Molecular architecture of a sodium channel S6 helix: radial tuning of the Nav1.7 activation gate

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Running title: Molecular architecture of Nav1.7 DII/S6

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Background: In-frame deletion mutation (Del-Leu955) in Nav1.7 sodium channel from a kindred with erythromelalgia hyperpolarizes activation.

Results: Del-Leu955 twists the S6 helix, displacing the Phe960 activation-gate. Replacement of Phe960 at the correct helical position depolarizes activation.

Conclusion: Radial tuning of activation-gate is critical to the activation of Nav1.7.

Significance: Structural tuning guided electrophysiology reveals the functional importance of radial tuning of the S6 segment.

Summary

Voltage-gated sodium (Nav) channels are membrane proteins that consist of 24 transmembrane segments organized into four homologous domains, and are essential for action potential generation and propagation. Although the S6 helices of Nav channels line the ion conducting pore and participate in channel activation, their functional architecture is incompletely understood. Our recent studies show that a naturally-occurring in-frame deletion mutation (Del-Leu955) of Nav1.7 channel, identified in individuals with a severe inherited pain syndrome (inherited erythromelalgia, IEM) causes a substantial hyperpolarizing shift of channel activation. Here we took advantage of this deletion mutation to understand the role of the S6 helix in the channel activation. Based on the recently published structure of a bacterial NaV channel (NavAb), we modeled the WT and Del-Leu955 channel. Our structural model showed that Del-Leu955 twists the DII/S6 helix, shifting location and radial orientation of the activation gate residue (Phe960). Hypothesizing that these structural changes produce the activation shift of Del-Leu955 channels, we restored a phenylalanine in wild-type orientation by mutating Ser961 (Del-Leu955/Ser961Phe), correcting activation by ~10 mV. Correction of the displaced Phe960 (Phe960Ser), together with introduction of the rescuing activation gate residue (Ser961Phe) produced an additional ~6 mV restoration of activation of the mutant channel. A simple point mutation in the absence of a twist (Leu955Ala) did not produce a radial shift, and did not hyperpolarize activation. Our results demonstrate the functional importance of radial tuning of the sodium channel S6 helix for the channel activation gate.

Introduction

The Nav1.7 channel, a member of the voltage-gated sodium channel family preferentially expressed in dorsal root ganglia (DRG) and sympathetic ganglia neurons, plays a critical role in pain signaling (1,2). The NaV1.7
channel amplifies subthreshold membrane depolarizations, contributes to the generation of action potentials (3,4), and may facilitate neurotransmitter release at the central terminals of DRG neurons within the spinal cord (5). Loss-of-function NaV1.7 channel mutations cause congenital indifference to pain (CIP) (6) while gain-of-function missense NaV1.7 mutations cause several painful disorders including inherited erythromelalgia (IEM) (7-9), Paroxysmal Extreme Pain Disorder (PEPD) (10,11) and Small Fiber Neuropathy (SFN) (12-14). These gain-of-function missense mutations represent experiments of nature that may shed light on the structural basis of sodium channel function.

The large pore-forming α-subunit of the mammalian NaV1.7 channel, similar to all members of this channel family, consists of four homologous domains (I-IV) linked by three intracellular loops (L1-L3). Each domain of the α-subunit has six transmembrane helices (S1-S6). The S1-S4 helices form the voltage sensor domain whereas the S5 and S6 helices, together with a membrane-reentrant pore loop (P-loop) between S5 and S6, form the ion-conducting pathway. The S6 helices are the backbone of the ion-conducting pathway, and multiple residues in the S6 helices have been implicated in channel activation and inactivation. However, there is still not a full understanding of the contribution of the S6 helices to channel activation. We recently identified a gain-of-function in-frame deletion (Del-Leu955) within S6 of domain II of Nav1.7 from an IEM family, which causes a robust hyperpolarizing shift of activation and slow-inactivation (15). Here, we took advantage of the structural change within the S6 helix produced by Del-Leu955 to investigate the contribution of the S6 helix to the channel activation. Using structural modeling-guided mutagenesis and patch-clamp electrophysiology, we demonstrate that the radial orientation of the DII activation gate residue (Phenylalanine-960, Phe960) within S6 is essential for channel activation. The displaced Phe960 in the Del-Leu955 mutant channel disrupts the activation gate, leading to a disease-causing hyperpolarizing shift of channel activation. Our results demonstrate the importance of radial tuning of the S6 helix for the NaV1.7 channel activation gate, and suggest that structural modeling-guided mutagenesis can contribute to our understanding of the functional architecture of voltage-gated sodium channels.

