Synergetic Action of Domain II and IV Underlies Persistent Current Generation in Nav1.3 as revealed by a tarantula toxin

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The persistent current (INaP) through voltage-gated sodium channels enhances neuronal excitability by causing prolonged depolarization of membranes. Nav1.3 intrinsically generates a small INaP, although the mechanism underlying its generation remains unclear. In this study, the involvement of the four domains of Nav1.3 in INaP generation was investigated using the tarantula toxin α-hexatoxin-MrVII (RTX-VII). RTX-VII activated Nav1.3 and induced a large INaP. A pre-activated state binding model was proposed to explain the kinetics of toxin-channel interaction. Of the four domains of Nav1.3, both domain II and IV might play important roles in the toxin-induced INaP. Domain IV constructed the binding site for RTX-VII, while domain II might not participate in interacting with RTX-VII but could determine the efficacy of RTX-VII. Our results based on the use of RTX-VII as a probe suggest that domain II and IV cooperatively contribute to the generation of INaP in Nav1.3.
also confirm that $I_{NaP}$ is the intrinsic property of Na$_v$s themselves, although the molecular determinants of $I_{NaP}$ in Na$_v$s are largely unknown.

In the present study, we identified a tarantula toxin named $\alpha$-hexatoxin-MrVII (RTX-VII) that enhances the $I_{NaP}$ of Na$_v$1.3 and used it as a probe to examine the involvement of each domain of Na$_v$1.3 in the generation of $I_{NaP}$. Our results reveal that domains II and IV work in a synergetic manner to determine the toxin-induced $I_{NaP}$ of Na$_v$1.3.

**Results**

**RTX-VII enhances the $I_{NaP}$ of Na$_v$1.3.** Macrothele raveni (Figure 1a, inset) venom was collected by using an electro-pulse stimulator as described previously$^{34}$. The lyophilized crude venom was fractionated by RP-HPLC (Figure 1a). A comprehensive screening of each eluted fraction against Na$_v$1.3 transiently expressed in HEK293T cells indicated that the fraction with a retention time of 44.6 min inhibited the fast inactivation of this channel (Figure 1b). This fraction contained a peptide with a molecular weight of 4064.71 Da as determined by MALDI-TOF MS, which was then further purified by analytical RP-HPLC (Supplementary Fig. S1a). Sequence of the peptide was determined by combining Edman degradation (Supplementary Fig. S1j) and cDNA sequencing (Supplementary Fig. S1b), and the toxin was named $\alpha$-hexatoxin-MrVII (RTX-VII). Blasting the full amino acid sequence of RTX-VII showed that it share 92% identity to a previously known spider toxin Magi-6 (Supplementary Fig. S1c). However, Magi-6 did not compete with the scorpion toxin LqhII in binding site 3 of Na$_v$s, and the symptoms caused by injection of pure Magi-6 to mice could not be directly linked to a particular ion channel receptor$^{35}$. This raise the possibility that the subtle amino acid sequence variation brought by RTX-VII makes it active on mammalian Na$_v$s, as that of APETx3 and APETx1, in which a single amino acid substitution between them confer these two toxins different ion channel selectivity$^{36}$. RTX-VII contains eight cysteine residues forming four disulfide bonds, as the measured molecular weight was 8 Da less than the theoretical one. The conserved arrangement of the cysteine residues in RTX-VII indicated that it contains a cystine knot (ICK) motif (Supplementary Fig. S1c).

As shown in Figure 1b, RTX-VII had three effects on current of Na$_v$1.3: (1) it increased the $I_{NaT}$ amplitude at the depolarizing voltage of $-10$ mV; (2) it inhibited the fast inactivation of the channel as determined by $I_{5ms}/I_{NaT}$ ratio; (3) and it induced a large $I_{NaP}$ as revealed by the $I_{45ms}/I_{NaT}$ ratio. At a depolarization of $-10$ mV, the $I_{NaP}$ generated by Na$_v$1.3 accounted for little of the $I_{NaT}$ under control conditions, whereas the treatment with 0.1 μM RTX-VII enhanced the $I_{NaP}$ to approximate 25% of the $I_{NaT}$. The $I_{NaP}$ evoked by the toxin lasted for several seconds and large tail current was
observed when cell membrane was repolarized (Supplementary Fig. S1d), distinguishing this toxin from certain α-scorpion toxins. The time course for 0.2 μM RTX-VII activating the \( I_{\text{NaP}} \) of Nav1.3 was characterized by a slow onset of action (\( t_{\text{off}} = 40.9 \pm 11.3 \) s) and a slow recovery upon washing (\( t_{\text{off}} = 162.8 \pm 39.7 \) s) (Figure 1c). The activation of the \( I_{\text{NaP}} \) of Nav1.3 by RTV-VII was dose-dependent, with an apparent EC\(_{50}\) of 120 nM (Figure 1d). The activity and selectivity of RTX-VII were examined against three Nav subtypes (Nav1.4, 1.5 and 1.7) expressed in HEK293T cells, TTX-R Navs of rat dorsal root ganglion neurons and Navs in neonatal rat hippocampal neurons. Among these channels, Navs of neonatal rat hippocampal neurons were sensitive to this toxin (Supplementary Fig. S1i), whereas the others were not (Supplementary Fig. S1e–h).

**Kinetics of RTX-VII action on Nav1.3.** The current-voltage (I–V) relationships of the \( I_{\text{NaT}} \) and \( I_{\text{NaP}} \) of Nav1.3 before and after the application of 0.2 μM RTX-VII were explored (Figure 2, Supplementary Fig. S2). Compared with the control, RTX-VII modified the I–V relationship of the \( I_{\text{NaT}} \) as follows: (1) the activation of the \( I_{\text{NaT}} \) was potentiated by the toxin at voltages ranging from −45 mV to 10 mV, while no potentiation was observed at voltages > 10 mV (Figure 2a); (2) the activation voltage of the maximum \( I_{\text{NaT}} \) was shifted from 5 mV in the control to −10 mV in the presence of the toxin (Figure 2a, Supplementary Fig. S2d). Although RTX-VII did not alter the reversal voltages (approximate 65 mV) of Nav1.3, toxin application did negatively shift channels’ initial activation voltage (Figure 2a and c). These data indicate that the toxin treatment may increase the opening probability of Nav1.3 channels in cell membrane and facilitate their activation at weak depolarizing voltages. The I–V curves of the \( I_{\text{NaP}} \) before and after the application of toxin indicate that the enhancement of the \( I_{\text{NaP}} \) by the toxin occurred across the depolarizing voltages tested (Figure 2a, Supplementary Fig. S2a) and the activation voltage of the maximum \( I_{\text{NaP}} \) was at about −10 mV. Regarding the I–V curves of the \( I_{\text{NaP}} \) and \( I_{\text{NaT}} \) in the presence of toxin, if the amplitudes of the \( I_{\text{NaP}} \) and \( I_{\text{NaT}} \) at each depolarizing voltage were normalized to their maximum amplitude, respectively, they overlapped completely (Figure 2b), suggesting that the \( I_{\text{NaP}} \) and \( I_{\text{NaT}} \) in the presence of the toxin share rather similar activation voltage. Similar to some α-scorpion toxins\(^{37}\), RTX-VII removed the fast inactivation of Nav1.3 in a voltage-independent way at depolarizing voltages ranging from −20 mV to +30 mV (Supplementary Fig S2b).

