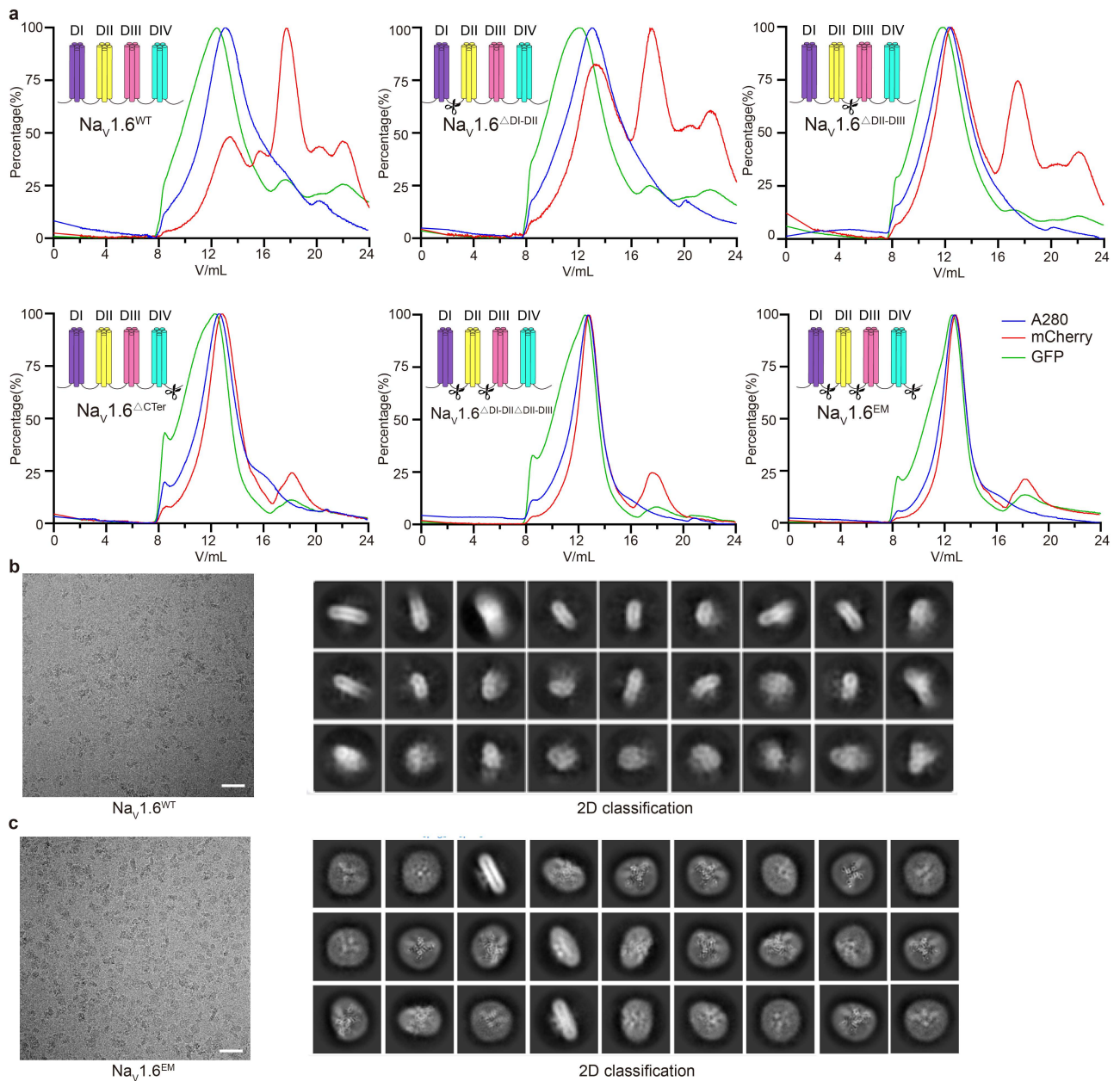Supplementary Information for

Structure of human Na$_V$1.6 channel reveals Na$^+$ selectivity and pore blockade by 4,9-anhydro-tetrodotoxin

Authors

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This file contains Supplementary Figure 1-10 and Table 1-3.

Supplementary Figure 1. Construct optimization of human Na$_V$1.6/β1/β2

a. Fluoresent SEC profiles of the Na$_V$1.6 WT and variants. The blue, red and green peaks represent singals from total protein absorbance at 280 nm, mCherry fluorecence and GFP fluoresence, respectively. Scissors indicate the truncation positions. **b-c**. Representative cryo-EM micrographs (Bar = 400 Å) and selected 2D class averages for Na$_V$1.6^{WT} (**b**) and Na$_V$1.6^{EM} (**c**). The experiments were repeated independently for more than 3 times with similar results.
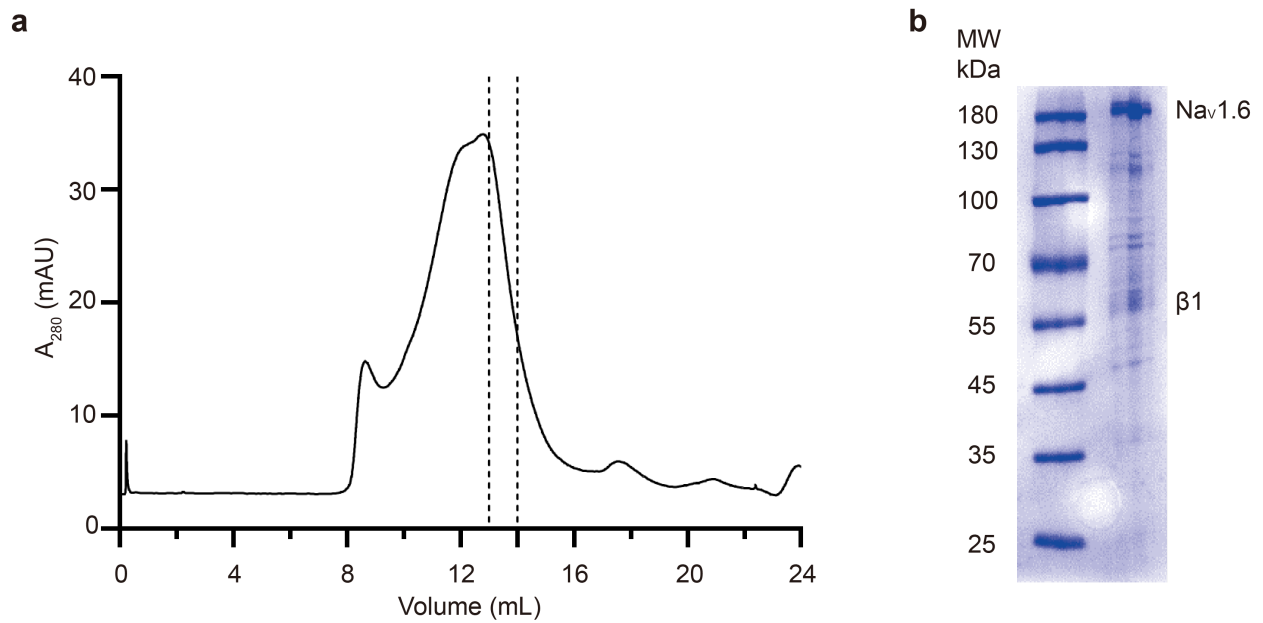
Supplementary Figure 2. Purification of human $Na_V1.6^{EM}/\beta1/\beta2$ complex