**Experimental Procedures:**

**Structural modeling**

Structural modeling was performed as previously described (16,17). Briefly, a two-step method was used for construction of hNaV1.7 channel. First, four transmembrane domain structural models were generated by a membrane-bound protein predication algorithm GPCR-ITASSER (18,19). Then, each single domain model was aligned to the corresponding domain of the recently solved bacterial sodium channel (PDB ID: 3RVY) (20) by TM-align (21). The four transmembrane domains models were assembled in a clockwise order viewed from extracellular side as suggested previously (22,23). The resulting four domain complex structural model was finally refined by FG-MD to remove inter-domain clashes and improve model quality (19,24,25).

**Plasmid preparation and HEK293 cell transfection**

TTX-resistant human NaV1.7 wild-type (WT) channel (hNaV1.7r) was constructed based on the hNaV1.7 (mRNA: NM_002977.3; protein: NP_002968.1 of NCBI database)(26). Del-Leu955 and other mutant channels were constructed on the hNaV1.7r background. These channels were transfected into HEK293 cells together with human β-1 and β-2 subunits (27) using Lipofectamine (Invitrogen, Carlsbad, CA, USA), as previously described (16). HEK293 cells were maintained in 1:1 Dulbecco’s modified Eagle’s media (DMEM)/F-12 supplemented with 10% fetal bovine serum (FBS, Hyclone) in a humidified 5% CO2 incubator at 37 °C. HEK293 cells were seeded onto poly-L-lysine coated glass coverslips (BD Biosciences, San Jose, CA, USA) in a 24 well plate 1 day before recording. The functions of all the mutant constructs of Nav1.7 channel were screened using the PatchXpress automated parallel patch-clamp system (Molecular Devices) (28).
Voltage-clamp recording

Whole-cell voltage-clamp recordings were obtained after 1 days of transfection as described previously (16) (29). The extracellular solution contained the following (in mM): 140 NaCl, 3 KCl, 1 MgCl$_2$, 1 CaCl$_2$, 20 Dextrose and 10 HEPES, pH=7.3 with NaOH (320 mOsm adjusted with dextrose). The pipette solution contained the following in (mM): 140 Cs-Fluoride, 10 NaCl, 1.1 EGTA, 10 HEPES, 20 Dextrose, pH=7.3 with CsOH (310 mOsm adjusted with dextrose). The pipette solution contained the following in (mM): 140 Cs-Fluoride, 10 NaCl, 1.1 EGTA, 10 HEPES, 20 Dextrose, pH=7.3 with CsOH (310 mOsm adjusted with dextrose). Patch pipettes had a resistance of 1-2 mΩ when filled with pipette solution. After achieving whole-cell recording configuration, the pipette and cell capacitance were manually minimized using the Axopatch 200B (Molecular Devices) compensation circuitry. Series resistance and prediction compensation (80-90%) were applied to reduce voltage errors. Recorded currents were digitized using pClamp software and a digidata 1440A interface (Molecular Devices) at a rate of 50 kHz after passing through a low-pass Bessel filter setting of 10 kHz. The recording was initiated after a 5 min equilibration period after establishing whole-cell configuration. To generate activation curves, cells were held at -140 mV and stepped to potentials of -80 to +40 mV in 5 mV increments for 100 ms. Peak inward currents were automatically extracted by Origin and fitted with Boltzmann function to determine the half activation ($V_{1/2}$), activation curve slope at half activation ($Z$) and reversal potential ($E_{Na}$) for each recording. Conductance was calculated as $G=I/(V_m-E_{Na})$ and were normalized by the maximum conductance value and fit with a Boltzmann equation.