The conductance-voltage (G–V) relationship and the steady-state inactivation of Nav1.3 before and after the application of RTX-VII were explored (Figure 2d). Compared with the control, RTX-VII increased the conductance of the cell membrane at depolarizing voltages below 10 mV as revealed by an approximate 16 mV negative shift of Nav1.3 channels’ activation curve induced by the toxin (\( V_{a} = -12.72 \pm 4.92 \) mV for control and \( V_{a} = -29.64 \pm 6.21 \) mV for the toxin treatment), which is in accordance with the negative shift of the activation voltage for maximum \( I_{\text{NaT}} \) observed in the I–V curve; the toxin did not significantly alter the slope factor of the activation curve (\( K_{a} = 6.83 \pm 0.98 \) mV for control and \( K_{a} = 5.66 \pm 1.12 \) mV for the toxin treatment). The I–V and G–V relationships

![Figure 2](https://www.nature.com/scientificreports/figure/kinetics-for-rtx-vii-interacting-with-nax1-3-a-i-v-curves-of-the-inat-and-inap-of-nav1-3-before-and-after-0-2-mum-rtx-vii-treatment-each-current-component-was-normalized-to-the-maximum-inat-in-control-n-11b-i-v-curves-of-the-inap-in-the-presence-or-absence-of-0-2-mum-rtx-vii-c-g-v-curves-of-the-inap-and-inat-of-nav1-3-9241-d-250.png)
of Na\textsubscript{1.3} before and after RTX-VII application were acquired with stringent controls of the uncompensated series resistance (Rs) caused depolarizing voltage error (the maximum tolerable voltage error was less than 5 mV, the mean maximum Rs-caused voltage error was 2.34 ± 1.08 mV, p < 0.001 when compared to the V \text{a} shifted amplitude). A steady-state component (approximately 20% of the INaT) that was resistant to inactivation was observed in the steady-state inactivation (SSI) curve when conditional voltages were above −20 mV, which should represent the INaP elicited by conditional pulses. A significant change of V \text{h} and K \text{h} were observed (V \text{h} = −44.58 ± 2.73 mV for control and V \text{h} = −51.12 ± 5.00 mV for the toxin treatment, p < 0.05; K \text{h} = −6.98 ± 1.44 mV for control and K \text{h} = −12.49 ± 0.41 mV for the toxin treatment, p < 0.001). The hyperpolarization shift of the G–V curve and a non-inactivated component in the SSI curve together resulted in an enlarged voltage range for generation of window current, indicating a slower development of closed state inactivation (CSI) in the toxin-treated channels.

The effect of RTX-VII on the repriming kinetics (recovery from fast inactivation) of Na\textsubscript{1.3} was also investigated. As shown in Figure 2e, the I\textsubscript{NaP} of Na\textsubscript{1.3} recovered gradually from fast inactivation with the repolarizing time (recovery time) increasing in the absence (control) and presence (toxin) of RTX-VII. The I\textsubscript{NaP} induced by the toxin was observed at all recovery time. The I\textsubscript{NaP} of the toxin-treated channels fully recovered at the recovery time of 0 ms, but no I\textsubscript{NaP} recovery was observed (Figure 2e, toxin). The recovery ratios of Na\textsubscript{1.3} I\textsubscript{NaP} before and after the application of toxin were plotted as a function of recovery time (Figure 2f), showing most of channels (>80%) recovered from fast inactivation in 4 ms in both conditions. An apparently faster repriming of the toxin-treated channels than that of control channels within 4 ms was observed, which could be associated with the existence of the I\textsubscript{NaP}. If the I\textsubscript{NaP} was subtracted from the I\textsubscript{NaT} in toxin treated channels, the residual current would exhibit the same repriming kinetics as that of the control (Supplementary Fig. S2c).

The molecular mechanism of RTX-VII as an excitatory toxin. The enhancement of I\textsubscript{NaP} of Na\textsubscript{a,s} in hippocampal neurons by RTX-VII may have led to excitatory toxic in mouse. Intracerebroventricular injection of 20 ng RTX-VII dissolved in 20 μl saline caused seizure-like symptoms, as described by circular running in the first several minutes following by involuntary body twitching, while animals in control group injected with 20 μl saline behaves normal (n = 5 in each group, Supplementary video). We therefore investigated the mechanism of RTX-VII as excitatory toxin. Na\textsubscript{1.3} is upregulated in the peripheral nervous system in response to nerve injury, and contributes to the hyperexcitability of nociceptive neurons under neuropathic conditions\textsuperscript{38,39}. The fast repriming kinetics and slow development of CSI of Na\textsubscript{1.3} make it suitable for generating a large response to slowly developing depolarizing inputs (ramp stimuli)\textsuperscript{40}. We first tested the effect of RTX-VII on the ramp current (I\textsubscript{ramp}) of Na\textsubscript{1.3} evoked by various ramp stimuli (Figure 3a p1). Consistent with previous studies\textsuperscript{38}, Na\textsubscript{1.3} expressed in HEK293T cells produced a large inward Na\textsuperscript{+} current in response to a linearly increasing voltage ramp from −100 mV to 20 mV at the ramp rate of 1.2 mV/ms; of the two I\textsubscript{ramp} peaks shown, the first one (I\textsubscript{ramp1}) but not the second one (I\textsubscript{ramp2}) was ramp rate-dependent, with higher rate leading to larger I\textsubscript{ramp1} (Figure 3b, black trace). The application of 0.5 μM RTX-VII increased the amplitude of both I\textsubscript{ramp1} and I\textsubscript{ramp2} generated by Na\textsubscript{1.3} at all ramp rates tested along with a hyperpolarization shift of the initial activation voltage for I\textsubscript{ramp} (Figure 3b, red trace); the maximum tolerable voltage error was less than 5 mV, the mean maximum Rs-caused depolarizing voltage error was 3.45 ± 1.26 mV. The negative shift of I\textsubscript{ramp1} of Na\textsubscript{1.3} was consistent with the channels’ negatively shifted activation. The enhanced activation of I\textsubscript{ramp1} of Na\textsubscript{1.3} may have been derived from the larger potential gradient (caused by the negative shift of activation voltage of I\textsubscript{ramp1}) that drives Na\textsuperscript{+} to cross the membrane as well as a slowed CSI which makes more channels available for activation.