a. SEC profile of human $Na_V1.6^{EM}/\beta1/\beta2$ complex. Fractions between two black dashed lines were pooled and concentrated for cryo-EM analysis. **b**. SDS-PAGE gel of the purified $Na_V1.6^{EM}/\beta1/\beta2$ sample stained by Coomassie blue. Bands for $Na_V1.6^{EM}$ and $\beta1$ were labelled. The experiments were repeated independently for more than 3 times with similar results. Source data are provided as a Source Data file.
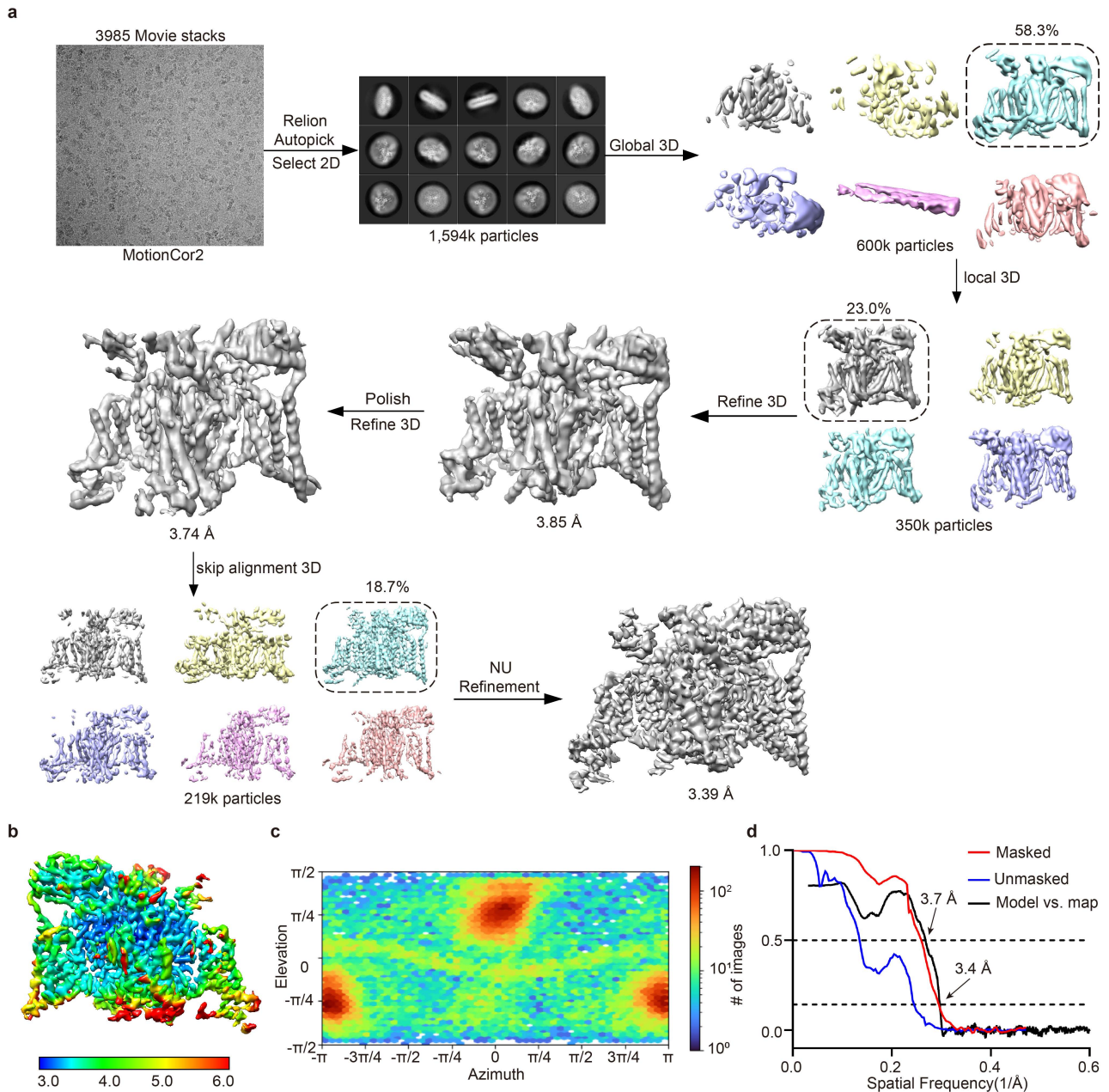
Supplementary Figure 3. Cryo-EM data processing of human Na$_V$1.6^{EM}/β1/β2 complex