Data analysis

Data were analyzed with Clampfit 9.2 (Molecular Devices) and OriginPro 8.5 (Microcal Software). Student’s t-test was used and statistical significance was accepted when $p<0.05$. Data was presented as means ± SEM.

Results

The Del-Leu955 deletion mutation hyperpolarizes activation of Na$\text{\textsubscript{v}}$.1.7 channel.

Our previous work has demonstrated that an in-frame deletion (Na$\text{\textsubscript{v}}$.1.7 Del-Leu955) within the DII/S6 of the Na$\text{\textsubscript{v}}$.1.7 channel (Fig.1A) produces inherited erythromelalgia (IEM) (15). This deletion occurs in the highly conserved S6 region (Fig.1A) and results in a dramatic (~25 mV) hyperpolarizing shift in the voltage-dependence of activation (15). We evaluated channel activation from cells expressing WT (Fig.1B) and Del-Leu955 mutant channels (Fig.1C) using a recording protocol for voltage-dependence of activation. We confirmed a robust shift in the activation $V_{1/2}$ (voltage at half-activation) for Na$\text{\textsubscript{v}}$.1.7 Del-Leu955 channel (Del-Leu955: -50 ± 1.6 mV, n=11) compared to Na$\text{\textsubscript{v}}$.1.7 WT channel (-23.8 ± 1.4 mV, n=7, $P<0.001$, Fig.1D). Inactivation kinetics was also analyzed. Within the range more positive than -45 mV, where WT channels begin to activate, inactivation kinetics was more rapid for Del-Leu955 mutant channel (e.g. fast inactivation time constant $\tau = 1.53 ± 0.17$ at -35 mV, n=7) compared to WT ($\tau = 2.79 ± 0.32$ at -35 mV, n=6, $P<0.01$). Between -35 mV to 0 mV, where Del-Leu955 and WT channel are both activated, no significant differences were found. This result is consistent with our previous report (15).

Structural modeling reveals radial displacement of the activation gate (Phe960) in the Na$\text{\textsubscript{v}}$.1.7 Del-Leu955 mutant channel.

To understand the structural basis of this change, we constructed an atomic-level structural model of the wild-type (WT) and Del-Leu955 Na$\text{\textsubscript{v}}$.1.7 channel based on the bacterial voltage-gated sodium channel NavAb (3RVY) (20) using our previously published methods (16). As can be seen from the structural models (Fig. 2A), the activation gate in WT Na$\text{\textsubscript{v}}$.1.7 channel consists of four aromatic residues, one from each S6 helix (DI: Tyr405, DII: Phe960, DIII: Phe1449, and DIV: Phe1752) (30). Key residues investigated in this study are colored and circled for highlighting: Phe960 as red and Ser961 as yellow. Leu955 is located 5 residues upstream of the activation gate residue (Phe960). When the Leu955 residue is deleted, the orientation of Phe960 (red) shifts radially towards the S6 of domain III (Fig. 2B). Del-Leu955 also rotates Serine 961 (Ser961, yellow) to the previous location of Phe960 so that the side chain of Ser961 now points to the pore, facing the other three activation gate residues.
(Fig. 2B). Side (Fig. 2C) and top (Fig. 2D) views of the four S6 helices of Del-Leu955 channel show that the hydrophobic ring which forms the intact activation gate in WT channels is disrupted in Del-Leu955 channels because of the displaced Phe960 (Fig. 2C, D). According to our model, the DII/S6 helix extends a few residues below the activation gate residue and becomes a flexible loop as it transitions to L3 (the third intracellular loop). As we previously demonstrated that the intact activation gate is essential for wild-type channel gating (30), we hypothesized that the radial displacement of Phe960 is a structural substrate that contributes strongly to the altered activation of the Del-Leu955 channel.

