Size Matters: Erythromelalgia Mutation S241T in Nav1.7 Alters Channel Gating*

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The Nav1.7 sodium channel is preferentially expressed in most nociceptive dorsal root ganglion neurons and in sympathetic neurons. Inherited erythromelalgia (IEM, also known as erythermalgia), an autosomal dominant neuropathy characterized by burning pain in the extremities in response to mild warmth, has been linked to mutations in Nav1.7. Recently, a substitution of Ser-241 by threonine (S241T) in the domain I S4-S5 linker of Nav1.7 was identified in a family with IEM. To investigate the possible causative role of this mutation in the pathophysiology of IEM, we used whole-cell voltage-clamp analysis to study the effects of S241T on Nav1.7 gating in HEK293 cells. We found a hyperpolarizing shift of activation midpoint by 8.4 mV, an accelerated time to peak, slowing of deactivation, and an increase in the current in response to small, slow depolarizations. Additionally, S241T produced an enhancement of slow inactivation, shifting the midpoint by −12.3 mV. Because serine and threonine have similar biochemical properties, the S241T substitution suggested that the size of the side chain at this position affected channel gating. To test this hypothesis, we investigated the effect of S241A and S241L substitutions on the gating properties of Nav1.7. Although S241A did not alter the properties of the channel, S241L mimicked the effects of S241T. We conclude that the linker between S4 and S5 in domain I of Nav1.7 modulates gating of this channel, and that a larger side chain at position 241 interferes with its gating mechanisms.

Naturally occurring mutations, frequent single amino acid substitutions, in voltage-gated sodium channels can cause disorders of excitable tissues such as epilepsy (1, 2), cardiac arrhythmia (3, 4), muscle diseases (5, 6), and the inherited painful neuropathy, inherited erythromelalgia (IEM,3 also known as erythermalgia) (7). Recently, IEM was linked to mutations in the voltage-gated sodium channel Nav1.7 (8–12). Nav1.7 is preferentially expressed in dorsal root and sympathetic ganglion neurons (13–15).

The Nav1.7 IEM mutations, which have been investigated to date, shift the midpoint of activation to hyperpolarized potentials, except for F1449V, all slow channel deactivation time constants, and increase the response to slow, small depolarizations (ramp currents) (8, 11, 16–18). Expression of F1449V and L858H mutant Nav1.7 channels in dorsal root ganglion neurons leads to an increase in excitability as measured by a reduced threshold and increased firing frequency, as would be expected from the changes in gating properties (8, 17).

The seven missense mutations in Nav1.7, which have been described to date (8–12, 17), alter residues that are highly conserved among all sodium channels. Except for two mutations in S4 and S6 of domain I (DI), the other five mutations change residues in cytoplasmic linkers, with one mutation in the S4-S5 linker of domain I (DI S4-S5), three mutations in the DII S4-S5, and one mutation in the loop between DIII and DIV (7). Ser-241 to threonine (S241T) mutation in DI S4-S5 was identified in several generations of a Flemish family with IEM (10). The S241T substitution might be considered a conservative change because of the similar chemical properties of the side chain, which can serve as a phosphoacceptor site. We reasoned that the increase in size of the side chain by one methyl group might alter the local folding of the linker or cause a long-range allosteric effect in other structures, which could affect properties of the channel leading to neuronal hyperexcitability. We used whole-cell patch-clamp methods to study the biophysical effects of the naturally occurring S241T mutation and then tested the hypothesis that these effects are attributable to the longer side chain of threonine by investigating the effect of substituting Ser-241 by alanine (S241A) and leucine (S241L). Our data show that residue 241 altered channel gating and suggest that channel function was sensitive to the size of the amino acid residue at that position.

**EXPERIMENTAL PROCEDURES**

Plasmids and Stable Cell Lines—The S241T, S241A, and S241L mutations were introduced individually into hNav1.7R.

