1   **FRONT MATTERS**
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3   **Title**

4   Intrinsic Gating Behavior of Voltage-Gated Sodium Channels Predetermines Regulation
5   by Auxiliary β-subunits

6
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23
24   **Abstract**

25   Voltage-gated sodium (Nav) channels mediate rapid millisecond electrical signaling in
26   excitable cells. Auxiliary subunits, β1-β4, are thought to regulate Nav channel function
27   through covalent and/or polar interactions with the channel's voltage-sensing domains.
28   How these interactions translate into the diverse and variable regulatory effects of β-
29   subunits remains unclear. Here, we find that the intrinsic movement order of the voltage-
30   sensing domains during channel gating is unexpectedly variable across Nav channel
31   isoforms. This movement order dictates the channel's propensity for closed-state
32   inactivation, which in turn modulates the actions of β1 and β3. We show that the
33   differential regulation of skeletal muscle, cardiac, and neuronal Nav channels is explained
34   by their variable levels of closed-state inactivation. Together, this study provides a unified
35   mechanism for the regulation of all Nav channel isoforms by β1 and β3, which explains
36   how the fixed structural interactions of auxiliary subunits can paradoxically exert variable
37   effects on channel function.

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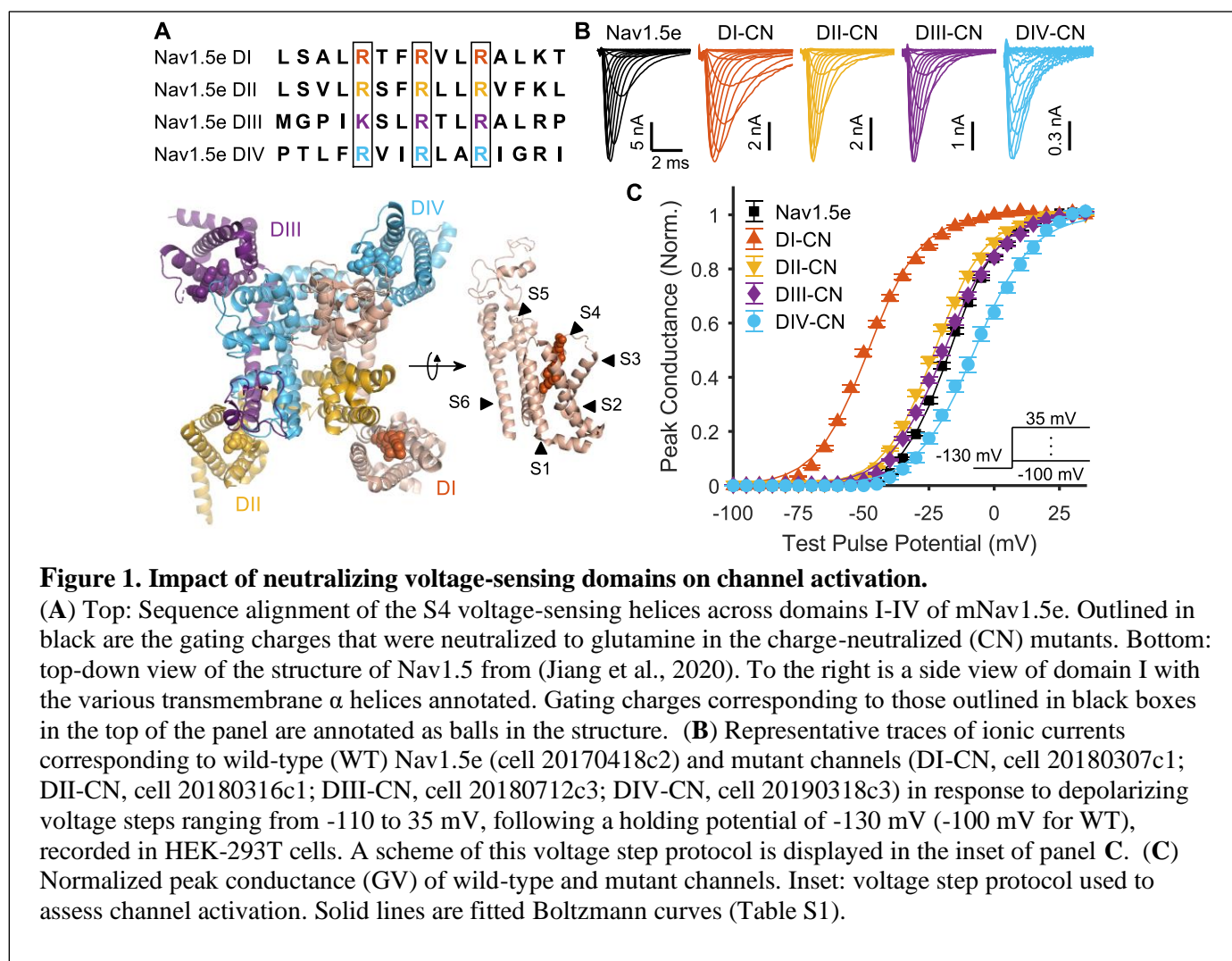43   **Supplemental Figures**: 4, **Supplemental Tables:** 3
44

MAIN TEXT

Introduction

Almost all ligand- and voltage-gated ion channels expressed in excitable tissue assemble as a signaling complex consisting of pore-forming and auxiliary subunits (Dolphin, 2018; Edelheit et al., 2009; Gonzalez-Perez and Lingle, 2019; Hull and Isom, 2018; Twomey et al., 2019). For example, the voltage-gated sodium (Nav) channel complex assembles from one of nine possible pore-forming α-subunits (Nav1.1-Nav1.9) with 1-2 auxiliary β-subunits (β1 to β4) that together shape the rapid and varied kinetics of action potentials in brain, heart and skeletal muscle tissue (Catterall et al., 2005; Hull and Isom, 2018). Individual Nav channel α-subunits form a tetrameric structure around a central Na^+-selective pore from four non-identical domains (DI-DIV). Each domain contains 6 transmembrane segments (S1-S6) that are either responsible for voltage detection (S1-S4) or formation of the pore structure (S5-S6) (Ahern et al., 2016; Jiang et al., 2020; Pan et al., 2018). Biochemical and electrophysiology studies as well as recent full-length cryo-EM Nav structures have revealed that these domains are selectively targeted by β-subunits: β1 and β3 subunits form polar interactions with DIII or DIV (Hsu et al., 2017; Hull and Isom, 2018; Pan et al., 2018; Yan et al., 2017) whereas β2 and β4 establish covalent links with DI and/or DII (Das et al., 2016; Hull and Isom, 2018; Isom et al., 1995, 1992; Shen et al., 2019). Together, these findings establish a common structural view of the interactions between α- and β-subunits.

Despite this, the effects of β1 and β3 on channel function remain undetermined. Although numerous electrophysiological studies have characterized the effects of β1, in the two most heavily studied Nav channels Nav1.4 and Nav1.5 – β1 has been shown to produce large (Bendahhou et al., 1995; Zhu et al., 2017), moderate (Malhotra et al., 2001; Nuss et al., 1995), or even no changes (Ferrera and Moran, 2006; Nuss et al., 1995) to channel gating. This variability, even within a single Nav channel isoform, demonstrates that the emerging structural view of β-subunits has been, so far, insufficient to explain their effects on channel function.

Here, we attempted to reconcile this apparent disconnect between structural and electrophysiological studies. Because β-subunits have been shown to alter voltage sensor movements to produce their functional changes in Nav1.5 channels (Zhu et al., 2017), we began by investigating the contributions of each voltage sensor to the gating of Nav1.5e, a neonatal form of Nav1.5 that is expressed in the brain (Wang et al., 2017). Using voltage-sensor neutralization experiments, voltage-clamp fluorometry (VCF), and kinetic modelling, we find that the functional contributions of each voltage sensor are not fixed. Instead, we demonstrate that the sequence of voltage sensor movements is variable, which in turn modulates the contributions of each voltage sensor to channel activation and inactivation. We further show that this mechanism determines the functional consequences of β1 and β3 association with cardiac Nav1.5 channels, skeletal muscle Nav1.4 channels, and neuronal Nav1.6 channels. Finally, through an analysis of previously published studies, we find that this mechanism successfully explains the variable regulation of all Nav channel isoforms by β1. We conclude that β1 and β3 impose a defined structural influence on all Nav channels and that their variable allosteric effects are determined by the intrinsic dynamics of the channel's voltage sensors.

Figure 1. Impact of neutralizing voltage-sensing domains on channel activation.
(**A**) Top: Sequence alignment of the S4 voltage-sensing helices across domains I-IV of mNav1.5e. Outlined in black are the gating charges that were neutralized to glutamine in the charge-neutralized (CN) mutants. Bottom: top-down view of the structure of Nav1.5 from (Jiang et al., 2020). To the right is a side view of domain I with the various transmembrane α helices annotated. Gating charges corresponding to those outlined in black boxes in the top of the panel are annotated as balls in the structure. (**B**) Representative traces of ionic currents corresponding to wild-type (WT) Nav1.5e (cell 20170418c2) and mutant channels (DI-CN, cell 20180307c1; DII-CN, cell 20180316c1; DIII-CN, cell 20180712c3; DIV-CN, cell 20190318c3) in response to depolarizing voltage steps ranging from -110 to 35 mV, following a holding potential of -130 mV (-100 mV for WT), recorded in HEK-293T cells. A scheme of this voltage step protocol is displayed in the inset of panel **C**. (**C**) Normalized peak conductance (GV) of wild-type and mutant channels. Inset: voltage step protocol used to assess channel activation. Solid lines are fitted Boltzmann curves (Table S1).

Results

Voltage Sensor Charge Neutralization Identifies the Dominant Role of DI in Channel Activation

Voltage-detection in Nav channels is mediated by gating charges (Arg or Lys residues) on each S4 segment (Fig. 1A) which together promote movement in individual S3-S4 voltage sensors following changes in the membrane electric field. To study the contribution of individual S3-S4 voltage sensors to channel gating, the first three gating charges in each S4 segment of the mouse Nav1.5e channel were mutated to glutamines to generate four charge-neutralized (CN) mutant channels: DI-CN, DII-CN, DIII-CN, DIV-CN (Fig. 1A). We reasoned that neutralization of individual voltage sensors would render the domain insensitive to changes in the membrane potential and, as such, would inform us about the biophysical properties of Nav1.5e, as reported for other voltage-gated sodium and potassium channels (Bao et al., 1999; Capes et al., 2013, 2012; Gagnon and Bezanilla, 2009; Sheets et al., 1999). Nav1.5e channels and the charge-neutralized mutants were characterized in HEK-293T cells transiently transfected with wildtype and mutant cDNAs.
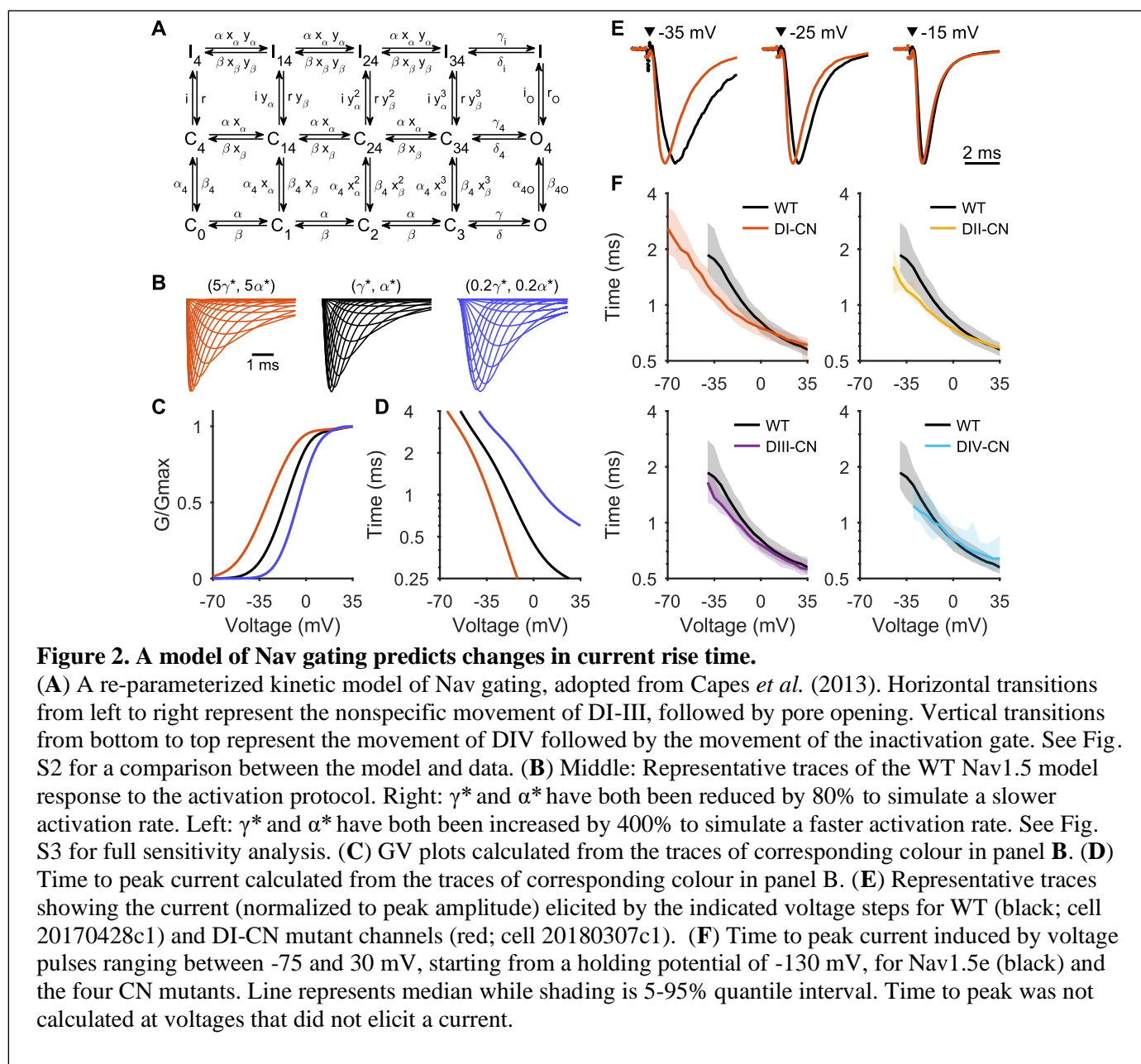
To assess channel activation, macroscopic Na^+ currents were recorded by applying depolarizing voltage steps in increments of +5 mV (Range, -110 and 55 mV), each from a holding potential of -130 mV (-100 mV for wildtype channels) (Fig. 1B). The voltage-dependence of channel activation for wildtype and mutant channels was then determined by fitting the peak conductance (G/V) with a Boltzmann function (Fig. 1C).