Na\textsubscript{1.3} intrinsically produces small I\textsubscript{NaP}, and the relationship between I\textsubscript{NaP} and I\textsubscript{ramp2} was investigated in a previous study in which a close correlation between them was observed\textsuperscript{37}. To clarify the relationship between the RTX-VII evoked I\textsubscript{NaP} and I\textsubscript{ramp2}, the protocol p2 described in Figure 3a was used to elicit two type currents of Na\textsubscript{1.3} (Figure 3c). Note only I\textsubscript{ramp2} could be evoked at the ramp rate of 0.2 mV/ms (Figure 3b and Figure 3c). RTX-VII dose-dependently enhanced I\textsubscript{NaP} (I\text{45ms}) and I\textsubscript{ramp2} of Na\textsubscript{1.3} (Figure 3c). The apparent EC\textsubscript{50} for RTX-VII activating I\textsubscript{ramp2} was 320 nM, as revealed by plotting I\textsubscript{ramp2}/I\textsubscript{NaT} Ratios as a function of toxin concentrations (Figure 3d). This EC\textsubscript{50} value did not differ much from that of RTX-VII activating the I\textsubscript{NaP} of Na\textsubscript{1.3} (120 nM). Furthermore, the correlation coefficient between I\textsubscript{NaP} and I\textsubscript{ramp2} was 0.9947 (Figure 3e), indicating a close correlation between them. Thus, data derived from the toxin study further confirmed the conclusion described above.

The effect of RTX-VII on the ramp current of Na\textsubscript{a,s} in neonatal hippocampal neurons was also examined. As shown in Figure 3f, both I\textsubscript{ramp1} and I\textsubscript{ramp2} of hippocampal Na\textsubscript{a,s} were evoked by a linearly increasing voltage ramp from −100 mV to 20 mV at the ramp rate of 1.2 mV/ms (Figure 3a, p3); both components of the I\textsubscript{ramp} of hippocampal Na\textsubscript{a,s} were greatly enhanced by toxin (Figure 3f, upper). As shown in Figure 3f (below), both I\textsubscript{ramp1} and I\textsubscript{ramp2} of hippocampal Na\textsubscript{a,s} in control displayed voltage-dependent inactivation by reverse ramp (R-ramp) stimulation following forward ramp (F-ramp) stimulation (Figure 3a, p4); as I\textsubscript{ramp1} disappeared, the amplitude of I\textsubscript{ramp2} decreased in the R-ramp compared with that in the F-ramp. On the contrary, 1 μM RTX-VII treatment removed the voltage-dependent inactivation of I\textsubscript{ramp2} but not I\textsubscript{ramp1}, as toxin induced a nearly unchanged I\textsubscript{ramp2} in both the forward and reverse ramps, while I\textsubscript{ramp1} was absent in the R-ramp. This finding indicates that the amplitude of I\textsubscript{ramp2} in the presence of the toxin is only dependent on the transmembrane potential, and this population of Na\textsubscript{a,s} generating I\textsubscript{ramp2} should maintain a continuous open state during the entire time course of AP. The toxin negatively shifted I\textsubscript{ramp1} and the enhanced activation of I\textsubscript{ramp2} might lower the threshold and increase the frequency of AP in hippocampal neurons, respectively, which possibly triggers the spontaneous AP firing in hippocampal neurons at a physiological resting potential. Current-clamp experiments showed that 2 μM RTX-VII triggered spontaneous high frequency AP firing in hippocampal neurons (Figure 3g, upper; Supplementary Fig. S3), a mechanism underlying toxin-induced seizure-like symptom in mice. By contrast, the spontaneous AP firing was rare in hippocampal neurons under control conditions (Figure 3g, below; Supplementary Fig. S3).

Domains II and IV of Na\textsubscript{1.3} are critical for I\textsubscript{NaP} generation by RTX-VII. Because Na\textsubscript{a,1.5} is resistant to RTX-VII (Figure 4b), a chimera strategy was used to screen the critical modules [voltage sensor domains (VSD) or pore domains (PD)] responsible for the toxin-induced I\textsubscript{NaP} of Na\textsubscript{1.3}. Each module from the four domains of Na\textsubscript{1.3} was substituted with the corresponding Na\textsubscript{a,1.5} module (Supplementary Fig. S4). The nomenclature of a specific chimeric channel was defined as follows: for example, Na\textsubscript{1.3/1.5 DII-VSD chimera is a hybrid channel in which the DI-DVSD of Na\textsubscript{1.3} was replaced with that of Na\textsubscript{a,1.5}. Eight Na\textsubscript{1.3} derived chimeric channels were constructed. All chimeric channels except the Na\textsubscript{1.3/1.5 DII-VSD chimera were functionally expressed in HEK293T cells; therefore, the hybrid channel Na\textsubscript{1.3/1.5 DII} was generated instead of the Na\textsubscript{1.3/1.5 DII-VSD chimera (Supplementary Fig. S4). To assess the potency and efficacy of RTX-VII for I\textsubscript{NaP} generation in each wt- or chimeric channel, a 300-ms depolarization to 10 mV from a holding potential of −100 mV was applied to evoke the I\textsubscript{NaT} and I\textsubscript{NaP} of a specific channel in the absence and presence of various concentrations of
Figure 3 | The effects of RTX-VII on the ramp currents of Na\(_{\text{a}}\)1.3 and Na\(_{\text{as}}\) of hippocampal neurons. (a) Protocols are used in the experiments described in this figure. (b) A series of inward Na\(_{\text{a}}\)1.3 currents are evoked by using linearly increasing ramp voltage from -100 mV to 20 mV with different ramp rate (Figure 3a, p1), the ramp time ranges from 100 ms to 600 ms, 100 ms/step. The Na\(_{\text{a}}\)1.3 ramp current (\(I_{\text{ramp1}}\)) displays two peaks with the first one (\(I_{\text{ramp1}}\)) but not the second one (\(I_{\text{ramp2}}\)) being sensitive to ramp rate in control (black traces). 0.5 \(\mu\)M RTX-VII enhances the amplitudes of both peaks and causes a hyperpolarized shift of the initial activation voltage for \(I_{\text{ramp1}}\) (red traces); numbers labeled above the traces indicate the ramp rate (mV/ms) (\(n = 10\)). (c) Representative traces show that RTX-VII dose-dependently enhances the \(I_{\text{NaP}}\) (\(I_{\text{NaP}}\)) and \(I_{\text{ramp2}}\) of Na\(_{\text{a}}\)1.3 elicited by the protocol p2 shown in Figure 3a (\(n = 5\)). (d) Dose-response curve for RTX-VII activating the \(I_{\text{ramp2}}\) of Na\(_{\text{a}}\)1.3, the apparent EC\(_{50}\) is determined as approximate 0.3 \(\mu\)M; the maximum and the minimum response of Na\(_{\text{a}}\)1.3 to RTX-II is 63.03\% and 2.15\%, respectively (\(n = 5\)). (e) The \(I_{\text{NaP}}/I_{\text{NaT}}\) ratio was plotted as a function of the \(I_{\text{ramp2}}/I_{\text{NaT}}\) ratio at each toxin concentration (data from Figure 3c). A linear fit of the dots shows the close correlation between the \(I_{\text{NaP}}\) and \(I_{\text{ramp2}}\) of Na\(_{\text{a}}\)1.3 (\(R^2 = 0.9947\)) (\(n = 5\)). (f) Compared with control, 1 \(\mu\)M RTX-VII evidently enhances both peaks (\(I_{\text{ramp1}}\) and \(I_{\text{ramp2}}\)) of the ramp current of Na\(_{\text{as}}\) in rat hippocampal neurons (upper). Protocol p3 shown in Figure 3a was used (\(n = 5\)); Representative traces (below) show that the \(I_{\text{ramp}}\) of Na\(_{\text{as}}\) in hippocampal neurons is elicited by protocol p4 shown in Figure 3a (\(n = 5\)). (g) Spontaneous AP firing in a neonatal rat hippocampal neurons in the absence (below) and presence (upper) of 2 \(\mu\)M RTX-VII (\(n = 8\)).
toxin, and the INaP was measured at the time point of 295 ms (Figure 4a) because the currents of chimeric channels reached a macroscopic steady state at the time point of 300 ms. To compare data derived from different channels, the relative values of INaP/INaT (both from the same current trace) after treatment with different concentrations of toxin were calculated, and the potency of RTX-VII on a specific channel was defined as the EC50 value, while the efficacy of RTX-VII was determined by steady-state INaP/INaT ratio at the saturated concentration of toxin.