Restoring Phe in the activation gate location depolarizes activation.

Our structural model reveals that in the Del-Leu955 channel, the side chain of Ser961 at DII/S6 becomes aligned with the other activation gate residues from DI, DIII and DIV (Fig. 2B). The side chain of Ser961, however, is not likely to contribute to maintaining the hydrophobic ring needed for an effective activation gate (Fig. 2B) (30). We hypothesized that the disrupted activation gate contributes to the hyperpolarizing shift of activation in Del-Leu955 channel. We tested this hypothesis by asking whether restoration of the hydrophobic ring, by mutating Ser961 back to Phe, would depolarize activation of the Del-Leu955 channel. Structural modeling (Fig. 3A) shows that this newly introduced Phe (yellow) would be situated in a location similar to the original Phe, so that it would be expected to interact with the other three hydrophobic residues to restore the activation gate (Fig. 3A). Note that the displaced Phe960 is in red. For this experiment, we created Del-Leu955/Ser961Phe double mutation and compared it with Del-Leu955 channel. Nav1.7 WT channel activation is shown for comparison (-23.8 ± 1.4 mV, n=7). As shown in Fig. 3B, Del-Leu955/Ser961Phe mutant channel displayed a robust ~+10 mV depolarizing shift in activation compared with that of Del-Leu955 (Del-Leu955: -50 ± 1.6 mV, n=11, vs Del-Leu955/Ser961Phe: -40.2 ± 1.5 mV, n=11, P<0.001), suggesting that introduction of a Phe to restore the activation gate depolarizes channel activation.

Correcting the displaced Phe further depolarizes activation.

Structural modeling also suggested that the displaced Phe960 (red) may influence the conformation of S6 helix of DIII that contributes to altered activation of the Del-Leu955 channels (Fig. 3A). To test this hypothesis, we mutated this displaced Phe960 into Ser (red, Fig. 3C), which was the original residue at this location. Voltage-clamp analysis showed that this additional mutation further shifted the activation $V_{1/2}$ by ~+5 mV (Del-Leu955/Ser961Phe: -40.2 ± 1.5 mV, n=11; Del-Leu955/Ser961Phe/Phe960Ser: -35.1 ± 1.5 mV, n=9, P<0.05) (Fig. 3D). To further test the effect of residue size at this site, we mutated the Phe960 to Alanine (Ala), a small residue predicted to have a minimal structural effect, and created a Del-Leu955/Ser961Phe/Phe960Ala mutation. The activation $V_{1/2}$ of this mutation was comparable to that of Del-Leu955/Ser961Phe/Phe960Ser mutation, shifting the $V_{1/2}$ by ~6 mV (-34.4 ± 1.1 mV, n=9, P<0.01 compared to Del-Leu955/Ser961Phe) (Fig. 3D). These results suggest that a displaced bulky Phe may influence the adjacent DIII/S6 helix lining the pore, thus affecting channel activation. Taken together, introducing a new Phe into the appropriate location within the activation gate and replacement of the displaced Phe with a smaller residue corrected the activation $V_{1/2}$ of Del-Leu955 channel by more than 60%.

A point mutation of residue 955, in the absence of a twist, does not cause a hyperpolarizing shift of activation.

As we hypothesized that the strong depolarizing shift of the Del-Leu955 channel is due to an in-frame deletion causing the twist of S6 helix, especially the displacement of the activation gate residue Phe960 at the more distal portion of the S6 helix, we further asked whether a point mutation of Leu955, in the absence of a twist, could hyperpolarize channel activation. If a point mutation of Leu955 were not able to hyperpolarize channel activation, it would further suggest that the twist of the S6 helix by
Leu955 deletion, rather than a residue change of Leu955, is likely to be the underlying mechanism of the activation shift. Alanine was chosen due to its small effect on the protein structure and because it has been found to be frequently located in transmembrane helices (31). Structural modeling showed that Leu955 in WT channel pointed towards a space formed by S4-5 linker and S6 helix (Fig. 4A). When Leu955 was mutated to Ala, the side chain was smaller and the structural effect was subtle (Fig. 4A, B). Interestingly, the activation of Leu955Ala channel (Leu955Ala: -19.1 ± 2.2 mV, n=7) was close to that of WT channel (WT: -23.8± 1.4 mV, n=7, P>0.05, Fig. 4C), which is markedly different from Del-Leu955/Ser961Phe/Phe960Ala channel or all the other mutant channels introduced in this study (P<0.01, Fig. 4C). Therefore, our data strongly suggest that the twist of the S6 helix, rather than the local change of the Leu955 residue, underlies the strong shift of activation seen in Del-Leu955 channels.