Charge neutralization led to statistically significant (see methods) shifts in the activation profile of all mutant Nav1.5e channels, with DI charge neutralization producing the greatest impact (Fig. 1C). Fits of peak G/V relationships estimated the voltage for half-maximal activation ($V_{1/2}$) to be -16.8 ± 0.45 mV (n = 51) for wildtype channels compared to -48.1 ± 0.55 mV (n = 24) for DI-CN (Fig. 1C, Table S1), representing a 30 mV hyperpolarizing shift in channel activation. The slope factor (k) was similar for wildtype (k = 9.0 ± 0.13 mV, n = 51) and DI-CN channels (k = 9.9 ± 0.13 mV, n = 24). In contrast, charge neutralization of DII and DIII had a more modest effect on channel activation with $V_{1/2}$ values of -22.6 ± 0.54 mV (n = 26) and -19.0 ± 0.58 mV (n = 23), respectively (Fig. 1C), corresponding to hyperpolarizing shifts in activation of about 6 and 2 mV compared to wildtype Nav1.5e (Table S1). Finally, charge neutralization of DIV had the opposite effect on channel activation, shifting the $V_{1/2}$ value to -7.4 ± 1.26 mV (n = 14) (Fig. 1C), representing a 10 mV *depolarizing* shift in channel activation compared to wildtype Nav1.5e (Table S1). Similar relative shifts in channel activation were observed when DI through to DIV voltage sensors were charge neutralized in the adult form of Nav1.5 (i.e. mH1), demonstrating that our observations are not specific to the neonatal form of Nav1.5 (Fig. S1). Our observations on Nav1.5 are comparable with previous findings on skeletal muscle Nav1.4 channels, with the exception of DIV-CN which did not depolarize activation in Nav1.4 (Capes et al., 2013).

DI Movement Is the Rate-Limiting Step for Pore Opening

The canonical Nav channel gating model postulates that DI-III movements are necessary for pore opening and DIV movement is sufficient and rate-limiting for inactivation (Ahern et al., 2016). To understand why neutralizing DIV affected activation (Fig. 1C), we attempted to explain our Nav1.5e data using a mathematical implementation of this gating model (Fig. 2A) (Capes et al., 2013). The model successfully captured the wildtype Nav1.5e data after the rate constants were re-parametrized using a custom-made evolutionary-type fitting algorithm (Fig. S2, Table S2) (see Methods).
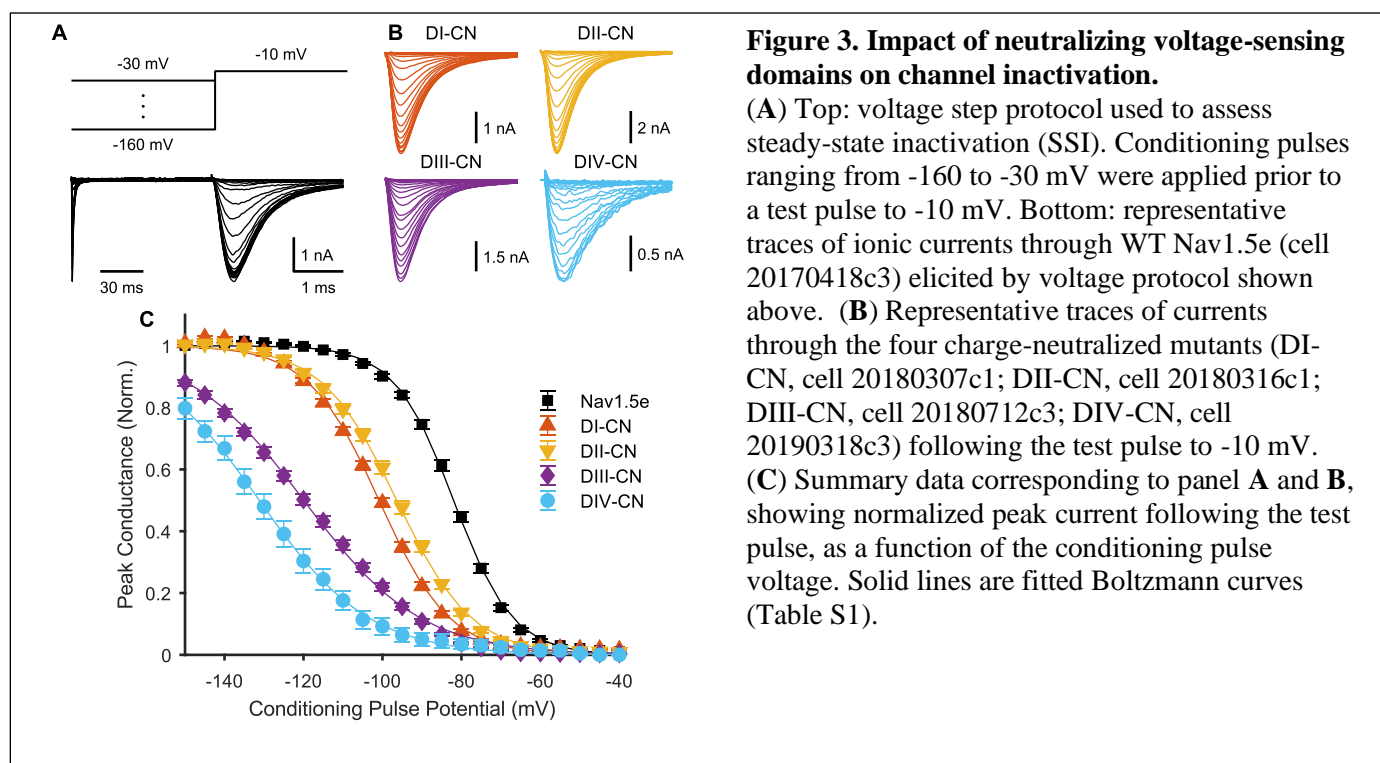
We hypothesized that DI-CN hyperpolarizes G/V because DI movement is normally rate-limiting for pore opening and that, conversely, DIV-CN might depolarize the G/V relationship by slowing the rate of pore opening. In the kinetic gating model, the rates that determine the transitions from the resting state to the open state are α and γ (Fig. 2A). To investigate how the voltage-dependence of the G/V relationship depends on these rates, we performed a sensitivity analysis with respect to parameters α* and γ* (Table S2). This analysis indicated that if domain neutralization accelerates or slows the rate of pore opening, the G/V relationship should be hyperpolarized or depolarized, respectively (Fig. S2E, F). For example, decreasing α and γ by 80% led to a depolarizing shift in the G/V relationship while increasing the rates by 400% led to a hyperpolarizing shift (Fig. 2B, C), consistent with our expectations.

Figure 2. A model of Nav gating predicts changes in current rise time.
(**A**) A re-parameterized kinetic model of Nav gating, adopted from Capes *et al.* (2013). Horizontal transitions from left to right represent the nonspecific movement of DI-III, followed by pore opening. Vertical transitions from bottom to top represent the movement of DIV followed by the movement of the inactivation gate. See Fig. S2 for a comparison between the model and data. (**B**) Middle: Representative traces of the WT Nav1.5 model response to the activation protocol. Right: γ^* and α^* have both been reduced by 80% to simulate a slower activation rate. Left: γ^* and α^* have both been increased by 400% to simulate a faster activation rate. See Fig. S3 for full sensitivity analysis. (**C**) GV plots calculated from the traces of corresponding colour in panel **B**. (**D**) Time to peak current calculated from the traces of corresponding colour in panel B. (**E**) Representative traces showing the current (normalized to peak amplitude) elicited by the indicated voltage steps for WT (black; cell 20170428c1) and DI-CN mutant channels (red; cell 20180307c1). (**F**) Time to peak current induced by voltage pulses ranging between -75 and 30 mV, starting from a holding potential of -130 mV, for Nav1.5e (black) and the four CN mutants. Line represents median while shading is 5-95% quantile interval. Time to peak was not calculated at voltages that did not elicit a current.

149   Additionally, our simulations revealed that a faster or slower rate of pore opening
150   should accelerate or slow the response risetime, respectively (Fig. 2B, D). This observation
151   is in line with single-channel studies, which have reported that the time course of
152   macroscopic currents is primarily determined by the latency to first opening (Aldrich et al.,
153   1983). To validate our hypotheses about DI and DIV, we therefore measured the risetime to
154   peak current of each mutant Nav channel (Fig. 2E, F). As anticipated, DI-CN channels
155   displayed faster risetimes at hyperpolarized membrane potentials (Fig. 2F), suggesting that
156   DI movement is normally rate-limiting for pore opening. The risetimes of DII-CN and DIII-
157   CN mutant channels were likewise consistent with their more modest impacts on the $V_{1/2}$ of
158   channel activation (Fig. 1C; Fig. 2F). Unexpectedly, DIV-CN mutants did not display

Figure 3. Impact of neutralizing voltage-sensing domains on channel inactivation.
(**A**) Top: voltage step protocol used to assess steady-state inactivation (SSI). Conditioning pulses ranging from -160 to -30 mV were applied prior to a test pulse to -10 mV. Bottom: representative traces of ionic currents through WT Nav1.5e (cell 20170418c3) elicited by voltage protocol shown above. (**B**) Representative traces of currents through the four charge-neutralized mutants (DI-CN, cell 20180307c1; DII-CN, cell 20180316c1; DIII-CN, cell 20180712c3; DIV-CN, cell 20190318c3) following the test pulse to -10 mV. (**C**) Summary data corresponding to panel **A** and **B**, showing normalized peak current following the test pulse, as a function of the conditioning pulse voltage. Solid lines are fitted Boltzmann curves (Table S1).

159 slower response risetimes relative to WT channels (Fig. 2F), suggesting that neutralizing
160 DIV does not slow the rate of pore opening. We therefore concluded that DIV-CN must
161 shift the G/V relationship through a separate mechanism. Since DIV is intrinsically
162 involved in Nav1.5 inactivation (Jiang et al., 2020), we hypothesized that charge
163 neutralization of DIV may affect the voltage-dependence of activation indirectly because of
164 the interdependence of the two processes. As explained below, we examined this by
165 measuring the impact of charge neutralization on steady-state inactivation.

