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Spider venom-derived peptide induces hyperalgesia in Na\textsubscript{v}1.7 knockout mice by activating Na\textsubscript{v}1.9 channels

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The sodium channels Na\textsubscript{v}1.7, Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9 are critical for pain perception in peripheral nociceptors. Loss of function of Na\textsubscript{v}1.7 leads to congenital insensitivity to pain in humans. Here we show that the spider peptide toxin called HpTx1, first identified as an inhibitor of Kv4.2, restores nociception in Na\textsubscript{v}1.7 knockout (Na\textsubscript{v}1.7-KO) mice by enhancing the excitability of dorsal root ganglion neurons. HpTx1 inhibits Na\textsubscript{v}1.7 and activates Na\textsubscript{v}1.9 but does not affect Na\textsubscript{v}1.8. This toxin produces pain in wild-type (WT) and Na\textsubscript{v}1.7-KO mice, and attenuates nociception in Na\textsubscript{v}1.9-KO mice, but has no effect in Na\textsubscript{v}1.8-KO mice. These data indicate that HpTx1-induced hypersensitivity is mediated by Na\textsubscript{v}1.9 activation and offers pharmacological insight into the relationship of the three Na\textsubscript{v} channels in pain signalling.

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Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, and is a serious public health issue. However, pain is also a part of the body’s warning mechanism, cautioning humans to take action to prevent further tissue damage. The voltage-gated sodium channel (Nav) Na\textsubscript{1.7} is known to play critical roles in the regulation of peripheral pain. Homozygous or compound heterozygous loss-of-function mutations in Na\textsubscript{1.7} lead to congenital insensitivity to pain (CIP)\textsuperscript{12,13}, whereas gain-of-function mutations cause episodic pain (i.e., primary erythromelalgia and paroxysmal extreme pain disorder) in humans\textsuperscript{14-19}. This evidence indicates that selectively blocking Na\textsubscript{1.7} may be useful to relieve pain. Sustained efforts have been made to develop selective inhibitors of this channel, some of which have shown efficacy in clinical studies, although larger clinical trials are needed to definitively assess efficacy\textsuperscript{10-13}. On the other hand, compared with painful, painfulness may be more serious. CIP individuals cannot distinguish between sharp and dull stimuli, leading to self-mutilation and painless fractures, and some individuals are even unable to detect temperature differences\textsuperscript{2,5,6}. Therefore, therapeutic compounds that restore the pain responses in Na\textsubscript{1.7}-related CIP could be useful. Notably, Na\textsubscript{1.7} might not be a suitable direct target to achieve such an effect because of its loss of function in affected individuals; therefore, an alternative strategy should be developed. Na\textsubscript{1.7} and two other Na\textsubscript{v} subtypes, Na\textsubscript{1.8} and Na\textsubscript{1.9}, are preferentially expressed in the peripheral terminals of sensory neurons\textsuperscript{14}. More recently, genetic and functional studies have illustrated that mutations in Na\textsubscript{1.8} and Na\textsubscript{1.9} cause human pain disorders, providing direct clinical evidence linking these two channels to human pain\textsuperscript{15-17}. Considering that the three channels play distinct roles in the generation and propagation of action potentials (APs)\textsuperscript{18-20}, they might regulate pain signaling cooperatively, and the elucidation of their relationship in pain regulation may be helpful for the treatment of Na\textsubscript{1.7}-related CIP.

Animal venom is a rich source of Na\textsubscript{v} modulators and potential therapeutic compounds\textsuperscript{21-26}. Specific modulators are not only novel drug candidates for therapeutics but also powerful pharmacological tools to probe the physiological roles of Na\textsubscript{v}23,25-28. In this study, we screened pain-inducing compounds from animal venoms and discovered that a spider peptide toxin, HpTx1, was able to rescue the pain response in Na\textsubscript{1.7} knockout (Na\textsubscript{1.7}-KO) mice by activating Na\textsubscript{1.9}. HpTx1 was previously identified as an inhibitor of the voltage-gated potassium channel (K\textsubscript{v}) K\textsubscript{4.2} 29, but our study showed that it is also a Na\textsubscript{v} modulator. Here, we provide pharmacological evidence that cross talk among Na\textsubscript{1.7}, Na\textsubscript{1.8}, and Na\textsubscript{1.9} may affect AP firing and pain signaling, thereby establishing an important role for Na\textsubscript{1.9} in pain perception.

