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Crystal structure of a voltage-gated sodium channel in two potentially inactivated states

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Abstract

In excitable cells, voltage-gated sodium (Na_v) channels activate to initiate action potentials and then undergo fast and slow inactivation processes that terminate their ionic conductance^{1,2}. Inactivation is a hallmark of Na_v channel function and is critical for control of membrane excitability³, but the structural basis for this process has remained elusive. Here we report crystallographic snapshots of the wild-type NavAb channel from *Arcobacter butzleri* captured in two potentially inactivated states at 3.2 Å resolution. Compared to previous structures of NavAb S6-cysteine mutants⁴, the pore-lining S6 helices and the intracellular activation gate have undergone significant rearrangements in which one pair of S6 segments has collapsed toward the central pore axis and the other S6 pair has moved outward to produce a striking dimer-of-dimers configuration. An increase in global structural asymmetry is observed throughout our wild-type NavAb models, reshaping the ion selectivity filter at the extracellular end of the pore, the central cavity and its residues analogous to the mammalian drug receptor site, and the lateral pore fenestrations. The voltage-sensing domains also shift around the perimeter of the pore module in NavAb, and local structural changes identify a conserved interaction network that connects distant molecular determinants involved in Na_v channel gating and inactivation. These potential inactivated-state structures provide new insights into Na_v channel gating and novel avenues to drug development and therapy for a range of debilitating Na_v channelopathies.

Voltage-gated ion channels (VGICs) share a common architecture consisting of a central ion-conducting pore module (PM) and four peripheral voltage-sensing domains (VSDs)^{4,5}. Voltage-gated potassium (K_v) and bacterial Na_v channels are homotetramers of subunits containing six transmembrane segments (S1–S6)^{5,6}, whereas vertebrate Na_v and calcium (Ca_v) channels contain four linked homologous domains in a single polypeptide⁷. The S5 and S6 segments of four subunits (or domains) form the PM⁷. The VSDs (S1–S4) place highly conserved S4 gating charges in the membrane electric field, where depolarization causes their outward movement during channel activation⁷. S4 movement is coupled through the S4–S5 linker to the intracellular activation gate to open the pore^{4,5}. In mammalian Na_v channels, two physically distinct inactivation processes control the activity

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Author contributions

W.A.C. and N.Z. are co-senior authors. J.P., N.Z., and W.A.C. conceived and J.P. conducted the protein purification and crystallization experiments. J.P. and N.Z. determined and analyzed the structure of NavAb. T.M.G., T.S., and W.A.C. conceived, T.M.G. conducted, and T.M.G., T.S., and W.A.C. analyzed the electrophysiology experiments. All authors contributed to writing the manuscript.

of Nav channels. Fast inactivation operates on the millisecond time scale and is quickly reversed upon repolarization, permitting Nav channels to be rapidly available for reactivation⁷. A tethered cytoplasmic inactivation gate connecting the third and fourth homologous domains confers fast inactivation through a hinged-lid mechanism⁷. Fast inactivation can be removed by intracellular protease treatment⁸ or mutations of the inactivation gate⁹ and can be restored by addition of inactivation-gate peptides¹⁰. By contrast, slow inactivation develops much more slowly during repetitive firing of action potentials and opposes high-frequency spike generation^{2,3}. Its essential physiological role is highlighted by disease mutations that affect slow-inactivation³ and clinically relevant channel blocking drugs bind to and stabilize slow-inactivated states^{11,12}. In contrast to fast inactivation, the structures and mechanisms involved in slow inactivation of Nav channels remain poorly defined.