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3 The abbreviations used are: IEM, inherited erythromelalgia; DI, domain I; DRG, nociceptive dorsal root ganglion; WT, wild type.
which carried a tetrodotoxin-resistant version of human
Nav1.7 cDNA (19) using QuickChange XL site-directed
mutagenesis reagents (Stratagene, La Jolla, CA). Human
embryonic kidney cells (HEK293) were transfected with
the individual mutant channel construct using Lipofectamine 2000
(Invitrogen) according to the recommendations of the manufac-
turer. Transfected HEK293 cells, grown under standard cul-
ture conditions (5% CO₂, 37 °C) in 50% Dulbecco's modified
Eagle's medium, 50% F-12 supplemented with 10% fetal bovine
serum, were treated with G418 for several weeks to derive stable
cell lines that expressed the mutant channel. We have previ-
ously demonstrated that the gating properties of Nav1.7 in
transfected HEK293 cells are similar to those of Nav1.7 in
native DRG neurons (19). Stable cell lines were chosen to min-
imize variability in transient transformations. Nevertheless,
the current density for cell lines expressing all three mutant deriv-
avatives of Nav1.7 were lower (318.7 ± 29.8 pA/pF for S241T,
210.8 ± 21.7 pA/pF for S241A, and 344.0 ± 39.0 pA/pF for
S241L) than for wild type Nav1.7r (556.2 ± 41.6 pA/pF), likely
because of the site of integration of the plasmid within the
HEK293 genome; however, we cannot rule out the possibility
that these substitutions affect single channel properties or sta-
bility of the channel within the cell membrane.

Electrophysiology—Whole-cell voltage-clamp recordings
(20) of HEK293 cells stably expressing the sodium channels
Nav1.7r, S241T, S241A, or S241L derivatives were performed with
an EPC-9 amplifier (HEKA Electronics, Lambrecht/Pfalz,
Germany) using fire polished 1–1.5 MΩ electrodes (World Pre-
cision Instruments, Inc., Sarasota, FL). The pipette solution
contained (in mM): 140 CsF, 10 NaCl, 1 EGTA, and 10 HEPES;
302 mosmol (pH 7.4, adjusted with CsOH), and the extracellu-
lar bath contained (in mM): 140 NaCl, 3 KCl, 10 glucose, 10
HEPES, 1 MgCl₂, 1 CaCl₂, 0.0003 tetrodotoxin; 310 mosmol
(pH 7.4, adjusted with NaOH). Tetrodotoxin was added to the
bath solution to block all endogenous voltage-gated sodium
currents, which might be present in HEK293 cells (21) and
thereby study Nav1.7r in isolation. All recordings were con-
ducted at room temperature (−21 °C). The pipette potential
was adjusted to zero before seal formation, and the voltages
were not corrected for liquid junction potential. Capacity tran-
sients were cancelled, and series resistance was compensated
by 65–95%. Leakage current was subtracted digitally online using
hyperpolarizing potentials applied after the test pulse (P/4 pro-
cedure), except for ramp current recordings. Currents were
acquired using Pulse software (HEKA Electronics), filtered at
2.9 kHz, and sampled at a rate of 20 kHz.

Voltage protocols were carried out 4 min after establishing
cell access. Briefly, standard current-voltage (I-V) families were
obtained using 40 ms pulses from a holding potential of −100
mV to a range of potentials (−100 to +60 mV) in 10 mV steps
with 10 s between pulses. The peak value at each potential was
plotted to form I-V curves. Activation curves were obtained by
calculating the conductance (G) at each voltage (V).

\[
G = \frac{I}{V - V_{rev}} \tag{Eq. 1}
\]

with \(V_{rev}\) being the reversal potential, determined for each cell
individually. Activation curves were fitted with the following
Boltzmann distribution equation,

\[
G_{Na} = \frac{G_{Na,max}}{1 + e^{-\frac{V_m - V_{1/2}}{k}}} \tag{Eq. 2}
\]

where \(G_{Na}\) is the voltage-dependent sodium conductance,
\(G_{Na,max}\) is the maximal sodium conductance, \(V_{1/2}\) is the potential
at which activation is half-maximal, \(V_m\) is the membrane poten-
tial, and \(k\) is the slope factor. Ramp currents were elicited by
slowly depolarizing voltage ramps, ranging from −100 to +20
mV at a rate of 0.2 mV/ms. Size of absolute ramp currents was
measured as the difference between first current downward
reflection and the peak of the ramp current.