166
167 **Voltage Sensors of All Domains Contribute to Steady-State Inactivation of Nav1.5e**
168 **Channels**

169 Steady-state inactivation (SSI) of wildtype and mutant Nav1.5e channels was determined by
170 applying a 100 ms-long conditioning pulse (range, -160 to -5 mV) followed by a test pulse
171 of -10 mV to elicit Na^+ currents (Fig. 3A, B). SSI plots were then constructed by fitting the
172 peak response at each test potential with a Boltzmann function (Fig. 3C). In agreement with
173 previous work on Nav1.4 (Capes et al., 2013), neutralizing DIV had the largest effect on
174 SSI, shifting the $V_{1/2}$ from -82.0 ± 0.52 mV in wildtype channels to -131.1 ± 2.55 mV (n =
175 13) in DIV-CN mutants (Table S1). The slope factor for the inactivation curve was
176 significantly flatter for DIV-CN mutants (k = -13.7 ± 1.46, n = 13) than for wildtype
177 Nav1.5e channels (k = -7.2 ± 0.14, n = 49), indicating a lower sensitivity to membrane
178 potential. Charge neutralization of DIII affected SSI in a manner similar to neutralizing
179 DIV, hyperpolarizing the $V_{1/2}$ of inactivation by 40 mV to -120.0 ± 1.09 mV (n = 23) and
180 flattening the SSI slope factor to -14.4 ± 0.15 (n = 23). Although measurements of SSI for
181 DI and DII-CN mutants differed from wildtype Nav1.5e, the shift was less than for DIII and
182 DIV-CN mutants. The $V_{1/2}$ of inactivation was estimated to be -100.9 ± 0.54 mV (n = 23)
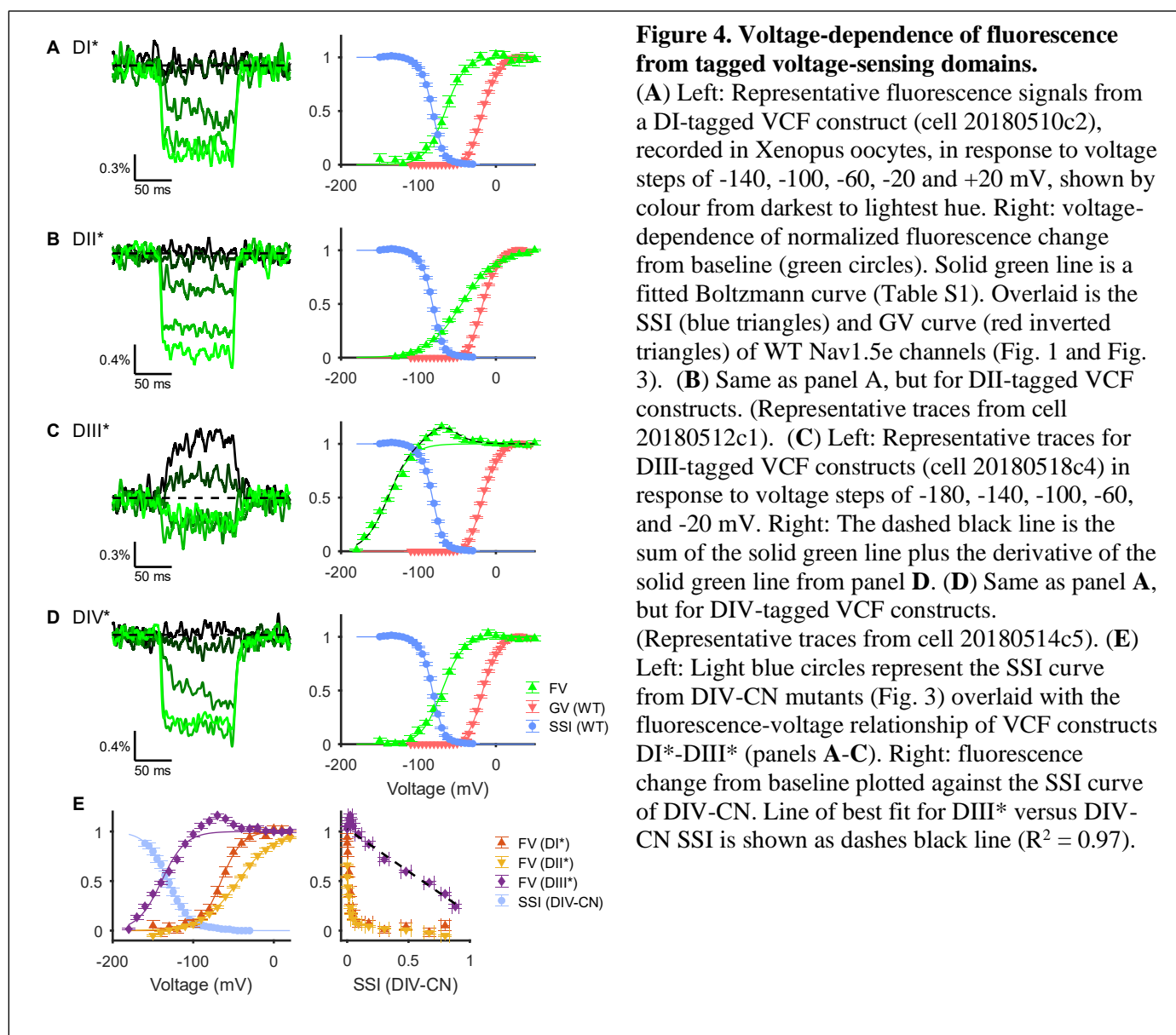
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183   and -96.5 ± 0.82 mV (n = 25) for DI and DII-CN mutants, respectively (Fig. 3C, Table S1)
184   corresponding approximately to 20 mV and 15 mV hyperpolarizing shifts. Similar relative
185   shifts in SSI were also observed from charge-neutralized adult Nav1.5 channel mutants
186   (Fig. S3), again demonstrating that our observations are not specific to the neonatal form of
187   Nav1.5. However, these results were starkly different from past studies on Nav1.4 (Capes et
188   al., 2013), where only DIV-CN mutants displayed altered SSI.

Voltage-Clamp Fluorometry Reveals That Domains III and IV Are Necessary for Inactivation

192   The charge neutralization experiments suggest that, at least for Nav1.5, both DIII and DIV
193   may be involved directly in inactivation, since their neutralization led to both a dramatic
194   hyperpolarization of SSI and a decreased sensitivity of inactivation to changes in membrane
195   potential. Although a role for DIII in inactivation has been proposed previously (Armstrong,
196   2006; Armstrong and Hollingworth, 2018; Cha et al., 1999), charge neutralization data of
197   Nav1.4 channels suggests that DIV alone is sufficient for inactivation (Capes et al., 2013).
198   The argument favouring sufficiency is that neutralizing DIV in Nav1.4 leads to a shift in
199   SSI that is so hyperpolarized, domains with intact voltage sensors must be in their
200   deactivated state (Ahern et al., 2016; Capes et al., 2013). However, recent VCF data has
201   shown that DIII activates at more hyperpolarized potentials in human Nav1.5 channels than
202   in Nav1.4 channels (Chanda and Bezanilla, 2002; Hsu et al., 2017). To test whether this is
203   also the case for the neonatal Nav1.5 used in this study, we quantified the intrinsic voltage-
204   sensitivity of each domain in Xenopus oocytes using VCF. Fluorescent probes were
205   conjugated to each domain, thereby engineering four domain-tagged VCF constructs: DI*,
206   DII*, DIII* and DIV*. Steady-state fluorescence was then measured at voltage steps
207   ranging from -150 mV (-180 mV for DIII*) to 50 mV.

208   In agreement with other VCF studies of Nav1.5 channels, the fluorescence-voltage (F-
209   V) curve for DI* was more hyperpolarized than that for DII* (Fig. 4A, B) (Hsu et al., 2017;
210   Varga et al., 2015). The voltage-dependence of the fluorescence signal of both DI* and
211   DII* exhibited $V_{1/2}$ values of -65.4 ± 2.3 mV (n = 13) and -44.1 ± 0.7 mV (n = 17) (see also,
212   Table S1), respectively, which were approximately 10 mV more depolarized than V1/2
213   values reported for adult Nav1.5 channels (Hsu et al., 2017; Varga et al., 2015). This
214   difference is in keeping with the more depolarized threshold for activation of Nav1.5e
215   channels compared to the adult Nav1.5 splice variant (Onkal et al., 2008). Interestingly, the
216   F-V plot of DII* had a shallower slope (k = 22.2 ± 0.6, n = 17) compared to DI* (k = 12.3 ±
217   1.2, n = 13), indicating that DII has a lower sensitivity to changes in membrane potential
218   (Fig. 4A, B).

219   The normalized F-V relationship for DIII* was significantly more hyperpolarized than
220   DI* and DII* with a $V_{1/2}$ value of -137.4 ± 1.1 mV (n = 27) and a slope factor of 15.9 ± 0.6
221   (Fig. 4C). Interestingly, the fluorescence signal was biphasic, reaching a maximum at about
222   -50 mV and declining in intensity at more depolarized potentials (Fig. 4C). This finding
223   suggests that the voltage sensor of DIII may exhibit two distinct movements, analogous to
224   the dynamics of the voltage sensor reported for Shaker K+-channels (Cha and Bezanilla,
225   1997) and DIII of Nav1.4 channels (Cha et al., 1999).

Figure 4. Voltage-dependence of fluorescence from tagged voltage-sensing domains.
(**A**) Left: Representative fluorescence signals from a DI-tagged VCF construct (cell 20180510c2), recorded in Xenopus oocytes, in response to voltage steps of -140, -100, -60, -20 and +20 mV, shown by colour from darkest to lightest hue. Right: voltage-dependence of normalized fluorescence change from baseline (green circles). Solid green line is a fitted Boltzmann curve (Table S1). Overlaid is the SSI (blue triangles) and GV curve (red inverted triangles) of WT Nav1.5e channels (Fig. 1 and Fig. 3). (**B**) Same as panel A, but for DII-tagged VCF constructs. (Representative traces from cell 20180512c1). (**C**) Left: Representative traces for DIII-tagged VCF constructs (cell 20180518c4) in response to voltage steps of -180, -140, -100, -60, and -20 mV. Right: The dashed black line is the sum of the solid green line plus the derivative of the solid green line from panel **D**. (**D**) Same as panel **A**, but for DIV-tagged VCF constructs. (Representative traces from cell 20180514c5). (**E**) Left: Light blue circles represent the SSI curve from DIV-CN mutants (Fig. 3) overlaid with the fluorescence-voltage relationship of VCF constructs DI*-DIII* (panels **A**-**C**). Right: fluorescence change from baseline plotted against the SSI curve of DIV-CN. Line of best fit for DIII* versus DIV-CN SSI is shown as dashes black line ($R^2 = 0.97$).

The F-V relationship observed for DIV* fluorescence occurred over a similar voltage range as SSI in WT channels (Fig. 4D) in keeping with the role of this domain in inactivation. The $V_{1/2}$ value was -69.5 ± 1.3 mV (n=15) and the slope factor was 13.2 ± 0.5 mV. Interestingly, at the potentials at which we observed DIV movement, the F-V relationship of DIII* deviates distinctly from a Boltzmann function. We observed that the fluorescence change of DIII was well fit by a Boltzmann function plus the derivative of the DIV* Boltzmann fit (Fig. 4C). This observation might suggest an interaction between DIII and DIV movement, or between DIII and the binding of the inactivation motif.