Bacterial Nav channels share key physiological properties with their more complex vertebrate descendants, including voltage-dependent activation, inactivation, and pharmacological sensitivity⁶. However, their simple homotetrameric structure leaves bacterial Nav channels without the fast inactivation gate found in mammalian Nav channels. Therefore, bacterial Nav channels are thought to undergo an inactivation process similar to slow inactivation¹³. Mutations near the selectivity filter^{13,14}, along the pore-lining S6 helices^{15–17}, and within VSDs of bacterial Nav channels have dramatic effects on inactivation, similar to slow inactivation in mammalian Nav channels (Supplementary Discussion). We previously reported the structure of NavAb from *Arcobacter butzleri* captured in a potentially pre-open state with four activated VSDs and a closed PM⁴. In space group *I*222, the structural model of NavAb-I217C was nearly four-fold symmetric with two very similar molecules in the asymmetric unit. The ion conductance pathway displayed a selectivity filter with a central orifice of ~4.6 Å lined by four Glu177 side-chains, followed by two sequential carbonyl sites fit to coordinate a Na⁺ ion in complex with a square planar array of hydrating waters⁴. The nearly four-fold symmetrical S6 segments formed a large central cavity and a tightly closed intracellular activation gate⁴. Four lateral pore fenestrations of similar size and shape were seen connecting the hydrophobic membrane phase to the central cavity, and the activated VSDs were arranged in a square array around the PM⁴. Here, through crystallographic and electrophysiological studies, we now describe the structure of NavAb in two potentially inactivated states.

When expressed in *Trichopulsia ni* cells, the wild-type NavAb channel (NavAb-WT) activates and inactivates during 7-ms depolarizing pulses from –180 mV to –40 mV (Fig. 1a). Repetitive 7-ms pulses cause a late phase of slow inactivation that is dependent on the frequency of depolarization and nears completion in 600 s at 0.2 Hz (Fig. 1a). By comparison, NavAb-I217C enters this deep slow-inactivated state more slowly and less completely, and its voltage-dependence of activation is shifted toward more negative potentials in a manner that is consistent with stabilization of the pre-open state (Supplementary Fig. 1, Supplementary Tables 1 and 2; Supplementary Results). The unusually strong, negatively shifted, and slowly reversible inactivation of NavAb-WT in *Trichopulsia ni* cells suggests that it might enter the late slow-inactivated state during our purification and crystallization procedures and never recover from it.

To gain insights into the native structure of NavAb-WT, we solubilized and purified it in a mild detergent and reconstituted it into a phosphatidylcholine-based bicelle system as described for NavAb-I217C⁴. Merohedrally twinned crystals formed in space group *P*4₂ and the NavAb-WT structure was phased and refined to 3.2 Å resolution (Supplementary Table 3). NavAb-WT channels are arranged in the crystals as though each is embedded in a phospholipid membrane bilayer (Supplementary Fig. 2). Remarkably, the structure of NavAb-WT differs substantially from our previously reported S6-cysteine mutant channels⁴

(Fig. 1b–e). Four molecules in the $P4_2$ asymmetric unit give rise to two independent NavAb-WT channels composed of dimers of AB and CD subunit conformations, respectively (NavAb-AB and NavAb-CD; Fig. 1b, c). The NavAb-AB and NavAb-CD models are each unique and strikingly asymmetric in structure (Fig. 1d and Supplementary Figs. 3–5). All VSDs are in an activated conformation, and the PM of both channel appears occluded by collapse of the S6 helices of subunits B or C toward the central axis (Supplementary Figs. 3–5).

We aligned the WT NavAb-AB and NavAb-CD models onto the selectivity filter of NavAb-I217C, revealing conformational adjustments that include asymmetric collapse of the S6 activation gate (Fig. 1d), narrowing of the selectivity filter, reshaping of the central cavity and lateral pore fenestrations, and displacement of the VSDs around the PM (see below). These structural features fit well with expectations of a Na_V channel captured in a slow-inactivated state (Supplementary Discussion). Multiple slow-inactivated conformations are predicted from kinetic analyses of Na_V channels³, and NavAb exhibits at least two inactivated states with different kinetics of recovery from inactivation, and differential effects of the S6 mutation I217C (Supplementary Fig. 1). The observation of two discrete conformations of NavAb-WT provides a potential structural basis for these functional properties.

The inactivated state of a Na_V channel is expected to be non-conductive. The closed inner ends of the S6 segments in NavAb-I217C form a nearly square array (red circle, Fig. 1e) and superimpose well upon other closed-pore tetrameric ion channel structures^{4,18}. By contrast, the intracellular activation gate in NavAb-AB and NavAb-CD has closed in an unprecedented way. Two S6 segments from diagonally opposed subunits have moved closer to the central pore axis, while the adjacent S6 segments have shifted farther away (red oval, Fig. 1d), asymmetrically collapsing the S6 activation gate in these potentially inactivated states. This finding is consistent with biophysical studies^{3,15–17} of bacterial and mammalian Na_V channels as well as pathological mutations that have implicated this pore region in slow-inactivation gating³. This novel activation gate structure may represent a hallmark of the slow-inactivated state in Na_V channels, and is in sharp contrast to the dilated activation gate observed in inactivated KcsA potassium channels¹⁹.