**Results**

**Hptx1 induces pain responses in Na\textsubscript{1.7}-KO mice.** To discover peptide activators that can induce pain and recover pain responses in Na\textsubscript{1.7}-related CIP, we first fractionated 15 crude venoms (from ten spiders and five snakes) using semipreparative reversed-phase high-performance liquid chromatography (RP-HPLC) and collected a total of 110 fractions (5–10 fractions per venom). Six of these fractions were identified to have pain-inducing activity, with a fraction from the venom of the spider H. venatoria exhibiting the strongest efficacy (Fig. 1a). By further purifying this fraction by analytical RP-HPLC, we identified a component with such a pain-inducing efficacy and named it HpTx1 (rational nomenclature: k-sparatoxin-Hv1a). The molecular weight of HpTx1 was 3910.8 Da as determined by matrix-assisted laser desorption/ionization-time of-flight mass spectrometry (MALDI–TOF MS) (Supplementary Fig. 1a). HpTx1 was found to have some sequence similarity, especially a conserved cysteine pattern, with some spider peptide toxins adopting an inhibitor cystine knot (ICK) motif (Fig. 1b), suggesting that the space structure of HpTx1 might contain a typical ICK motif.

We next examined whether HpTx1 could produce pain sensation in mice lacking Na\textsubscript{1.7}. According to methods reported previously\textsuperscript{30,31}, we generated Na\textsubscript{1.7}-KO mice by crossing Na\textsubscript{1.7} floxed mice (Na\textsubscript{1.7} in C57BL/6 genetic background) with Na\textsubscript{1.8}-Cre mice, which led to the specific deletion of Na\textsubscript{1.7} in Na\textsubscript{1.8}-positive sensory neurons (tissue-restricted Na\textsubscript{1.7} knockout, Na\textsubscript{1.7}-KO). These mice showed deficits in mechanical pain responses but no alteration in thermal pain behavior (Fig. 1d, e), consistent with the results reported by Minett et al.\textsuperscript{31}. As shown in Fig. 1c, injection of 10 μM HpTx1 into the hind paws of Na\textsubscript{1.7}-KO mice or control (Na\textsubscript{1.7}) littermate mice triggered robust nocifensive behaviors, such as licking and biting of the injected paws. Furthermore, the pain-inducing effect was further validated in evoked pain models (Fig. 1d, e). Na\textsubscript{1.7}-KO mice treated with 10 μM HpTx1 recovered the deficit in mechanical pain caused by Na\textsubscript{1.7} ablation (Fig. 1d); injection of 10 μM HpTx1 also reduced thresholds for thermal pain in Na\textsubscript{1.7}-KO mice, paralleling the effect of HpTx1 on fNa\textsubscript{1.7} mice (Fig. 1e).

Unlike intraplantar injection of 10% formalin, which elicited robust neurogenic inflammation in the injected hind paws, HpTx1 injection failed to produce neurogenic inflammation, as revealed by the Evans blue test (Fig. 1f, g). No swelling was observed in hind paws injected with 50 μM HpTx1, whereas serious edema was found with injection of 10% formalin (Fig. 1h). These results suggested that HpTx1 evoked pain behaviors in fNa\textsubscript{1.7} and Na\textsubscript{1.7}-KO mice, but failed to trigger neurogenic inflammation.

HpTx1 activates some small DRG neurons in Na\textsubscript{1.7}-KO mice. The effects of HpTx1 on membrane excitability were examined in small (<30 μm) dorsal root ganglion (DRG) neurons from WT mice by using current-clamp recordings. Four parameters related to AP firing, including resting membrane potential (RMP), current threshold (rheobase), amplitude, and firing frequency, were determined in the experiments. As shown in Fig. 2a, 0.75 μM HpTx1 significantly depolarized RMP by ~2.0 mV (control: −50.4 ± 1.2 mV; HpTx1: −48.4 ± 1.3 mV; n = 30, P < 0.0001) (Supplementary Table 1). Treatment with 0.75 μM HpTx1 remarkably decreased the rheobase to evoke an AP by 9.3 pA (control: 41.3 ± 3.9 pA; HpTx1: 32.0 ± 3.4 pA; n = 30, P = 0.0026) (Fig. 2b; Supplementary Table 1). Importantly, 15 out of the 30 DRG neurons tested (50.0%) exhibited a decrease in rheobase in the presence of HpTx1, whereas 10% exhibited an increase, and 40% showed no change (Supplementary Table 2). However, no significant changes in AP amplitude were observed in the presence of 0.75 μM HpTx1 (control: 117.8 ± 1.3 mV; HpTx1: 116.8 ± 1.4 mV; n = 30, P = 0.1) (Fig. 2c; Supplementary Table 1). In 10 out of the 15 neurons with reduced rheobase, the reduction in rheobase was also associated with a prominent increase in firing frequency in response to depolarizing currents (Fig. 2d). The input resistance remained unchanged by HpTx1 treatment (Supplementary Table 1). Similar effects of HpTx1 on membrane excitability were observed in some small DRG neurons from fNa\textsubscript{1.7} mice (Fig. 2a–c; Supplementary Table 1).