The substitution of the VSD/PD of Nav1.3 with that of Nav1.5 had different effects on the potency and efficacy of RTX-VII. Compared with the wt-channel, five chimeric channels, namely Nav1.3/1.5 DI-VSD, DI-PD, DII-PD, DIII-VSD and DIII-PD produced a large INaP in response to RTX-VII, whereas the other three chimeric channels, Nav1.3/1.5 DII, DIV-VSD and DIV-PD displayed a smaller INaP (Figure 4a). The apparent EC50 values of RTX-VII on these channels were further determined from dose-response curves (Figure 4c and d). The bar diagrams shown in Figure 4e and f indicate the changes in the potency and efficacy of RTX-VII on each Nav1.3 derived chimeric channel compared with that for wt-Nav1.3 (n = 7–11). (f) Bars show ratios of the steady-state INaP/INaT of wt- and Nav1.3 derived chimeric channels in the presence of the saturated concentration of toxin. These values are 45.88 ± 4.78%, 52.78 ± 8.32%, 48.87 ± 6.09%, 24.46 ± 5.27%, 40.64 ± 5.47%, 53.95 ± 8.13%, 49.00 ± 5.88%, 7.16 ± 2.33% and 24.48 ± 7.52% for the chimeric channels Nav1.3, 1.3/1.5 DI-VSD, 1.3/1.5 DI-PD, 1.3/1.5 DII, 1.3/1.5 DII-PD, 1.3/1.5 DIII-VSD, 1.3/1.5 DIII-PD, 1.3/1.5 DIV-VSD and 1.3/1.5 DIV-PD, respectively (***p < 0.001, when compared with wt-1.3) (n = 7–11).

Figure 4 | Domain II and IV of Na\(_{\alpha,1.3}\) are synergistically involved in INaP generation. (a) A 300-ms recording of currents of wt-Na\(_{\alpha,1.3}\) and Na\(_{\alpha,1.3}\) derived chimeric channels before and after the application of various concentration of RTX-VII. Chimeric channels were constructed as follows: the voltage-sensor domain (VSD) or pore domain (PD) of DII, DIII or DIV of Nav1.3 was substituted with the corresponding domain of Nav1.5 (see Supplementary Fig. S4). Here, INaP was measured at time point of 295 ms. Note DII, DIV-VSD, DIV-PD substitutions in Na\(_{\alpha,1.3}\) resulted in the reduction of toxin induced INaP compared with wt- and other chimeric channels (n = 7–11). (b) Representative traces show that Na\(_{\alpha,1.5}\) is resistant to RTX-VII (n = 4). (c) Dose-response curves for RTX-VII activating the INaP of wt-Na\(_{\alpha,1.3}\) channel and Na\(_{\alpha,1.3}\) derived chimeric channels that did not or slightly changed toxin potency (EC50) or efficacy (steady-state INaP/INaT ratio at the saturated concentration of the toxin) (n = 7–11). (d) Dose-response curves for RTX-VII activating the INaP of wt-Na\(_{\alpha,1.3}\) and chimeric channels that dramatically changed toxin potency and/or efficacy (n = 7–11). (e) Bars show the fold changes of the apparent EC50 of RTX-VII on each Na\(_{\alpha,1.3}\) derived chimeric channel compared with that for wt-Na\(_{\alpha,1.3}\) (n = 7–11). (f) Bars show ratios of the steady-state INaP/INaT of wt- and Na\(_{\alpha,1.3}\) derived chimeric channels in the presence of the saturated concentration of toxin. These values are 45.88 ± 4.78%, 52.78 ± 8.32%, 48.87 ± 6.09%, 24.46 ± 5.27%, 40.64 ± 5.47%, 53.95 ± 8.13%, 49.00 ± 5.88%, 7.16 ± 2.33% and 24.48 ± 7.52% for the chimeric channels Nav1.3, 1.3/1.5 DI-VSD, 1.3/1.5 DI-PD, 1.3/1.5 DII, 1.3/1.5 DII-PD, 1.3/1.5 DIII-VSD, 1.3/1.5 DIII-PD, 1.3/1.5 DIV-VSD and 1.3/1.5 DIV-PD, respectively (***p < 0.001, when compared with wt-1.3) (n = 7–11).
channel Na, 1.3/1.5 DII was not caused by loss of toxin binding but was rather associated with an intrinsic limitation of this hybrid channel in generating a larger I_{NaP}.

