

**The tarantula toxins ProTx-II and HWTX-IV differentially interact with human
Na_v1.7 voltage-sensors to inhibit channel activation and inactivation**

Yucheng Xiao, Kenneth Blumenthal, James O. Jackson II, Songping Liang and Theodore
R. Cummins

Department of Pharmacology and Toxicology, Indiana University School of Medicine,
Indianapolis, Indiana 46202 (Y.X., J.O.J., T.R.C.),

Department of Biochemistry, School of Medicine and Biomedical Sciences, State
University of New York, Buffalo, New York 14214, USA (K.B.)

and

Key Laboratory of Protein Chemistry and Developmental Biology of the Ministry of
Education, College of Life Sciences, Hunan Normal University, Changsha, Hunan
410081, China (Y.X., S. L.)

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Corresponding author: Dr. Theodore R. Cummins, Department of Pharmacology and Toxicology, Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, Indiana 46202, USA. Email: trcummin@iupui.edu.

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Abbreviations:

HWTX-IV, huwentoxin-IV; TTX, tetrodotoxin; Na_v, voltage-gated sodium channel; WT, wild type; HEK293, human embryonic kidney 293; domains I-IV, DI-DIV; transmembrane segments 1-6, S1-S6

Abstract

The voltage-gated sodium channel $\text{Na}_v1.7$ plays a crucial role in pain, and drugs that inhibit $\text{hNa}_v1.7$ may have tremendous therapeutic potential. ProTx-II and huwentoxin-IV (HWTX-IV), cystine knot peptides from tarantula venoms, preferentially block $\text{hNa}_v1.7$. Understanding the interactions of these toxins with sodium channels could aid the development of novel pain therapeutics. While both ProTx-II and HWTX-IV have been proposed to preferentially block $\text{hNa}_v1.7$ activation by trapping the domain-II voltage-sensor in the resting configuration, we show that specific residues in the voltage-sensor paddle of domain-II play substantially different roles in determining the affinities of these toxins to $\text{hNa}_v1.7$. The mutation E818C increases ProTx-II's and HWTX-IV's IC_{50} for block of $\text{hNa}_v1.7$ currents by 4- and 400-fold, respectively. In contrast, the mutation F813G decreases ProTx-II affinity by 9-fold, but has no effect on HWTX-IV affinity. Importantly, we also show that ProTx-II, but not HWTX-IV, preferentially interacts with $\text{hNa}_v1.7$ to impede fast-inactivation by trapping the domain-IV voltage-sensor in the resting configuration. Mutations E1589Q and T1590K in domain-IV each decreased ProTx-II's IC_{50} for impairment of fast-inactivation by ~6-fold. In contrast mutations D1586A and F1592A in domain-IV increased ProTx-II's IC_{50} for impairment of fast-inactivation by ~4-fold. Our results show that 1) while ProTx-II and HWTX-IV binding determinants on domain-II may overlap, domain-II plays a much more crucial role for HWTX-IV and 2) contrary to what has been proposed to be a guiding principle of sodium channel pharmacology, molecules do not have to exclusively target the domain-IV voltage-sensor in order to influence sodium channel inactivation.

Introduction

Voltage-gated sodium channels play important roles in action potential generation and propagation. As $\text{Na}_v1.7$ is a crucial contributor to pain sensation (Cox et al., 2006; Cummins et al., 2007), drugs that selectively target human $\text{Na}_v1.7$ ($\text{hNa}_v1.7$) could be ideal analgesics. Unfortunately drugs targeting sodium channels typically have broad spectrum sodium channel activity and narrow therapeutic windows (Cummins and Rush, 2007). Therefore there is substantial interest in identifying compounds that selectively target $\text{hNa}_v1.7$ and determining their molecular mechanisms of action.

ProTx-II and Huwentoxin-IV (HWTX-IV) are tarantula toxins that target voltage-gated sodium channels. These toxins belong to the inhibitory cystine knot family and are stabilized by the same disulfide frame (C1-C4, C2-C5 and C3-C6) (Middleton et al., 2002; Peng et al., 2002). However they show limited sequence similarity (Fig. 1A). Although ProTx-II inhibits multiple sodium channel subtypes ($\text{Na}_v1.1$ -1.8), it has been reported to be ~100-fold more selective for $\text{Na}_v1.7$ (Schmalhofer et al., 2008; Smith et al., 2007). HWTX-IV preferentially inhibits tetrodotoxin (TTX)-sensitive neuronal subtypes (including $\text{Na}_v1.7$), does not inhibit TTX-resistant neuronal subtypes, and has little effect on skeletal muscle ($\text{Na}_v1.4$) and cardiac ($\text{Na}_v1.5$) subtypes (Xiao et al., 2008). Although these toxins have divergent properties, both are classified as voltage-sensor modifiers (Sokolov et al., 2008; Xiao et al., 2008).

Voltage-sensor modifiers target the voltage sensors of ion channels. The pore-forming sodium channel α -subunit consists of four domains (DI - DIV), each having six transmembrane segments (S1-S6) (Catterall et al., 2003). The S5-S6 segments form the channel pore and the S1-S4 segments form voltage sensor modules. The S4 segments, rich in positive residues, sense membrane depolarization and move outward to induce channel gating. Scorpion toxins have been extensively characterized as voltage-sensor modifiers, and understanding the molecular determinants of their interactions with voltage-gated sodium channels has provided invaluable insight into channel structure-function relationships. Scorpion α -toxins

interact with the DIV S3-S4 linker to stabilize DIV-S4 in the closed state, impeding fast inactivation. Scorpion β -toxins bind to the DII S3-S4 linker, trapping the DII-S4 in the activated state and enhancing channel activation. The binding sites for these scorpion toxins are defined as neurotoxin receptor sites 3 and 4, respectively (Cestele and Catterall, 2000). These data, in conjunction with other studies, indicate that the S4 segments in DI, DII and DIII are determinants of channel activation while that of DIV is predominantly involved in channel inactivation (Cestele et al., 2001; Cha et al., 1999; Sheets and Hanck, 2007). Both ProTx-II and HWTX-IV have been proposed to inhibit activation by trapping the DII voltage-sensor in the resting configuration. However, their binding determinants may not be identical. While HWTX-IV may selectively bind to neurotoxin site 4 (Xiao et al., 2008), ProTx-II may interact with novel binding sites on $\text{Na}_v1.5$ (Smith et al., 2007). Recently it was suggested that ProTx-II inhibits activation of $\text{rNa}_v1.2a$ by interacting with the voltage-sensor “paddles” (S3b-S4 motifs) of DI, DII and DIV (Bosmans et al., 2008). This finding was somewhat surprising given the presumed role of DIV in inactivation, leading to the proposal that for a toxin to alter inactivation it must exclusively interact with the voltage-sensor paddle of DIV (Bosmans et al., 2008).

We investigated the interactions of ProTx-II and HWTX-IV with the voltage-sensor paddles in $\text{hNa}_v1.7$ DI, DII and DIV. Our data show that these two tarantula toxins differ substantially in their interactions with $\text{hNa}_v1.7$. Although they may not be ideal as analgesic drugs, understanding the molecular determinants of their complicated interactions with voltage-gated sodium channels should aid the development of novel $\text{hNa}_v1.7$ blockers.

Materials and Methods

Toxins - ProTx-II was recombinantly produced as described by Smith et al. (Smith et al., 2007). HWTX-IV was purified from the crude venom of the female tarantula *O. huwena* as described by Peng *et al.* (Peng et al., 2002). The purity of ProTx-II and HWTX-IV was determined to be over 99% by high pressure liquid chromatography and matrix-assisted laser desorption ionization time-of-flight analysis.

Plasmids of sodium channels - The cDNA genes encoding rat (r) Na_v1.2, rNa_v1.3 and rNa_v1.4 were inserted into the vectors pRC-CMV, pcDNA3.1-mod and pRBG4, respectively (Cummins et al., 2001; O'Leary, 1998; Ukomadu et al., 1992). The cDNA genes encoding human (h) Na_v1.5 and hNa_v1.7 were subcloned into the vectors pcDNA3.1 and pcDNA3.1-mod, respectively (Klugbauer et al., 1995). Auxiliary subunits hβ1 and hβ2 were inserted into an internal ribosome entry site vector (Lossin et al., 2002).

Site-directed mutagenesis of Na_v1.7 - All hNa_v1.7 mutations in this study were constructed using the QuikChange II XL Site-Directed Mutagenesis kit according to the manufacture's instruction. All constructs were sequenced to confirm that the appropriate mutations were made.

Transient Transfection - Transient transfections of hNa_v1.7 wild type (WT) and mutant constructs into human embryonic kidney 293 (HEK293) cells were performed using the calcium phosphate precipitation method. HEK293 cells were grown under standard tissue culture conditions (5% CO₂ and 37 °C) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The WT and mutant hNa_v1.7 channels were cotransfected with the hβ1 and hβ2 subunits to increase the current density. The calcium phosphate-DNA mixture (channel constructs and a green fluorescent protein reporter plasmid) was added to the cell culture medium and left for 3 h, after which the cells were washed with fresh medium. Cells with green fluorescent protein fluorescence were selected for whole-cell patch clamp

recordings 36-72 h after transfection. Stably transfected cell lines containing WT rNa_v1.2, rNa_v1.3, rNa_v1.4 and hNa_v1.5 without any β subunit or green fluorescent protein reporter plasmid were prepared using the method described previously (Xiao et al., 2008).