Conformational shifts of the S5 and S6 segments in NavAb are hinged at the extracellular side of the PM near where these segments connect to the pore (P)-helix and P2-helix, respectively (Supplementary Fig. 4c–d). Two S6 segments in NavAb-AB engage an interaction with the S4–S5 linker from a neighboring subunit near the intracellular activation gate (Fig. 1d). Electron density suggests the side-chain of Asn211 forms a stabilizing inter-subunit hydrogen bond with a backbone carbonyl in the S4–S5 linker (Fig. 1d), which is not formed in NavAb-I217C (Fig. 1e). Notably, Asn211 is the only universally conserved S6 residue in all Na_V channels (Supplementary Fig. 6), yet its close interaction with the S4–S5 linker is seen only in two of the four subunits in NavAb-AB due to structural asymmetry. Mutations of the equivalent S6 Asn residue in domains I and III of vertebrate Na_V channels, but not in domains II and IV, have dramatic effects on slow-inactivation^{20,21}. Thus, NavAb may offer the first structural views of a conserved interaction occurring during slow inactivation in the Na_V channel family.

Structural adjustments throughout the PM in NavAb-AB and NavAb-CD culminate in dramatic effects at the selectivity filter, where accumulating evidence implicates molecular rearrangements in slow inactivation gating in bacterial and mammalian Na_V channels (Supplementary Discussion). The selectivity filter in NavAb-I217C is rigidly anchored by a hydrogen bond (~3.0 Å) between Thr175 and Trp179 of neighboring subunits⁴. This landmark interaction forces the Thr175 and Leu176 carbonyls to point towards the ion

conduction pathway and positions the Glu177 side-chains squarely against the P2-helix (Fig. 2a, b). In NavAb-AB, two of the key Thr175-Trp179 interactions have become unlatched, since only a very weak hydrogen bond (~ 3.8 Å) could exist between these partners (Fig. 2a, b). In the unlatched subunits, Ser180 has also flipped its conformation to engage the Glu177 carboxylate of a neighboring subunit (Fig. 2a, b), and formation of this new hydrogen bond may correlate with entry into the inactivated state¹³. Concerted structural changes distort the geometry along the Thr175-Leu176 carbonyl funnel, which was perfectly sized to coordinate and conduct a fully hydrated square-planar Na^+ complex in NavAb-I217C (Supplementary Figs. 7 and 8). Analysis of the NavAb-WT pore diameters indicates a 1–2 Å narrowing and distortion of the backbone carbonyl geometry in the central and inner Na^+ coordination sites of the selectivity filter (Fig. 2a, Supplementary Figs. 7 and 8), suggesting that NavAb would be less permissive to the conduction of optimally hydrated Na^+ ions in these inactivated states.

Consistent with the existence of multiple inactivated states in NavAb (Fig. 1, Supplementary Fig. 1), the Thr175-Trp179 interaction network remains intact in NavAb-CD, as does the predominant side-chain conformation of Ser180 (Fig. 2a, b). However, the Gln172 side-chain, which makes a strong interaction with the Glu177 backbone carbonyl in NavAb-I217C, is disengaged from this interaction in the NavAb-CD channel (Fig. 2a, b). Loss of this supporting interaction would destabilize the selectivity filter, similar to unlatching the Thr175-Trp179 network described above in NavAb-AB. In fact, comparison of crystallographic temperature factors suggests that the entire P-helix displays increased mobility in these inactivated states (Fig. 2c). Therefore, destabilization of the selectivity filter and concomitant remodeling of the outer pore vestibule in NavAb-WT (Fig. 2; Supplementary Figs. 5 and 8) may correlate with entry into the inactivated state. This conclusion would be consistent with the effects of toxin binding, permeant ions, and mutations in the selectivity filter of mammalian Na_v channels on slow inactivation (Supplementary Discussion)³.