**Domain II of Na, 1.3 is not involved in interacting with RTX-VII.** Further experiments were performed to clarify the roles of Na, 1.3 DII and DIV. First, we examined whether RTX-VII binds to Na, 1.3 DII. Neurotoxins acting on DII of Na, 5 often cause a negative or positive shift of the activation kinetics of targeted channels \(^2\). The substitution of the DII of Na, 1.3 with that of Na, 1.5 should affect the RTX-VII-induced negative shift of activation kinetics of Na, 1.3 if the toxin binds to Na, 1.3 DII, as RTX-VII did not affect the I–V curve of Na, 1.5 (Supplementary Fig. S5a). Therefore, the activation kinetics of the chimeric channel Na, 1.3/1.5 DII was investigated before and after the application of 2 μM RTX-VII. RTX-VII negatively shifted the voltage-dependent activation of the Na, 1.3/1.5 DII chimera and increased the I_{NaP} at voltages ranging from −50 mV to 5 mV (Figure 5a). In addition, 2 μM RTX-VIII caused an approximate 14 mV negative shift of the G–V curve of the Na, 1.3/1.5 DII chimera without changing the slope factor (V \(_{\text{m}}\) = −18.00 ± 1.71 mV for control and V \(_{\text{m}}\) = −32.09 ± 1.99 mV for the toxin treatment; K \(_{\text{m}}\) = 7.23 ± 1.22 mV for control and K \(_{\text{m}}\) = 6.82 ± 1.30 mV for the toxin treatment; the maximum tolerable voltage error was less than 5 mV, the mean maximum Rs-caused depolarizing voltage error was 2.92 ± 2.10 mV, p < 0.001 when compared to the Vo shifted amplitude) (Figure 5b). This raises the possibility that the toxin might not interact with Na, 1.3 DII, which was further confirmed by using a competitive assay. HNTX-III is a tarantula toxin that inhibits the I_{NaT} of Na, 1.3 and Na, 1.7. It was found that this toxin targeted DIII S3–S4 linker of Nav1.7.\(^7\) The wt-Nav1.5 channel and the Na, 1.3/1.5 DII chimera were resistant to 1 μM HNTX-III treatment, whereas the Na, 1.3/1.5/1.5 DII-PD chimera was inhibited by 1 μM HNTX-III (Supplementary Fig. S5 b–d). Reconstruction of the DII of Nav1.3 to Nav1.5 (Nav, 1.5/1.3 DII chimera) conferred the inhibitory activity of HNTX-III to this channel (Supplementary Fig. S5 e). These evidences indicate HNTX-III inhibit Nav1.3 by binding to its DII-VSD. If RTX-VII also targeted DII-VSD of Na, 1.3, its binding should prevent the interaction of HNTX-III with Na, 1.3 because of steric hindrance, which would result in an attenuation of the inhibitory potency of HNTX-III on Na, 1.3. As shown in Figure 5c, the inhibitory effects of HNTX-III on Na, 1.3 I_{NaT} did not differ between 0.5 μM RTX-VIII-pretreated and -untreated channels. The dose-response curves were also superimposed well (Figure 5d), providing evidence to rule out the binding of RTX-VII to Na, 1.3 DII. Next, we determined the molecular determinant in DIV of Na, 1.3 for RTX-VII binding. Since Na, 1.5 is resistant to RTX-VII, the residues in S1–S2 and S3–S4 extracellular loops of Na, 1.3 were mutated to the corresponding residues of Na, 1.5, respectively (Figure 5e). A total of seven residues were mutated and six of them were functionally expressed except V1566F. The kinetics for the activation and SSI of all mutants were listed in supplementary Table S1. Compared with wt-Na, 1.3, Four mutant channels (K1503P, M1505K, L1507N) carrying mutations in the S3–S4 linker led to a 4–12 folds increase in EC_{50} values, whereas the E1562Q and E1562R mutation in the S3–S4 linker resulted in an approximate 5 folds and 20 folds increase of the apparent EC_{50} values (Figure 5f and 5g). These data indicate that multiple residues located in Na, 1.3 DIV were involved in interacting with RTX-VII, and that E1562 was the most important residue for the interaction.

Reverse reconstruction of Na, 1.3 DII and DIV into Na, 1.5 fully restores toxin efficacy. Considering the critical role of the DII and DIV of Na, 1.3 in the RTX-VII-induced I_{NaP}, we assumed that reverse reconstruction of Na, 1.3 DII and DIV into Na, 1.5 might restore the efficacy of the toxin. A reversal chimeric strategy was used as follows: four domains of Na, 1.3 were stepwise reconstructed into the scaffold of Na, 1.5 (Supplementary Fig. S6). The nomenclature of a chimeric channel was defined as follows: for example, Na, 1.5/1.3 DI is a chimeric channel in which the DI of Na, 1.5 was substituted with that of Nav1.3. A total of 11 chimeric channels were constructed and their I_{NaP} generation by the toxin was compared. Again, I_{NaP} was measured at the time point of 295 ms (Figure 6a). The substitution of all four domains of Na, 1.5 with those of Na, 1.3 (Nav1.5/1.3 DI-DII-III-IV) almost fully restored the efficacy of RTX-VII, thus eliminating the involvements of the intracellular loops of Na, 1.3 in the toxin-induced I_{NaP}. Of the four single domain replaced chimeric channels, Na, 1.5/1.3 DI, Na, 1.5/1.3 DII and Na, 1.5/1.3 DIV chimera were resistant to RTX-VII, similar to wt-Na, 1.5, whereas Na, 1.5/1.3 DIV chimera was sensitive to RTX-VII. Furthermore, the toxin slowed the inactivation and induced a small steady-state I_{NaP} in this chimeric channel, indicating that Na, 1.3 DIV is important but not sufficient for RTX-VII inducing large I_{NaP}. Of the two triple domain replaced chimeric channels, Na, 1.5/1.3 DI-DII-IV chimera did not fully restore toxin efficacy but Nav1.5/1.3 DI-DII-IV did, which indicates that the DII but not the DI and DIII of Na, 1.3 is required for toxin inducing large I_{NaP}. Of the three double domain replaced chimeric channels, the reconstruction of the DI or DIII of Na, 1.3 into the scaffold of Na, 1.5/1.3 DIV chimera (Nav1.5/1.3 DII-IV chimera or Na, 1.5/1.3 DIV-IV chimera) had a limited effect on restoring toxin efficacy, whereas the reconstruction of the DII of Na, 1.3 into Na, 1.5/1.3 DIV chimera (Nav1.5/1.3 DIV-IV chimera) almost fully restored toxin efficacy, suggesting the assembly of the DI and DIV of Nav1.3 should be sufficient for RTX-VII inducing large I_{NaP}. Additionally, the chimeric channel Na, 1.5/1.3 DI-III-IV&DII PD, where only the DIII-PD but not the whole DII of Na, 1.3 was present, also attenuated the efficacy of RTX-VII compared with that of Na, 1.5/1.3 DI-III-IV chimera, which strongly supports that the DIII-VSD of Nav1.3 plays a vital role in toxin-induced I_{NaP} generation.

The apparent EC_{50} values of RTX-VII on the Na, 1.5 derived chimeric channels containing Na, 1.3 DIV were estimated from the dose-response curves (Figures 6b and c), and the changes in the potency and efficacy of RTX-VII on these chimeric channels were showed in Figures 6d and e, respectively. Of the Na, 1.3 DIV-containing chimeric channels, the Na, 1.3 DII-containing ones (Nav1.5/1.3 DI-DII-IV, DI-DII and DIV and Na, 1.7–1.9), but not those without reconstruction of DI (Nav1.5/1.3 DI or DIV-VSD), DI or DIII (Nav1.5/1.3 DI-DII-VSD, Nav1.5/1.3 DI-DII-IV, DI-III, DIV and DIII-IIV-DII-PD chimera), produced a large steady-state I_{NaP} comparable to that of wt-Na, 1.3 in the presence of saturated concentration toxin (Figure 6e). On the other hand, the toxin potency on these Na, 1.3 DIV-containing chimeric channels were only slightly weaker than that of wt-Na, 1.3, although the greatest fold change of EC_{50} was observed in Na, 1.5/1.3 DI-DII-DIV chimera (8 folds) (Figure 6d). Moreover, the incorporation of Na, 1.3 DI into Nav1.5/1.3 DI-DIV chimera (Nav1.5/1.3 DI-DII-DIV chimera) led to an evident enhancement of toxin potency, which is comparable to that of wt-Na, 1.3 channel. The results are consistent with the interpretation that the DIV of Na, 1.3 was the main toxin binding site, while the DI-PD of Na, 1.3 might construct the low affinity binding site for RTX-VII. Overall, combining data in Figures 4 and 6 confirmed the cooperative involvement of DI and DIV in the toxin-induced I_{NaP} of Na, 1.3.