Finally, the voltage dependence of the F-V plot for DIII* was strongly correlated with measurements of steady-state inactivation in DIV-CN mutants (Fig. 4E, left). In fact, plotting the SSI of DIV-CN mutants versus the fluorescence signal of DIII* at each membrane potential displayed a strong linear correlation (Fig. 4E, right). In contrast, the F-
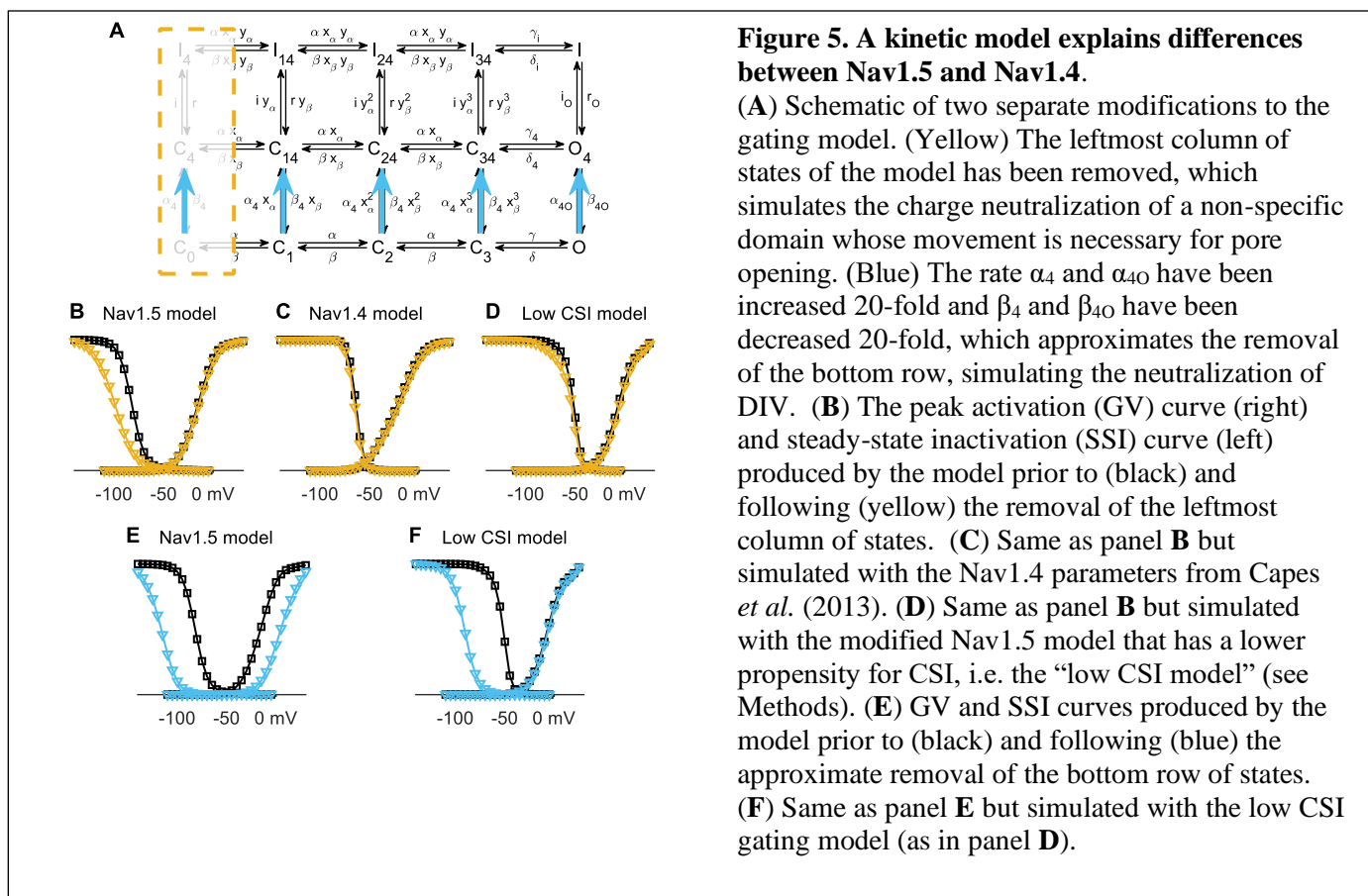
238   V curves for DI* and DII* were too depolarized to be correlated to SSI in DIV-CN mutants
239   (Fig. 4E). This finding indicates that in the absence of DIV gating charges, DIII movement
240   determines the voltage-dependence of SSI. Together with our previous results (Fig. 3C),
241   this observation strongly suggests that in Nav1.5, both DIII and DIV are intrinsically
242   necessary for channel inactivation, whereas DI and DII are not.

243

244   **Order of Voltage Sensor Movement Determines Closed-State Inactivation**

245   The identified role of DIII in inactivation explains why DIII-CN affects SSI similarly to
246   DIV-CN (Fig. 3C). We were thus left with two unexplained observations: why does
247   neutralizing DI and DII hyperpolarize SSI (Fig. 3C) if they are not involved in inactivation
248   (Fig. 4), and why does neutralizing DIV affect activation (Fig. 1C) if DIV movement is not
249   necessary for pore opening (Fig. 2)? Since these effects did not occur in Nav1.4 channels
250   (Capes et al., 2013), to understand our observations, we compared the outcomes of our
251   reparametrized Nav1.5 model (Table S2) to the Capes *et al.* Nav1.4 model (Capes et al.,
252   2013). We simulated DII-CN by removing the leftmost column of the model, consisting of
253   the states C0, C4, and I4 (Fig. 5A). This is equivalent to assuming that the first step in the
254   activation process is already complete; that is, the relevant domain has been biased towards
255   its "active" conformation. Removal of states C0, C4, and I4 caused a leftward shift in the
256   SSI curve without affecting activation in the Nav1.5 model (Fig. 5B). This shift in SSI was
257   due to positive cooperativity between gating transitions that lead to channel activation and
258   movement of the DIV voltage sensor ($x_\alpha/x_\beta > 1$, see Table S2). Notably, such cooperativity
259   between voltage sensors has been reported in past VCF studies (Campos et al., 2007;
260   Chanda et al., 2004). As a result of this cooperativity, neutralizing DI and DII increases the
261   probability of inactivation directly from closed states, i.e. closed-state inactivation (CSI),
262   even though they are not themselves necessary for inactivation.

263   Repeating our numerical experiment with the Nav1.4 parameters estimated by Capes
264   et al. (Capes et al., 2013) revealed that the Nav1.4 model predicted their experimental
265   observations, i.e. SSI was not affected (Fig. 5C). This was not because of a lack of
266   interdomain coupling. In fact, the Nav1.4 model has similar levels of interdomain coupling
267   as the Nav1.5 model (Nav1.4: $x_\alpha/x_\beta = 8.33$, Capes et al., 2013; Nav1.5: $x_\alpha/x_\beta = 8.01$, Table
268   S2). However, we noted that in the Nav1.4 model, DIV moved exclusively following DI-III,
269   whereas this was not true in the Nav1.5 model. Since DIV movement is necessary for
270   inactivation, if DIV moves exclusively following pore opening, then CSI is not possible. To
271   confirm that this difference was sufficient to explain the outcomes of neutralizing DI and
272   DII in Nav1.4 and Nav1.5 channels, we modified the Nav1.5 parameters to force DIV to
273   move only after pore opening (see Methods). Indeed, simulating DII-CN in this modified
274   "low CSI" Nav1.5 model did not appreciably alter gating (Fig. 5D), consistent with
275   experiments on Nav1.4. Altogether, these results suggest that Nav1.5 channels have an
276   intrinsically higher propensity for CSI compared to Nav1.4 channels, which in turn
277   modulates the functional consequences of neutralizing DI and DII.
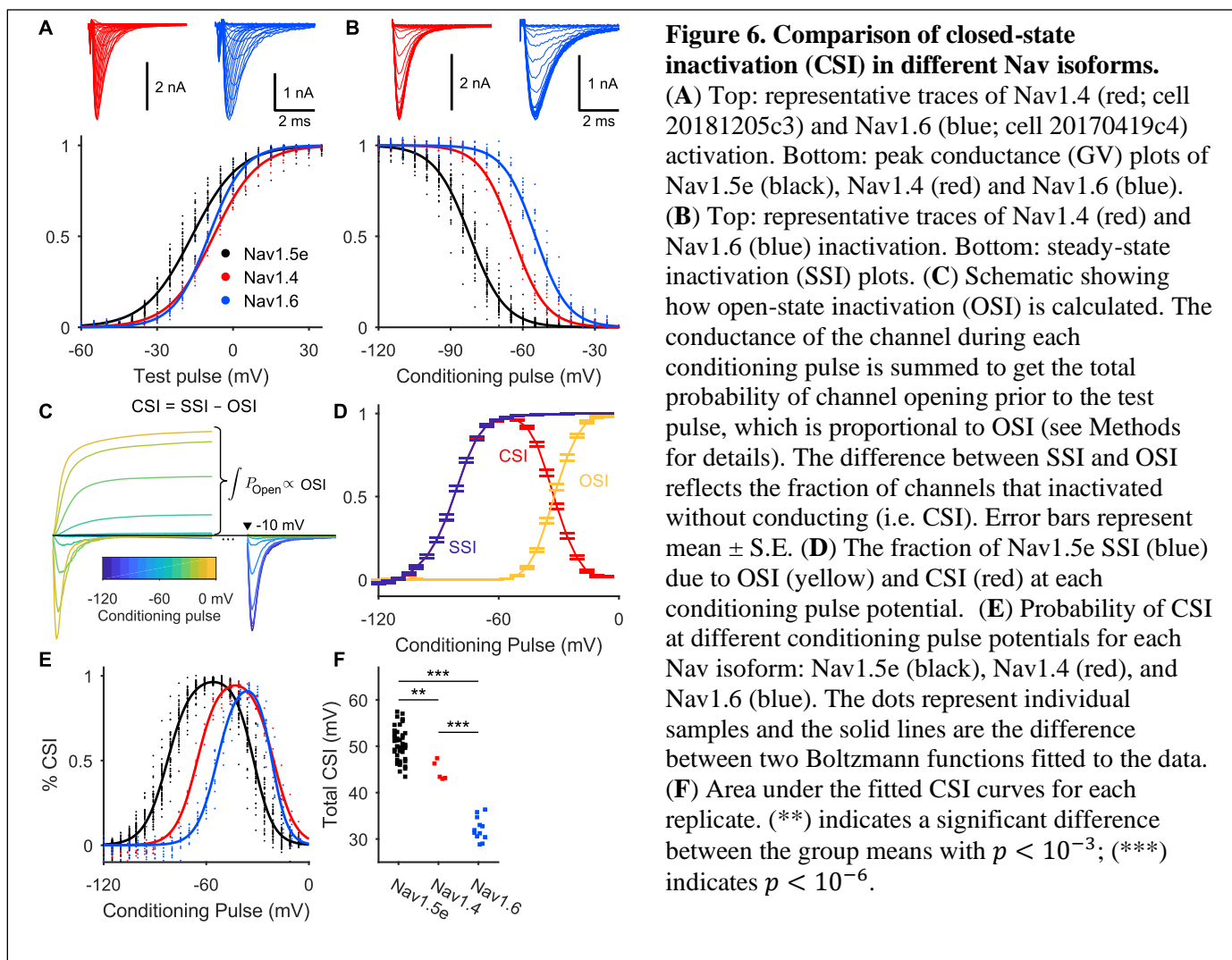
278   We next simulated the effects of DIV-CN. Removing the entire bottom row of the
279   model led to unrealistic conductance profiles (data not shown). We therefore elected to
280   simulate DIV-CN by drastically biasing the transition probability from the bottom row to

Figure 5. A kinetic model explains differences between Nav1.5 and Nav1.4.
(**A**) Schematic of two separate modifications to the gating model. (Yellow) The leftmost column of states of the model has been removed, which simulates the charge neutralization of a non-specific domain whose movement is necessary for pore opening. (Blue) The rate α_4 and α_{4O} have been increased 20-fold and β_4 and β_{4O} have been decreased 20-fold, which approximates the removal of the bottom row, simulating the neutralization of DIV. (**B**) The peak activation (GV) curve (right) and steady-state inactivation (SSI) curve (left) produced by the model prior to (black) and following (yellow) the removal of the leftmost column of states. (**C**) Same as panel **B** but simulated with the Nav1.4 parameters from Capes *et al.* (2013). (**D**) Same as panel **B** but simulated with the modified Nav1.5 model that has a lower propensity for CSI, i.e. the "low CSI model" (see Methods). (**E**) GV and SSI curves produced by the model prior to (black) and following (blue) the approximate removal of the bottom row of states. (**F**) Same as panel **E** but simulated with the low CSI gating model (as in panel **D**).

281   the middle row, by increasing the rates α_4 and α_{4O} 20-fold and decreasing the rates $\beta 4$ and
282   β_{4O} 20-fold (Fig. 5A). In the Nav1.5e model, simulating DIV-CN led to a hyperpolarization
283   of the SSI curve and depolarization of the GV curve (Fig. 5E), consistent with our
284   experimental observations from Nav1.5e DIV-CN mutants (Fig. 1C and Fig. 3C). In the
285   modified "low CSI" model, the same manipulation hyperpolarized SSI, with no effect on
286   channel activation (Fig. 5F), again consistent with the effects of neutralizing DIV in Nav1.4
287   (Capes et al., 2013). These observations can be explained as follows. In the Nav1.5e (high
288   CSI) model, the voltage-dependence of activation is set by a competition between pore
289   opening and (closed-state) inactivation. When DIV is neutralized, inactivation is faster,
290   shifting the balance towards CSI and thus requiring more depolarized voltages for pore
291   opening to outcompete inactivation. In the low CSI gating model, activation is much faster
292   than inactivation at all voltages, such that the voltage-dependence of activation represents
293   the steady-state probability of pore opening, and thus is largely unaffected by a faster
294   inactivation rate. In summary, both of the aforementioned differences in Nav1.4 and Nav1.5
295   CN mutants can be explained by differences in their intrinsic propensities for CSI.