Overlaying the structural models of NavAb-AB and NavAb-CD onto NavAb-I217C provides insight into a network of structurally coupled residues unique to Na_v channels that scaffold the selectivity filter and line the surrounding S5 and S6 segments (Fig. 3a–b). Structure- based sequence alignment of the four linked domains from mammalian Na_v channels pinpoints analogous residues in the PM of NavAb: Phe144 and Phe152 in the S5 segment, Leu170 and Phe171 in the P-helix, Trp179 in the P2-helix, and Phe198 and Ile202 in the S6 segment (Fig 3a–b and Supplementary Fig. 6). Notably, substitution of the Leu170 equivalent or the Ile202 equivalent in $\text{Na}_v1.4$ dramatically alters its slow-inactivation^{22,23}. These comparisons highlight an evolutionarily conserved network of residues coupling the conformation of the intracellular activation gate to the selectivity filter through a molecular mechanism that results in collapse of the pore into a prominent dimer-of-dimers arrangement for all of the functional elements in NavAb-WT.

The structural changes observed in NavAb-WT also alter the central cavity, where amino acid side-chains analogous to those involved in drug binding in mammalian Na_v channels^{24–27} have a different spatial arrangement due to the asymmetric collapse of two S6 segments (Fig. 3c). Local anesthetics and related pore blockers of mammalian Na_v channels block the NavAb homologue NaChBac in a state-dependent manner^{6,16}. If similar asymmetric conformational changes occur during inactivation of mammalian Na_v channels (Supplementary Discussion), they could rationalize why pore-blocking drugs bind to and stabilize inactivated states of Na_v channels through interactions with only three of their four S6 segments^{25,26}.

Four striking lateral pore fenestrations in the PM of NavAb-I217C revealed a hydrophobic access pathway to the central cavity⁴. Compared to the nearly identical pore fenestrations in NavAb-I217C, two diagonal fenestrations have narrowed in NavAb-AB, while the adjacent two fenestrations have opened wider (Fig. 3b, c and Supplementary Figs. 9, 10). In NavAb-CD, both pairs of pore fenestrations are smaller than in NavAb-I217C (Fig. 3b, c and Supplementary Fig. 9, 10). Therefore, different-sized drugs and other hydrophobic molecules could potentially gain access to the NavAb central cavity through these differentially-sized pore fenestrations (Fig. 3c and Supplementary Figs. 9, 10). If mammalian Nav channels have similar pore fenestrations, our NavAb structures predict they would provide dynamic drug access to the central cavity during different stages of channel gating, as postulated by the modulated receptor hypothesis of drug action²⁸.

We can now compare the VSD structures from a single VGIC captured in different pore conformations for the first time. The S4 segments in all NavAb structures have a similar 3_{10} helical conformation from R1 to R4 (Fig. 4a), suggesting S4 does not undergo a major conformational change during inactivation. Surprisingly, crystallographic temperature factors indicate that the S4 segment is the most well-ordered region of the VSD (Fig. 4b). Upon superimposing the pore domains of NavAb, a hinge point is seen at the foot of the S5 segment (Fig. 4c), as reported previously when comparing closed-pore NavAb-I217C and open-pore Kv1.2 structures⁴. Hence, movements at this S5 gating hinge may be involved in both pore-opening⁴ and inactivation gating. Our NavAb structures do not provide evidence for transition of the S4 segment into a “relaxed” conformation²⁹; however, we do observe repositioning of the entire VSD around the PM (Fig. 4d, e). This movement of the VSD with respect to the PM is likely to be required for entry into the potentially inactivated states represented by the NavAb-AB and NavAb-CD models. Perhaps pivoting of the VSD around the PM at the S5 gating hinge forces collapse of two S6 segments into an asymmetric dimer-of-dimers conformation at the activation gate. Some gating-modifier toxins have binding determinants in both the VSD and neighboring PM of ion channels (Supplementary Discussion), including Nav channels³⁰, suggesting that nature has evolved a strategy to trap specific gating intermediates by binding toxins at this interface and locking the VSD and PM in fixed relative positions. This gating movement is a potential target for design of next-generation Nav blocking drugs that could have increased voltage-dependence and improved subtype selectivity.