a. The flowchart of cryo-EM data processing for Na$_V$1.6^{EM}/β1/β2 complex. A total of 1,594k particles were picked from 3,985 micrographs. Two rounds of 2D classification and two rounds of 3D classification were performed to remove bad particles, followed by AutoRefine, Bayesian polish and contrast transfer function (CTF) refinement in Relion to improve the map quality. The final EM density map was generated by the non-uniform (NU) refinement in CryoSPARC. **b.** Local resolution distribution of the final map of the Na$_V$1.6^{EM}/β1/β2 complex. **c.** Angular distribution of the cryo-EM reconstruction of Na$_V$1.6^{EM}/β1/β2 complex used for final refinement. **d.** Fourier shell correlations (FSC) curves of the Na$_V$1.6^{EM}/β1/β2 complex.
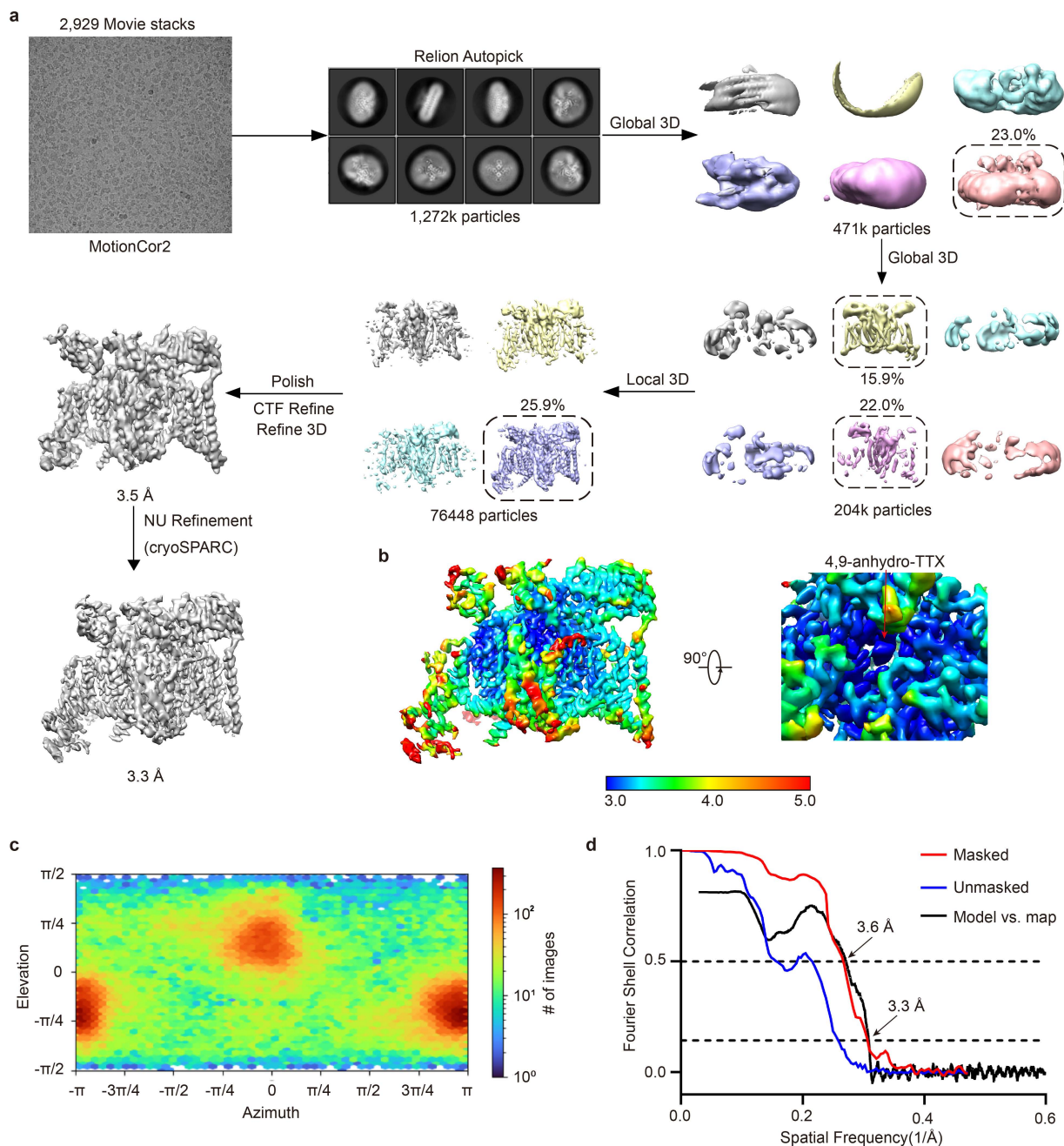
Supplementary Figure 4. Cryo-EM data processing of the Na$_V$1.6$^{4,9\text{-ahTTX}}$ complex

a. The flowchart of the Na$_V$1.6^{EM}/β1/β2 and 4,9-ah-TTX complex data processing. A total of 1,272k particles were picked from 2,929 micrographs. Two rounds of global 3D classification and a further round of local 3D classification were performed to remove bad particles, followed by AutoRefine, Bayesian polish and contrast transfer function (CTF) refinement in Relion to improve the map quality. The final EM density map was genera ted by the non-uniform (NU) refinement in CryoSPARC. **b.** Local resolution distribution of the final 3D map. The EM density of 4,9-anhydro-TTX was labelled with a red arrow. **c.** Angular distribution of the particles used for the final 3D reconstruction. **d.** Fourier shell correlations (FSC) curves of the Na$_V$1.6$^{4,9\text{-ahTTX}}$ complex.