Discussion

In this study, we took advantage of a naturally occurring deletion (Del-Leu955) mutation of the Na$_v$1.7 sodium channel that produces a large hyperpolarizing shift in channel activation, and employed a combined approach of structural modeling, mutagenesis and voltage-clamp electrophysiology to understand the contribution of the functional architecture of the S6 transmembrane helix to channel activation. Using structural modeling, we found that this in-frame deletion results in a twist of the DII S6 helix beginning several residues above the activation gate residue (Phe960), causing a radial shift of this residue towards the S6 helix of DIII instead of the ion-conducting pore. Virtual mutagenesis predicted that the substitution Ser961 to Phe (Del-Leu955/Ser961Phe) might restore the WT composition of the activation gate and thus partially rescue activation. This manipulation indeed depolarized activation $V_{1/2}$ by ~10 mV, supporting our hypothesis. As modeling further suggested that the originally displaced Phe960 might have a functional effect on the S6 of DIII, we additionally mutated the displaced Phe960 into a Ser (Del-Leu955/Ser961Phe/Phe960Ser) to mimic the original residue composition of this part of the DII/S6. This substitution resulted in an additional ~5 mV shift of activation $V_{1/2}$ towards the WT value. Consistent with a steric influence, we observed a depolarization of the activation $V_{1/2}$ with Del-Leu955/Ser961Phe/Phe960Ala. To understand whether the local change of the Leu955 residue contributes to this hyperpolarizing shift of activation, we created and assessed a point mutation (Leu955Ala) that did not introduce a twist. This manipulation placed the activation $V_{1/2}$ within the range of that of the WT Na$_v$1.7 channel, with a slight depolarizing tendency that did not reach statistical significance. Taken together, these results indicate that the structural integrity of the helix rather than a single amino acid change in this region is responsible for the large hyperpolarizing shift of activation. Our results support a model in which radial orientation of critical residues within S6 plays a key role in shaping channel activation.

The S6 helix directly lines the ion-conducting pathway, and contributes to several aspects of channel gating. Point mutations of the Na$_v$1.7 channel in patients with IEM reported so far all hyperpolarize activation (1,2). These point mutations, while informative for understanding channel gating, do not provide insight into the functional architecture of the S6 helix. An in-frame deletion such as Del-Leu955, on the other hand, causes a more significant change to the overall architecture. We therefore used this deletion as a tool to identify key features of the S6 helix and demonstrated that a radial displacement of Phe960 in the Del-Leu955 mutant channel disrupted the activation gate, and that the displaced bulky Phe appears to influence a neighboring helix. Interestingly, in our previous study, we found that direct mutation of activation gate Phe960 (Phe960Val) leads to a ~6 mV hyperpolarizing shift of activation (30), emphasizing the importance of intact activation gates. The current study reinforces this finding, and additionally shows that for the DII activation gate, radial orientation is essential. Interestingly, our earlier study suggests that replacing Phe with a larger Trp would not produce additional effects regarding the channel activation (30). Although the
mutations we introduced in this study depolarize activation significantly towards WT channel, the effect is incomplete. This may be because the in-frame deletion may cause more profound changes to channel structure in additional to the twist of the activation gate (e.g. the orientation of all the residues of DII/S6 helix downstream of Phe960 was shifted). Addition of another residue after the Phe would not resolve this issue, since neither crystal structure nor molecular biophysical analysis have definitively identified the end of the S6 helix of hNav1.7 channel.