The effects of HpTx1 on membrane excitability were also assessed in small DRG neurons from Na\textsubscript{1.7}-KO mice. As shown in Fig. 2e, 0.75 μM HpTx1 significantly depolarized RMP by 3.2 mV (control: −49.6 ± 1.0 mV; HpTx1: −46.4 ± 1.1 mV; n = 28, P = 0.006) and decreased the rheobase by 16.6 pA (control:
48.3 ± 4.6 pA; HpTx1: 31.7 ± 3.4 pA; n = 29, P = 0.0003), but did not alter AP amplitude (control: 108.2 ± 1.3 mV; 0.75 μM HpTx1: 106.5 ± 1.3 mV; n = 29, P = 0.09) (Supplementary Table 1) or input resistance (Supplementary Table 1). A total of 65.5% of neurons (19 out of 29) showed a decrease in rheobase in the presence of HpTx1 (Supplementary Table 2), and these neurons that exhibited a reduction in rheobase also showed enhanced AP firing in response to depolarizing currents (Fig. 2f, g). These results indicated that HpTx1 might enhance the membrane excitability of some small DRG neurons from WT, fNav1.7, and Na1.7-KO mice by depolarizing RMP, decreasing current threshold, and increasing AP firing.

**HpTx1 is an inhibitor of Na1.7 and an activator of Na1.9.** Sanguinetti et al. demonstrated that HpTx1 is a blocker of the K4.2 channel. Our study confirmed HpTx1 inhibition of K4.2 currents, with a half-maximal inhibitor concentration (IC50) value measured at 1.2 ± 0.3 μM (Supplementary Fig. 1b). K4.2 is
crucial for the ERK-dependent modulation of dorsal horn neuronal excitability and the ERK-dependent hyperalgesia, but it is seldomly expressed in peripheral sensory neurons.

We found that HpTx1 had no evident effect on voltage-gated potassium channels in DRG neurons, and the current-voltage curves were not altered by HpTx1 treatment (Supplementary Fig. 1c, d). Therefore, we assumed that potassium channel inhibition might not be the primary reason for pain reactions induced by HpTx1. Moreover, HpTx1 did not change voltage-gated calcium channels in DRG neurons (Supplementary Fig. 1e). Therefore, we investigated the effect of HpTx1 on Na$_{\text{v}}$1.7 in Na$_{\text{v}}$1.7-induced pain responses were not distinct between the WT and Na$_{\text{v}}$1.7-KO mice, suggesting that the responses are independent of Na$_{\text{v}}$1.7. Unexpectedly, HpTx1 inhibited the human Na$_{\text{v}}$1.7 (hNa$_{\text{v}}$1.7) currents expressed in human embryonic kidney (HEK) 293T cells with an IC$_{50}$ value of 0.51 ± 0.12 μM (Fig. 3a) without altering the steady-state activation (control: V$_{1/2}$ = −21.9 ± 1.9 mV and k = 5.2 ± 0.2 mV; HpTx1: V$_{1/2}$ = −22.1 ± 3.3 mV and k = 6.9 ± 0.3 mV; n = 4, P > 0.05) or inactivation (control: V$_{1/2}$ = −70.7 ± 1.9 mV and k = −4.8 ± 0.1 mV; HpTx1: V$_{1/2}$ = −73.5 ± 1.7 mV and k = −4.7 ± 0.1 mV; n = 7, P > 0.05) of this channel (Fig. 3b; Supplementary Table 3). In addition, examination of the effect of HpTx1 on tetrodotoxin-sensitive (TTX-S) Na$_{\text{v}}$ channels in WT mouse small DRG neurons demonstrated that 1 μM HpTx1 inhibited TTX-S Na$_{\text{v}}$ currents by 62.9 ± 7.8% (Fig. 3c). In addition to Na$_{\text{v}}$1.7, the TTX-S channel Na$_{\text{v}}$1.6 is also found within small DRG neurons. Indeed, HpTx1 had inhibitory activity on Na$_{\text{v}}$1.6 with an IC$_{50}$ of 5.63 ± 0.13 μM, indicating that HpTx1 was tenfold less potent at Nav1.6 than at Nav1.7 (Supplementary Fig. 1f). Vasylyev et al. 34 estimated that ~70% TTX-S Na$_{\text{v}}$ currents are mediated by Na$_{\text{v}}$1.7 in mouse small DRG neurons. These data suggested that the observed effect on TTX-S Na$_{\text{v}}$ currents mainly arise from Na$_{\text{v}}$1.7 inhibition by HpTx1.