Our NavAb crystal structures provide insight into conformational changes that may underlie the process of slow inactivation, a conserved property of Nav channels from bacteria to man (Supplementary Discussion). During the conformational changes that we propose lead to slow inactivation, the NavAb channel dramatically alters its central pore, moving from a nearly square arrangement in the selectivity filter, pore-lining S6 segments, and activation gate, to a strikingly asymmetric arrangement in which the four subunits morph into two pairs of conformations. This structural transition has dramatic consequences for all functional elements of NavAb, providing new templates for understanding the slow-inactivation process, the effects of disease mutations, and the complex properties of drugs that block mammalian Nav channels.

Methods Summary

WT NavAb was expressed in *Trichopulsia ni* insect cells, purified using anti-Flag resin and size exclusion chromatography, reconstituted into DMPC:CHAPSO bicelles, and crystallized over an ammonium sulphate solution containing 0.1 M Na-citrate, pH 4.75. A SAD data set from a SeMet-substituted protein crystal enabled phase determination and guided initial rigid body refinement protocols. Standard refinement procedures accounting

for merohedral twinning were performed. Electrophysiological experiments were carried out on WT NavAb in *Trichopulsia ni* cells using standard protocols.

Full Methods and associated references are available in the online version of the paper at www.nature.com/nature.

Methods

Protein expression and purification

To the best of our knowledge³¹, NavAb represents the only prokaryotic membrane protein to be over-expressed in a eukaryotic expression system for structural studies to date. NavAb was cloned into the pFASTBac-Dual vector preceded by an N-terminal Flag-tag. Recombinant baculovirus were generated using the Bac-to-Bac system (Invitrogen). *Trichopulsia ni* insect cells were harvested 72 h post-infection and resuspended in 50 mM Tris pH 8.0, 200 mM NaCl (Buffer A) supplemented with protease inhibitors and DNase. Following sonication, digitonin (EMD Biosciences) was added to 1% and solubilization was carried out for 1–2 h at 4° C. Clarified supernatant was agitated with anti-Flag M2-agarose resin (Sigma) pre-equilibrated with Buffer B (Buffer A supplemented with 0.12% digitonin) for 1–2 h at 4° C. Flag-resin was washed with ten column volumes of Buffer B and eluted with Buffer B supplemented with 0.1 mg/mL Flag peptide. NavAb was subsequently passed over a Superdex 200 column (GE Healthcare) in 10 mM Tris pH 8, 100 mM NaCl and 0.12% digitonin and peak fractions were concentrated using a Vivaspin (30K MWKO) centrifugal device. Selenomethionine-labeled proteins were expressed as previously described⁴ and purified as above.

NavAb crystallization and data collection

NavAb was concentrated to ~20 mg/mL and reconstituted into DMPC:CHAPSO (Anatrace) bicelles according to standard protocols³². The NavAb-bicelle preparation was mixed in a 1:1 ratio and setup in a hanging-drop vapor-diffusion format over a well solution containing ~2 M ammonium sulfate, 100 mM Na-citrate pH 4.75. Native and SeMet-labeled proteins crystallized under essentially identical conditions. Crystals were passed through solutions containing 2 M ammonium sulfate, 100 mM Na-citrate pH 4.75 and 28% glucose (wt/v) in increments of ~6% glucose during harvesting. As previously suggested³³, the inclusion of nicotinic acid at saturating concentration in the cryo solution was found to prolong the lifetime of our NavAb crystals in the x-ray beam. Crystals were plunged into liquid nitrogen and maintained at 100 K during all data collection procedures.

Over 1,500 NavAb crystals were screened at the Advanced Light Source (BL8.2.1 and BL8.2.2), but most WT NavAb crystals did not diffract beyond 3.5 Å. A single anomalous dispersion (SAD) data set collected near the selenium absorption edge ($\lambda = 0.9792$ Å) from a SeMet-labeled crystal was used to determine initial experimental phases and proved to be our best data set. In addition to nicotinic acid treatment³³, special care was taken to minimize exposure times and orient the NavAb crystals in order to maximize data completeness and quality.