Following the initial report of the structure of the bacterial voltage-gated sodium channel (NavAb, 3RVY) (20), the crystal structures of other bacterial NaV channels (NavRh, 4DXW) (32) and (NavM, 4F4L) (33), as well NavAb in an inactivated state (4EKW) (34) have been reported. These new structures suggest an asymmetric organization of the four S6 helices, despite the fact that these are homotetrameric channels. With the availability of new structures, we asked how they may affect our modeling of the activation gate region of the NaV1.7 channel. In figure 5, we align both the WT and Del-Leu955 NaV1.7 structural models with NavRh (4DXW). Within this particular region, the activation gate residues (Tyr405 of DI, Phe960 of DII, Phe1449 of DIII, and Phe1752 of DIV) of NaV1.7 align reasonably well with those of NavRh (Leu219), although it is clear that when the helices extend towards the extracellular direction, they begin to separate. Given that NavRh and NavAb are different bacterial sodium channels, it is understandable that notable differences exist between NavRh and NavAb (35), and between our NaV1.7 structural model and NavRh or NavAb. Nevertheless, within the activation gate region, the structural elements are relatively conserved: therefore it is not surprising that our modeling provided guidance for our mutagenesis and patch-clamp experiments, which permitted us to dissect the architecture of an S6 helix.

Traditional, systematic mutagenesis using Alanine (36), cysteine (37) or tryptophan scanning (38,39) has yielded important information regarding the structure-function relationship of sodium channels. However, this approach is relatively time- and resource-consuming. In the present study, structural modeling provided insights regarding which residues to change via site-directed mutagenesis to test our hypothesis. With the increasing availability of sodium channel crystal structures (20,32,34), structural modeling-guided mutagenesis may provide an approach that is direct, and time- and resource-efficient.

In summary, our studies indicate that a naturally occurring gain-of-function deletion mutation (Del-Leu955) causes a radial shift of the S6 helix of NaV1.7 channel, resulting in a disruption of the activation gate and displacement of a bulky residue which affects the neighboring DIII S6 helix. Our results demonstrate an important contribution of radial tuning of S6 to activation and suggest that structural modeling-guided mutagenesis may provide a useful tool for understanding the functional architecture of voltage-gated sodium channels.
References:


Figure legends:

**Figure 1.** The Del-Leu955 deletion mutation hyperpolarizes activation of Nav1.7 channel

A, Schematic of the human Nav1.7 channel topology showing residue Leu955. All the rescue mutations we introduced in this study were listed in below. B-C, Representative traces of current families recorded from HEK293 cells expressing WT (B) and Del-Leu955 (C) channel. D, Del-Leu955 mutant channel was studied in parallel with WT channel in HEK293 cells using a voltage-dependence of activation protocol. (Del-Leu955: -50 ± 1.6 mV, n=11, Nav1.7 WT: -23.8 ± 1.4 mV, n=7, P<0.001). Stimulus protocol for activation is also shown as an insert.

**Figure 2.** Radial displacement of the activation gate (Phe960) in Del-Leu955 channel

A, Structural model of WT Nav1.7 channel shows four residues, one from each domain (Tyr405 of DI, Phe960 of DII, Phe1449 of DIII, and Phe1752 of DIV) forms the activation gate. Phe960 is colored red and Ser961 is colored yellow; both of them are circled for highlighting. B, In the Del-Leu955 structural model, when Leu955 is deleted, the orientation of Phe960 rotates radially towards the S6 of domain III. The Leu955 deletion also rotates Ser961 to the previous location of Phe960 so that the side chain of Ser961 now points to the pore, facing the other three activation gate residues. Phe960 of Del-Leu955 channel is shown in red and Ser961 is shown in yellow. Both are circled for highlighting. C, The intramembrane view of four S6 helices of the Del-Leu955 mutant channel structural model. All other structural elements are omitted for clarity. D, Extracellular view of the four S6 helices of the Del-Leu955 mutant channel structural model.