Whole-cell Patch Clamp Recordings - Whole-cell patch clamp recordings were carried out at room temperature (~21 °C) using an EPC-10 amplifier (HEKA, Lambrecht, Germany). Data were acquired on a Pentium IV computer using the Pulse program (version 8.31; HEKA). Fire-polished electrodes were fabricated from 1.7-mm capillary glass (VWR, West Chester, PA) using a P-97 puller (Sutter, Novato, CA). The standard pipette solution contained (in mM): 140 CsF, 1 EGTA, 10 NaCl and 10 HEPES, pH 7.3. The standard bathing solution was (in mM) 140 NaCl, 3 KCl, 1 MgCl₂, 1 CaCl₂ and 10 HEPES, pH 7.3. After filling with pipette solution, the access resistance of electrode pipette ranged from 0.8 to 1.4 MΩ and the average resistance was 0.98 ± 0.02 MΩ (n=250). The liquid junction potential for these solutions was <8 mV; data were not corrected to account for this offset. The offset potential was zeroed before contacting the cell. After establishing the whole-cell recording configuration, the resting potential was held at -100 mV for 5 min to allow adequate equilibration between the micropipette solution and the cell interior. Linear leak subtraction, based on resistance estimates from four to five hyperpolarizing pulses applied before the depolarizing test potential, was used for all voltage clamp recordings. Membrane currents were usually filtered at 5 kHz and sampled at 20 kHz. Voltage errors were minimized using 80% series resistance compensation, and the capacitance artifact was canceled using the computer-controlled circuitry of the patch clamp amplifier. The stock solutions for ProTx-II and HWTX-IV were made at 1 mM using bathing solution containing 1 mg/ml BSA, and aliquots were stored at -20 °C. Before use, the solution was diluted to the concentrations of interest with fresh bathing solution. Toxin was diluted into the recording chamber (volume of 300 μl) and mixed by repeatedly pipetting 30 μl to achieve the specified final concentration. The extent of the inhibitory effect of toxin was typically assessed around 10 - 20 min after toxin treatment.

Data Analysis - Data were analyzed using the Pulsefit (HEKA) and GraphPad Prism 4 (GraphPad Software) programs. All data points are shown as mean \pm S.E. and n is presented as the number of the separate experimental cells. Steady-state activation and inactivation curves were fitted using Boltzmann equation: $y = 1/(1+\exp((V_{1/2}-V)/k))$, in which $V_{1/2}$, V and k represented midpoint voltage of kinetics, test potential and slope factor, respectively. Concentration-response curves to determine IC_{50} values were fitted using the Hill equation: $y = f_{\text{bottom}}(1 - f_{\text{bottom}})/(1 + ([Tx]/IC_{50})^{nH})$, where nH is Hill coefficient, IC_{50} is half maximal inhibitory concentration, and f_{bottom} is the fraction of current resistant to inhibition at high toxin (Tx) concentration. For HWTX-IV, the nH was set to 1 because our mutagenesis data have shown that the toxin had a single high affinity binding site in hNa_v1.7. For ProTx-II slowing fast-inactivation, the nH was also set to 1 because only sodium channel DIV is involved in channel inactivation gating.

Results

ProTx-II and HWTX-IV block hNa_v1.7 channels at nanomolar concentrations. Although ProTx-II and HWTX-IV have previously been tested against hNa_v1.7 channels (Schmalhofer et al., 2008; Xiao et al., 2008), their activity has not been directly compared. Therefore we first compared the effects of ProTx-II and HWTX-IV on wild-type (WT) hNa_v1.7 channels expressed in HEK293 cells using whole cell voltage-clamp recordings. Although both toxins blocked the peak transient sodium currents conducted by hNa_v1.7, ProTx-II exhibited a 30-fold higher affinity for WT hNa_v1.7 than did HWTX-IV (Fig. 1D). The IC₅₀ values for ProTx-II and HWTX-IV were determined to be 0.7 and 22.7 nM, respectively. Consistent with previous findings (Schmalhofer et al., 2008; Smith et al., 2007), 100 nM ProTx-II not only shifted channel activation in the depolarizing direction by 31.1 mV, but also increased the slope factor by twofold. In contrast, in the presence of 100 nM HWTX-IV, neither the activation nor the slope factor was obviously modified in the range of voltages tested in the present study (Fig. 1BC and Table 1-2). Because 100 nM is not a saturating concentration of HWTX-IV, it is likely that the residual currents shown in Fig. 1B result from channels that did not bind HWTX-IV. Indeed, in our previous study we showed that saturating concentrations of HWTX-IV shift the voltage-dependence of activation by at least 200 mV in the depolarizing direction (Xiao et al., 2008).

Comparison of the electrophysiological properties of WT and mutant hNa_v1.7 channels. To explore the molecular determinants of ProTx-II and HWTX-IV interactions with hNa_v1.7, we made specific mutations in the S3-S4 regions of DI, DII and DIV. We mainly focused on residues in the S3-S4 regions that previous studies have indicated were important for the inhibitory activity of either ProTx-II or HWTX-IV. The electrophysiological properties of mutant channels, expressed in HEK293 cells, were characterized under the whole-cell recording configuration and the voltage-dependent properties were compared to WT hNa_v1.7. The electrophysiological parameters of activation and steady-state inactivation, estimated by fitting the data with Boltzmann equations, are summarized in Supplemental Table S1. These

data indicate that acidic residues in extracellular S3-S4 linkers of DI and DII can modulate voltage-dependent activation of hNa_v1.7. Although the mutations L201V/N206D, F813G and F204A/F813G shifted the midpoint potentials of activation of hNa_v1.7 by less than +5 mV, the mutations of acidic residues E203K/E818C, E818C and F813G/E818C shifted activation by +17.6, +9.8 and +9.2 mV, respectively. This finding is consistent with previous reports that voltage sensors of DI and DII are important for channel activation (Cestele et al., 2001; Cha et al., 1999). By contrast, consistent with the finding that DIVS4 is mainly responsible for fast-inactivation (Cestele et al., 2001; Sheets and Hanck, 2007), mutations of most residues (D1586A, D1586E, E1589Q, T1590K, F1592A, and D1586A/T1590K) in DIV had limited effect on channel activation. Only the double mutation T1590K/F1592A substantially shifted channel activation, by +8.2 mV (Supplemental Table S1). None of mutations significantly altered steady-state inactivation. The slope factors for steady-state activation and inactivation also did not change compared to WT hNa_v1.7 (Supplemental Table S1).

Because the inhibition of hNa_v1.7 by ProTx-II is voltage dependent in the range of physiological voltages (Smith et al., 2007), the shifting of the current-voltage relationship caused by channel mutation might affect the assessment of toxin affinity when measured at the same test pulse potential. To precisely measure toxin affinity under similar activation conditions, the test pulse potential to activate hNa_v1.7 WT and mutant construct channels was set between -10 and +10 mV for the various constructs to ensure that ~90% channel conductance was available.

Mutations in DIIS3-S4 linker differentially decreased toxin affinities for hNa_v1.7. In previous studies, two residues in the DIIS3-S4 linker, F813 and E818 (Supplemental Fig. S1C), were shown to be important for hNa_v1.7 block by ProTx-II and HWTX-IV, respectively (Schmalhofer et al., 2008; Xiao et al., 2008). However, it is not known if F813 is important for HWTX-IV block or if E818 is important for ProTx-II block of hNa_v1.7. To determine whether these two tarantula toxins share the same binding site on the DIIS3-S4 linker of hNa_v1.7, we measured the IC₅₀ values of the two toxins on two single

mutations, F813G and E818C using the whole-cell patch-clamp technique. As shown in Fig. 2, the F813G mutation decreased ProTx-II affinity for hNa_v1.7 by 9-fold with the IC₅₀ value estimated to be 6.0 nM, but the value of HWTX-IV (28.2 nM) for this mutant was close to that (22.7 nM) for WT channels (see Tables 1 and 2). This result indicates that the residue F813 in hNa_v1.7 might interact structurally with ProTx-II but not HWTX-IV. Our previous work demonstrated that the neutralizing mutation E818Q could decrease HWTX-IV affinity by 63-fold (Xiao et al., 2008). Interestingly, when this acidic residue was substituted with Cys (E818C) in our present study, hNa_v1.7 current became substantially more resistant to HWTX-IV. Even when exposed to the toxin at concentrations up to 10 μM, hNa_v1.7-E818C current was only inhibited by 50.3 ± 3.0% (*n* = 3). The IC₅₀ value was estimated to be 9.1 μM (Fig. 2B and Table 2), indicating that the E818C mutation decreased the sensitivity of hNa_v1.7 to HWTX-IV by at least 400-fold. By contrast, this mutation was found to only decrease ProTx-II affinity by 4-fold, with an IC₅₀ value of 2.9 nM (Table 1). Given the weak decrease of ProTx-II block by E818C and the proximity of F813 to E818, it is possible that the decrease caused by the E818C mutation results from a change in the orientation of F813 within the DIIS3-S4 linker. To further examine this possibility, we constructed a double mutant F813G/E818C. The IC₅₀ value of ProTx-II for the double mutant was estimated to be 29.8 nM in Fig. 2A (see Table 1). The decrease in ProTx-II affinity (42-fold) for the double mutant F813G/E818C is additive relative to the effects of the two single mutations from which it derived. Overall, these data strongly indicate that although the binding determinants of ProTx-II and HWTX-IV may partially overlap, they are not identical on hNa_v1.7 DII.

Mutations in DIS3-S4 linker did not significantly change toxin affinities for hNa_v1.7. As DII mutations only partially reduced ProTx-II block, and a previous study (Bosmans et al., 2008) showed that ProTx-II is likely to interact with multiple voltage sensors of rNa_v1.2a including DI, we next asked if the extracellular DIS3-S4 linker contributes to the sensitivity of hNa_v1.7 for these two toxins. We first focused on E203 and F204 in DIS3-S4 of hNa_v1.7 (Supplemental Fig. S1C) because mutation of the corresponding residues in rNa_v1.2a (E207 and F208) was reported to reduce the binding affinity of ProTx-

II for rNa_v1.2a by 2.7- and 13.5-fold, respectively (Bosmans et al., 2008). Here we constructed two double mutants of hNa_v1.7 E203K/E818C and F204A/F813G, with the expectation that mutations that reduced binding at DII might help identify the contributions of residues in DI. The IC₅₀ values of ProTx-II for E203K/E818C (4.6 nM) and F204A/F813G (8.2 nM) were not different from those of single mutants E818C (2.9 nM) and F813G (6.0 nM), respectively (Fig. 2A; Tables 1 and 2). These data suggested that although hNa_v1.7-E203 may have a weak interaction with ProTx-II, hNa_v1.7-F204 does not seem to play a role in ProTx-II inhibition of hNa_v1.7.