Supplementary Figure 5. Representative EM densities for Na$_V$1.6^{EM}/β1/β2 complex.

a. The EM densities for the S1–S6 segments in each domain were shown as gray surface. b. The EM densities for the Pore loop segments in each domain were shown as gray surface. Side-chains of selected residues were shown as sticks.

a

```
                                           N289  N295                          N308                  N326
hNaV1.6_ECLI  286 ------------------INFNESYLEN---------------GTKGFDWEEYINNKTNFYTVPGMLEPLLCGNSSDAG 331
hNaV1.1_ECLI  283 ---TNASLEEHSIE-KNITVNYNGTLINE---------------TVFEFDWKSYIQDSRYHYFLEGFLDALLCGNSSDAG 343
hNaV1.2_ECLI  284 ---DNSSFEINITSFFNNSLDGNGTTFNR---------------TVSIFNWDEYIEDKSHFYFLEGQNDALLCGNSDAG  345
hNaV1.3_ECLI  283 ---SDSAFETNTTSYFNGTMDSNGTFVNV---------------TMSTFNWKDYIGDDSHFYVLDGQKDPLLCGNGSDAG 344
hNaV1.4_ECLI  290 FNDTNTTWYSNDTWYGNDTWYGNEMWYGNDSWYANDTWNSHASWATNDTFDWDAYISDEGNFYFLEGSNDALLCGNSSDAG 367
hNaV1.5_ECLI  283 ----------------NFTALNGTNGSV------------EADGLVWESLDLYLSDPENYLLKNGTSDVLLCGNSSDAG  333
hNaV1.7_ECLI  276 ---------------FRNSLENNETLESI---------MNTLESEEDFRKYFYYL---------EGSKDALLCGFSTDSG 322
hNaV1.8_ECLI  277 --------------VKNDMAVNE-----------------TTNYSSHRKPDIYINKR------GTSDPLLCGNGSDSG  317
hNaV1.9_ECLI  288 ----------------------------------------CKNISNPEA---Y---DHCFEKKENSPEFKMCGIWMGNS 320
hNaX_ECLI     273 ------------------ENENETLHNR------------TGNPY-------YIRETENFYYLEGERYALLCGNRTDAG  314
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b

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                       P1I                  P2I                             P1II              P2II
hNaV1.6  356 FSWAFLALFRLMTQDYWENLYQLTLRAA 383      922 FFHSFLIVFRVLCGEWIETMWDCMEVA 948
hNaV1.1  368 FSWAFLSLFRLMTQDFWENLYQLTLRAA 395      937 FFHSFLIVFRVLCGEWIETMWDCMEVA 963
hNaV1.2  370 FSWAFLSLFRLMTQDFWENLYQLTLRAA 397      928 FFHSFLIVFRVLCGEWIETMWDCMEVA 954
hNaV1.3  369 FSWAFLSLFRLMTQDYWENLYQLTLRAA 396      929 FFHSFLIVFRVLCGEWIETMWDCMEVA 955
hNaV1.4  392 FSWAFLALFRLMTQDYWENLFQLTLRAA 419      747 FFHSFLIVFRILCGEWIETMWDCMEVA 773
hNaV1.5  358 FAWAFLALFRLMTQDCWERLYQQTLRSA 385      884 FFHAFLIIFRILCGEWIETMWDCMEVS 910
hNaV1.7  347 FSWAFLALFRLMTQDYWENLYQQTLRAA 374      913 FFHSFLIVFRVLCGEWIETMWDCMEVA 939
hNaV1.8  342 FAWAFLSLFRLMTQDSWERLYQQTLRTS 369      835 FFHSFLIVFRILCGEWIENMWACMEVG 861
hNaV1.9  345 FGWSFLAMFRLMTQDSWEKLYQQTLRTT 371      754 FWHSFLVVFRILCGEWIENMWECMQEA 780
hNaX     339 FGWALFALFRLMAQDYPEVLYHQILYAS 366      674 FFHSFLNVFRILCGEWVETLWDCMEVA 700

                       P1III                P2III                           P1IV              P2IV
hNaV1.6  1399 VGAGYLALLQVATFKGWMDIMYAAVDS 1425     1691 FGNSMICLFQITTSAGWDGLLLPIL 1715
hNaV1.1  1418 VGFGYLSLLQVATFKGWMDIMYAAVDS 1444     1710 FGNSMICLFQITTSAGWDGLLAPIL 1734
hNaV1.2  1408 VGLGYLSLLQVATFKGWMDIMYAAVDS 1434     1700 FGNSMICLFQITTSAGWDGLLAPIL 1724
hNaV1.3  1403 VGAGYLALLQVATFKGWMDIMYAAVDS 1429     1695 FGNSMICLFQITTSAGWDGLLAPIL 1719
hNaV1.4  1230 VGLGYLSLLQVATFKGWMDIMYAAVDS 1256     1522 FGNSIICLFEITTSAGWDGLLNPIL 1546
hNaV1.5  1405 VGAGYLALLQVATFKGWMDIMYAAVDS 1431     1697 FANSMLCLFQITTSAGWDGLLSPIL 1721
hNaV1.7  1392 VGLGYLSLLQVATFKGWTIIMYAAVDS 1418     1684 FGNSMICLFQITTSAGWDGLLAPIL 1708
hNaV1.8  1353 VAMGYLALLQVATFKGWMDIMYAAVDS 1379     1647 FANSMLCLFQITTSAGWDGLLSPIL 1671
hNaV1.9  1243 VGNAYLALLQVATFKGWMDIIYAAVDS 1269     1537 FASSMLCLFQISTSAGWDSLLSPML 1561
hNaX     1128 VGNGFLSLLQVATFNGWITIMNSAIDS 1154     1420 FGNSMLCLFQVAIFAGWDGMLDAIF 1444
```

c, **d**, **e** (concentration-response curves)

- **c** — Normalized Current vs TTX (nM):
  Na$_V$1.6^{EM} IC$_{50}$ = 1.9 nM (red); Na$_V$1.2^{WT} IC$_{50}$ = 4.9 nM (blue); Na$_V$1.7^{WT} IC$_{50}$ = 16.7 nM (black)
- **d** — Normalized Current vs 4,9-anhydro-TTX (nM):
  Na$_V$1.6^{EM} IC$_{50}$ = 52.0 nM; Na$_V$1.6^{L1712A} IC$_{50}$ = 61.1 nM; Na$_V$1.6^{ECL1} IC$_{50}$ = 7773 nM; Na$_V$1.6^{ECL3} IC$_{50}$ = 137.2 nM
- **e** — Normalized Current vs TTX (nM):
  Na$_V$1.6^{EM} IC$_{50}$ = 1.9 nM; Na$_V$1.6^{ECL1} IC$_{50}$ = 167.0 nM; Na$_V$1.6^{ECL3} IC$_{50}$ = 2.20 nM

Supplementary Figure 6. Sequence alignments of the human Na$_V$ channels and functional characterizations of human Na$_V$ channels by TTX analogs.