296

297   **Auxiliary β-Subunits Selectively Target Closed-State Inactivation**

298   The above results demonstrate that the functional contributions of each voltage sensor to
299   channel gating depends both on the intrinsic coupling of the voltage sensor to the activation
300   and/or inactivation processes *and* the sequential order of voltage sensor movements. In

Figure 6. Comparison of closed-state inactivation (CSI) in different Nav isoforms. (**A**) Top: representative traces of Nav1.4 (red; cell 20181205c3) and Nav1.6 (blue; cell 20170419c4) activation. Bottom: peak conductance (GV) plots of Nav1.5e (black), Nav1.4 (red) and Nav1.6 (blue). (**B**) Top: representative traces of Nav1.4 (red) and Nav1.6 (blue) inactivation. Bottom: steady-state inactivation (SSI) plots. (**C**) Schematic showing how open-state inactivation (OSI) is calculated. The conductance of the channel during each conditioning pulse is summed to get the total probability of channel opening prior to the test pulse, which is proportional to OSI (see Methods for details). The difference between SSI and OSI reflects the fraction of channels that inactivated without conducting (i.e. CSI). Error bars represent mean ± S.E. (**D**) The fraction of Nav1.5e SSI (blue) due to OSI (yellow) and CSI (red) at each conditioning pulse potential. (**E**) Probability of CSI at different conditioning pulse potentials for each Nav isoform: Nav1.5e (black), Nav1.4 (red), and Nav1.6 (blue). The dots represent individual samples and the solid lines are the difference between two Boltzmann functions fitted to the data. (**F**) Area under the fitted CSI curves for each replicate. (**) indicates a significant difference between the group means with $p < 10^{-3}$; (***) indicates $p < 10^{-6}$.

301   particular, we found that, with the exception of DIII, the voltage sensors in Nav1.4 and
302   Nav1.5 are intrinsically coupled to essentially the same gating processes; however, in
303   Nav1.5 a disposition for early DIV movement, and thus CSI, alters the consequences of
304   voltage sensor neutralization. To extend these results beyond voltage sensor neutralization,
305   we used the high and low CSI gating models developed in the previous section to analyze a
306   range of more general gating perturbations, and found that the high and low CSI models
307   responded in fundamentally different ways (Fig. S4). For example, stabilizing the resting
308   state of DIV in the low CSI model did not alter activation or inactivation, whereas in the
309   high CSI model the same perturbation depolarized SSI (Fig. S4). This was intriguing,
310   because β1 and β3 subunits are thought to stabilize the resting state of DIV (Zhu et al.,
311   2017). Our results thus predict that β1 and β3 subunits should differentially regulate high
312   and low CSI channels, not because β1 and β3 interact with the voltage sensors differently,
313   but simply because their CSI levels are different. This would suggest an unappreciated,
314   physiological role for the sequence of voltage sensor movements in that it determines CSI
315   levels. To test this, we sought to investigate the effects of β1 and β3 on Nav channels with
316   different levels of CSI.

We first collected data from three different Nav channels: namely skeletal muscle Nav1.4 channels, cardiac Nav1.5 channels, and neuronal Nav1.6 channels (Fig. 6A, B). To experimentally quantify their propensities for CSI, we adapted the method proposed by Armstrong (Armstrong, 2006); briefly, the total conductance observed during a conditioning pulse was compared to the fraction of available channels during a subsequent test pulse (Fig. 6C, see also Methods). This analysis estimates the amount of open-state inactivation (OSI) occurring during the conditioning pulse, with CSI being defined as the fraction of SSI that cannot be accounted for by open state inactivation (OSI) (Fig. 6D). The estimated fraction of SSI occurring from closed states for Nav1.5e peaked at 100% and remained high for a large range of voltages (Fig. 6D, E). This was consistent with the Nav1.5e gating model where CSI can be determined exactly by adding up all the inactivation that occurs from closed states. To quantitatively compare CSI levels between isoforms, we fit the CSI curve with the difference of two Boltzmann curves; the integral of this function was defined to be the total amount of CSI. This analysis revealed that the three isoforms exhibited distinct levels of CSI (Fig. 6F), with Nav1.5e displaying the most, followed by Nav1.4 and finally Nav1.6 (Fig. 6F).

If $\beta1$ and $\beta3$ regulate gating by stabilizing the resting state of DIV (Zhu et al., 2017), we would expect each of these isoforms to be differentially regulated, due to their distinct levels of CSI (see above). Coexpression of each isoform with either $\beta1$ or $\beta3$ confirmed this hypothesis. $\beta1$ and $\beta3$ led to depolarizing shifts in SSI of Nav1.5e, with no effects on channel activation (Fig. 7A). Consequently, the amount of total CSI was significantly reduced by both β-subunits (Fig. 7B). Nav1.4, which in our hands exhibited moderate levels of CSI (Fig. 6F), displayed moderate depolarizing shifts in SSI when coexpressed with $\beta1$ or $\beta3$ (Fig. 7C). CSI was reduced (Fig. 7D), but to a lesser degree than for Nav1.5e. Finally, activation and inactivation of Nav1.6 was unaffected by β-subunit coexpression (Fig. 7E), and the amount of total CSI was not altered (Fig. 7F). In summary, $\beta1$ and $\beta3$ depolarized SSI in each channel to a degree commensurate with the level of CSI displayed by the α-subunit alone (Fig. 6 and Fig. 7A-F), validating the regulatory mechanism proposed above.

It is well known that the effects of β-subunits on channel gating varies substantially between studies. We asked whether this variability could be explained by differences in CSI. To do this, we compared the reported effects of $\beta1$ on Nav1.1-Nav1.8 (Table S3). (We did not find sufficiently many studies to perform the same analysis for $\beta3$.) This cross-study comparison did not reveal strong evidence for $\beta1$ altering the voltage-dependence of activation (Table S3). However, the voltage-dependence of SSI exhibited large shifts following $\beta1$ coexpression (Fig. 7G, left). These shifts were extremely variable across studies, even between studies on the same α-subunit (Fig. 7G, left), suggesting that the varied effects of $\beta1$ are not due to isoform differences. Since we could not calculate CSI without access to the current recordings, we defined a "naïve" measure of CSI for each report as the difference between the $V_{1/2}$ of activation and SSI, which we denote CSI* (Fig. 7G, right). CSI* of the α-subunits alone varied significantly across studies (Fig. 7G, right), but was nevertheless linearly correlated with the shifts in SSI following coexpression with $\beta1$ ($R^2=0.7$). These observations indicate that the regulation of all Nav channel isoforms by $\beta1$ is determined by CSI. Altogether, these results suggest that the seemingly paradoxical reports of $\beta1$ regulation in the literature are, in fact, consistent with a singular mechanism of action.

Figure 7. Comparison of β-subunit regulation on various Nav isoforms.
(**A**) Left: peak activation as a function of voltage (G/V) for Nav1.5e alone (black, inverted triangles), and coexpressed with β1 (red, circles) and β3 (blue, squares). Right: steady-state inactivation (SSI). (**B**) Total CSI, as in Fig. 6F, for Nav1.5e alone and when coexpressed with β1 and β3. Each dot represents a sample. (***) indicates that $p < 10^{-4}$. (**C-F**) Same as panel A and B, but for Nav1.4 and Nav1.6. (**) indicates $p < 10^{-2}$. (**G**) Left: Change in the difference between reported $V_{1/2}$ values for activation and steady-state inactivation (ΔSSI) following coexpression of β1 with various Nav isoforms (Nav1.1-Nav1.8). Data taken from past publications (Table S3). Circles represent data from mammalian cells, while triangles represent data from Xenopus oocytes. Lines represents standard error. Right: ΔSSI is plotted against CSI* measurement from the α-subunit alone. Nav isoform is colour-coded as in the left plot. R^2 and p value for the line of best fit is reported in the bottom right.

Discussion

The present study advances our understanding of voltage-gated sodium (Nav) channels in several important and interrelated ways. First, we show that the functional contributions of the various voltage sensing domains to channel gating depend on the channel's propensity for closed-state inactivation (CSI), a gating property which reflects the movement order of the voltage sensors. Second, this study reveals that Nav channels do not follow a single, prototypical gating sequence. Instead, our experiments demonstrate that the gating of Nav channels is better explained as a continuum of gating behaviours defined by each channel's propensity for CSI. Third and finally, we show that CSI is selectively targeted by β1 and β3 auxiliary subunits to exert their allosteric effect on skeletal muscle, cardiac, and neuronal Nav channels. In fact, we provide strong evidence that this mechanism extends to every Nav channel isoform, suggesting a novel form of channel regulation. In sum, we propose that the weak to strong modulation of all Nav channel isoforms by β1 and β3 is not a direct result of

376 structural differences, per se, but is rather a natural consequence of variability in the
377 intrinsic dynamics of channel gating.

378
379 **Differing contributions of voltage sensing domains to Nav channel activation and**
380 **inactivation**

381 Previous charge neutralization experiments have reported disparate changes to the voltage-
382 dependence of activation when comparing skeletal muscle (Capes et al., 2013; Chahine et
383 al., 1994), neuronal (Kontis and Goldin, 1997), and cardiac (Chen et al., 1996) Nav
384 channels. Here, we show that changes to the voltage-dependence of activation is an
385 unreliable marker for a domain's role in activation, whereas the latency to peak current is
386 more informative (Fig. 2). Using this measure, we found that in the neonatal sodium
387 channel, Nav1.5e, DI movement is likely rate limiting for pore opening and DII movement
388 contributes significantly, but to a lesser degree. Although DIII movement is thought to be
389 necessary for channel activation in Nav1.4 skeletal muscle channels (Chanda and Bezanilla,
390 2002), neutralizing DIII in Nav1.5 did not alter channel activation (Figs. 1 & 2). This is
391 likely because DIII is already in its activated position in wildtype Nav1.5 channels at the
392 voltage range used in our protocols (Fig. 4) (Hsu et al., 2017; Zhu et al., 2017).
393 Consequently, neutralizing the domain's voltage sensor would not be expected to
394 significantly alter the overall activation process. Finally, although DIV-CN Nav1.5 mutants
395 exhibit an altered voltage-dependence of activation, the observed effects are more consistent
396 with an increased inactivation rate (Fig. 2F & Fig. S4). Considering this, we did not find
397 evidence that DIV movement is necessary for activation, consistent with previous
398 experiments on Nav1.4 channels that were mutated to prevent inactivation (Goldschen-Ohm
399 et al., 2013). In summary, our results suggest that DI and DII movements determine the rate
400 of activation, whereas DIII, at least in Nav1.5, likely plays little role in activation at
401 physiological membrane potentials.

402 Charge-neutralization experiments on Nav1.4 have suggested that DIV is uniquely
403 sufficient for inactivation (Capes et al., 2013), whereas recent VCF experiments on Nav1.5
404 have strongly implicated both DIII and DIV in inactivation (Hsu et al., 2017). Here, we
405 found that neutralizing either DIII or DIV led to a similar hyperpolarizing shift and
406 flattening of the SSI curve. Furthermore, we observed that SSI in DIV-CN Nav1.5 mutants
407 is strongly correlated with DIII movement (Fig. 4E). Together, this suggests that the Nav1.5
408 inactivation is intrinsically coupled to DIII movement. Notably, DIV-CN Nav1.4 mutants
409 inactivate at voltages too hyperpolarized to be caused by the movement of DIII (Capes et
410 al., 2013; Chanda and Bezanilla, 2002), and DIII-CN Nav1.4 mutants did not display
411 significantly altered SSI (Capes et al., 2013). Accordingly, we conclude that DIII is coupled
412 to inactivation differently in Nav1.4 and Nav1.5 channels, which could explain the
413 divergent views surrounding the role of DIII in inactivation (Ahern et al., 2016; Armstrong
414 and Hollingworth, 2018). Finally, although we found that in Nav1.5 neutralizing DI and DII
415 significantly affected inactivation, these observations could be explained by interdomain
416 coupling with DIV. Nevertheless, our observations suggest that DI and DII movements can
417 functionally contribute to channel inactivation, even if they are not necessary for
418 inactivation, per se. Overall, our results highlight unappreciated variability in the molecular
419 basis of channel gating across Nav channel isoforms.

420

Closed-state inactivation modulates the contributions of voltage sensing domains

It is well established that Nav channels can inactivate from closed states (Aldrich et al., 1983; Armstrong, 2006; Bean, 1981; Lawrence et al., 1991; Vandenberg and Horn, 1984), which is thought to occur when DIII and DIV (possibly just DIV in Nav1.4) move prior to channel activation (Armstrong, 2006). Nevertheless, the prevailing idea is that DIV movement is substantially slower than the other domains across all voltages (Armstrong, 2006; Bosmans et al., 2008; Capes et al., 2013; Chanda and Bezanilla, 2002), implying that inactivation occurs predominantly from the open state. Interestingly, almost all evidence for this delayed DIV movement is from Nav1.4 channels (Capes et al., 2013; Chanda and Bezanilla, 2002; Goldschen-Ohm et al., 2013). In contrast to Nav1.4, however, we observed a high propensity for CSI in wildtype Nav1.5 channels (Fig. 6D), indicating a gating sequence that is preferential to early DIV movement. We showed that such a difference in gating sequence is sufficient to explain the contrasting outcomes of voltage sensor neutralization between Nav1.4 and Nav1.5 channels. Furthermore, we found that this apparent dichotomy of low and high CSI gating in fact defines a *continuum* of gating behaviours along which all Nav channel isoforms lie (Fig. 7 & 8). This study thus suggests that the gating sequence is a variable parameter which modulates the contributions of each voltage sensor to activation and inactivation across Nav channels. More generally, our modelling predicts that CSI should modulate the effects of any gating modifiers which target the movements of these voltage sensing domains. Indeed, this mechanism succeeded in predicting the effects of auxiliary β subunits (discussed below), highlighting the unappreciated importance of CSI in the biological functionality of all Nav channels.