We were somewhat surprised that the E203K and F204A mutations did not significantly alter the effect of ProTx-II on hNa_v1.7, given the reported effect of the corresponding mutations on rNa_v1.2a. One possibility was that the difference between the relative impact of the DIS3-S4 substitutions in rNa_v1.2a and hNa_v1.7 could be a result of overall sequence differences in the DIS3-S4 linker region. Although this linker region is highly conserved among voltage-gated sodium channel isoforms, rNa_v1.2a differs from the hNa_v1.7 construct that we used at several positions in the DIS3-S4 linker. Interestingly, this linker is subject to alternative splicing in both rNa_v1.2 and hNa_v1.7 (Raymond et al., 2004). Splicing of exon 5 changes L201 and N206 (present in the variant that we have been testing) to Val and Asp in the DIS3-S4 linker, respectively (Chatelier et al., 2008). Sequence alignment shows that the V201 and D206 in the alternative splice variant hNa_v1.7a are conserved at the corresponding positions in rNa_v1.2a (V204 and D209) (see Supplemental Fig. S1). The effect of Ala-substitutions at these residues on rNa_v1.2a sensitivity to ProTx-II was previously examined and, although the V204A mutation did not affect ProTx-II block of rNa_v1.2a/Kv2.1 chimeras, the D209A mutation decreased block in the chimeric channels by ~threefold (Bosmans et al., 2008). Therefore we examined if alternative splicing of DIS3-S4 of hNa_v1.7 could impact the sensitivity to ProTx-II. Fig. 2A shows that the IC₅₀ value of ProTx-II was measured to be 1.0 nM for the variant hNa_v1.7a (L201V/N206D), which is close to the value for WT hNa_v1.7. Although our results do not completely rule out the possibility that ProTx-II interacts with DIS3-S4 in hNa_v1.7, it indicates that such an interaction is less important than in rNa_v1.2a.

As shown in Fig. 2B, the concentration dependencies of HWTX-IV inhibition almost completely overlap for the mutants E818C and E203K/E818C, as did the curves for WT, F813G and F204A/F813G Na_v1.7 channels. Our data also indicate that alternative splicing of DIS3-S4 in hNa_v1.7 does not alter block by HWTX-IV (Fig. 2B and Table 1). Overall these data suggest that the DIS3-S4 linker is not a major determinant of either ProTx-II or HWTX-IV interactions with hNa_v1.7.

ProTx-II preferentially interacts with hNa_v1.7 to increase sustained currents. In the presence of 100 nM ProTx-II, $4.9 \pm 0.6\%$ of sodium channels could still be activated at -10 mV (Fig. 2A, n = 4). In an attempt to completely eliminate the hNa_v1.7 sodium current, we increased the ProTx-II concentration to 1 μ M. However, no further block was observed, suggesting that the toxin effect on hNa_v1.7 activation saturates at a concentration of around 100 nM. Intriguingly, 1 μ M ProTx-II was detected to significantly increase sustained currents generated by WT hNa_v1.7 (Fig. 3AB). The sustained currents did not decay completely during at least 50 ms. By contrast, in the presence of 1 (or even 10) μ M HWTX-IV, no alternation of fast-inactivation of WT (or mutant E818C) Na_v1.7 channels was detected (see Supplemental Fig. S2). Since hNa_v1.7 currents induced at -10 mV inactivate completely within 10 ms in the absence of ProTx-II, we assayed the efficacy of toxin impeding fast-inactivation by measuring the $I_{10\text{ ms}}/I_{\text{peak}}$ ratio, which gives an estimate of the probability for the channel to generate sustained currents after 10 ms. Sustained currents induced by 1 μ M ProTx-II were detectable at voltages ranging from -40 to +70 mV, but at voltages more positive than +75 mV sustained currents were not evident (Fig. 3BD), indicating that the ProTx-II enhancement of sustained currents in hNa_v1.7 was voltage-dependent. Importantly, the sustained currents were blocked completely by 200 nM TTX, providing evidence that these sustained ionic currents were indeed fluxing through the hNa_v1.7 channel pore (Fig. 3CE).

We next wanted to estimate the concentration-response relationship of the apparent ProTx-II effect on the sustained current on multiple sodium channel subtypes expressed in HEK293 cells. Fig. 4A shows

representative current traces for five subtypes (rNa_v1.2a, rNa_v1.3, rNa_v1.4, hNa_v1.5 and hNa_v1.7) before and after application of 1 μM ProTx-II. As reported previously (Schmalhofer et al., 2008; Smith et al., 2007), the toxin IC₅₀ for inhibition of activation of hNa_v1.7 was ~70-fold higher than for the other four subtypes rNa_v1.2a-hNa_v1.5 (Fig. 4B). In addition, while the sustained currents induced by 1 μM ProTx-II in hNa_v1.7 was 85.3 ± 3.4% of the peak current (*n* = 7), in rNa_v1.2a, rNa_v1.3, rNa_v1.4 and hNa_v1.5 it was only 18.0 ± 2.5%, 14.5 ± 1.1%, and 19.2 ± 4.0% (*n* = 3-4) of the peak current, respectively. As can be seen in Fig. 4C, fitting the data on the relative amplitude of the sustained currents induced by ProTx-II with the Hill equation yielded apparent IC₅₀ values of 4.5, 5.6, 4.2, 4.1 and 0.24 μM for rNa_v1.2a, rNa_v1.3, rNa_v1.4, hNa_v1.5 and hNa_v1.7, respectively (Supplemental Table S2). It is difficult to accurately determine the IC₅₀ values for apparent inhibition of inactivation. Comparisons of the apparent IC₅₀ values are further complicated here because ProTx-II inhibits activation to different degrees for the different sodium channel isoforms. Despite these caveats, our data clearly indicate that ProTx-II preferentially induces sustained currents in hNa_v1.7 in addition to the preferential inhibition of hNa_v1.7 activation.

The voltage-dependent induction of sustained currents in hNa_v1.7 by ProTx-II is somewhat similar to the voltage-dependent inhibition of activation by ProTx-II. One explanation for the increased sustained currents is that ProTx-II might induce what appears to be sustained current by variably prolonging the latency to activation. However, the sustained currents are also similar to those induced by scorpion α-toxins that inhibit inactivation of voltage-gated sodium channels (Strichartz and Wang, 1986). Therefore an alternative explanation is that ProTx-II, in addition to its ability to inhibit activation, also inhibits inactivation, possibly by interacting with the hNa_v1.7 DIV voltage-sensor associated with inactivation in a manner similar to that of scorpion α-toxins (Rogers et al., 1996). Although all of the previous studies on ProTx-II have indicated that ProTx-II inhibits only activation of voltage-gated sodium channels (Bosmans et al., 2008; Middleton et al., 2002; Schmalhofer et al., 2008; Smith et al., 2007; Sokolov et al., 2008), Bosmans et al. reported that ProTx-II could interact with the DIV voltage sensor of rNa_v1.2a. Surprisingly, they found that specific substitutions in the S3-S4 linker region of DIV of rNa_v1.2a

substantially reduce the ability of ProTx-II to inhibit activation of rNa_v1.2a channels expressed in *Xenopus* oocytes. Our results suggested that, at least for sodium channels expressed in mammalian cells, ProTx-II might be inducing sustained currents by interacting with DIV. If this effect is due to binding of DIV, the apparent affinity of ProTx-II binding for hNa_v1.7 DIV may be ~17-fold higher than for other subtypes.

Mutations in DIV alter ProTx-II's impact on sustained currents generated in hNa_v1.7. To further investigate how ProTx-II preferentially induced sustained sodium currents in hNa_v1.7, we compared the amino acid sequences of DIVS3-S4 linkers from eight sodium channel subtypes Na_v1.1-Na_v1.8 (Fig. 5). The most striking difference in this region is the unique presence of Thr-1590 in hNa_v1.7; the residue at the corresponding position in rNa_v1.1-hNa_v1.6 is Lys and Gln in hNa_v1.8. The residue at this position can be important in modulating the effect of scorpion α -toxins on sodium channels (Leipold et al., 2004). Importantly, D1586, E1589 and F1592 in hNa_v1.7 are conserved at the corresponding positions among six other subtypes (Na_v1.1-Na_v1.6). The residues at these positions are interesting as previous studies have determined that the first is crucial for the ability of site 3 scorpion α -toxins and sea anemone toxins to modify sodium channel inactivation and the latter two have been implicated in rNa_v1.2a interactions with ProTx-II (Bosmans et al., 2008; Rogers et al., 1996). Therefore we next investigated if single substitutions at these four residues in hNa_v1.7 (D1586A, E1589Q, T1590K and F1592A) might be important determinants of ProTx-II preferentially inducing sustained currents in hNa_v1.7.