a. Sequence alignment of the ECL$_I$ between human Na$_V$ channel subtypes. The identical residues were marked in blue. Asn linked glycosylation sites were marked in red and labelled. **b**. Sequence alignment of P1 and P2 of human Na$_V$ channels. The main residues involved in 4,9-ah-TTX binding are highlighted in yellow. Positively charged residues and Lys/Ala were marked in red and purple, respectively. **c**. The concentration-response curves for the blockade of Na$_V$1.6^{EM} (red), Na$_V$1.2^{WT} (blue), and Na$_V$1.7^{WT} (black) by TTX. Na$_V$1.6^{EM}, n = 5; Na$_V$1.2^{WT}, n = 5; Na$_V$1.7^{WT}, n = 4. **d**. The concentration-response curves for the blockade of Na$_V$1.6^{EM} and its variants by 4,9-ah-TTX. Na$_V$1.6^{EM}, n = 5; Na$_V$1.6^{L1712A}, n = 4; Na$_V$1.6^{ECL1}, n = 5; Na$_V$1.6 ^{ECL3}, n = 4. **e**. The concentration-response curves for the blockade of Na$_V$1.6^{EM} and its variants by TTX. Na$_V$1.6^{EM}, n = 5; Na$_V$1.6^{ECL1}, n = 7; Na$_V$1.6 ^{ECL3}, n = 4. Data are presented as mean ± SEM. n biological independent cells. Source data are provided as a Source Data file.
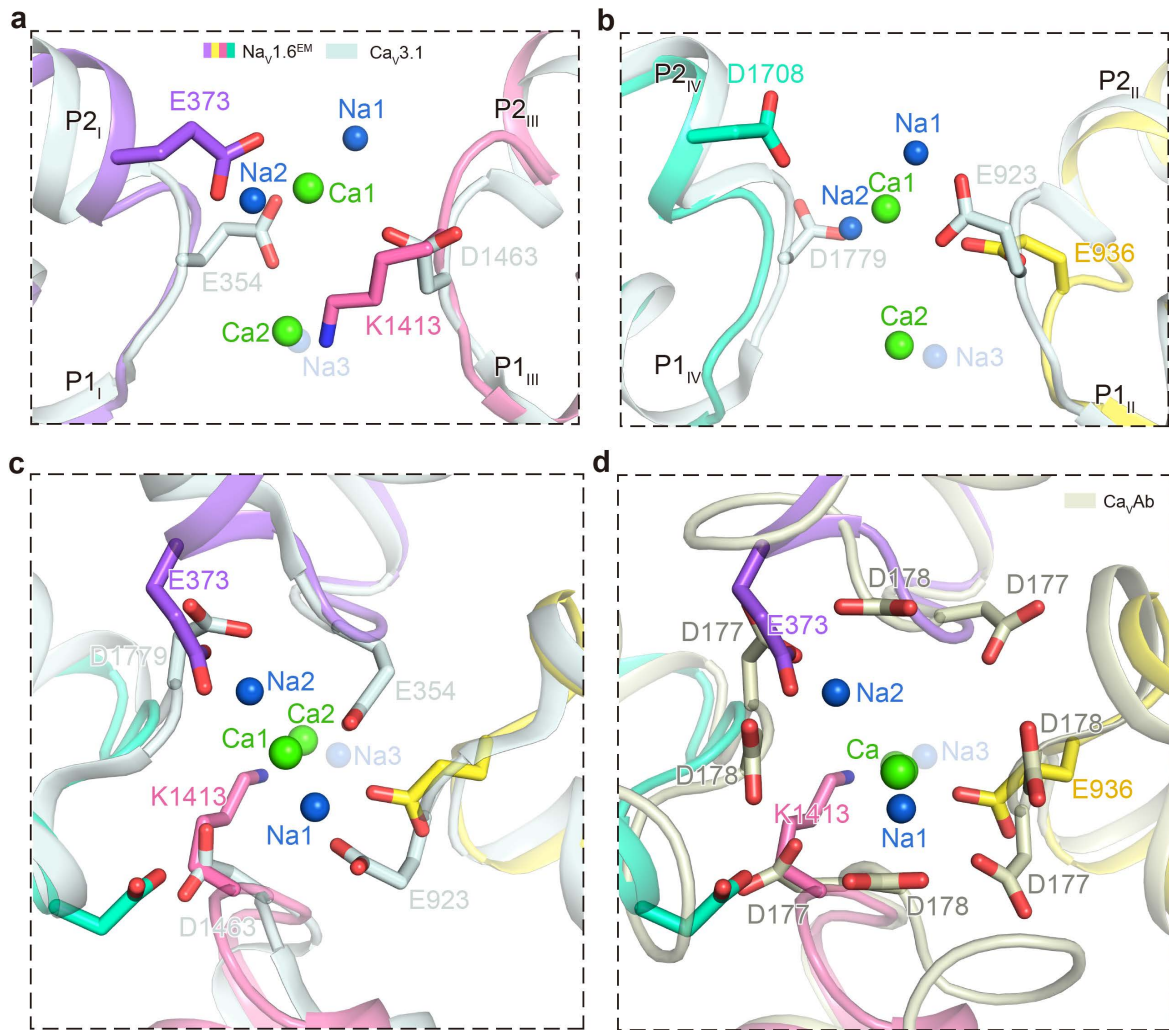
Supplementary Figure 7. Structural comparison of Na$^+$ binding sites of Na$_V$1.6^{EM} and Ca^{2+} binding sites of Ca$_V$3.1.