Protocols for assessing steady-state fast inactivation con-
sisted of a series of prepulses (−130 to −10 mV) lasting 500 ms
from the holding potential of −100 mV followed by a 40 ms
depolarization to −10 mV to assess the noninactivated tran-
sient current. The normalized curves were fitted using a Boltz-
mann distribution equation,

\[
\frac{I_{Na}}{I_{Na,max}} = \frac{1}{1 + e^{-\frac{V_m - V_{1/2}}{k}}} \tag{Eq. 3}
\]

where \(I_{Na,max}\) is the peak sodium current elicited after the most
hyperpolarized prepulse, \(V_{1/2}\) is the preconditioning pulse
potential, \(V_m\) is the half-maximal sodium current, and \(k\) is the
slope factor. The decay time constants were obtained by fitting
a single exponential function to the current traces.

Steady-state slow inactivation was determined using 30 s
prepulses ranging from −130 mV to +10 mV followed by a
brief hyperpolarization to −120 mV for 100 ms to remove fast
inactivation. Available channels were then tested with a 20 ms
test pulse to −10 mV. Elicited currents were normalized to the
maximal peak current and fitted using a Boltzmann distribu-
tion with an added constant (offset).

The rate of deactivation was measured using a short (0.5 ms)
depolarizing pulse to −20 mV followed by a 50 ms repolarizing
pulse to potentials ranging from −100 to −40 mV. Decaying
current was then fitted with a single exponential function using
PulseFit software (HEKA Electronics).

Statistical analysis was carried out using SPSS software (SPSS
Inc., Chicago, IL) performing one-way analysis of variance, and
significance for multiple comparisons was tested using Tam-
hane T2 post hoc analysis. Results revealing \(p < 0.05\) were con-
sidered significant. All data are presented as mean ± S.E.

RESULTS

S241T Lowers Threshold for Activation, Slows Deactivation,
and Increases Ramp Responses of hNav1.7r. The amino acid
threonine is larger than serine by a methyl (CH₃) group, but
both have similar chemical reactivity at their reactive OH group
on the side chain, for example, phosphorylation. Nevertheless,
the S241T mutation has been linked to IEM (10). Therefore, we
investigated the effects of the S241T substitution on the bio-
physical properties of Nav1.7.

To characterize the effects of substitution of Ser-241 with
threonine on the biophysical properties of Nav1.7, we per-
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formed whole-cell patch-clamp recordings from HEK293 cell lines stably transfected with wild type Nav1.7<sub>R</sub> and the mutant derivative S241T. Voltage steps from a holding potential of −100 mV produced a fast activating and fast inactivating current for both constructs (Fig. 1A). The V<sub>1/2</sub> of activation (Fig. 1B and Table 1) was −25.6 ± 0.9 mV (n = 47) for Nav1.7<sub>R</sub> and −34.0 ± 1.1 mV (n = 39) for the S241T mutant channels, showing a shift of −8.4 mV. The V<sub>1/2</sub> of steady-state fast inactivation was not changed by this mutation (Fig. 1B and Table 1). Thus, the predicted window current would be larger, close to resting membrane potential of neurons.

Kinetics of activation were investigated by measuring the time to peak of the transient current. Considering the observed shift in V<sub>1/2</sub> of activation induced by the S241T mutation, we expected a faster activation depending on the step voltage. At −30 mV, activation of S241T was significantly faster than that of Nav1.7<sub>R</sub> (Fig. 1C; p < 0.05, one-way analysis of variance, Tamhane post hoc analysis). The same statistical analysis was performed for all indicated p values. Deactivation of S241T was significantly slower at −40 and −50 mV (Nav1.7<sub>R</sub>, 0.34 ± 0.03 ms; S241T, 1.55 ± 0.15 ms at −40 mV; p < 0.01 (Fig. 1D)). The inactivation decay time constants at these potentials were 12.0 ± 3.6 ms (−40 mV) and 5.3 ± 0.4 ms (−50 mV) for Nav1.7<sub>R</sub> and 8.4 ± 1 ms (−40 mV) and 3.2 ± 0.4 ms (−50 mV) for S241T. As the inactivation time constants were significantly slower than the deactivation time constants, contamination of channel closing by inactivation could be considered low.