Current traces for these mutant channels were elicited by a 20-ms depolarizing potential of -10 or -5 mV from a holding potential of -100 mV (Fig. 6A). It is important to note that none of the four mutations altered the ability of ProTx-II to block hNa_v1.7 activation. As shown in Fig. 6B, the fit of the Hill equation yielded the IC₅₀ values for ProTx-II inhibition of activation to be 1.0 nM (D1586A), 0.5 nM (E1589Q), 0.9 nM (T1590K) and 0.7 nM (F1592A). In contrast, these mutations had distinct effects on the ability of ProTx-II to induce hNa_v1.7 sustained currents (Fig. 6A and Supplemental Fig. S4). Two of

the mutations significantly decreased the enhancement of sustained currents by ProTx-II. The sustained currents induced by ProTx-II (1 μ M) in E1589Q and T1590K channels were $40.3 \pm 1.7\%$ ($n = 4$) and $37.2 \pm 10.0\%$ ($n = 3$) of the peak current, respectively. The other two DIV mutations that we tested increased the ability of ProTx-II to enhance sustained currents. When treated only with 100 nM toxin, the sustained currents generated in D1586A and F1592A channels was $74.5 \pm 2.6\%$ ($n = 4$) and $61.1 \pm 12.8\%$ ($n = 4$) of the peak current, respectively, which are close to the value obtained for 1 μ M ProTx-II on WT hNa_v1.7. As ProTx-II inhibits activation of the DIV mutant channels and WT channels to the same extent, we can confidently compare the relative effect on inhibition of inactivation by measuring the current amplitude 10 ms into the depolarizing pulse (I_{10ms}) and calculating the ratio (I_{10ms}/I_{peak}), where I_{peak} is the peak current remaining after ProTx-II treatment. In Fig. 6C, the apparent IC₅₀ values for ProTx-II inhibition of fast-inactivation were estimated to be 48.3 nM, 1.6 μ M, 1.4 μ M, 70.1 nM and 240 nM for D1586A, E1589Q, T1590K, F1592A and WT hNa_v1.7, respectively (see Supplemental Table S2). Therefore, our data show that while two mutations E1589Q and T1590K selectively decreased ProTx-II ability to induce sustained currents in hNa_v1.7 DIV by ~6-fold, the other two mutations D1586A and F1592A selectively increased ProTx-II ability to induce sustained currents by ~4-fold.

These findings are very different from those of a previous study which found that the conserved mutations E1614A and F1620A in rNa_v1.2a decreased ProTx-II inhibition of rNa_v1.2a *activation* by over 6-fold and decreased ProTx-II affinity for the rNa_v1.2a DIV paddle motif by over 10-fold (Bosmans et al., 2008). Again in these previous studies, carried out with rNa_v1.2a, ProTx-II reportedly had no effect on sustained currents or inactivation. As can be seen in Fig. 5, there are only 2 differences in the DIVS3-S4 linkers of hNa_v1.7 and rNa_v1.2a: Asp-1586 and Thr-1590 of hNa_v1.7 are substituted with Glu and Lys, respectively, in rNa_v1.2a. When we replaced Asp-1586 with Glu in hNa_v1.7, the ability of ProTx-II to inhibit channel activation or induce sustained currents were not changed compared to WT channels (Fig. 6BC and Table 1), indicating that the sequence difference at this residue is not important in determining the differences between our observations and those of Bosmans et al. (2008). However, it has been clearly shown that

DIV residues can have substantial combinatorial effects on the interaction of scorpion α -toxins with specific sodium channel isoforms (Leipold et al., 2004). Therefore, we next asked whether the residue at position 1590 (Thr versus Lys) was the key factor for reversing the structural interactions of D1586 and F1592 with ProTx-II. Using the mutant T1590K as a model, we constructed two double mutations D1586A/T1590K and T1590K/F1592A. As seen in Fig. 7, the IC_{50} values for ProTx-II inducing sustained currents were estimated to be 0.26 μ M for D1586A/T1590K and 0.18 μ M for T1590K/F1592A, which were 5-fold and 7-fold smaller than the value for the single mutation T1590K, respectively (Supplemental Table S2). Therefore, together with the data on two single mutations D1586A and F1592A (Fig. 7C), these results suggest that the Thr to Lys exchange at position 1590 could not reverse the structural interaction of D1586 and F1592 with ProTx-II. In addition, neither double mutation altered the ability of ProTx-II to inhibit channel activation (Fig. 7B and see Table 1).

Finally, we asked whether decreasing the inhibition of action of $hNa_v1.7$ by ProTx-II would alter the apparent effects of ProTx-II on inactivation. Therefore we used the F813G/E818C double mutation, which reduces inhibition of activation by approximately 42-fold, and examined the impact of the E1589Q and F1592A mutations (Supplemental Fig. S5). In the F813G/E818C background, the E1589Q and F1592A mutations still have no effect on the inhibition of activation by ProTx-II, but the E1589Q mutation decreased the inhibition of inactivation by 5-fold and the F1592A mutation increased the inhibition of inactivation by 8-fold (Supplemental Fig. S5C). Thus these DIV mutations have nearly identical effects on ProTx-II inhibition of inactivation for WT and F813G/E818C mutant $Na_v1.7$ channels. Collectively, our data show that while mutations in DIV of $hNa_v1.7$ do not alter the inhibition of activation by ProTx-II, they do substantially modulate the ability of ProTx-II to induce sustained sodium currents in $hNa_v1.7$. This is similar to the DIV dependent inhibition of inactivation by scorpion α -toxins, indicating that ProTx-II can inhibit both activation *and* inactivation of voltage-gated sodium channels.

Discussion

We investigated the molecular determinants of the interactions of two tarantula toxins with hNa_v1.7. ProTx-II and HWTX-IV have a similar cysteine knot structure and both exhibit higher affinities for blocking hNa_v1.7 than for other subtypes (Schmalhofer et al., 2008; Xiao et al., 2008). However, these two toxins differ quite substantially in their non-cysteine sequence and our data indicate that they are very different in the extent and functional consequences of their interactions with hNa_v1.7.

Differential interactions of ProTx-II and HWTX-IV with hNa_v1.7 voltage-sensors. ProTx-II and HWTX-IV are classified as voltage-sensor modifiers and both have been proposed to selectively inhibit channel activation by trapping the DII voltage-sensor in the closed state (Sokolov et al., 2008; Xiao et al., 2008). Our results reveal that a single mutation (E818C) reduced the sensitivity of hNa_v1.7 for HWTX-IV by over 400-fold (Table 1). Our results further show that mutations of residues in the S3-S4 linkers of DI and DIV did not alter the inhibition of hNa_v1.7 by HWTX-IV. The DII voltage-sensor is clearly the main determinant of action for HWTX-IV inhibition of activation of hNa_v1.7.

In contrast, ProTx-II is likely to interact with multiple regions of sodium channels. In a study of ProTx-II interactions with Na_v1.5, we concluded that ProTx-II may not make critical interactions with extracellular linker regions of Na_v1.5, suggesting the existence of a novel toxin binding site (Smith et al., 2007). On the other hand, two studies with rNa_v1.2a (Sokolov et al., 2008) and hNa_v1.7 (Schmalhofer et al., 2008) suggested that ProTx-II might specifically interact with the DII voltage-sensor. However, using chimeric Kv2.1 channels containing the voltage sensor paddle regions of rNa_v1.2a, Bosmans et al. (2008) found that ProTx-II can interact with the voltage sensors from three domains (DI, DII and DIV) of rNa_v1.2a. Consistent with this finding, we demonstrate that ProTx-II interacts with the voltage sensors of two domains (DII and DIV) in hNa_v1.7. However, our data suggest that the DIS3-S4 linker is less important in determining hNa_v1.7 sensitivity to ProTx-II than might be predicted by the chimeric paddle approach

and raises the possibility that other regions of the DI voltage-sensor (such as the S1-S2 linkers) may influence the isoform specific interactions.

The binding determinants of ProTx-II and HWTX-IV partially overlap on hNa_v1.7 DII. ProTx-II is at least 70-fold more selective for hNa_v1.7 over other subtypes that we tested, consistent with previous findings (Schmalhofer et al., 2008; Smith et al., 2007). Our data supports the assertion (Schmalhofer et al., 2008) that this selectivity might result from the higher sensitivity of hNa_v1.7 DII for ProTx-II. In DII, F813 is unique in hNa_v1.7. When F813 is substituted with Gly, the corresponding residue in most other sodium channel subtypes, we found that ProTx-II affinity for hNa_v1.7 is decreased by 9-fold. This decrease is smaller than that previously reported, where the F813G mutation completely abolished selectivity of ProTx-II for hNa_v1.7. It is not clear what accounts for this quantitative difference; however in the previous study the reverse substitution in hNa_v1.2 (G839F) did not significantly increase ProTx-II inhibition of hNa_v1.2 (Schmalhofer et al., 2008), indicating that the residue at this position is not the only determinant of hNa_v1.7's high sensitivity to ProTx-II. Interestingly, F813 is specific for ProTx-II's interaction and does not seem to play any role in HWTX-IV's interactions. Furthermore, we show that although E818 interacts with both toxins, it is much more important for HWTX-IV. Therefore our data indicate that the DIIS3-S4 linker is important in determining the higher selectivity of these toxins for hNa_v1.7, but the binding determinants of ProTx-II and HWTX-IV only partially overlap in this region. This observation, in combination with the striking lack of identity between ProTx-II and HWTX-IV, suggests that DII of hNa_v1.7 may be an excellent target for development of hNa_v1.7 specific inhibitors.

Molecular mechanism for ProTx-II inhibition of fast-inactivation. We observed that ProTx-II induced sustained hNa_v1.7 currents. Because ProTx-II dissociates from hNa_v1.7 at depolarized potentials, one explanation for the sustained current could have been that ProTx-II simply prolonged the latency to channel opening without any effect on inactivation. However, mutations in hNa_v1.7 DIV that substantially modulated the sustained current induced by ProTx-II had no effect on the inhibition of

activation by ProTx-II, in both the WT and F813G/E818C backgrounds. Interestingly, no effect was seen on the voltage-dependence of steady-state inactivation with ProTx-II (Fig. 1C); however, it should be noted that disparate effects on the voltage-dependence of steady-state inactivation and the rate of open-state fast-inactivation are frequently observed for DIV manipulations (Bendahhou et al., 1999; Yang et al., 1994). Scorpion α -toxins often have no effect on the voltage-dependence of steady-state fast-inactivation but dramatically inhibit open-state fast-inactivation of voltage-gated sodium channels (Maertens et al., 2006) and this action is dependent on the DIVS3-S4 linker (Rogers et al., 1996). Based on these previous studies and our data showing that the DIV mutations selectively alter the ability of ProTx-II to induce sustained currents in hNa_v1.7, we conclude that ProTx-II not only has the ability to inhibit activation, but also inhibits fast-inactivation of sodium channels through a mechanism similar to neurotoxin receptor site 3 toxins such as α -toxins.