Intrinsic gating properties of Nav channels dictate auxiliary subunit regulation

The auxiliary subunits, β1 and β3, have surprisingly varied effects on Nav channel gating, even within a single isoform (Fig. 7G, Table S3). Consequently, this variability is likely not due to differences in channel structure (Jiang et al., 2020; Shen et al., 2019). Rather, the observations made here motivate an alternative explanation: β1 and β3 specifically target CSI such that their effects on channel gating are determined by the variable dynamics of the pore-forming subunit. Theoretically, we found that if β1 and β3 stabilize the resting state of DIV, as demonstrated in Nav1.5 using VCF (Zhu et al., 2017), then their effects should depend on the channel's propensity for CSI (Fig. S4). This prediction was confirmed by our experiments comparing Nav1.4, Nav1.5, and Nav1.6 channels (Fig. 7).

Intriguingly, the idea that the intrinsic gating properties of a channel predetermine the effects of allosteric modifiers has also recently been suggested for AMPA receptors (Dawe et al., 2019). Depending on the channel splice variant (flip or flop), gating modifiers, such as anions and auxiliary subunits, either exert an effect (flip) or no effect (flop), similar to what we observe for high and low CSI Nav channels. This switching in regulation was shown to be due to the mobility of the apo or resting state of the receptor (Dawe et al., 2019), suggesting a critical role for the intrinsic dynamics of the pore-forming subunits. These observations point to an over-arching principle of ion channel regulation which could be tested by exploring the potential role of the intrinsic gating to other ion channel families.

Notably, our predictions concerning the influence of CSI on Nav channel regulation extend to any gating modifiers which target the movements of specific voltage sensing domains. Interestingly, several studies on toxins that target voltage sensors – which include

466 certain scorpion, sea anenome, and cone snail toxins (Ahern et al., 2016) – have described
467 varied effects across Nav channel isoforms (Alami et al., 2003; Leipold et al., 2006;
468 Oliveira et al., 2004). Although many of these observations have been ascribed to structural
469 variation between the channels, it would be informative to assess the extent to which
470 differences in CSI may also contribute. Since any compounds which target voltage sensor
471 movements in Nav channels have potential as novel drugs for chronic pain, epilepsy, and
472 heart disorders (Bosmans and Swartz, 2010; Cardoso and Lewis, 2019), identifying sources
473 of CSI variability *in vivo* and understanding why CSI is so poorly controlled for *in vitro*
474 (Fig. 7G) may have particular importance for novel drug design. Finally, it has been
475 suggested that insights into channelopathies may be realized through the analysis of
476 "homologous" mutations across Nav channels (Loussouarn et al., 2016). Whether CSI alters
477 the consequences of other mutations as profoundly as the voltage sensor neutralizing
478 mutations performed here is clearly of interest for future study.

479

480 **Methods**

481 **Molecular biology**

482 The mouse mH1 pcDNA3.1(+)-plasmid was obtained from Dr. T. Zimmer (Camacho et al.,
483 2006). Exon 6a cDNA was amplified out of mouse brain homogenates, using the Access
484 RT-PCR System (Promega), and then exchanged with exon 6b in mH1 using the
485 Quikchange method of site-directed mutagenesis (SDM) (Braman et al., 1996), to generate
486 the Nav1.5e plasmid. A similar RT-PCR approach was also used to clone out the mouse β1
487 and β3 cDNA and insert it into a pcDNA3.1(+) vector.

488 Charge-neutralizing mutations of Nav1.5e were engineered using single-primer
489 reaction in parallel (SPRINP) SDM (Edelheit et al., 2009). Primers (Integrated DNA
490 Technologies) were designed containing both the amino acid exchanges of interest and a
491 silent restriction site. Following the PCR reaction, unmutated templates were digested using
492 0.4-0.8 U/μL of DpnI (New England Biolabs). The resulting PCR mixture was transformed
493 into house-grown competent DH5α. Colonies were grown overnight on agar plates and
494 picked for liquid culturing in 25 g/L Lysogeny Broth (Fisher Bioreagents). Plasmids were
495 harvested using QIAprep Spin Miniprep Kits (Qiagen). Mutations were screened via
496 restriction digest and gel electrophoresis, using the silent restriction sites initially designed
497 into the SDM primers. Plasmid sequences were verified with Sanger sequencing done by
498 the Innovation Centre of McGill University and Genome Quebec, using Sequencher 4.8 and
499 CLC Sequence Viewer 8.0.

500 Nav1.5e DIV-CN constructs were modified further by conjugating the Nav sequence
501 to an engineered GFP fluorophore called Mystik (Mys) via a P2A linker. The P2A allowed
502 for the stoichiometric 1:1 translation of the Mystik and Nav genes, so that cells showcasing
503 the strongest fluorescence were the most likely to express the Nav channel in higher
504 abundance (Ahier and Jarriault, 2014). The allowed us to overcome the poor expression of
505 Nav1.4 alone. Primers (Integrated DNA Technologies) with overhangs containing
506 fragments of the Nav plasmid's 5' untranslated region or part of the P2A-Nav sequence
507 were used to PCR amplify a double-stranded "megaprimer" containing (from 5' to 3'): the
508 5' UTR upstream of the Nav channel, the entire Mys gene, the P2A sequence, and the 5'
509 end of the Nav channel cDNA. This megaprimer was then isolated via gel electrophoresis,

510 extracted into solution using MinElute Gel Extraction Kits (Qiagen), and inserted into the
511 Nav channel plasmid by means of the Quikchange method of PCR discussed previously.

512

Cell culture

514 Human embryonic kidney cells mutated to over-express the SV40 large T-antigen (HEK-
515 293T cells) were used as the expression system for electrophysiological recordings. HEK-
516 293T cultures were maintained in minimal essential media containing Glutamax (MEM-
517 glutamax; Gibco), supplemented with 10% fetal bovine serum (FBS; Gibco), and kept at
518 conditions of 37°C, 100% humidity, and 5% CO_2 in a ThermoForma Series II Water-
519 Jacketed CO_2 Incubator (ThermoFisher). Main HEK-293T stocks were grown in T-25
520 Flasks (Corning) and twice a week passaged using Trypsin-EDTA solution (Gibco) into 35-
521 mm Tissue-Culture Treated Culture Dishes (Corning) for transfection purposes. The
522 calcium phosphate transfection protocol (Jordan et al., 1996) was used to transiently
523 transfect HEK-293T cells at least 24 hours before recordings. Each 35-mm culture dish was
524 loaded with 0.5 µg of the Nav channel plasmid, 0.2 µg of the transfection reporter Mys
525 (unless already present, as in the Mys-P2A-Nav constructs), and, if present, 1.5 µg of β1 or
526 β3. The cDNA mixture was dissolved in 560 mM of $CaCl_2$ (unless otherwise indicated, all
527 chemical reagents are from Sigma-Aldrich), and an equal volume of 2XBES solution (in
528 mM: 50 BES, 280 NaCl, and 1.5 Na_2HPO_4) was added to induce precipitate formation.
529 After roughly one minute, the DNA-calcium phosphate precipitates were added to a single
530 culture dish. The dish was then returned to the incubator where cells had time to be
531 transfected, typically over 6 to 9 hours. The reaction was quenched 6 to 9 hours later by
532 rinsing with phosphate-buffered saline (PBS, in mM: 137 NaCl, 2.7 KCl, 10.1 Na_2HPO_4,
533 and 2 NaH_2PO_4) containing 1 mM EDTA and rinsed with PBS containing 1 mM $MgCl_2$ and
534 1 mM $CaCl_2$. Transfected HEK-293T cells were left to recover overnight.

535

Electrophysiology

537 At least two hours before the start of recordings, transfected cells were dissociated from
538 their cultures using Accutase and then re-plated at a lower density. This step increased the
539 yield of isolated cells, minimizing gap junctions that form between adjacent HEK-293T
540 cells and optimizing the quality of the voltage-clamp conditions. Culture media was
541 replaced with external solution containing (in mM): 155 NaCl, 4 KCl, 5 HEPES, 1 $MgCl_2$,
542 and 1.8 $CaCl_2$, with pH adjusted to 7.3-7.4 using NaOH. Cells were patched with
543 microelectrodes containing an internal solution that was optimized for voltage-clamp
544 conditions, made up of (in mM): 115 CsCl, 5 HEPES, 5 Cs_4-BAPTA, 1 $MgCl_2$, 0.5 $CaCl_2$,
545 10 Na_2ATP, with pH adjusted to 7.3-7.4 using CsOH and sucrose added to keep the
546 osmolality matching that of the external solution, between 295-300 mOsm. Borosilicate
547 glass capillaries – with an inner diameter of 1.15 mm, an outer diameter of 1.65 mm, a
548 length of 100 mm, and a 0.1 mm filament (King Precision Glass, Inc) – were pulled using a
549 PP-830 vertical puller (Narishige), yielding microelectrodes with a pipette resistance of 1 to
550 5 MΩ. Microelectrode tips were then dipped into Bees-Wax Pure Natural (Integra Miltex)
551 and subsequently fire-polished with an MF-900 Micro Forge (Narishige), to reduce noise
552 and improve membrane seals. Dissociated cells were viewed using an Eclipse Ti-U Inverted
553 Microscope (Nikon). Transfected cells were identified by their green fluorescence, excited

554 by a DC4104 4-Channel LED Driver (Thorlabs). Positive pressure was applied orally to
555 electrodes before being lowered to the bottom of the recording chamber using an MP-285
556 micromanipulator (Sutter Instrument). Once the electrode tip was positioned just above the
557 surface of the cell, the release of the positive pressure was sufficient to form a glass-
558 membrane seal of at least 1 GΩ. Pulses of negative pressure were then delivered to improve
559 the strength of the seal and eventually break through, giving access to the whole-cell patch-
560 clamp configuration. All recordings were done at room temperature.

561 Voltage commands were delivered through an AxoPatch 200B Amplifier (Axon
562 Instruments). Capacitive transients from the pipette and from the cell were cancelled, cell
563 capacitance and series resistance were monitored to avoid changes exceeding 30% over the
564 course of the recording, and series resistance was compensated to the amplifier's maximum
565 (98% on the machine, but in practice probably closer to 80%). Currents were acquired at
566 100 kHz, low-pass Bessel filtered at 5 kHz using a Model 900 Tunable Active Filter
567 (Frequency Devices), and telegraphed via an Axon Digidata 1550 (Molecular Devices). All
568 data was collected and saved digitally using pClamp 10.7 software (Axon Instruments).

569

570 **Voltage-clamp protocols**

571 When cells were not being recorded, either between protocols or between sweeps within a
572 protocol, they were clamped at a holding potential of -60 mV. To assess channel activation,
573 cells were stepped down to the baseline potential for 300 ms, depolarized to a series of
574 potentials between -110 and +70 mV in increments of 5 mV for 100 ms, then returned to the
575 baseline potential for another 300 ms. This baseline potential was either -100 mV for
576 Nav1.5e, or -130 mV in the case of the charge-neutralized mutants, to counter the latter's
577 increased propensity for channel inactivation. To assess steady-state inactivation, cells were
578 stepped down to the baseline potential for 300 ms, given a pre-pulse that varied between -
579 160 mV and -5 mV in increments of 5 mV for 100 ms, pulsed directly to the test pulse
580 potential of -10 mV for 50 ms, then returned to -100 mV for an additional 300 ms. Leak
581 subtraction was performed on the raw data in a custom Igor Pro (Wavemetrics) program
582 post-hoc.

583

584 **Voltage-clamp fluorometry**

585 All experiments conducted on *Xenopus laevis* were done with the approval of and according
586 to the guidelines established by the Animal Care Committee of the National Institutes of
587 Natural Sciences, an umbrella institution of the National Institute for Physiological
588 Sciences. We obtained the Nav1.5 VCF constructs used by Varga et al. (Varga et al., 2015)
589 from Dr. Johnathon R. Silva and used site-directed mutagenesis, as described previously, to
590 convert them to their respective Nav1.5e variants (with the exception of position 215, which
591 was not mutated to leucine because it was used as a labelling cysteine). The four Nav genes,
592 inserted on pMax vectors, could be linearized using Pacl (TOYOBO) and used as a template
593 to generate cRNA using the mMESSAGE T7 RNA transcription kit (Thermo Fisher
594 Scientific).