From our understanding, inhibition of Na$_{\text{v}}$1.7 may produce analgesia rather than pain. This seemingly paradoxical result suggests that other ion channels might be involved in HpTx1-induced pain. Therefore, the two TTX-resistant (TTX-R) channels, Na$_{\text{v}}$1.8 and Na$_{\text{v}}$1.9, were examined. As shown in Fig. 3d, e and Supplementary Fig. 2a, HpTx1 robustly enhanced the current amplitude and inhibited the fast inactivation of TTX-R Na$_{\text{v}}$ channels in mouse small DRG neurons. Specifically, steady-state inactivation (SSI) was not significantly altered (Supplementary Fig. 2b), but a noninactivated component (~35–42% of the transient inward peak currents) was observed.
in the SSI curve in the presence of 0.75 μM HpTx1 (Fig. 3f). These effects were limited to DRG neurons expressing Na,1.9 (Fig. 3f; Supplementary Fig. 2b), which was further confirmed in ND7/23 cells heterologously expressing Na,1.8 or Na,1.9. HpTx1 significantly enhanced human Na,1.9 (hNa,1.9) currents in ND7/23 cells (Fig. 3g; Supplementary Fig. 2c) and potently inhibited the fast inactivation of this channel (Fig. 3g, h), consistent with the effect of HpTx1 on TTX-R Na,9 currents in mouse DRG neurons. The half-maximum effective concentration (EC50) of HpTx1 was determined to be 0.47 ± 0.08 μM (Fig. 3g). In contrast, HpTx1 did not affect rat Na,1.8 (rNa,1.8) currents expressed in ND7/23 cells (Supplementary Fig. 2d).

Further investigation showed that 0.75 μM HpTx1 had no effect on the voltage dependence of the steady-state activation of hNa,1.9 (control: V1/2 = −38.2 ± 3.6 mV and k = 8.9 ± 1.5 mV; HpTx1: V1/2 = −35.2 ± 3.0 mV and k = 10.0 ± 0.9 mV; n = 6, P > 0.05). However, the voltage dependence of the SSI was significantly shifted by approximately 13.4 mV in the presence of 0.75 μM HpTx1 (control: V1/2 = −69.9 ± 1.3 mV and k = −9.0 ± 0.7 mV; HpTx1: V1/2 = −56.5 ± 2.4 mV and k = −15.0 ± 0.6 mV; n = 8, P = 0.0009) (Fig. 3i; Supplementary Table 3). This result differed from the effect of HpTx1 on TTX-R Na,9 currents in DRG neurons, potentially due to differences between DRG neurons and ND7/23 cells and between mouse Na,1.9 (mNa,1.9)
and hNa1.9 sequences. Na1.9 channels in ND7/23 cells and DRG neurons have distinct posttranslational modifications and auxiliary subunits that are known to affect the pharmacological properties and physiological properties of Na1 channels.5,38. However, for both TTX-R Na currents in DRG neurons and Na1.9 currents in ND7/23 cells, their window currents were obviously improved in the presence of HpTx1 (Fig. 3i). Indeed, 0.75 μM HpTx1 robustly increased the peak of the ramp current of TTX-R Na in small DRG neurons from WT mice by 62.0 ± 8.8% (Supplementary Fig. 2e) and of hNa1.9 expressed in ND7/23 cells by 71.8 ± 14% (Supplementary Fig. 2f), potentially increasing Na+ influx and leading to enhanced excitability of DRG neurons.