Our data indicate that the effects on activation and inactivation are independent. Mutations in domain IV that substantially alter the effect on the inhibition of inactivation have no effect on the inhibition of activation. Mutations in domain II preferentially impact the inhibition of activation. This suggests that ProTx-II can simultaneously interact with two independent sites, one in DIV and the other possibly in DII. The estimated IC₅₀ for inhibition of activation is ~400-fold smaller than the apparent IC₅₀ for inhibition of inactivation, providing additional evidence that there are likely two independent interaction sites. Despite this differential in IC₅₀'s, the interaction with DIV is able to induce measurable sustained currents because the inhibition of activation is incomplete, even at relatively high concentrations.

These findings have some important implications. First, they point out potential limitations in the usefulness of ProTx-II as a research tool. Although ProTx-II may help determine if Na_v1.7 contributes to peak transient sodium currents in neurons, our results indicate that it would be problematic to use ProTx-II to determine the contribution of Na_v1.7 to sustained sodium currents. Second, it must also be used with care when examining the contribution of Na_v1.7 to action potential firing properties. Excitatory toxins

such as scorpion α -toxins and the tarantula toxin Jingzhaotoxin-I that impede fast-inactivation can prolong action potential duration and increase repetitive action potential firing (Rogers et al., 1996; Xiao et al., 2007; Xiao et al., 2005). Sustained $\text{Na}_v1.7$ currents induced by ProTx-II, even small ones, could be problematic. Sensory neurons that express $\text{Na}_v1.7$ often express other sodium channel isoforms such as $\text{Na}_v1.8$ that are less sensitive to ProTx-II (Middleton et al., 2002), and the persistent $\text{Na}_v1.7$ currents induced by ProTx-II in conjunction with $\text{Na}_v1.8$ currents could have complicated effects on excitability. The multiple effects of ProTx-II on $\text{hNa}_v1.7$ activation and inactivation would be expected to complicate, if not contraindicate, the use of ProTx-II administration to inhibit pain.

A previous report concluded that although ProTx-II interacts with the voltage-sensor paddles of DI, DII and DIV, ProTx-II only inhibited $\text{rNa}_v1.2a$ activation, not inactivation (Bosmans et al., 2008). As mutations in $\text{rNa}_v1.2a$ DI, DII and DIV paddles all affected the extent of inhibition of activation of $\text{rNa}_v1.2a$, it was concluded that drugs targeting any of the paddle motifs in the first three domains would only influence channel activation, regardless of any interaction with DIV. Furthermore, as DIV mutations altered the ability of ProTx-II to inhibit activation of $\text{rNa}_v1.2a$ channels expressed in *Xenopus* oocytes, it was concluded that toxins need to *exclusively* interact with the voltage-sensor of DIV in order to alter inactivation of voltage-gated sodium channels (Bosmans et al., 2008). Our results challenge the broad applicability of these conclusions. It is not entirely clear what accounts for the seemingly opposite actions of ProTx-II on the DIV's of $\text{rNa}_v1.2a$ and $\text{hNa}_v1.7$. We found that ProTx-II could impede fast-inactivation in multiple isoforms expressed in HEK293 cells, including $\text{rNa}_v1.2a$. Because the effects on fast-inactivation are much smaller in $\text{rNa}_v1.2a$, $\text{rNa}_v1.3$, $\text{rNa}_v1.4$ and $\text{hNa}_v1.5$ than in $\text{hNa}_v1.7$, they might have been overlooked in previous studies. Alternatively, the differential effects of ProTx-II could result from differences between *Xenopus* oocytes and mammalian cells. Differences in the lipid composition of the membrane could be a factor as ProTx-II exhibits substantial lipid binding activity (Smith et al., 2005). Indeed, the sensitivity of potassium channels to tarantula toxins can be modulated by differences in the

composition of the lipid bilayer (Schmidt and MacKinnon, 2008). Differences in posttranslational modifications, such as glycosylation, might also modulate the sensitivity of sodium channels to ProTx-II.

In summary, in this study we extensively investigated the interaction of two tarantula toxins with hNa_v1.7. HWTX-IV selectively inhibits activation of hNa_v1.7 and this is specifically determined by residues in the DII voltage sensor. ProTx-II also interacts with DII, but our data indicates that ProTx-II inhibits both activation and inactivation of hNa_v1.7. These data show that, contrary to what has previously been proposed to be a guiding principle of sodium channel pharmacology, toxins do not have to exclusively target the DIV voltage sensor in order to influence sodium channel inactivation. Molecules that interact with the multiple voltage-sensors of sodium channels can impede both activation and inactivation, and these complex interactions need to be carefully considered when targeting the voltage-sensors of sodium channels.

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Footnotes

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Legends for Figures

Figure 1. Effects of ProTx-II and HWTX-IV on WT Na_v1.7 expressed in HEK293 cells. (A), Sequence alignment of ProTx-II and HWTX-IV. Six conserved cysteines are identified by the encompassing rectangles in the sequence alignment. (B), Differential effects of two toxins on the current-voltage relationships of WT Na_v1.7. Cells were held at -100 mV. Na_v1.7 currents were elicited by 50-ms depolarization steps to various voltages ranging from -80 to +100 mV in 5-mV increments. Currents elicited before and after application of 100 nM ProTx-II (left panel) or 100 nM HWTX-IV (right panel) were normalized to the maximum amplitude of control peak current. (C), Effects of the two toxins on normalized steady-state activation and inactivation of WT Na_v1.7. Channel conductances before and after application of 100 nM ProTx-II or 100 nM HWTX-IV were calculated with the equation: $G(V) = I/(V-V_{rev})$, in which I , V and V_{rev} represented inward current elicited as described in (B), test potential and reversal potential, respectively. Data are plotted as a fraction of the maximum conductance. The voltage dependence of steady-state inactivation was estimated using a standard double-pulse protocol, in which a 20-ms depolarizing test potential of 0 mV followed a 500-ms prepulse at potentials that ranged from -130 to -10 mV with a 10-mV increment. Cells were held at -100 mV. All curves were fit with the Boltzmann equation as described under “Experimental Procedures”. (D), concentration-dependent inhibition of WT Na_v1.7 by two toxins. Data points (mean ± S.E., each from 3 - 4 cells) were fit with the Hill equation as described under “Experimental Procedures”. The values of IC₅₀, slope factor (nH) and f_{bottom} yielded were shown in Table 1 and Table 2.

Figure 2. Concentration-response inhibitory curves of ProTx-II (A) and HWTX-IV (B) on DI and DII mutant Na_v1.7 channels. Sodium current was induced at 5 s intervals by a 20-ms depolarization from a holding potential of -100 mV. The test pulse potentials to activate channels were set to -10 mV (WT and L201V/N206D), -5 mV (F204A/F813G, F204A/F813G, F813G, E1589Q), 0 mV (E818C and F813G/E818C) and +10 mV (E203K/E818C), respectively. The residual current after toxin treatment was plotted as fraction of the control current. Data points (mean ± S.E., each from 3 - 7 cells) were fit with a Hill equation as described under “Experimental Procedures”. The values of IC₅₀, slope factor (nH) and f_{bottom} yielded are shown in Table 1 and Table 2.

Figure 3. ProTx-II significantly impeded fast inactivation of WT Na_v1.7 expressed in HEK293 cells. Cells were held at -100 mV. Families of current traces before (A) and after application of 1 μM ProTx-II (B) or 200 nM TTX (C) were induced by 50-ms depolarizing steps to various potentials ranging from -100 to +100 mV in 5-mV increments. (D) and (E), Effects of 1 μM ProTx-II (D) or 200 nM TTX (E) on the current-voltage (I-V) relationship of WT Na_v1.7. All currents induced before and after toxin treatment were plotted as fraction of the maximum amplitude of control peak current. The dotted line indicates the control I-V curve. The red filled circles indicate the peak I-V curves after application of 1 μM ProTx-II. I_{10ms} (blue open diamond) was shown as the current inactivated at 10 ms after application of 1 μM ProTx-II. (E), Effects of 200 nM TTX on the current-voltage (I-V) relationship of WT Nav1.7 sustained currents induced by ProTx-II. All currents induced before and after toxin treatment were plotted as fraction of the maximum amplitude of control peak current. The dotted line indicates the control I-V curve. The green open circles indicate the I-V curve after application of 200 nM TTX. I_{10ms} (blue open diamond) was shown as the current inactivated at 10 ms after application of 1 μM ProTx-II.

Figure 4. ProTx-II differentially inhibited both activation and inactivation of sodium channel subtypes expressed in HEK293 cells. Cells were held at -100 mV. (A), Currents through WT Na_v1.2, Na_v1.3, Na_v1.4 and Na_v1.7 were induced by a 20-ms depolarizing potential of -10 mV. Na_v1.5 current was elicited at -30 mV. The dotted lines show the residual current in the presence of 1 μM ProTx-II after normalization to the maximum amplitude of control current. (B), Concentration-response inhibitory curves of ProTx-II on the activation of five sodium channel subtypes (Na_v1.2, Na_v1.3, Na_v1.4, Na_v1.5 and Na_v1.7). The residual current after ProTx-II treatment was plotted as a fraction of control current. Data points (mean ± S.E., each from 3-7 cells) were fit with Hill equation as described under “Experimental Procedure”. The IC₅₀ values were estimated to be 52.9 ± 1.1 (Na_v1.2a), 109.9 ± 7.1 (Na_v1.3), 107.6 ± 7.7 (Na_v1.4) and 79.4 ± 40.7 (Na_v1.5) nM, respectively. The slope factor (*nH*) ranged from 0.9 to 1.1. (C), Concentration-response inhibitory curves of ProTx-II on the fast-inactivation of five sodium channel subtypes. The I_{10ms} was plotted as a fraction of the residual current after ProTx-II treatment. Inhibition of fast-inactivation increases the ratio of I_{10ms}/I_{peak} . Data points (mean ± S.E., each from 3-7 cells) were

fit with Hill equation as described under “Experimental Procedure”. The IC_{50} values were shown in Supplementary Table 2.

Figure 5. Amino acid sequence alignment of the DIV S3-S4 linkers of seven α subunit isoforms from human.