595 The oocytes of *Xenopus laevis* were surgically harvested from anaesthetized animals
596 as described previously (Kume et al., 2018). Oocytes were separated ad defolliculated using

597 2 mg/ml collagenase treatment for 6.5 hours. Then oocytes were incubated overnight at
598 17°C in Ringer's solution containing (in mM): 88 NaCl, 1 KCl, 2.4 NaHCO$_3$, 0.3 Ca(NO$_3$)$_2$,
599 0.41 CaCl$_2$, 0.82 MgSO$_4$, and 15 HEPES, with pH adjusted to 7.4 with NaOH and use of
600 0.1% penicillin-streptomycin. The remaining follicular layers were manually peeled. 25 ng
601 of Nav channel cRNA was injected into the vegetal pole of oocytes using Nanoject II
602 (Drummond). Treated oocytes were then returned to their 17°C incubator and left for 1 to 3
603 days to express protein.

604 Oocytes were labelled for 20 minutes using 10 μM of methanethiosulfonate-
605 carboxytetrmethylrhodamine (MTS-TAMRA) dye on ice. Dye conjugation was done in
606 depolarizing solution (containing, in mM, 110 KCl, 1.5 MgCl2, 0.8 CaCl2, 0.2 EDTA and
607 10 HEPES, pH adjusted to 7.1 with KOH) in an effort to expose and label S4 helices.
608 Excess dye was removed by rinsing five times with fresh ND-96 solution (96 mM NaCl, 2
609 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, with pH adjusted to 7.4 wit
610 NaOH). Oocytes in solution were kept on ice until use. Eventually, oocytes were transferred
611 to the recording chamber filled with ND-96 solution, at room temperature, oriented in such
612 a way that fluorescent recordings were done on the animal pole. Voltage clamp for
613 macroscopic current recording was performed by using an amplifier (OC-725C, Warner
614 Instruments). Borosilicate glass capillaries (World Precision Instruments) were used with a
615 resistance of 0.1–0.3 MΩ when filled with 3 M KOAc and 10 mM KCl. The fluorescent
616 recordings were performed with the fluorescence microscope (Olympus BX51WI) equipped
617 with a water immersion objective lens (Olympus XLUMPLAN FL 20x/1.00). The light
618 source was emitted by a xenon arc lamp (L2194-01, Hamamatsu Photonics) and passed
619 through a band-pass excitation filter (520-550 nm). The intensity of the excitation light was
620 decreased to prevent the fluorophore from bleaching by ND filters (Olympus U-25ND6 and
621 U-25ND25). Emitted light was passed through a band-pass emission filter (580IF). The
622 emission signals were detected by a photomultiplier (Hamamatsu Photonics H10722-110).
623 The baseline signal was adjusted to 2 V. The detected current and fluorescent signal were
624 acquired by a Digidata 1332 (Axon Instruments) and Clampex 10.3 software (Molecular
625 Devices) at 100 kHz.

626 Oocytes were held at a baseline of -60 mV between sweeps and protocols. Once we
627 could detect a visible change in fluorescence upon depolarization of the oocyte from -120
628 mV to +60 mV, we could run the following activation protocol. The oocyte was stepped
629 down to -120 mV for about 300 ms, then hyperpolarized/depolarized to a series of
630 potentials between -180 mV and +60 mV in increments of 10 mV for 100 ms, then stepped
631 back to -120 mV for about 300 ms before returning to baseline. The protocol was run and
632 averaged 10 times to improve the signal-to-noise ratio in the fluorescent output. Voltage-
633 clamp fluorometry data was analyzed automatically using custom-made Igor Pro scripts.

634

635 **Literature search**

636 Values for the effect of β1 coexpression on Nav gating were gathered from published
637 studies found through searching keywords on Google Scholar and from papers cited in these
638 studies. The search was restricted to data collected in either HEK293 cells or Xenopus
639 oocytes. Following least-squares regression of ΔSSI against CSI*, one outlier was identified

640 using Cook's distance and subsequently excluded from the analysis (Nav1.8; Xenopus
641 oocytes; (Vijayaragavan et al., 2001)). All numerical values are reported in Table S3.

642

643 **Data analysis and statistics**

644 For activation, E_{Na} was determined by fitting the linear part of the current-voltage curve to a
645 line and extrapolating its x-intercept. Conductance was calculated as $I/(V-E_{Na})$, then fit with
646 a Boltzmann function

647
$$G(V) = \frac{G_{max}}{1 + e^{-\frac{V-V_{1/2}}{k}}},$$

648 where G_{max} is the maximum conductance, $V_{1/2}$ is the half-activation potential, and k is the
649 slope factor of voltage sensitivity. For steady-state inactivation, peak currents following the
650 test pulse were plotted against the corresponding pre-pulse potential; a Boltzmann function
651 was then fit to this I-V curve. To calculate time to peak, the maximum current occurring at
652 least 0.5 ms following the voltage step was identified (to avoid identifying capacitive
653 transients at low voltages). The latency from the moment of voltage step to this peak was
654 calculated.

655 To experimentally quantify the fraction of inactivation occurring from closed states
656 (i.e. closed-state inactivation), we adapted the method proposed by Armstrong (Armstrong,
657 2006). Briefly, open-state inactivation was estimated during a conditioning voltage step (-
658 100 ms long, ranging from -120 to -5 mV), and then compared to the fraction of available
659 channels during a subsequent test pulse (-10 mV), thereby estimating the fraction of steady-
660 state inactivation occurring through open states (Fig. 6C). Closed-state inactivation was
661 then defined as the complementary fraction. Specifically, open-state inactivation (OSI) was
662 estimated by the equation

663
$$OSI(V) \propto \sum_{t=0}^{\tau} g(t; V) \, \Delta t,$$

664 where $g(t; V)$ is the experimentally observed channel conductance at time t for voltage step
665 V. In the original method (Armstrong, 2006), OSI was scaled by assuming 100% of steady-
666 state inactivation occurs from open states at 0 mV. However, our data displayed
667 significantly more closed-state inactivation than was observed by Armstrong (Armstrong,
668 2006) and consequently OSI did not necessarily attain a maximum within the tested voltage
669 range. We therefore modified this method by fitting a Boltzmann function, $f(V) =$
670 $\alpha\left(1 + e^{-(V-V_{1/2})/k}\right)^{-1}$, to the unscaled OSI versus voltage curve to obtain a scaling factor,
671 i.e. α. This allowed open-state inactivation to attain its maximum outside of the observed
672 voltage range.

673 For statistical analysis, Tukey's method was used following a one-way ANOVA, with
674 a significance level of $p < 0.01$. All data was reported as mean ± SE. CSI* was calculated
675 as the difference between group means; the standard error estimate of CSI* (for plotting
676 purposes, see Fig. 7G) was thus defined in the sense of Welch's t-test, i.e. $SE =$
677 $\sqrt{s_1^2/N_1 + s_2^2/N_2}$.

678

Computational modelling

680 For simulations of Nav gating, the kinetic model of Nav1.4 developed by Capes et al.
681 (Capes et al., 2013) was reparametrized with a custom-made evolutionary fitting algorithm
682 programed in MATLAB 2017b (The MathWorks, Inc.). The first generation of models were
683 initialized with parameters drawn from a multivariate Gaussian centered at the Nav1.4
684 parameters with zero covariance and a coefficient of variation of 1. Each individual was
685 assessed on z-scores calculated with respect to our experimental observations of the
686 integral, peak amplitude, and time to peak of the activation and inactivation currents, as
687 well as the recovery from inactivation, steady-state inactivation, and IV curves (Fig. S2).
688 We ran the algorithm until the maximum fitness did not improve by at least 10% over 40
689 generations. When reparametrizing the model, we relaxed several assumptions made by the
690 original authors. Specifically, we removed the constraint that the voltage-dependent charge
691 of transitions reflecting movement of the same domain be the same and did not define any
692 parameters with respect to others, no longer forcing microscopic reversibility. Nonetheless,
693 the modelled conductances were well behaved during all simulations and our sensitivity
694 analyses demonstrated a smoothness of model output with respect to local perturbations
695 (Fig. S2E, F), suggesting that we did not overfit the model. The resulting parameter values
696 are reported in Table S2.

697 To produce the low CSI gating model (Fig. 5 & Fig. S4), the parameters where
698 adjusted to bias DIV to move following pore opening. This was done by decreasing the
699 forward rate of DIV movement while the channel was closed (α_4 was decreased 100 fold)
700 and increasing the forward rate following pore opening (α_{4O} was increased 100 fold).
701 Consequently, the probability of CSI was reduced.

702 All simulated protocols were identical to those applied experimentally. The modelled
703 current was calculated as $I_{mem} = (O + O_4)(V - E_{rev})$, where E_{rev} is the average reversal
704 potential calculated experimentally, O and O_4 are the open states of the channel, and V is
705 voltage. As for all protocols with applied step voltages, solutions to the model were
706 computed using the exponential of the transition matrix.

707

Data and materials availability

709 Summary data and all the code used in the generation of figures is publicly available at
710 https://github.com/niklasbrake/Nav2020. Raw data is available upon request from the
711 authors.

712

713

Acknowledgments

715

743   **References**

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Supplementary Figures & Tables
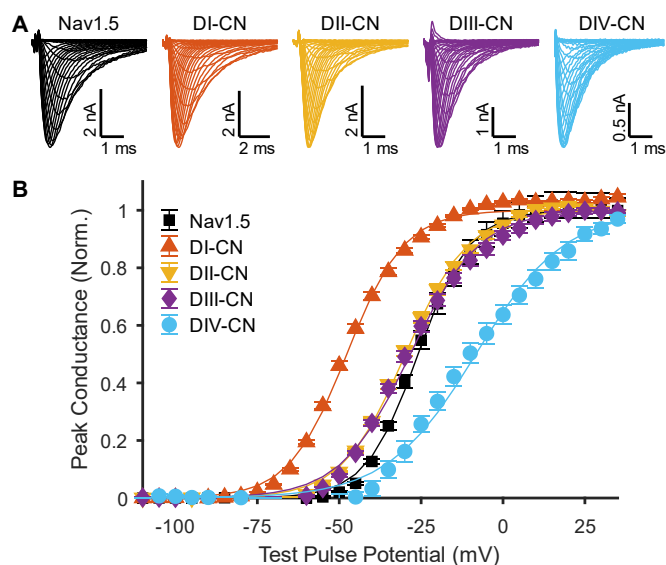
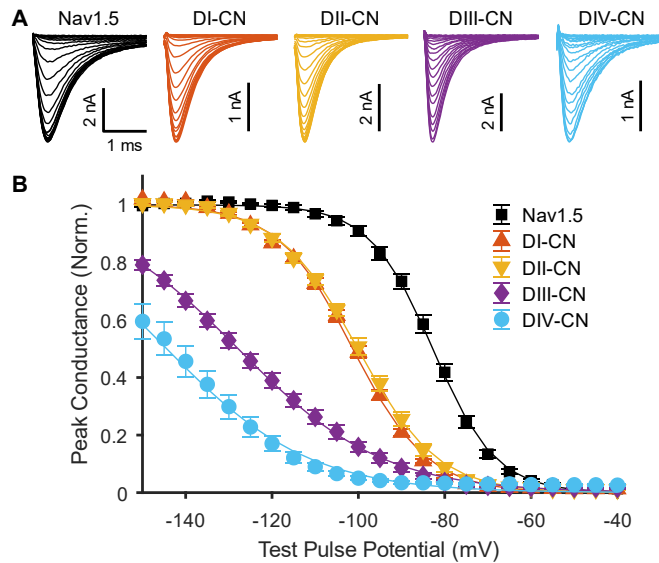**Fig. S1. Changes in peak GV following domain neutralization in adult form of Nav1.5, mH1.**