As a further test of toxin specificity, HpTx1 produced neither activation nor persistent inhibition when applied to other pain-related ion channels, including transient receptor potential vanilloid 1 (TRPV1) and acid-sensing ion channels (ASICs) (Supplementary Fig. 3). Taken together, these results suggested that HpTx1 enhanced Na1.9 activity by inhibiting its fast inactivation and that the mechanism by which HpTx1 improves membrane excitability and evokes pain in Na1.7-KO mice might be related to this enhanced Na1.9 activity.

HpTx1-triggered pain responses depend on Na1.9 activity. We next tested the effects of HpTx1 on the membrane excitability of small DRG neurons from Na1.9-KO mice. As shown in Fig. 4a, 0.75 μM HpTx1 did not change RMP (control: −50.2 ± 1.7 mV; HpTx1: −49.1 ± 1.7 mV; n = 29, P = 0.1828), but significantly increased rheobase by 10.4 pA (control: 40.0 ± 4.3 pA; HpTx1: 50.4 ± 5.6 pA; n = 25, P = 0.008). Furthermore, HpTx1 had no effect on AP amplitude (control: 110.6 ± 1.3 mV; HpTx1: 110.7 ± 1.7 mV; n = 25, P = 0.3132) or input resistance (Supplementary Table 1). The increased rheobase in the presence of HpTx1 led to an obvious suppression in the evoked AP firing frequency of small DRG neurons from Na1.9-KO mice with HpTx1 treatment (Fig. 4b, c, n = 25). These effects differed from those observed in WT and Na1.7-KO mice, and these differences could be interpreted to be due to the inhibition of Na1.7 currents by HpTx1.

In further in vivo experiments, the pain-related behaviors induced by injection of HpTx1 into hind paws observed in WT mice were not observed in Na1.9-KO mice (Fig. 4d). In contrast to the decrease in thermal and mechanical stimulus thresholds induced by HpTx1 in WT mice, HpTx1 had an analgesic effect in Na1.9-KO mice. In Na1.9-KO mice, HpTx1 significantly increased the mechanical threshold (Fig. 4e) and the latency of paw withdrawal under noxious heat stimulus (Fig. 4f), most likely by inhibiting Na1.7.

Our data demonstrated that HpTx1 enhanced the excitability of primary afferent neurons and elicited pain, depending on the expression of Na1.9 and that Na1.9 activation might be required for such an effect. In addition, Na1.9 expression in DRG neurons from WT mice showed an ~59.6% overlap with the expression of IB4, a marker for small, unmyelinated nonpeptidergic fibers (Supplementary Fig. 4). As mentioned above, 50–65% of small DRG neurons from WT, Na1.7 or Na1.7-KO mice exhibited decreased current thresholds for AP firing. These neurons may be the small nonpeptidergic neurons that express Nav1.9 and may transmit nociceptive signals from the periphery to the spinal dorsal horn57.

HpTx1 fails to affect pain responses in Na1.8-KO mice. Given the important roles of Na1.8 in AP generation and pain signaling, we sought to determine whether Na1.8 is also required for HpTx1-induced pain, although HpTx1 did not directly affect Na1.8. We investigated the effects of HpTx1 on Na1.8-KO and Na1.7/Na1.8 double-knockout (Na1.7/Na1.8-DKO) mice (simultaneous knockout of Na1.7 and Na1.8). As shown in Fig. 5a–d, 0.75 μM HpTx1 depolarized the RMP of small DRG neurons from Na1.8-KO mice by 2.6 mV (control: −46.9 ± 1.9 mV; HpTx1: −44.3 ± 1.8 mV; n = 18, P = 0.0037) without affecting rheobase (control: 40.0 ± 6.1 mV; HpTx1: 38.9 ± 8.0 mV; n = 18, P > 0.999), AP amplitude (control: 83.4 ± 3.2 mV; HpTx1: 82.5 ± 2.3 mV; n = 18, P = 0.9816) or firing frequency (Supplementary Table 1). In Na1.7/Na1.8-DKO mice, 0.75 μM HpTx1 significantly depolarized the RMP of small DRG neurons by 5.2 mV (control: −52.3 ± 1.4 mV; HpTx1: −47.1 ± 1.2 mV; n = 19, P < 0.0001) and decreased rheobase by 11.6 pA (control: 60.0 ± 6.3 pA; HpTx1: 48.4 ± 6.4 pA; n = 19, P = 0.0112) but had no effect on AP amplitude (control: 88.4 ± 2.3 mV; HpTx1: 87.8 ± 2.5 mV; n = 19, P = 0.5522) or firing frequency (Fig. 5a–e, Supplementary Table 1). These data suggested that HpTx1 failed to enhance small DRG neuron AP firing in Na1.8-KO and Na1.7/Na1.8-DKO mice, despite depolarizing the DRG neuron RMP in both mouse lines.