Structure determination and refinement

X-ray diffraction data were integrated and scaled with DENZO/SCALEPACK³⁴ and further processed with the CCP4 package³⁵. Initial efforts to determine and refine the NavAb-WT structure using our previous NavAb-I217C model and various protocols led to R_{free} stalling at ~40%, even after accounting for the perfect merohedral twinning characteristic of the NavAb-WT crystals. Fortunately, unbiased experimental phases could be obtained using a 3.2 Å SAD-data set collected from a single SeMet-labeled crystal with the PHENIX

software package³⁶. The NavAb-I217C model was manually placed into this experimentally-phased map. Despite limited map quality, approximate boundaries to define rigid bodies in subsequent refinement procedures were apparent (at the S5 gating hinge) and led to an immediate ~6 % drop in the R_{free} factor. Complete and partial poly-Ala models were used in combination with SAD phases (in PHENIX³⁶) to assist with model re-building and side-chain placement in the program O³⁷. Partial models were similarly used to assess and confirm the boundaries of our WT model. Both protein chains in the WT-AB channel extend from amino acid 1–219; in the WT-CD channel, chain C extends from amino acid 1–213, and chain D extends from amino acid 1–217. Only fragmented or weak electron density can be seen beyond these modeled S6 residues. At this point, the SeMet-labeled data set was reprocessed to lower redundancy (to ~3) and used as “native” data, leading to an overall improvement in data and map quality. Electron density for nine lipids and three water molecules were accounted for at this stage of model building. Application of TLS groups³⁸, as implemented in REFMAC^{39,40}, led to a ~1.5 % drop in the R_{free} and further improvement in map quality. Although examined, NCS restraints were never applied during the refinement procedure due to the asymmetry immediately apparent within the NavAb-WT model. Tight geometric restraints were maintained throughout refinement and the overall geometry of the final NavAb-WT model is excellent (Supplementary Table 3). Only Arg68 in the S2–S3 loop (chain A and C) and Ser93 in the S3–S4 loop (chain A, C and D) appear as outliers in the Ramachandran plot (5 residues of the 880 modeled).

In order to facilitate comparison between our NavAb structures (i.e. Fig 2), and because NavAb-I217C was originally refined using the CNS software⁴¹ to 2.7 Å resolution⁴, the final scaled data set and deposited NavAb-I217C coordinates were re-refined using similar REFMAC procedures^{39,40} described above for WT-NavAb (to effective resolutions of 2.7 Å and 3.2 Å). The R/R_{free} , overall map quality, geometry, and root-mean-square deviation of all refined NavAb-I217C models are highly comparable.

Structure analysis

The geometry of NavAb-WT structural models was assessed using PROCHECK⁴². The pore radius was calculated using standard settings in the MOLE software⁴³ for Supplementary Fig. 3C and Supplementary Fig. 8. More detailed representations of the pore were obtained using the HOLLOW⁴⁴ software for Fig 3d–e, Supplementary Fig. 7a, and Supplementary Figs. 9–10. Structural alignments were performed using LSQMAN⁴⁵ and O³⁷. Unless otherwise stated, all figures have been prepared with the WT-AB and WT-CD channels independently aligned onto the selectivity filter (residues Thr175-Leu176-Glu177) of the tetrameric NavAb-I217C channel model. All structural figures were prepared with the PyMol software⁴⁶.

Electrophysiology

Baculovirus containing the WT-NavAb construct used for crystallography (i.e. containing an N-terminal Flag tag) were used to infect *Trichopulsia ni* cells. After 24 h, whole cell sodium currents were recorded using an Axopatch 200 amplifier (Molecular Devices, Sunnyvale, CA) with glass micropipettes (2–5 MΩ). Capacitance was subtracted and series resistance was compensated using internal amplifier circuitry; 80% of series resistance was compensated. The intracellular pipette solution contained (in mM): 35 NaCl, 105 CsF, 10 EGTA, 10 HEPES, pH 7.4 (adjusted with CsOH). The extracellular solution contained (in mM): 140 NaCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, pH 7.4 (adjusted with NaOH). Voltage clamp pulses were generated and currents were recorded using Pulse software controlling an Instrutech ITC18 interface (HEKA, Great Neck, NY). Data were analysed using Igor Pro 6.2 (WaveMetrics, Lake Oswego, OR).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Structure and function of NavAb