**Discussion**

Neurotoxins produced by venomous animals, plants, and microorganisms are a valuable pool of molecular probes to investigate the structure-function relationship of Na, 5.\(^7\) RTX-VII robustly enhances the I_{NaP} of Na, 1.3 and discriminates Na, 5 subtypes Na, 1.4, Na, 1.5, and Na, 1.7–1.9 from Na, 1.3. The toxin-induced and the intrinsic I_{NaP} share some common features, such as sub-threshold activation, a close correlation with Iramp2, and triggering spontaneous high frequency AP firing. Furthermore, the brief late
Figure 5 | Domain IV but not domain II of Nav1.3 is involved in interacting with RTX-VII. (a) The I–V curves of Nav1.3/1.5 DII chimera before and after 2 μM RTX-VII treatment (n = 6). (b) 2 μM RTX-VII negatively shifts the G–V curve of Nav1.3/1.5 DII chimera without changing the slope factor (Vg = −18.00 ± 1.71 mV for control and Vg = −32.09 ± 1.99 mV for the toxin treatment; Kd = 7.23 ± 1.22 mV for control and Kd = 6.82 ± 1.30 mV for the toxin treatment) (n = 6). (c) Representative traces show 1 μM HNTX-III indiscriminately inhibits the INaT of 0.5 μM RTX-VII-untreated-(RTX-VII free) and -treated-(RTX-VII pretreated) NaV1.3 channel (n = 5). HNTX-III was dissolved in bath solution containing 0.5 μM RTX-VII. (d) The dose-response curve for HNTX-III inhibiting the INaT of 0.5 μM RTX-VII-treated or -untreated Nav1.3 channel shows that the potency of HNTX-III on both types of channel are the same (n = 5). The fractions which are resistant to the high dose of HNTX-III treatment account for 5.12% and 6.77% of the maximum INaT, respectively. (e) Sequence alignment of the DIV-VSD of Nav1.3 and Nav1.5, red arrows indicate amino acid residues in Nav1.3 which were mutated to their counterpart in Nav1.5. (f) Dose-response curves for RTX-VII enhancing the INaP of Nav1.3 mutants shown in Figure 5e demonstrate molecular determinants in Nav1.3 for interacting with RTX-VII (n = 5–8); the apparent EC50 values are 117.73 nM, 741.48 nM, 208.83 nM, 541.00 nM, 1209.76 nM, 1505.60 nM, 1399.27 nM, 635.04 nM, 2451, 32 nM for wt-NaV1.3, K1503P, Y1504E, M1505K, T1506I, L1507N, E1562Q and E1562R, respectively. (g) Bars show the fold changes of apparent EC50 values of RTX-VII for mutants compared with that for wt-NaV1.3 channel (n = 5–8).
opening and burst of openings of Navs may be the common mechanism underlying the origin of both types of INaP. However, the intrinsic INaP of Navs is small, which hampered the investigation of the mechanism underlying INaP generation. RTX-VII dramatically enhancing the INaP of Nav1.3 enabled detailed investigations of Nav1.3 INaP generation. In the present study, we clarified the roles of the four domains of Nav1.3 in INaP generation by using RTX-VII as a molecular probe.

Along with the enhancement of INaP, RTX-VII also facilitates Nav1.3 channel opening at weak depolarizations as revealed by the toxin potentiating INaT of Nav1.3 when depolarizing voltages are below 10 mV as well as the toxin negatively shifting channel’s steady-state activation. This observation is not without precedent, as some \( \alpha \)-scorpion toxins modulate Navs in a similar way. This phenomenon could be reasonably interpreted as an increase of the maximum opening probability of the toxin-treated channels. However, how the toxin-bound channels open with a greater probability at weak depolarizations remains unclear. Our data indicate that RTX-VII binds to the DIV-VSD instead of the DII-VSD of Nav1.3, which suggests that the potentiation of Nav1.3 activation by the toxin might not derive from the toxin facilitating DII activation. Two possible explanations for RTX-VII enhancing the INaP of Nav1.3 are proposed: (1) toxin treatment altered single channel conductance of Nav1.3. This interpretation seems unreasonable because the toxin does not alter the inward and outward Na\(^+\) current of Nav1.3 evoked by strong depolarizing voltages above 10 mV; (2) RTX-VII tends to stabilize the DIV-VSD of Nav1.3 in a partially activated state (pre-activated state), which is required for channel activation but not sufficient to trigger channel inactivation (the fully activated DIV-VSD is required for the fast inactivation of Navs). The vital difference is that \( \beta \)-scorpion toxins trap the DII-VSD but not DIV-VSD of Navs in the activated state. The latter interpretation seems plausible as emerging evidences support DIV is involved in Na\(^+\) activation. Furthermore, the voltage driving the outward movement of DIV-VSD is the later step in the activation sequence of Navs as well as the unique role of DIV-VSD in channel gating, we would like to suggest that RTX-VII might tend to trap and stabilize the DIV-VSD of Nav1.3 during channel activation. The reconstruction of Nav1.3 DII but not DI or DIII to Nav1.5/1.3 DIV chimera fully restored toxin efficacy, but it is interesting that RTX-VII did not bind to Nav1.3 DII. Therefore, the role of this domain in the toxin-induced INaP remains unclear. Previous studies