Small, slow depolarizations of Nav1.7 evoke ramp currents that mimicked weak natural stimuli, for example, generator potentials. The slow closed-state inactivation of Nav1.7 permits this channel to produce relatively larger ramp currents compared with other sodium channels (19, 22). HEK293 cells which express Nav1.7<sub>R</sub> channels produced
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Size of the Side Chain at Position 241 Modulates Gating Properties—Serine and threonine side chains differ by one methyl group (Fig. 2A), which could interfere with the packing of the S4-S5 linker secondary structure when the two amino acids are exchanged. To address the possibility that the different size of the side chain of the residue at position 241 contributes to the functional changes in the S241T mutant channels, we substituted Ser-241 by the relatively smaller residue alanine (S241A), which carried one less oxygen atom or the significantly larger residue leucine (S241L) (Fig. 2A). S241L substitution was chosen because it had a bulkier side chain compared with serine; also, a S246L mutation in Nav1.4 (Ser-246 in Nav1.4 corresponds to Ser-241 in Nav1.7) has been described in a patient with myasthenic syndrome (23). HEK293 cells, stably transfected with S241A and S241L, produced fast activating and inactivating voltage-gated sodium currents, similar to Nav1.7R and S241T channels (Fig. 2A).

We investigated the effect of S241A and S241L on voltage-dependence of activation and steady-state fast inactivation. S241A did not significantly alter activation properties of the channel (Fig. 2B, blue squares and Table 1). In addition, the midpoint of steady-state fast inactivation was not significantly different compared with Nav1.7R. The fast inactivation slope decreased to 6.2 ± 0.1 mV (n = 25; p < 0.05; slope for Nav1.7R, 6.6 ± 0.1 mV).

S241L currents, however, showed a significant hyperpolarizing shift in activation midpoint compared with Nav1.7R (by 4.9 mV; n = 21; p < 0.05 (Fig. 2B, yellow squares and Table 1)), similar to the effect of S241T; the activation midpoints of S241L and S241T were not significantly different. The slope of activation a ramp current as expected (peak ramp current as percentage of transient peak is 0.13% ± 0.01%, n = 46 (Fig. 1E)), S241T substitution caused a 3.6-fold increase in the ramp current (S241T, 0.49% ± 0.07% of peak transient current, n = 38; p < 0.001). The voltage at which the ramp current peaked was hyperpolarized for S241T as expected from the negative shift of the V1/2 of activation.

Enhanced slow inactivation has been observed for several IEM mutations (16, 18), including those showing increased neuronal excitability in dorsal root ganglion neurons (8, 17, 18). S241T enhances steady-state slow inactivation and shifts the V1/2 by −12.3 mV from −78.4 ± 2.1 mV (n = 21) for Nav1.7R to −90.7 ± 1.1 mV (n = 12) for S241T (p < 0.001) (Fig. 1F and Table 1) and reduces the slope factor from 11.9 ± 0.4 mV for Nav1.7R to 6.3 ± 0.3 mV for S241T channels (p < 0.001), thereby reducing the voltage dependence of slow inactivation steeper. However, the offset for slow inactivation of the S241T mutant channel (7.7% ± 0.8%) was not different from that of Nav1.7R channels (8.4% ± 1%).

| Channel       | Activation | Fast inactivation | Slow inactivation | Ramp current |
|---------------|------------|------------------|-------------------|--------------|
|               | Midpoint   | Slope n          | Midpoint          | Slope n      | Percentage | Voltage at peak n |
| Nav1.7R       | −25.6 ± 0.9| 6.02 ± 0.21      | −82.3 ± 0.9       | 6.6 ± 0.1    | 81.8 ± 0.8 | 6.2 ± 0.11      |
| S241T         | −34.0 ± 1.1| 6.52 ± 0.26      | −116.0 ± 1.1      | 6.7 ± 0.2    | 29.0 ± 0.2 | 6.3 ± 0.3       |
| S241A         | −24.0 ± 0.7| 6.55 ± 0.19      | −114.0 ± 0.9      | 6.2 ± 0.1    | 25.0 ± 0.2 | 9.5 ± 0.6       |
| S241L         | −30.5 ± 1.0| 7.8 ± 0.3        | −118.0 ± 1.2      | 6.1 ± 0.2    | 20.0 ± 0.2 | 6.6 ± 0.3       |

TABLE 1
Parameters of Boltzmann fits and values for ramp currents
All values are in mV, except for the data of the ramp currents, which are presented as percentage of transient peak currents.

*Significantly different from Nav1.7R, tested with one-way analysis of variance and Tamhane post hoc analysis, with p at least smaller than 0.05.