a, Use-dependent development of slow inactivation. Depolarizations from a holding potential of -180 mV to -40 mV, 7 ms in duration, were applied at 0.2 Hz (circles) or once per min (triangles), and the peak current elicited by each pulse was measured. Currents were normalized to the peak inward current during the first pulse. **b**, Main structural elements of NavAb. The nearest VSD and pore domain are removed for clarity. **c**, Packing arrangement in the WT NavAb crystals. Four crystallographically independent channel subunits are colored: purple (chain A), yellow (chain B), cyan (chain C) and pink (chain D). **d–e**, *Left*, red dashed lines indicate the C α location of D219 (the last S6 residue modeled in WT-AB), where the S6 helices are shown as cylinders. WT-chain A, purple; WT- chain B, yellow. *Middle and right*, space filling models in expanded boxes highlight Asn211 and the S6 interaction site of WT-chain A with Leu123 on the S4–S5 linker.

Figure 2. Structural changes in the selectivity filter of NavAb

a, Stick representation of the selectivity filter with a $2F_o - F_c$ map calculated at $3.2 \text{ \AA}$ resolution (grey mesh) contoured at 1.5σ for NavAb-I217C and 1.0σ for NavAb-AB and NavAb-CD. Symmetry-related subunits in WT-AB and WT-CD are colored white (Chains A and D) and cyan (Chains B and C), respectively. **b**, Hydrogen bonds ($< 3.5 \text{ \AA}$) supporting the selectivity, as discussed in the main text, are shown as dotted lines. **c**, Crystallographic b-factors are colored from low to high (blue to red) along the P-helix, selectivity filter, and P2-helix regions.

Figure 3. Conformational changes in the pore module of NavAb

a, Highly conserved residues in the PM of Nav channels are shown in stick and mesh representation. Mutations of the Leu170 and Ile202 side-chain equivalents (green and pink, respectively) in Nav1.4 alter slow-inactivation in Nav1.4. Asn211 (S6) is shown for reference. **b**, Superposition of the NavAb-I217C (grey) and NavAb-AB (yellow) PMs highlights a structurally-coupled, evolutionarily-conserved set of amino acid residues that communicate between the intracellular activation gate (S6), the central cavity (S5 and S6), and the selectivity filter (P- and P2-helices), and the selectivity filter. **c**, A view through the PM sectioned below the selectivity filter illustrates the lateral pore fenestrations, hydrophobic access to the central cavity, and structural asymmetry in the NavAb-AB and NavAb-CD pore domains. Phe203 side-chains are yellow sticks. NavAb residues implicated in drug binding in vertebrate Nav channels are colored: Thr206 (blue), Met209 (green), and Val213 (orange). **d–e**, Viewed from the plane of the lipid bilayer, the pore fenestrations provide dynamic access to the central cavity from the hydrophobic membrane phase. Phe203 side-chains are shown as stick and mesh representations (yellow). The yellow labels d and e on the structures in panel c show where the fenestrations illustrated in panels d and e are located in the complete structure. Note the positions of Phe203 illustrated in yellow in panels c-e for orientation.

Figure 4. Structure and coupling of the VSD in NavAb

a, Superposition of the S4 segment backbone from NavAb-I217C with WT-AB and WT-CD models (white, yellow, and salmon, respectively) shows high structural similarity. **b**, Crystallographic b-factors colored from low to high (blue to red) along one WT-AB subunit highlight the mobility of the S1–S3 segments relative to the S4 segment. **c**, The distribution of b-factors further suggests a structural coupling between the S4 segment, the S4–S5 linker, and the S5 segment. **c**, Superposition of the pore domains from NavAb-I217C (gray), WT-AB (yellow), and WT-CD (red) demonstrates a major hinge point at the base of the S5 segment. **d**, Selectivity filter-based superposition of the NavAb-AB and NavAb-CD channels illustrates a rolling motion of the VSDs around the PM during inactivation gating as viewed from the extracellular side of the membrane. Displacements of the VSDs measure up to ~ 5 Å. **e**, View from the plane of the membrane highlights the relative vertical and horizontal displacements of the WT NavAb VSDs during inactivation gating. Chain B, yellow; Chain C, red. **f**, Similar view of Chain A, yellow; Chain D, red. The labels on the structures in panel d show where the fenestrations illustrated in panels e and f are located in the complete structure.