Figure 6 | The substitution of the domain II and IV of Nav1.5 with those of Nav1.3 restores RTX-VII efficacy. (a) A 300-ms recording of the currents of Nav1.5 derived chimeric channels in the absence and presence of various concentration of RTX-VII (n = 6–9). The chimeric channels were constructed as follows: one or several domains (DI, DII, DIII or DIV) of Nav1.5 were substituted with the corresponding domain/s of Nav1.3 (see Supplementary Fig. S6). (b) Dose-response curves for RTX-VII enhancing the INaP of wt-Na1.3 and Nav1.5 derived chimeric channels that did not or slightly restored toxin efficacy (steady-state INaP/INaT ratio at the saturated concentration of toxin) (n = 6–9). (c) Dose-response curves for RTX-VII enhancing INaP of wt-Na1.3 and Nav1.5 derived chimeric channels that almost completely restored toxin potency and/or efficacy (n = 6–9). (d) Bars show the fold changes of the apparent EC50 of RTX-VII for each Nav1.5 derived chimeric channels compared with that for wt-Na1.3 (n = 6–9). (e) Bars show the steady-state INaP/INaT ratio of wt-Na1.3 and Nav1.5 derived chimeric channels in the presence of saturated concentrations of toxin. This values are 47.92 ± 5.43%, 46.37 ± 8.87%, 6.3 ± 1.82%, 10.60 ± 3.32%, 53.81 ± 5.56%, 12.68 ± 3.67%, 50.88 ± 8.33 and 20.41 ± 3.90% for Nav1.3, 1.5/1.3 DI-DII-DIV, 1.5/1.3 DI-DIII-DIV, 1.5/1.3 DI-DIV, 1.5/1.3 DI-DIV and 1.5/1.3 DIV-DIV and 1.5/1.3 DI-DIV-DIV&DIV-PD, respectively (***p < 0.001, N.S = not significant, when compared with wt-Na1,3) (n = 6–9).
showed that the inter-domain interactions of Na₅,s is necessary for channel gating⁴⁰,⁵¹. We proposed in this study that the DII and DIV of Na₅,1.3 might cooperate to trigger late brief opening and burst of opening to generate I₅₃₅, and RTX-VII should facilitate/amplify this cooperation to induce large I₅₃₅ in Na₅,1.3. The subtle amino acid sequence differences of the domain II between Na₅,1.3 and Na₅,1.5 greatly affect this cooperation, namely the DII of Na₅,1.3 can cooperate well with its own DIV, which is not the case for the DII of Na₅,1.5 with DIV of Na₅,1.3. The roles of Na₅,1.3 DII and DIII of in the toxin-induced I₅₃₅ generation were unclear. The fact that the replacement of the DI or DIII of Na₅,1.3 with that of Na₅,1.5 did not affect toxin efficacy could not exclude the possibility that both domains might involve in the I₅₃₅ generation, because high sequence similarity of DI and DIII between Na₅,1.3 and Na₅,1.5 is observed and probably the inter-domain interactions might not be interfered although these two domains were replaced.

RTX-VII induced large I₅₃₅ in Na₅,1.3 at the end of a 50-ms or a 300-ms depolarization, which differs from some scorpion toxins and sea anemone toxins that slow the inactivation of Na₅,s but the resultant current decay rapidly in 50 ms (Lqh2 as a representative)⁴⁸. What is the difference derived from? Theoretically, Lqh2 trapping the DIV-VSD of Na₅,s in the closed state should have induced large I₅₃₅, but the fact is not. How are the toxin-bound channels inactivated? Slow inactivation may not be the underlying mechanism. This is because that slow inactivation is rarely observed in a 50 ms depolarization (such a short depolarization is not sufficient to trigger this gating process). The repriming kinetics of the toxin-bound channels is the same as or even faster than that of the toxin-free channels⁴⁸, which is also inconsistent with the fact that the recovery of Na₅,s from slow inactivation is slow⁴⁰. Based on the unique role of DIV in fast inactivation, a model was proposed to clarify these two problems. Macroscopically, in this model, a depolarization would drive and maintain the first three domains of Na₅,s in an activated state; Lqh2 and RTX-VII could trap the DIV-VSD of Na₅,s in the closed⁴¹ and partially activated state, respectively. For Lqh2, such trapping is not very stable, as the depolarization prolongs, the toxin-bound DIV-VSD would be gradually activated, triggering channel inactivation. However, for RTX-VII, the DII of Na₅,1.3 might allosterically slow/inhibit this process, which therefore makes RTX-VII stably trap the DIV-VSD of Na₅,1.3 in the partially activated state and then the channels would maintain a persistent opening state. The understanding of this process in the single channel level could be as follows: the inactivation ball of a Na₅,s has an “on state” (blocking the pore) and an “off state” (free in cytosol) which are tightly coupled to the activated and resting state of DIV-VSD, respectively⁴⁰. Normally, DIV-VSD is immobilized in an outward conformation by activation⁴⁴,⁵⁵. The toxin-bound DIV-VSD could be activated by strong depolarization but not be stably immobilized as toxins tend to “drag” the DIV-VSD to its resting state (partially activated state for RTX-VII). Thus, when the toxin-bound DIV-VSD is activated, the inactivation ball is in the “on state” and the pore is occluded; when the toxin-bound DIV-VSD is in the resting state (partially activated state for RTX-VII), the channel just opens. The inactivation ball switches between the “on state” and the “off state” quickly and such inactivation ball movement should trigger the burst opening of the channel in single channel recording. For Lqh2, as the depolarization prolonged, the DIV-VSD of most channels would be stably immobilized and these channels were consequently trapped stably in the inactivated state. On the other hand, for RTX-VII, toxin binding to the DIV-VSD of Na₅,1.3 should allosterically affect the conformation of DIII-VSD, which would in turn interfere with the time-dependent immobilization of DIV-VSD. We proposed that such gating model underlies the generation of large I₅₃₅ in Na₅,1.3 by RTX-VII.

Methods

Venom and toxin purification. Spider Macrothele raveni were collected in Guangxi province, China. The spider has a body length of 3–5 cm and the venom was collected by an electric stimulation method as described in another work of our laboratory⁴⁶. The collected crude venom was lyophilized and preserved at −80 °C before use. The crude venom was dissolved in ddH₂O to a final concentration of 5 mg/ml and subjected to the first round of RP-HPLC purification (acetonitrile gradient: 1%–60%, at an increasing rate of 1% per minute). The fraction containing RTX-VII was then collected, lyophilized and subjected to the second round of RP-HPLC with a slower increasing acetonitrile gradient (acetonitrile at an increasing rate of 0.5% per minute) to obtain the purified toxin.

Toxin sequencing and cDNA of RTX-VII. Partial amino acid sequence of RTX-VII was determined by Edman degradation on an Applied Biosystems/PerkinElmer Life Science Procise 491-A protein sequencer. The cDNA of this toxin was obtained by blasting Edman degradation determined amino acid sequence of RTX-VII against the local cDNA library database of the spider Macrothele raveni (unpublished data).