Intraplantar injection of 10 μM HpTx1, a concentration that elicited acute pain in WT and Na1.7-KO mice, failed to affect the pain responses in Na1.8-KO mice (Fig. 5f). In addition, in contrast to the effects of HpTx1 in WT and Na1.7-KO mice, HpTx1-induced mechanical allodynia (Fig. 5g) and thermal hyperalgesia were abrogated in Na1.8-KO mice (Fig. 5h). Together, these data suggested that Na1.8 was also required for HpTx1-induced pain in mice.

The mechanism of HpTx1 acting on Na1.7 and Na1.9. At least six different neurotoxin receptor sites have been identified on Na1 channels so far, with site 3 and site 4 being hot spots for spider peptide toxins.38,39 The involvement of the domain IV (DIV) s3b-s4 segment in the formation of receptor site 3 is crucial for fast inactivation.22,39,40 In this study, our data showed that HpTx1 slowed the development of the fast inactivation of Na1.9 (Fig. 3g, h), implying that HpTx1 might target site 3. To identify the region of Na1.9 critical for the toxin-induced inhibition of fast inactivation, we constructed several chimaeric channels. Because Na1.8 was resistant to HpTx1, we made a chimera, in which the voltage-sensor domain DIV (DIV s1–s4) of Na1.9 was replaced with the corresponding domain of Na1.8 (named Na1.9/1.8 DIV s1–s4) (Supplementary Fig. 5a). In this chimaera channel, HpTx1 exhibited a significant decrease in efficacy (Supplementary Fig. 5b). Further investigation showed that replacing the DIV s3b-s4 P1 region of Na1.9 abolished the effects of HpTx1 on the channel (Fig. 6a, b; Supplementary Fig. 5c), but replacing the DIV s1-s2 linker did not (Supplementary Fig. 5d). Moreover, we confirmed that reverse construction of Na1.9 DIV s3b-s4 P1 into Na1.8 (Na1.8/1.9 DIV s3b-s4 P1) conferred the efficacy of HpTx1 (Fig. 6b; Supplementary Fig. 5e). These results indicated that HpTx1 might inhibit the fast inactivation of Na1.9 through binding to the DIV s3b-s4 region. In addition, site-directed mutagenesis analysis, in which a total of seven amino acid residues in DIV s3–s4 P1 of Na1.9 were replaced with the corresponding residues of Na1.8, showed that three mutations (T1444L, E1450L, and N1451K) significantly reduced the toxin activity (Fig. 6c; Supplementary Fig. 5f–h), suggesting that these residues might directly affect the interaction of Na1.9 with HpTx1.

HpTx1 inhibited Na1.7 current amplitude without altering steady-state activation and inactivation (Fig. 3b). However, as shown in Supplementary Fig. 5i, progressively longer strong depolarization (to +80 mV) led to an increase in the fraction of Na+ currents recovered from inhibition by HpTx1, and increased depolarization potentials were correlated with increased...
**Discussion**