(A), Crucial determinants of neurotoxin receptor 3 are located in the S3-S4 linker of sodium channel domain II. The positions of amino acid residues of interest are shaded in grey. (B), Schematic diagram of sodium channel α subunit. The voltage sensor (the 4th segment) of each domain is shaded in gray and marked with “+ +”. The amino acid sequence of DIV S3-S4 linker is shown in the square frame of (A) as indicated by arrows.

Figure 6. Mutations in DIV S3-S4 linker differentially alter the effect of ProTx-II on fast-inactivation of $hNa_v1.7$ channels expressed in HEK293 cells.

(A) Representative current traces for five mutant (D1586A, D1586E, E1589Q, T1590K and F1592A) $hNa_v1.7$ channels. The test pulse potential was -10 mV (D1586A and D1586E) and -5 mV (E1589Q, T1590K and F1592A), respectively. Cells were held at -100 mV. The dotted line shows the residual current in the presence of 0.1 or 1 μ M ProTx-II after normalization to the maximum amplitude of control current. (B), Concentration-response inhibitory curves of ProTx-II on the activation of WT and five mutant (D1586A, D1586E, E1589Q, T1590K and F1592A) $Na_v1.7$ channels. Residual current after toxin treatment was plotted as a fraction of control peak current amplitude. Data points (mean \pm S.E., each from 3-6 cells) were fitted with Hill equation as described under “Experimental Procedure”. The calculated values of IC_{50} , slope factor (nH) and f_{bottom} are shown in Table 1. (C), Concentration-response inhibitory curves of ProTx-II on fast-inactivation of WT and five mutant (D1586A, D1586E, E1589Q, T1590K and F1592A) $Na_v1.7$ channels. The I_{10ms} was plotted as a fraction of the residual current after ProTx-II treatment. Data points (mean \pm S.E., each from 3-6 cells) were fitted with Hill equation as described under “Experimental Procedure”.

Figure 7. Two mutations (D1586A and F1592A) enhanced the ProTx-II slowing fast-inactivation of $hNa_v1.7$ in the presence of K1590.

(A) Representative current traces for two double mutant (D1586A/T1590K and T1590K/F1592A) $hNa_v1.7$ channels. The test pulse potential was -5 mV for D1586A/T1590K and 0 mV for T1590K/F1592A, respectively. Cells were held at -100 mV. The dotted lines show the residual current in the

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presence of 1 μM ProTx-II after normalization to the maximum amplitude of control current. (B), Concentration-response inhibitory curves of ProTx-II on the activation of three mutant (T1590K, D1586A/T1590K and T1590K/F1592A) $\text{Na}_v1.7$ channels. Residual current after toxin treatment was plotted as a fraction of control peak current amplitude. The calculated values of IC_{50} , slope factor (nH) and f_{bottom} are shown in Table 1. (C), Concentration-response inhibitory curves of ProTx-II on fast-inactivation of three mutant (T1590K, D1586A/T1590K and T1590K/F1592A) $\text{Na}_v1.7$ channels. The $I_{10\text{ms}}$ was plotted as a fraction of the residual current after ProTx-II treatment. The IC_{50} values are shown in Supplementary Table 2. In (B) and (C), data points (mean \pm S.E., each from 3 - 4 cells) are fit with a Hill equation as described under “Experimental Procedure”.

Table 1

Channel	Voltage dependence of activation						
	control		ProTx-II				
	$V_{1/2}$, mV	k , mV	$V_{1/2}$, mV	k , mV	IC ₅₀ , nM	nH	f_{bottom}
hNa _v 1.7	-41.5 ± 0.8	6.5 ± 0.7	-10.4 ± 0.9	14.5 ± 0.8	0.7 ± 0.2	1.1 ± 0.2	0.05 ± 0.02
L201V/N206D	-42.5 ± 0.4	5.3 ± 0.4	-12.1 ± 1.0	17.1 ± 0.9	1.0 ± 0.3	1.1 ± 0.2	0.03 ± 0.01
F813G	-39.6 ± 0.2	5.5 ± 0.2	-23.0 ± 2.7	18.7 ± 2.8	6.0 ± 2.1	1.0 ± 0.2	0.09 ± 0.04
E818C	-33.0 ± 1.2	8.1 ± 1.1	-0.6 ± 1.2	15.3 ± 1.1	2.9 ± 0.1	1.0 ± 0.1	0.13 ± 0.03
E203K/E818C	-14.2 ± 0.4	9.0 ± 0.4	14.2 ± 1.8	19.2 ± 1.5	4.6 ± 0.7	1.2 ± 0.2	0.16 ± 0.02
F204A/F813G	-41.1 ± 1.3	7.2 ± 1.1	-26.3 ± 1.2	14.9 ± 1.2	8.2 ± 1.1	1.3 ± 0.2	0.08 ± 0.03
F813G/E818C	-34.0 ± 1.2	8.1 ± 1.1	-20.5 ± 1.4	15.3 ± 1.3	29.8 ± 3.6	1.0 ± 0.2	0.10 ± 0.05
D1586A	-33.0 ± 0.8	7.5 ± 0.7	-7.1 ± 1.2	16.6 ± 1.1	1.0 ± 0.1	1.2 ± 0.4	0.08 ± 0.04
D1586E	-40.6 ± 0.7	7.0 ± 0.6	-13.6 ± 1.9	17.5 ± 1.9	0.4 ± 0.03	1.0 ± 0.2	0.09 ± 0.02
E1589Q	-40.0 ± 1.6	8.4 ± 1.4	-11.7 ± 1.9	17.2 ± 1.8	0.5 ± 0.1	1.0 ± 0.1	0.06 ± 0.02
T1590K	-44.2 ± 0.5	5.2 ± 0.4	-16.2 ± 0.8	14.0 ± 0.7	0.9 ± 0.4	1.1 ± 0.3	0.07 ± 0.04
F1592A	-34.1 ± 1.1	8.0 ± 0.9	-5.3 ± 2.1	19.3 ± 2.1	0.7 ± 0.3	1.1 ± 0.4	0.04 ± 0.04
D1586A/T1590K	-40.5 ± 0.3	6.9 ± 0.2	-2.5 ± 0.8	18.5 ± 0.8	0.7 ± 0.4	0.8 ± 0.4	0.04 ± 0.01
T1590K/F1592A	-35.1 ± 0.2	6.9 ± 0.2	-8.3 ± 2.6	25.2 ± 2.9	0.8 ± 0.4	1.1 ± 0.1	0.06 ± 0.01

Table 1 Characterization of activation of hNa_v1.7 mutants in the presence of 100 nM ProTx-II.

Cells were held at -100 mV. Families of currents were induced by 50-ms depolarizing steps to various potential ranging from -80 to +40 mV. Recording currents from WT and mutant Na_v1.7 started at ~20 min after establishing whole cell configuration. All data come from 3-4 cells. The half-activation potential ($V_{1/2}$) and slope factor (k) were determined with Boltzmann fits. The values of IC₅₀, slope factor (nH) and f_{bottom} were determined with Hill equation in Fig. 2A, Fig. 6B and Fig. 7B.

Table 2

Channel	Voltage dependence of activation				
	control		HWTX-IV		
	$V_{1/2}$, mV	k , mV	$V_{1/2}$, mV	k , mV	IC_{50} , nM
hNa _v 1.7	-38.8 ± 0.8	6.8 ± 0.7	-40.2 ± 0.7	7.3 ± 0.6	22.7 ± 6.3
L201V/N206D	-40.4 ± 1.3	7.9 ± 1.2	-36.7 ± 1.2	9.6 ± 1.1	20.8 ± 3.6
F813G	-32.7 ± 1.0	6.7 ± 0.9	-36.3 ± 1.4	8.0 ± 1.3	28.2 ± 5.7
E818C	-41.6 ± 0.8*	7.1 ± 0.7*	-39.7 ± 0.9*	8.9 ± 0.8*	9120.1 ± 1400.0
E203K/E818C	-20.5 ± 0.3*	8.5 ± 0.3*	-16.0 ± 1.1*	13.8 ± 1.0*	8892.0 ± 1135.2
F204A/F813G	-46.7 ± 0.4	4.5 ± 0.3	-42.7 ± 0.4	6.1 ± 0.3	26.7 ± 6.4

Table 2 Characterization of activation of hNa_v1.7 mutants in the presence of 100 nM HWTX-IV.

Cells were held at -100 mV. Families of currents were induced by 50-ms depolarizing steps to various potentials ranging from -80 to +40 mV. Recording currents from WT and mutant Na_v1.7 started at ~20 min after establishing whole cell configuration. Note that the asterisk indicates that the concentration of HWTX-IV was 10 μM. All data come from 3-4 cells. The half-activation potential ($V_{1/2}$) and slope factor (k) were determined with Boltzmann fits. IC_{50} values were determined with Hill equation in Fig. 2B, in which slope factor was set to 1. The f_{bottom} values yielded were 0 on both wild type and mutant Na_v1.7 channels and therefore are not listed.