![FIGURE 2. S241T shifts steady-state activation to more hyperpolarized potentials.](image-url)

A. S241  
B. S241T  

FIGURE 2. S241T shifts steady-state activation to more hyperpolarized potentials. A, representative I-V families recorded from HEK293 cells stably expressing Nav1.7R (Ser-241; upper left panel) or the mutations S241T (upper right panel), S241A (lower left panel), and S241L (lower right panel). Currents were evoked by depolarizing the membrane from the holding potential of −100 mV to potentials ranging from −100 to 40 mV in 10 mV steps. In this figure and Figs. 3 and 4, data for Nav1.7R and S241T are the same as in Fig. 1 and are included for comparison. The structure of the amino acid at position 241 in the S4-S5 linker in domain I is shown as structural model; the stick model highlights the side chain of serine for Nav1.7R, threonine for S241T, alanine for S241A, and leucine for S241L. B, voltage dependence of steady-state fast inactivation and activation are shown for Nav1.7R (gray squares; activation, n = 47 and inactivation, n = 51), S241T (pink squares; activation, n = 39 and inactivation, n = 29), S241A (blue squares; activation, n = 35 and inactivation, n = 25), and S241L (yellow squares; activation, n = 21 and inactivation, n = 20). Steady-state fast inactivation was obtained by measuring the current evoked by a 40 ms pulse to −10 mV after inducing fast inactivation with a 500 ms prepulse to potentials ranging from −130 to −10 mV. Conductance curves were deduced from current-voltage families normalized and fitted with a Boltzmann equation, as described under "Experimental Procedures." Data points are connected with straight lines and represent means ± S.E.
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**DISCUSSION**

We show in this study that the IEM mutation S241T of the human voltage-gated sodium channel Nav1.7 alters the biophysical properties of the channel, consistent with the pathophysiology of this disorder. Activation of S241T was shifted to hyperpolarized potentials, time to peak was faster, deactivation was slower, and response to slow, small depolarization (ramp currents) was increased. All these changes would be expected to increase the excitability of nociceptive dorsal root ganglion neurons harboring the mutation, thus contributing to the pain in IEM (8, 17). Slow inactivation, on the other hand, was enhanced in the S241T mutant channels, similar to previously reported IEM mutations (8, 16, 18), but its contribution to the pathophysiology of erythromelalgia is not known at this time. This change would theoretically render mutations expressing DRG neurons hypoxicitable. Current clamp investigations on the F1449V mutation in DRG neurons, however, showed that the expression of the mutant channel in DRG neurons, although enhancing slow inactivation, renders the neurons hyperexcitable, as reflected by a lower threshold for firing and an increased frequency of firing of DRG neurons expressing the mutant channel compared with those expressing WT channels (8). Similarly, L858H mutation, which also shows enhanced slow inactivation (16), renders DRG neurons expressing the mutant channel hyperexcitable (17). Thus, the effect of slow inactivation on the channel appears to be outweighed by other gating changes.

Serine and threonine are polar amino acids that share similar biochemical characteristics because of their reactive OH group in their side chains. Thus, substitutions such as S241T are generally expected to maintain the function of the protein. Although Ser-241 in the context of the S4-S5 linker (SVKK) is a potential phosphoacceptor site for protein kinase C, it is reasonable to expect that TVKK would be an equally probable substrate. However, Ser-241 is a highly conserved residue in the DI S4-S5 linker in all the vertebrate sodium channels, and because S241T has been identified in patients in whom sensory neurons are hyperexcitable, a substitution at this position would be predicted to affect the gating properties of the channel, similar to other IEM mutations. Threonine side chain, however, is larger than that of serine by a methyl group. To assess the role of the size of the side chain at position 241 in hNav1.7, we mutated serine to the slightly smaller amino acid alanine (S241A) and to the larger amino acid leucine (S241L), both of which are nonpolar, unlike serine or threonine. S241A showed characteristics similar to wild type Nav1.7R, whereas S241L induced changes comparable with those observed for the naturally occurring mutation S241T. Thus, polarity of the res-
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FIGURE 4. S241L enhances slow inactivation of Nav1.7, similar to S241T. The mutations S241L (yellow squares, n = 7) and S241T (pink squares, n = 12) shift voltage-dependence of slow inactivation to hyperpolarized potentials compared with Nav1.7a (gray squares, n = 21) and S241A (blue squares, n = 13). Steady-state slow inactivation was assessed using a 20 ms pulse to −10 mV after a 30 s prepulse at potentials ranging from −140 to −10 mV followed by a 100 ms pulse to −120 mV to remove fast inactivation. See inset in Fig. 1F for protocol. Data points are connected with straight lines and represent means ± S.E. Right panels, bar graphs show the results of a Boltzmann fit (midpoint of slow inactivation and slope). *, significant difference to Nav1.7a with p < 0.05; ***, p < 0.001.