a-b. Side view of the Na$^+$ sites compared with the Ca^{2+} sites of Ca$_V$3.1 (PDB code: 6KZO, colored in light blue). The diagonal repeats of DI and DIII (**a**), DII and DIV (**b**) are shown separately for clarity. **c-d**. Top-down view of the Na$^+$ sites of Na$_V$1.6 compared with the Ca^{2+} sites of Ca$_V$3.1 (PDB code: 6KZO, colored in light blue) (c) and that of Ca$_V$Ab (PDB code: 4MS2, colored in gray) (d), respectively. Key residues were shown side-chains in sticks. Ions were shown as spheres.
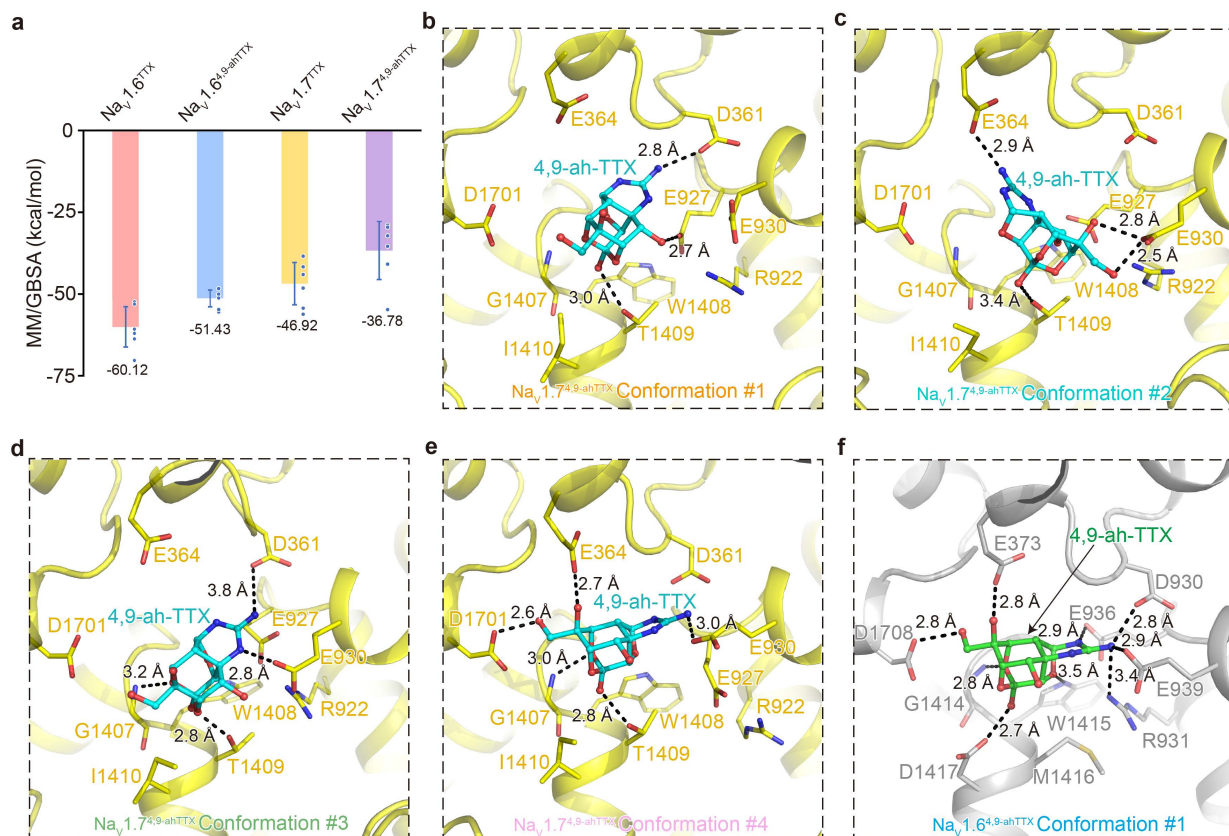
Supplementary Figure 8. Different conformations of 4,9-ah-TTX binding to Na$_V$1.6 and Na$_V$1.7 from MD.

a. The binding affinity of TTX and 4,9-ah-TTX to Na$_V$1.6 and Na$_V$1.7 calculated by MM/GBSA from the MD simulations. MD simulations for Na$_V$1.6^{TTX}, Na$_V$1.6$^{4,9-ahTTX}$, Na$_V$1.7^{TTX}, and Na$_V$1.7$^{4,9-ahTTX}$ were repeated 6 times independently. Data are presented as mean±SEM. **b-e**. Detailed binding modes of the four major conformations of 4,9-ah-TTX binding in Na$_V$1.7 from the MD simulations. **f**. The only major binding mode of 4,9-ah-TTX in Na$_V$1.6 from the MD simulations. Source data are provided as a Source Data file.