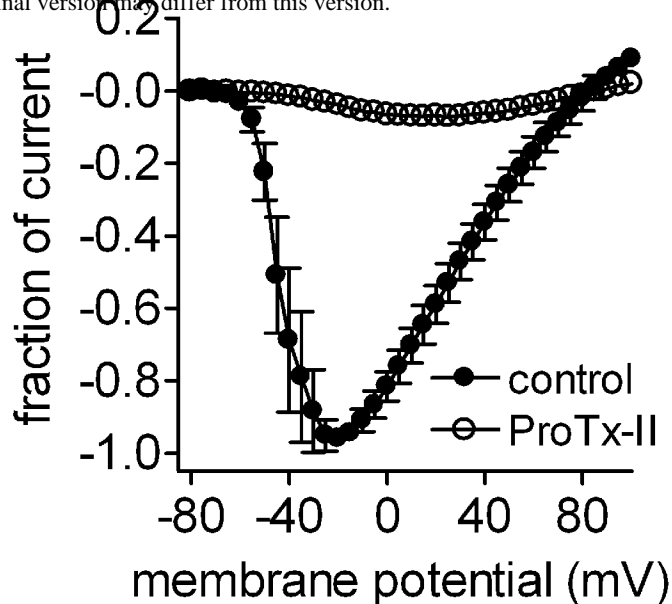
Figure 1

A

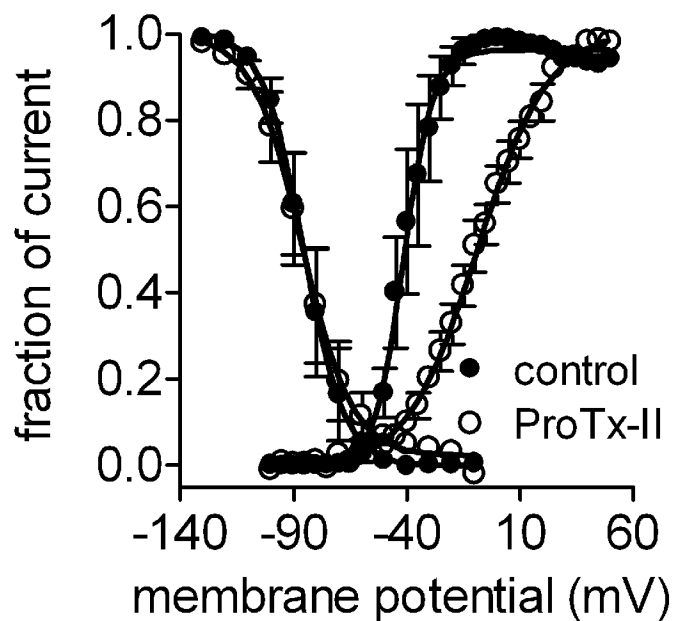
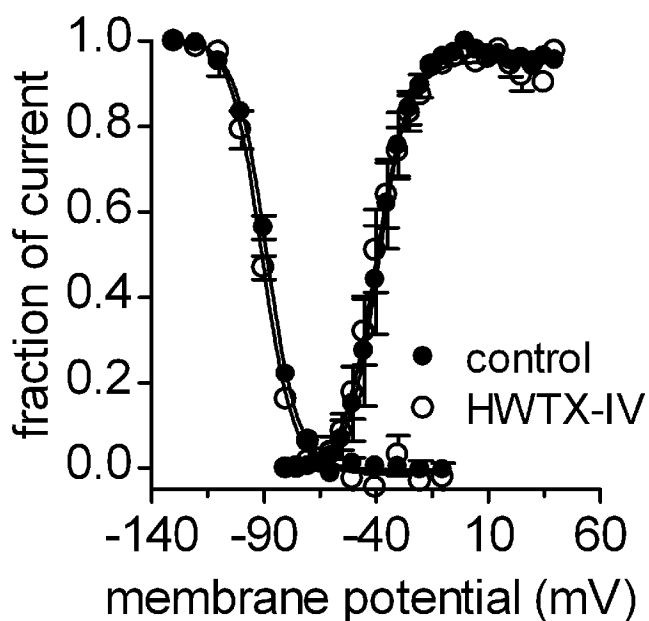
ProTx-II YCQKWMWTC¹D-SERK²CCEG--MVCR---LW³CKKKLW
HWTX-IV E¹CLEIFKAC²NPSNDQ³CKSSKLV⁴CSRKTRW⁵CKYQI-

B

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C



D

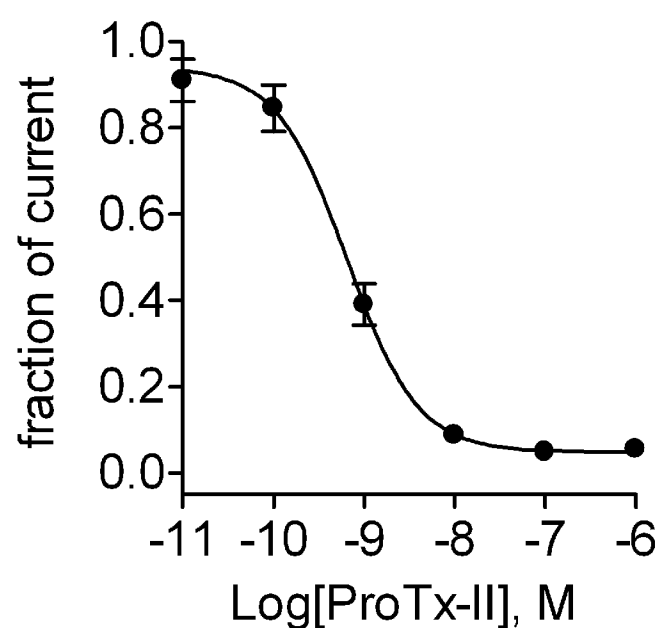
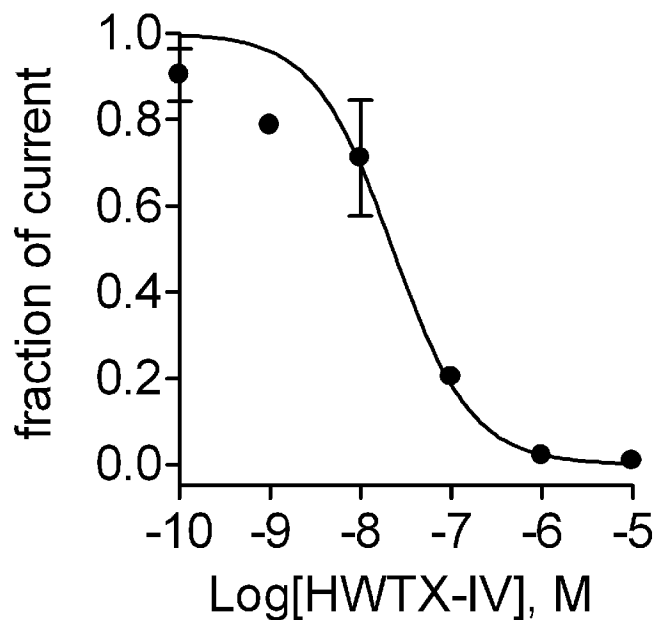