**Figure 3.** Restoring the activation gate and removing the displaced Phe depolarize activation

A, Structural modeling suggests that mutating Ser961 to Phe may restore the activation gate composition. Phe960 is shown in red and Ser961Phe is shown in yellow; both of them are circled for highlighting. Note
that Phe960 (red) is in a displaced position. 

B, Del-Leu955/Ser961Phe mutant channel was created and studied in parallel with Del-Leu955 channel in HEK293 cells. Del-Leu955/Ser961Phe displayed a robust ~+10 mV depolarizing shift in activation $V_{1/2}$ compared with that of Del-Leu955 (Del-Leu955: -50 ± 1.6 mV, n=11, vs Del-Leu955/Ser961Phe: -40.2 ± 1.5 mV, n=11, P<0.001). Both Del-Leu955 and WT curves (here and in the following figures) are a re-plot of Figure 1. C, Structural model shows the correction of a displaced Phe960 into Ser (circled and red) together with an introduced Phe (circled and yellow) in the activation gate helical position. D, The displaced Phe960 was mutated to Ser, which was the original residue at this location. This additional mutation further shifted activation $V_{1/2}$ by ~5 mV (Del-Leu955/Ser961Phe: -40.2 ± 1.5 mV, n=11; Del-Leu955/Ser961Phe/PHe960Ser: -35.1 ± 1.5 mV, n=9; P<0.05). Phe960 was also mutated to Ala (Del-Leu955/Ser961Phe/Phe960Ala) and the activation $V_{1/2}$ of this mutation (-34.4 ± 1.1 mV, n=9) was comparable to that of Del-Leu955/Ser961Phe/Phe960Ser mutation but significantly different from Del-Leu955/Ser961Phe mutation (P<0.01).

**Figure 4. A point mutation of residue 955 that does not introduce a twist does not depolarize activation.**

A-B, Structural model of WT and Leu955Ala mutant channel. Leu955 is highlighted in white and circled (A). Ala955 is highlighted in grey and circled (B). No obvious structural difference was observed. C, The Leu955Ala point mutation was created and studied. The activation $V_{1/2}$ of Leu955Ala mutant channel (-19.1 ± 2.2 mV, n=7) was comparable to that of WT channel (-23.8± 1.4 mV, n=7, P>0.05) but very much different from Del-Leu955/Ser961Phe/Phe960Ala or all the other mutant channels introduced in this study (P<0.01).

**Figure 5. Alignment of NaV1.7 structural model with NavRh structures**

A, Cytoslic view of the alignment of NaV1.7 structural model with that of NavRh. NaV1.7 is shown in wheat and NavRh is shown in grey. Tyr405 of DI, Phe960 and Ser 961 of DII, Phe1449 of DIII, and Phe1752 of DIV is shown in stick configuration. Activation gate of NavRh (Leu219) was also shown in stick configuration. B, Extracellular view of the alignment of NaV1.7 structural model with NavRh. C, Cytoslic view of the alignment of Del-Leu955 mutant channel structural model with NavRh. Del-Leu955 is shown in cyan and NavRh is shown in grey. D, Extracellular view of the alignment of Del-Leu955 mutant channel structural model with NavRh.

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**Disclosure:** The authors report no conflicts of interest.
Fig. 1 Yang et al

A

residues

Nav1.7 WT  951- LFLALLSSFSSDNL -965
Nav1.7 Del-Leu955  951- LFLA-LLSSFSSDNL -965

Mutation abbreviations used:
Del-L  Deletion-Leu955
Del-L/SF  Deletion-Leu955/Ser961Phe
Del-L/SF/FS  Deletion-Leu955/Ser961Phe/Phe960Ser
Del-L/SF/FA  Deletion-Leu955/Ser961Phe/Phe960Ala
LA  Leu955Ala

B

C

3 nA

3 ms

2 nA

5 ms

D

Normalized conductance

Voltage (mV)
Fig. 2 Yang et al
Fig. 3 Yang et al
Fig. 4 Yang et al