The S4-S5 linker of ion channels has been implicated in the regulation of gating properties of channels, and mutations in these linkers cause a number of neurological and muscular disorders. Sodium channel mutations in the S4-S5 linkers in domains II–IV have been shown to affect gating of several channels: Nav1.7 (16), Nav1.6 (11, 24), Nav1.5 (25), and Nav1.4 (26–32), and Nav1.2 (33). There is evidence for an important functional role of the S4-S5 linker from mutations of other ion channels. The S218L mutation in the DI S4-S5 linker of voltage-gated calcium channel Cav2.1 induces a gain of function and causes familial hemiplegic migraine (34). Interaction between the residue Asp-540 in S4-S5 and Arg-665 in S6 is suggested to contribute to stabilization of the closed conformation in hERG channels (35). The hyperpolarization-activated anion channel HCN2 is shown to display disrupted gating because of mutations in S4-S5 linker (36). Thus, the S4-S5 linker appears to modulate gating properties of a diverse group of ion channels.

Recently published models of voltage-gated ion channels (37–45) depict a voltage sensor consisting of segments S1–S4, whereas segments S5 and S6 form the pore, control ion selectivity, and opening of the channel. The voltage sensor complex size of a side chain within the S4-S5 linker that possibly faces S6 might be expected to facilitate bending of S6 and thus lower the threshold for channel activation.

The midpoint of steady-state fast inactivation was not affected by S241T, S241A, or S241L. This is consistent with the idea that the S4-S5 linkers in DI and DII play only a minor role in fast inactivation, whereas the S4-S5 linkers of DIII and DIV are postulated to be the receptor site for the fast inactivation gate (30, 33, 49–52). Several mutations in S4-S5 linker of domain III of sodium channels Nav1.4 (53), Nav1.5 (25), and Nav1.6 (24) have been shown to affect fast inactivation. The suggested minor role of the DI S4-S5 linker in fast inactivation might explain why we did not observe significant changes in steady-state fast inactivation of S241T; only S241L, which replaced serine with a much larger amino acid, produced a small negative shift, suggesting that the function of the receptor site for the inactivation gate might have been enhanced by a larger residue.

The data presented here show that S241T alters Nav1.7 gating in a manner that could underlie dorsal root ganglion neuron hyperexcitability (8, 17). We have also shown that S241L induces gating changes similar to those of S241T and would thus be predicted to cause a mutant phenotype in patients who might carry this mutation. Paradoxically, the S246L substitution in Nav1.4 (which is equivalent to Ser-241 in Nav1.7) observed in a family with myasthenic syndrome, did not in itself induce a disease phenotype, which instead was linked to the mutation V1442E in the DIV S3-S4 extracellular linker of the channel (23). Nav1.4/S246L caused a 7 mV hyperpolarizing shift of steady-state fast inactivation of Nav1.4 and shifted the V1/2 of steady-state slow inactivation to the left, comparable with our results for Nav1.7/S241L. Interestingly, it also increased the population of channels that did not undergo slow inactivation at positive potentials and did not cause a change in V1/2 of activation (23). The difference in the effects on activation of analogous mutations in Nav1.7 and Nav1.4 and in the clinical effects of these mutations (disease-inducing for Nav1.7/S241L...
but not for Nav1.4/S246L) suggests an isomor-specific role of the S4–S5 linker and cautions against over-generalization of the limited experimental results from heterologous expression systems. Because Nav1.4 is mainly expressed in skeletal muscle cells (54, 55), whereas Nav1.7 is expressed in sensory neurons and sympathetic neurons (13–15), it is not surprising that these analogous mutations in Nav1.4 and Nav1.7 have different functional effects. As shown recently, cell background can have a significant influence on the effects of mutant-channel electrogensis (17).

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