Residues interacting with ligands	Nav1.6^4,9-ahTTX		Nav1.7^4,9-ahTTX					Nav1.6^TTX				Nav1.7^TTX			
	c.1	c.2	c.1	c.2	c.3	c.4	c.5	c.1	c.2	c.3	c.4	c.1	c.2	c.3	c.4
Cluster population (ns)	599	2	342	105	82	67	5	555	32	11	2	479	87	30	3
P [GLU936 (GLU927)]	100%	100%	87%	97%	100%	28%	60%	100%	84%	100%	100%	100%	100%	100%	100%
HA [GLU936 (GLU927)]	100%	100%	71%	98%	80%	22%	80%	92%	9%	91%	100%	100%	100%	100%	100%
HA [GLU373 (GLU364)]	100%	100%	55%	48%	7%	97%	60%	100%	100%	82%	50%	100%	100%	100%	100%
P [GLU939 (GLU930)]	98%	100%	21%		87%	78%		91%	34%	73%	100%	39%	76%	43%	33%
P [ASP370 (ASP361)]	97%	100%	53%	1%	91%	100%	20%	100%	100%	100%	100%	100%	100%	100%	100%
HA [ASP370 (ASP361)]	96%	100%	52%	1%	80%	100%		100%	100%	100%	100%	98%	100%	100%	100%
HA [GLU939 (GLU930)]	93%	50%	46%	91%	91%	78%	100%	92%	31%	82%	100%	62%	70%	73%	67%
HD [MET1416 (THR1409)]	92%	100%	92%	54%	100%	97%	80%	99%	100%	100%	100%	100%	100%	100%	100%
HD [ASP1417 (ILE1410)]	79%	100%	30%		60%	60%		25%	100%	18%		65%	18%	70%	100%
HD [GLY1414 (GLY1407)]	75%	100%	92%	5%	98%	100%	20%	41%	100%	55%	100%	100%	100%	100%	100%
HA [ASP1417 (ILE1410)]	73%	50%						20%	100%	9%					
HD [TRP1415 (TRP1408)]	65%	50%	45%	10%	24%	97%	60%	46%	100%	45%		84%	85%	70%	67%
AR [TYR371 (TYR362)]	54%		21%	53%		61%	60%	32%	6%	9%		8%	15%		
HA [ASP1708 (ASP1701)]	51%	100%	37%		9%	99%		52%	100%			37%	54%	30%	
HD [ARG931 (ARG922)]	47%	50%	48%	65%	45%		80%	10%		9%		53%	2%	87%	100%
HY [TYR371 (TYR362)]	39%	50%	58%	82%		30%	60%	37%	9%	18%		23%	24%	7%	
HD [GLY1706 (GLY1699)]	12%		1%					36%	22%	18%		71%	84%	80%	100%
HA [GLY1414 (GLY1407)]	11%	50%	0%					19%	3%	18%					
HA [PHE1412 (PHE1405)]	5%		16%	27%				4%	31%	9%		11%	11%	20%	
HD [GLY1709 (GLY1702)]	5%	50%						23%		9%					
P [GLU373 (GLU364)]	5%		38%	54%	20%		60%	34%		9%		73%	2%	100%	100%
HY [LYS1413 (LYS1406)]	1%														
HA [GLY1706 (GLY1699)]	1%							5%							
HA [TYR371 (TYR362)]	1%		7%				40%	1%			50%	2%	1%		
HD [TYR371 (TYR362)]	0%		3%					65%	100%	91%	100%	21%	91%	3%	
HD [ASN374 (ASN365)]	0%							4%							
HD [TRP1707 (TRP1700)]	0%														
HA [GLY317 (GLY308)]			1%												
HD [LEU319 (LYS310)]			2%	6%								0%			
HA [GLN369 (GLN360)]			5%			7%	20%	1%	16%			1%	9%		
HA [CYS934 (CYS925)]			1%		1%										
HA [GLY935 (GLY926)]			47%			93%						1%	16%		
HD [LYS1413 (LYS1406)]			1%					10%	100%			19%	57%		
AR [TRP1415 (TRP1408)]			1%	3%											
HA [MET1416 (THR1409)]			78%	6%	100%	97%		0%				99%	100%	100%	100%
P [ASP1708 (ASP1701)]			13%	1%								0%			
HA [THR314 (TYR305)]				1%											
HD [TRP917 (TRP908)]				4%			20%								
HD [TYR1420 (TYR1413)]				21%			60%								
HD [TRP372 (TRP363)]								5%		9%					
HA [LYS1413 (LYS1406)]								20%		27%					

Supplementary Figure 9. Ligand-protein contact analysis based on MD study.

The type and frequency of interactions between protein and ligand are listed for each conformation cluster of a protein-ligand system. C.# indicates the index of the cluster. The digits associated with green-white color scheme indicate the appearance frequency of the interaction within the cluster. For residues interacting with ligands, the annotation follows the format of "Interaction Type [Residue in Nav1.6 (Counterpart the residue in Nav1.7)]". The interaction identification and interaction type definition follow the study described by Daria et al[1]. A general annotation for interaction types is listed below: IP (salt bridges), HY (hydrophobic interactions), HA (hydrogen bond, ligand atom as acceptor), HD (hydrogen bond, ligand atom as donor), AR (aromatic system related stacking).
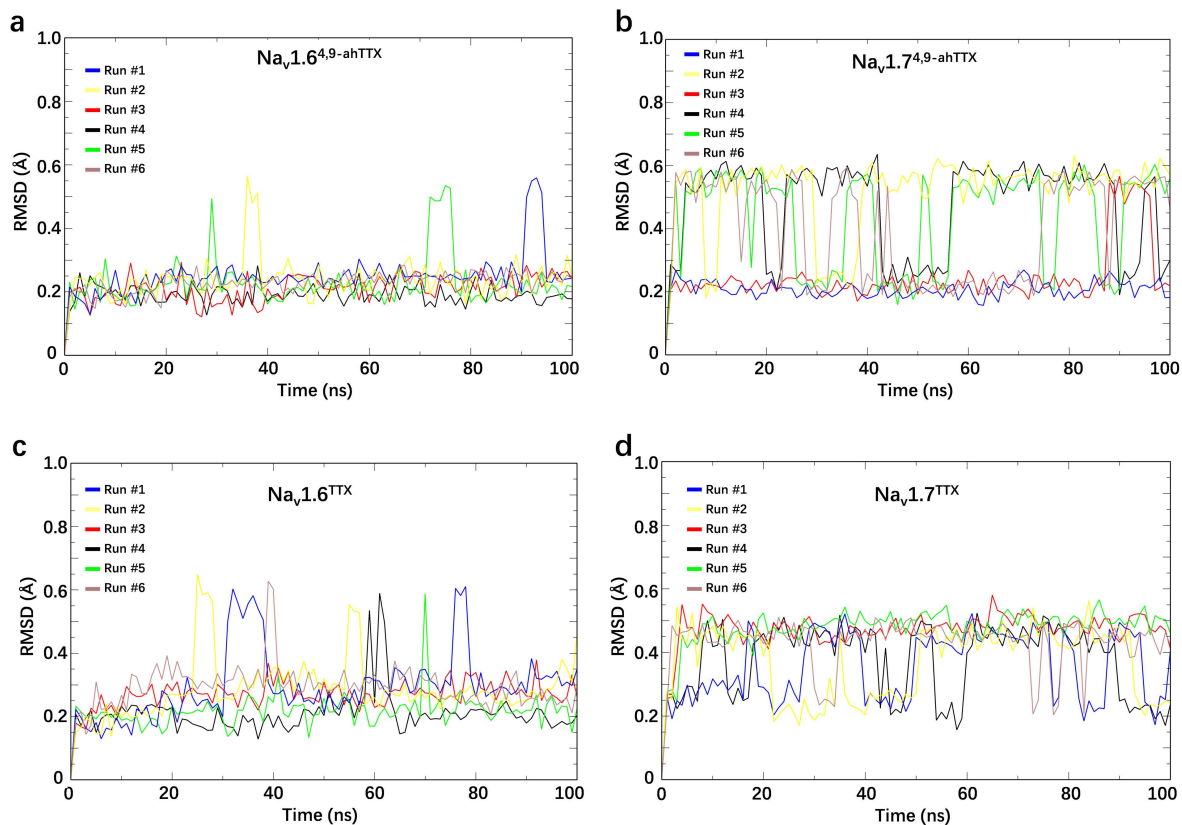
Supplementary Figure 10. Ligand dynamics in MD study.

a to **d**, The ligand RMSD plots for each replicate of the simulations for $Na_v1.6^{4,9\text{-}ahTTX}$ (a), $Na_v1.7^{4,9\text{-}ahTTX}$ (b), $Na_v1.6^{TTX}$ (c), and $Na_v1.7^{TTX}$ (d). Before RMSD calculation, all the structures in each trajectory were aligned with the initial structure of that trajectory by using Least Squares algorithm.