Figure 2

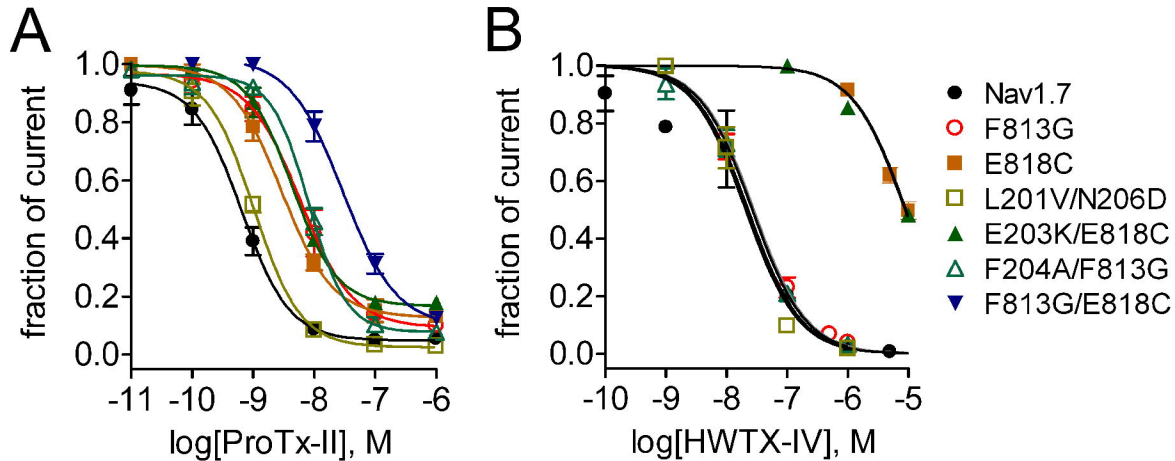


Figure 3

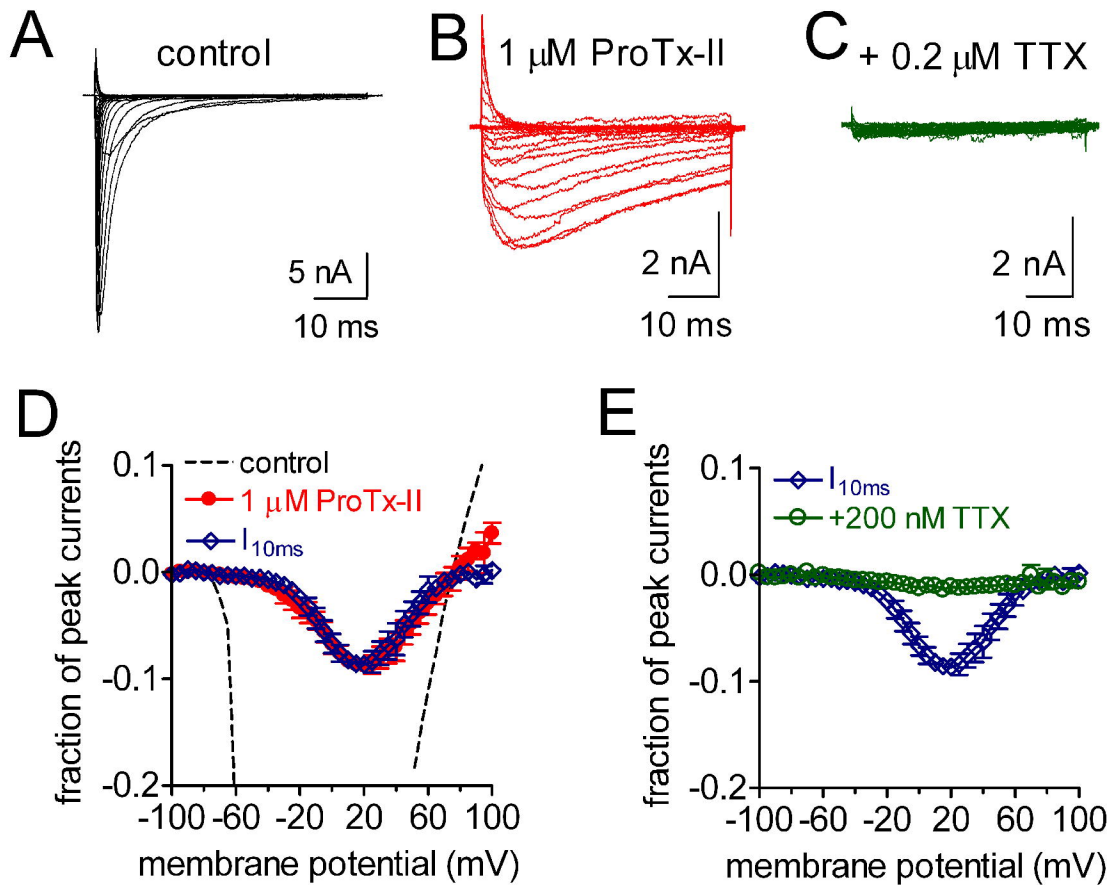


Figure 4

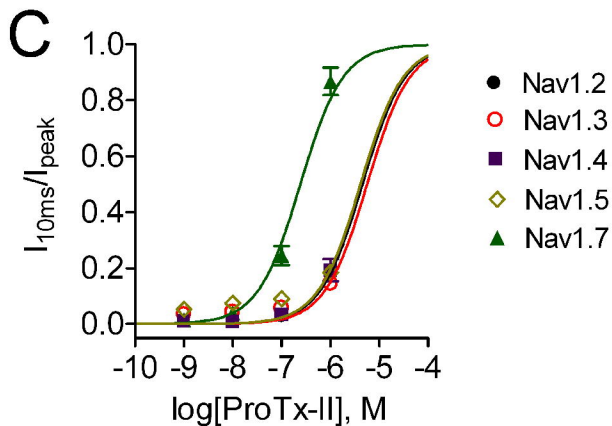
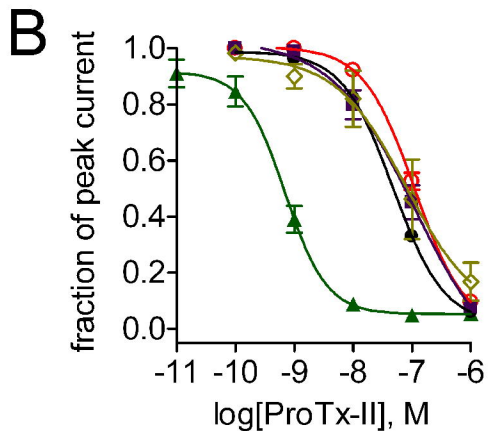
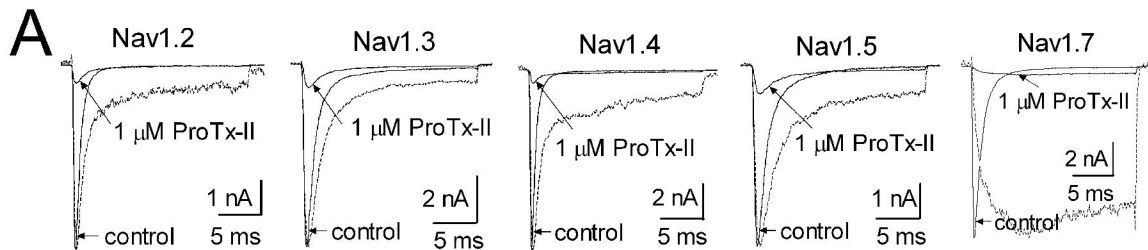


Figure 5

A

		DIVS3	S3-S4 linker	DIVS4	
rNav1.1	1615	SIVGMFLAELIE	--KYFVSP	TLFRVIRLARI	1643
rNav1.2a	1604	SIVGMFLAELIE	--KYFVSP	TLFRVIRLARI	1632
rNav1.3	1551	SIVGMFLAELIE	--KYFVSP	TLFRVIRLARI	1579
rNav1.4	1419	SIVGLALS	DLIQ--KYFVSP	TLFRVIRLARI	1447
hNav1.5	1601	SIVGTVLS	DIIQ--KYFFSP	TLFRVIRLARI	1629
hNav1.6	1596	SIVGMFLADIIE	--KYFVSP	TLFRVIRLARI	1624
hNav1.7	1578	SIVGMFLADLIE	--TYFVSP	TLFRVIRLARI	1606
rNav1.7	1578	SIVGMFLAEMIE	--KYFVSP	TLFRVIRLARI	1606
hNav1.8	1550	SIASLIIFS	AILKSLQSYFSP	TLFRVIRLARI	1580

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B

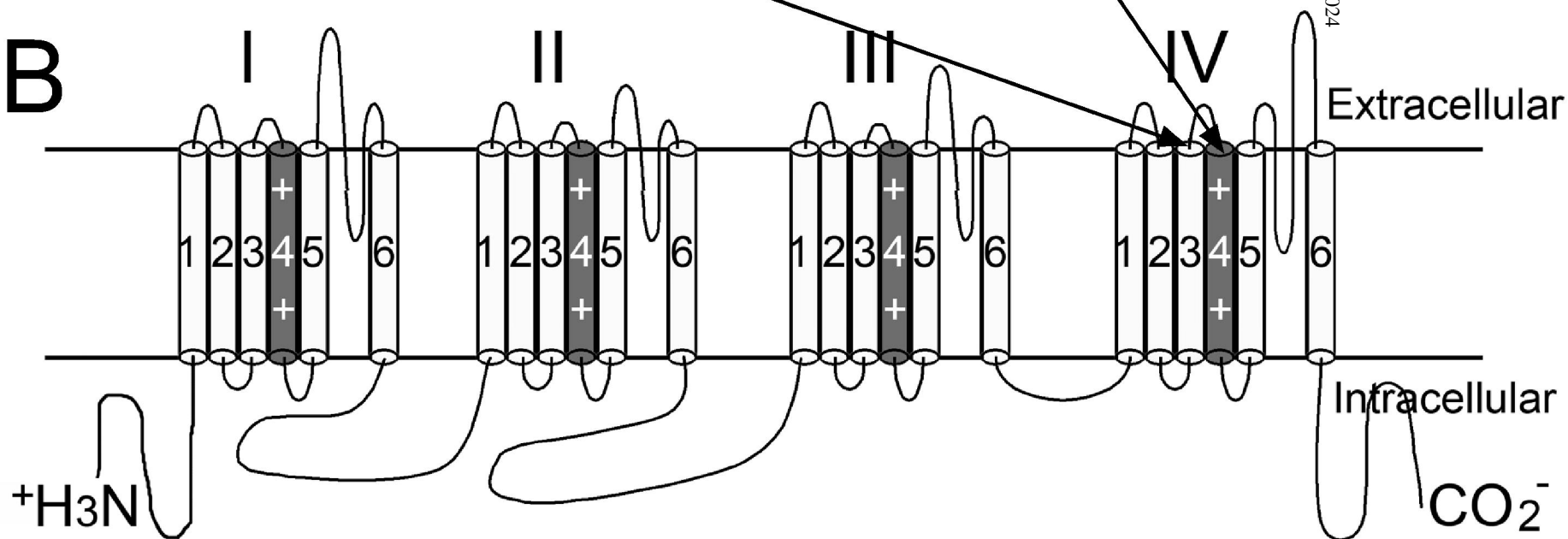


Figure 6

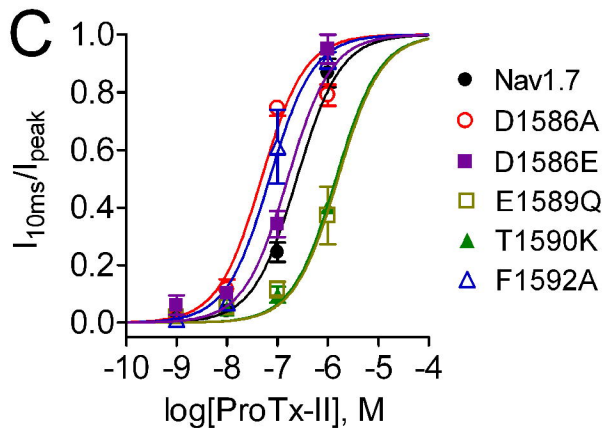
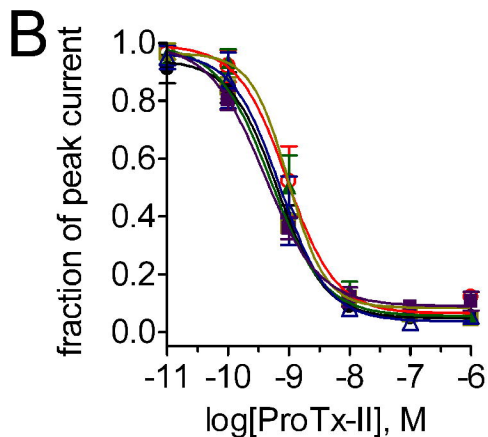
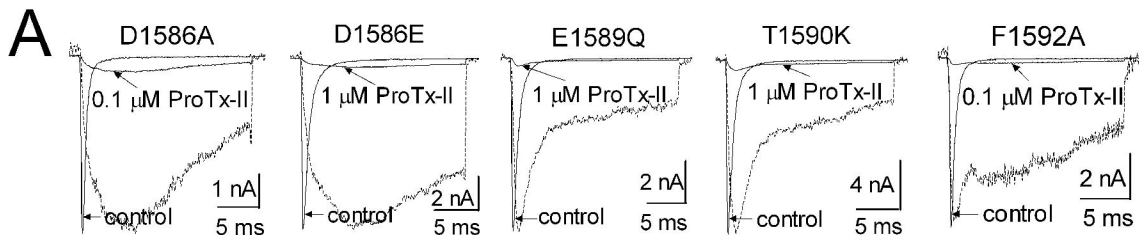


Figure 7

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