(**A**) Representative traces of ionic currents corresponding to wild-type (WT) Nav1.5 (cell 20170311c3) and mutant channels (DI-CN, cell 20180301c6; DII-CN, cell 20180711c3; DIII-CN, cell 20180309c12; DIV-CN, cell 20181022c7) in response to depolarizing voltage steps ranging from -110 to 35 mV, following a holding potential of -130 mV (-100 mV for WT), recorded in HEK-293T cells. (**B**) Summary data corresponding to panel **A**, showing normalized peak current following the test pulse, as a function of the test pulse voltage.
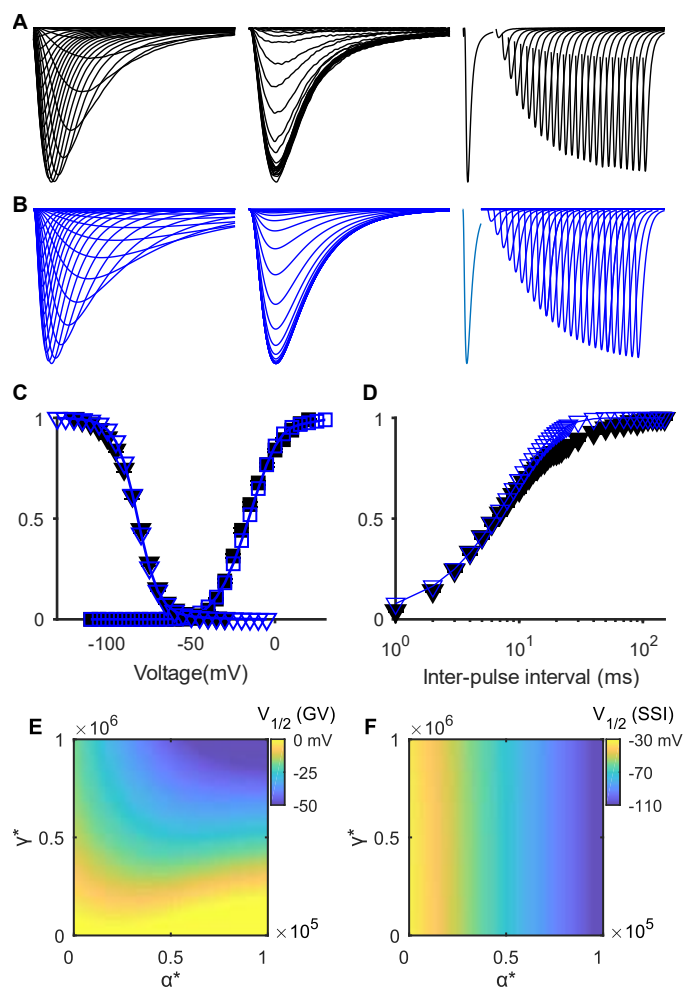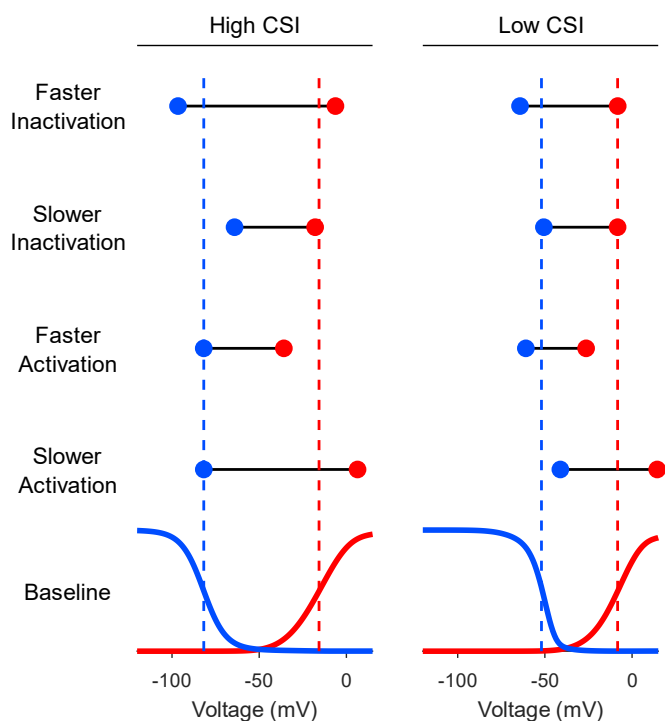
Fig. S2. Comparison of reparametrized Nav1.5e model to data.
(**A**) From left to right, averaged currents recorded from WT Nav1.5e channels during the activation protocol (as in Fig. 1), inactivation protocol (as in Fig. 3), and recovery from inactivation protocol (currents elicited by a test pulse of -10 mV, following a holding potential of -10 mV and a hyperpolarizing inter-pulse of -120 mV which lasted between 1-150 ms). (**B**) Modelled currents with the gating model in Fig. 2A and parameters from Supplementary Table S2. (**C**) Steady-state inactivation (inverted triangles) and GV (square) curves of WT Nav1.5e channel (black) and model (blue). (**D**) Fraction of recovered current as a function of inter-pulse interval for the WT channel (black) and model (blue). (**E**) Heat-map showing the sensitivity of the $V_{1/2}$ of GV. At low values of α^*, changes in γ^* have little effect on the $V_{1/2}$ of GV, and vice versa. However, as both are increased, the $V_{1/2}$ of GV becomes more hyperpolarized. (**F**) Heat-map showing the sensitivity of the $V_{1/2}$ of SSI to changes in α^* and γ^*. As α^* increases, a hyperpolarizing shift in the $V_{1/2}$ of SSI is observed, whereas changes in γ^* produce almost no effects on SSI. Both heat-maps are color-coded based on the color-bars to the right of each panel.

975
976 **Fig. S3. Changes in steady-state inactivation following domain neutralization in adult**
977 **form of Nav1.5, mH1.**
978 (**A**) Representative traces of ionic currents corresponding to wild-type (WT) Nav1.5 (cell
979 20170311c3) and mutant channels (DI-CN, cell 20180301c6; DII-CN, cell 20180711c3;
980 DIII-CN, cell 20180309c12; DIV-CN, cell 20190305c4) in response to a test pulse to -10
981 mV following conditioning pulses ranging from -160 to -30 mV. (**B**) Summary data
982 corresponding to panel **A**, showing normalized peak current following the test pulse, as a
983 function of the conditioning pulse voltage.

Fig. S4. Comparison of models with high and low propensities for closed-state inactivation (CSI).

High CSI refers to the reparametrized Nav1.5e model (Fig. 2A and Table S2). Low CSI refers to the same model, where inactivation has been biased towards open-state inactivation (see Methods). The GV (red) and SSI (blue) curves of these two models are depicted at the bottom (baseline). Above these curves are depicted the changes in the $V_{1/2}$ of GV (red circle) and SSI (blue circle) following four different parameter modifications, with the dashed lines providing a reference to the baseline values: faster inactivation, α_4 increased 10-fold; slower inactivation, α_4 decreased 10-fold; faster activation, γ increased 10-fold; slower inactivation, γ decreased 10-fold.

Table S1. Boltzmann parameters for Nav1.5e.

Nav1.5e	$V_{1/2}$	k	n
WT[†]	-16.8 ± 0.5	9.0 ± 0.1	51
	-82.0 ± 0.5	-7.2 ± 0.1	49
DI-CN	-48.1 ± 0.6	9.9 ± 0.1	24
	-100.9 ± 0.5	-8.7 ± 0.1	23
DII-CN	-22.6 ± 0.5	9.6 ± 0.2	26
	-96.5 ± 0.8	-9.2 ± 0.2	25
DIII-CN	-19.0 ± 0.6	10.3 ± 0.2	23
	-120.0 ± 1.1	-14.4 ± 0.2	23
DIV-CN	-7.4 ± 1.3	10.7 ± 0.3	14
	-131.1 ± 2.6	-13.7 ± 1.5	13
$+ \beta_1$	-14.8 ± 0.7	8.6 ± 0.1	14
	-73.5 ± 1.0	-6.1 ± 0.3	11
$+ \beta_3$	-13.8 ± 0.8	8.9 ± 0.2	18
	-74.5 ± 0.6	-6.2 ± 0.2	17
DI*-VCF[‡]	-65.4 ± 2.3	12.3 ± 1.2	13
DII*-VCF	-44.1 ± 0.7	22.2 ± 0.6	17
DIII*-VCF	-137.4 ± 1.1	15.9 ± 0.6	27
DIV*-VCF	-69.5 ± 1.3	13.2 ± 0.5	15

[†]Top row for each channel contains estimated Boltzmann parameters for peak activation (G/V) and bottom row contains parameters for steady-state inactivation (SSI). [‡]Parameters for VCF constructs are fits to the fluorescence-voltage (F-V) curve. Data report mean $\pm$ S.E.

1001 **Table S2. Parameters for Nav1.5e gating model.**

Rate constant	k^*	q	Cooperativity factor	
α	17,000	-0.23	x_α	0.73
β	830	1.4	x_β	0.092
α_4	1,000	-0.22	y_α	4.1
β_4	3.0×10^6	-0.43	y_β	0.65
α_{4o}	1,800	-0.7		
β_{4o}	74	1.7		
γ	10,000	-3.0		
γ_i	51,000	-5.2		
γ_4	17,000	-6.2		
δ	910	-1.9		
δ_4	690	-2.2		
δ_i	3,000	-0.81		
i	25,000	-0.013		
r	5,200	0.054		
i_o	11,000	0.11		
r_o	0.001	0		

1002 Parameter values for the fit shown in Fig. S2. The rate, k, of each transition is computed as $k = k^*$
1003 $\exp(-qV/KT)$, where V is the membrane voltage, K is Boltzmann's constant, T is the temperature,
1004 and k* and q are fitted parameters. The cooperativity factors x and y reflect the degree of coupling
1005 between the activation of DI–III and DIV activation or channel inactivation, respectively.
1006
1007

Table S3. Summary for β1 effects on activation and inactivation.

Isoform	Inactivation		Activation		Cell	Ref.
	α	+ β1	α	+ β1		
Nav1.1	-55.5 ± 1.3	-54.2 ± 0.8	-18.3 ± 1.5	-19.0 ± 1.8	HEK	Patino et al. (2009)
	-35.0 ± 1.0	-43.0 ± 1.0	-17.0 ± 1.0	-18.0 ± 1.0	Oocyte	Smith and Goldin (1998)
Nav1.2	-51.7 ± 2.6	-58.1 ± 1.3	-18.9 ± 1.4	-20.1 ± 3.1	Oocyte	Patino et al. (2009)
	-42.0 ± 2.0	-52.0 ± 1.0	-18.0 ± 2.0	-21.0 ± 2.0	Oocyte	Smith and Goldin (1998)
Nav1.3	-64.9 ± 1.5	-59.9 ± ??	-25.5 ± 1.6	-25.5 ± ??	HEK	Cummins et al. (2001)
Nav1.4	-54.0 ± 1.0	-60.0 ± 1.0	-27.0 ± 1.0	-26.0 ± 1.0	Oocyte	Nuss et al. (1995)
	-54.0 ± 0.4	-62.0 ± 0.1	-25.0 ± 0.5	-33.0 ± 0.3	Oocyte	Sánchez-Solano et al. (2017)
	-66.1 ± 0.6	-66.5 ± 0.5	-22.8 ± 0.9	-23.9 ± 1.2	HEK	Ferrera and Moran (2006)
	-74.2 ± 1.9	-65.3 ± 1.6	-24.8 ± 1.2	-24.7 ± 2.3	HEK	Bendahhou et al. (1995)
	-67.7 ± 1.3	-66.0 ± 2.2	-19.2 ± 2.0	-19.1 ± 4.7	HEK	Hayward et al. (1996)
	-64.0 ± 0.7	-59.9 ± 0.5	-8.0 ± 0.7	-6.1 ± 0.6	HEK	This study
Nav1.5	-76.0 ± 1.0	-78.0 ± 1.0	-36.0 ± 1.0	-37.0 ± 1.0	Oocyte	Nuss et al. (1995)
	-52.0 ± 4.2	-56.1 ± 3.5	-27.8 ± 2.3	-28.7 ± 1.6	Oocyte	Qu et al. (1995)
	-65.3 ± 0.9	-65.9 ± 0.8	-18.6 ± 4.2	-23.3 ± 2.0	Oocyte	Fahmi et al. (2001)
	-84.8 ± 2.5	-74.0 ± 2.4	-35.8 ± 1.4	-34.6 ± 1.9	Oocyte	Zhu et al. (2017)
	-77.1 ± 0.5	-72.9 ± 1.0	-25.6 ± 1.0	-24.9 ± 1.0	HEK	Malhotra et al. (2001)
	-82.0 ± 0.5	-73.5 ± 1.0	-16.8 ± 0.5	-14.8 ± 0.7	HEK	This study
Nav1.6	-74.3 ± 2.3	-72.2 ± 0.6	-36.7 ± 1.1	-34.8 ± 1.7	HEK	Zhao et al. (2011)
	-51.5 ± 0.4	-50.8 ± 0.3	-13.4 ± 0.8	-14.2 ± 0.3	Oocyte	Tan and Soderlund (2011)
	-54.2 ± 0.7	-50.7 ± 1.3	-11.5 ± 0.5	-11.6 ± 0.6	HEK	This study
Nav1.7	-70.9 ± 0.5	-65.7 ± 0.5	-18.6 ± 0.4	-17.4 ± 1.8	HEK	Laedermann et al. (2013)
	-68.2 ± 0.4	-69.2 ± 0.4	-22.0 ± 2.7	-27.7 ± 1.3	Oocyte	Vijayaragavan et al. (2001)
Nav1.8	-43.2 ± 2.0	-47.8 ± 1.5	-12.5 ± 1.7	-16.5 ± 1.4	HEK	Bendahhou et al. (1995)
	-54.8 ± 1.7	-62.6 ± 2.3	4.7 ± 0.7	-3.3 ± 1.0	Oocyte	Vijayaragavan et al. (2001)