Supplementary Table 1. Concentration-response inhibition statistics of 4,9-ah-TTX or TTX for different subtypes and variants.

Human subtypes	Compounds	IC50 (nM)	95% CI	n
$Na_V1.6^{EM}$	4,9-ah-TTX	52.0	44.33 to 61.09	5
$Na_V1.2^{WT}$	4,9-ah-TTX	257.9	217.4 to 305.9	6
$Na_V1.7^{WT}$	4,9-ah-TTX	1340	1209 to 1486	6
$Na_V1.6^{ELC1}$	4,9-ah-TTX	7773	4538 to 13313	5
$Na_V1.6^{ELC3}$	4,9-ah-TTX	137.2	123.1 to 152.9	4
$Na_V1.6^{L1712A}$	4,9-ah-TTX	61.1	55.45 to 67.23	4
$Na_V1.6^{M1416T/D1417I}$	4,9-ah-TTX	256.5	224.0 to 293.7	5
$Na_V1.6^{EM}$	TTX	1.9	1.76 to 2.13	5
$Na_V1.2^{WT}$	TTX	4.9	4.43 to 5.46	5
$Na_V1.7^{WT}$	TTX	16.7	14.54 to 19.18	4
$Na_V1.6^{ELC1}$	TTX	167.0	115.2 to 242.3	7
$Na_V1.6^{ELC3}$	TTX	2.2	2.01 to 2.41	4

Supplementary Table 2. Primers used in this study.

Name	Sequence (5' to 3')
$Na_V1.6$ F	atggcagcgcggctgcttg
$Na_V1.6$ R	acacttggattctctgacctctttttgt
$Na_V1.6^{\triangle DI\text{-}DII}$ F	tcccctcggagctctcggaaggacagaatcaacagtata
$Na_V1.6^{\triangle DI\text{-}DII}$ R	gattctgtccttccgagagctccgagggggagcccccctcc
$Na_V1.6^{\triangle DII\text{-}DIII}$ F	acagaggatgttagcggcaagtcttggtggatcctgcgg
$Na_V1.6^{\triangle DII\text{-}DIII}$ R	ccaccaagacttgccgctaacatcctctgtgttgaggtt
$Na_V1.6^{\triangle CTer}$ F	caccgggagaaaaaagagctggaagttctgttccaggggccatggtgagcaa
$Na_V1.6^{\triangle CTer}$ R	ctggaacagaacttccagctctttttttctcccggtgtgtgcctccattctccagc
$Na_V1.6^{L1712A}$ F	gatggcctgctgGCGcccatcctaaaccgcccccctgact
$Na_V1.6^{L1712A}$ R	ggtttaggatgggCGCcagcaggccatcccaaccagctga
$Na_V1.6^{ECL1}$ F	ctgcagctgttcatggggaaccttaagcataaatgttttcgaaattca
$Na_V1.6^{ECL1}$ R	agtgtcaaaacttgtgtaaccataatcagggtttctgccaattttcac
$Na_V1.6^{ECL3}$ F	ttcagcatcatgggagttaacttgtttgctggcaagttctatgagtgt
$Na_V1.6^{ECL3}$ R	aagaagggccaggtatcctgccccgacattatcaaagttcactttcag
$Na_V1.6^{M1416T/D1417I}$ F	ccttcaaaggctggacgattatcatgtatgcagctgtagattcccggaagcctg
$Na_V1.6^{M1416T/D1417I}$ R	cagctgcatacatgataatcgtccagcctttgaaggttgctacttgaagaagggc

Supplementary Table 3. Cryo-EM data collection, refinement and validation statistics

	$Na_V1.6^{EM}/\beta1/\beta2$ (EMDB-34387) (PDB: 8GZ1)	$Na_V1.6^{4,9-ahTTX}$ (EMDB-34388) (PDB: 8GZ2)
Data collection and processing		
Magnification	130,000 ×	130,000 ×
Voltage (kV)	300	300
Electron exposure (e–/Å^2)	60	60
Defocus range (μm)	−1.2 ~ −2.2	−1.2 ~ −2.2
Pixel size (Å)	1.04	1.04
Symmetry imposed	C1	C1
Initial particle images (no.)	1594472	1272486
Final particle images (no.)	41387	76448
Map resolution (Å)	3.4	3.3
FSC threshold	0.143	0.143
Map resolution range (Å)	3.0 ~ 5.0	3.0 ~ 5.0
Refinement		
Initial model used (PDB code)	6J8I	$Na_V1.6$-β1-β2
Model resolution (Å)	3.7	3.6
FSC threshold	0.5	0.5
Map sharpening B factor (Å^2)	-69.0	-58.1
Model composition		
Non-hydrogen atoms	11912	11984
Protein residues	1445	1467
Ligands	19	18
B factors (Å^2)		
Protein	90.46	79.36
Ligand	98.52	75.52
R.m.s. deviations		
Bond lengths (Å)	0.003	0.005
Bond angles (°)	0.623	0.818
Validation		
MolProbity score	1.89	2.03
Clashscore	10.55	12.37
Poor rotamers (%)	0.00	0.00
Ramachandran plot		
Favored (%)	94.98	93.62
Allowed (%)	5.02	6.38
Disallowed (%)	0.00	0.00

SUPPLEMENTARY REFERENCES

1   Kokh, D. B. *et al.* A workflow for exploring ligand dissociation from a macromolecule: Efficient random acceleration molecular dynamics simulation and interaction fingerprint analysis of ligand trajectories. *J Chem Phys* **153** (2020).