Constructs and transfection. All Na₅ clones and beta subunit clones were kindly gift from Dr Theodore R.Cummings (Department of pharmacology and Toxicology, Stark Neurosciences Research Institute, Indiana University School of Medicine, USA). cDNA genes encoding rat Na₅,1.3 and rat Na₅,1.4 were subcloned into the vectors pcDNA3.1 and pRCGb416,56, respectively; the cDNA genes encoding human Na₅,1.5 and human Na₅,1.7 were subcloned into the vectors pcDNA3.1 and pcDNA3.1-mod52, respectively. Auxiliary β1 and β2 subunits both were cloned from human and inserted into an internal ribosome entry site vector⁴⁴. All site mutations of Na₅,1.3 were constructed by using the QuickChange II XL Site-directed Mutagenesis kit (Agilent Technologies) according to the manufacturer’s instruction. The cytosolic boundaries of two adjacent transmembrane segments and two adjacent domains of Na₅,1.3 or Na₅,1.5 were determined by proteins’ topological information deposited in NCBI protein database (for Na₅,1.3, the website is http://www.ncbi.nlm.nih.gov/protein/ and for Na₅,1.5, the website link is http://www.ncbi.nlm.nih.gov/ protein/NP_932173.1). The protein sequence location of each voltage sensor (VSD)/ pore domain (PD) of all four domains of Na₅,1.3 and Na₅,1.5 are as listed in Supplementary Table S4. A homologous recombination strategy was employed to generate the chimeric channels using the In-Fusion®HD Cloning kit (Clontech Laboratories) or CloneEZ® PCR Cloning kit (GenScript). For example, for the construction of Na₅,1.3/1.5 DIII-VSD chimera, the DI-VS was amplified by PCR using a pair of primers with their 5' end extended by a 15 bp long joint which is homologous or reverse complement to the upstream or downstream flanking sequence of DI-VSD of Na₅,1.3. A pair of oppositely directed primers was used to linearize the whole Na₅,1.3 cloned plasmid with the DI-VS of Na₅,1.3 deleted. The PCR amplified segment and the linearized plasmid were subjected to 1% agarose gel electrophoresis, respectively. The corresponding bands were recycled using a DNA gel extraction kit (Sangon biotech) and ligated using the In-Fusion®HD Cloning kit (Clontech Laboratories) or CloneEZ® PCR Cloning kit (GenScript). Before being transformed to E.coli Top10 competent cell, the ligated product was subjected to FastDigest DpnI (Thermo Scientific) treatment at 37 °C for 1 hour to remove the template plasmid. The transformants were verified by colony-PCR using a pair of gene specific primer for each inserted segment and then sequencing (Genscript). The primers used for vector linearization and amplification of the following constructs were listed in Supplementary Table S2 and Table S3. HEK293 cells (ATCC) were grown under the standard cell culture conditions (5% CO₂ and 37 °C) in Dulbecco’s Modified Eagle Medium (DMEM, Life technologies) supplemented with 10% fetal bovine serum. These Na₅ constructs were co-transfected with plasmid containing β1 subunit and PEGFP-N1 to HEK293T cells using Lipofectamine 2000 (Invitrogen) (manufacturer’s instruction). HepG2 cells containing Na₅,1.3 mutants and Na₅,1.3 derived chimeric channels, 3 μg Na₅, plasmid, 1 μg plasmid containing β1 subunit and 0.5 μg PEGFP-N1 plasmid were co-transfected for wt. Na₅,1.5 and Na₅,1.5 derived chimeric channels, 1 μg Na₅, plasmid, 0.3 μg plasmid containing β1 subunit and 0.5 μg PEGFP-N1 plasmid were co-transfected. For wt. test, Na₅,1.3 was co-transfected with plasmid containing β1 subunit and plasmid, containing β2 subunit²⁵. Cells were 80%–90% confluent before transfection, and cells were seeded on a poly-L-lysine coated Microscope Cover Glass (Fisher scientific) 4–6 hours after transfection. 24 hours after seeding, cells were ready for patch-clamp analysis.

Primary culture of DRG and hippocampal neurons and toxicity test of animals. Animals (Sprague-Dawley rats and Kunming mice) were used according to the guidelines of the National Institutes of Health for care and use of laboratory Animals. The experiments were approved by the Animal Care and Use Committee of the College of medicine, Hunan Normal University. Acutely dissociated dorsal root ganglion (DRG) cells were prepared from 4 weeks old Sprague-Dawley rats and maintained in short-term primary culture using the method described by Hu, H.Z and Li, Z.W⁵⁹. The dissociated cells were cultured for 3–24 h were used in the patch experiments. Experiments were conducted at room temperature (20–25 °C). For primary culture of hippocampal neurons, hippocampal tissues of neonatal rats were dissected and treated with 0.25% trypsin in Ca²⁺–Mg²⁺–free Hank’s Buffered Salt solution at 37 °C for 15 min, and then were disassociated by trituration with a glass Pasteur pipette and seeded on poly-L-lysine-coated Microscope Cover Glass placed in a cell culture dish (35 × 10 mm, corning) and incubated at 37 °C in an atmosphere of 5% CO₂. Cells cultured for 3–24 h were used in the patch experiments. Experiments were conducted at room temperature (20–25 °C). For primary culture of hippocampal neurons, hippocampal tissues of neonatal rats were dissected and treated with 0.25% trypsin in Ca²⁺–Mg²⁺–free Hank’s Buffered Salt solution at 37 °C for 15 min, and then were disassociated by trituration with a glass Pasteur pipette and seeded on poly-L-lysine-coated Microscope Cover Glass placed in a cell culture dish (35 × 10 mm, corning). Approximately 3.5×10⁴ cells in DMEM containing 10% fetal bovine serum were plated in each dish. The culture medium were replaced with serum-free Neurobasal®.
medium (Life Technologies) supplemented by 2% B27 (Life Technologies) on the technique using an EPC 10 USB Patch Clamp Amplifier (HEKA Elektronik). Cells were seeded in a glass coverslip were placed in a perfusion chamber in which rapid exchange of solutions around cells could be performed. The recording pipettes were made from glass capillary (thickness 0.225 mm) using a PC-10 puller (NARISHIGE). The pipet resistance was controlled at 1.5–2.0 MΩ by adjusting the pulling temperature. The standard pipet solution contained (in mM): 140 NaCl, 1 MgCl2, 5 KCl, 2 CaCl2, 10 HEPES, and 10 glucose, pH 7.3 (adjusted with NaOH). During the recording, no current was injected to neurons. Data were presented as Mean ± SD. n is presented as the number of the separate experimental cells. Dose response curves were fitted using the following Hill equation: y = (ymax – ymin) × (1 + (x/EC50)n), where fmax and fmin represent the maximum and minimum response to channel, [Tx] represent the Hill coefficient for effectiveness of selected channel to toxin and EC50 represent the concentration of toxin to half maximal response. The Hill coefficient was set to 1 except where indicated otherwise. This is reasonable based on our mutagenesis analysis, which indicated a single high affinity binding site in Na,3.1 for RTX-VII. Statistical significance was assessed with Microsoft excel 2010 using one-Way ANOVA. Statistical significance was accepted at P values less than 0.05.

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Author contributions

C.T. and Z.L. designed experiments; C.T., X.Z., Y.Z., Z.X., Y.H., B.C., Z.H. and C.Z. performed experiments; C.T. constructed mutant and chimeric channels, C.T. and X.Z. conducted patch clamp analysis; C.T., Z.L. and S.L. contributed to manuscript preparation; C.T. and Z.L. wrote the manuscript.

Additional information

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