In this study, we discovered that enhancing Na1.9 activity may recover the pain deficit in CIP caused by loss-of-function of Na1.7, which was revealed by the intensive study of HpTx1 as a probe. HpTx1 modulates Na6 activities through an action mode distinct from previously reported peptide toxins, inhibiting Na1.7 and activating Na1.9, but not affecting Na1.8. Consequently, the effects of HpTx1 on pain responses depend on the expression of these three channels in small DRG neurons. HpTx1 causes pain in WT and Na1.7-KO mice and analgesia in Na1.9-KO mice, but is ineffective in Na1.8-KO mice. The results at the channel level and the data at the animal phenotype level are well connected by the action of HpTx1 on the membrane excitability of small DRG neurons. In other words, this interesting discovery establishes a link among Na1.7, Na1.8 and Na1.9 in the pain dissociation. The data indicated that the binding of HpTx1 was reversed by prolonged strong depolarization that activated the voltage sensor, similar to our previous results with HWTX-IV41 and HNTX-III42, which are site 4 toxins that interact with Na1.7 domain II s3b-s4 (DII s3b-s4). Accordingly, chimerae channels of Na1.7 were constructed. A substitution of the DII s3b-s4 region of Na1.7 with the corresponding region of Na1.8 (Na1.7/1.8 DII s3b-s4) caused this channel to be completely insensitive to HpTx1 (Fig. 6d–f). Similarly, site-directed mutagenesis analysis indicated that D816 and E818 observably affected the efficacy of the HpTx1-Na1.7 interaction (Fig. 6e, f). In particular, mutating E818 to arginine, an oppositely charged amino acid residue located in the corresponding position of Na1.9, resulted in an ~20-fold reduction in HpTx1 activity (Fig. 6e, f), potentially explaining why HpTx1 did not depress Na1.9 currents through binding to DII s3–s4. In addition, two mutants of Na1.7 (F813S and G819S) increased the efficacy of HpTx1 (Fig. 6e, f). Furthermore, the reverse chimerae (Na1.7/8Na1.17 DII s3b-s4) conferred toxin sensitivity with an IC50 value of 3.0 ± 0.6 µM (Fig. 6e). However, site-directed mutants, in which seven amino acid residues in DII s3–s4 of Na1.8 were replaced with the corresponding residues of Na1.7, were insensitive to HpTx1 (Supplementary Fig. 6). These results suggested that multiple amino acid residues in Na1.7 DII s3–s4 might be synergistically involved in the interaction with HpTx1.
signaling pathway and provides a helpful strategy for therapeutic development for Na<sub>1.7</sub>-related CIP.

Our study revealed that the activity of Na<sub>1.9</sub> determines the responses of pain or no pain in mice treated with HpTx1. Enhanced activation of Na<sub>1.9</sub> led to hyperexcitability of some small DRG neurons and pain responses in mice regardless of whether Na<sub>1.7</sub> was lacking or inhibited by HpTx1, but Na<sub>1.8</sub> was required in this process. HpTx1 evoked robust pain responses and produced profound hyperexcitability to mechanical and thermal stimuli in Na<sub>1.7</sub>-KO mice (Fig. 1c–e), as revealed by the depolarized RMP, reduced rheobase and increased AP firing in some small DRG neurons with Na<sub>1.9</sub> activation by HpTx1 (Fig. 2c–g). In contrast, HpTx1 failed to induce pain in Na<sub>1.9</sub>-KO mice, but rather decreased DRG neuron excitability, blocked neuronal signaling, and induced analgesia (Fig. 4), consistent with Na<sub>1.7</sub> inhibition by HpTx1. These observations demonstrate that the two phenotypes derived from the dual activities of HpTx1 (inhibiting Na<sub>1.7</sub> and activating Na<sub>1.9</sub>) are manifested in these two mouse mutants. However, WT mice, which express Na<sub>1.7</sub> and Na<sub>1.9</sub>, displayed pain responses with HpTx1 treatment, indicating that enhanced Na<sub>1.9</sub> activity might conceal the analgesic activity derived from the inhibition of Na<sub>1.7</sub>. Our data suggest that the inhibition of Na<sub>1.7</sub> by HpTx1 treatment may be considered analogous to KO of Na<sub>1.7</sub> to some extent. One possible explanation for the ability of HpTx1-induced Na<sub>1.9</sub> activation to overshadow the analgesic activity derived from HpTx1-induced Na<sub>1.7</sub> inhibition based on the role of Na<sub>1.7</sub> and Na<sub>1.9</sub> as AP threshold channels<sup>18,20,43,44</sup> is that HpTx1-bound Na<sub>1.9</sub> largely depolarized the RMP of DRG neurons, potentially compensating for the absence of Na<sub>1.7</sub> and leading to AP generation. Studies have proven that a moderately depolarized RMP contributes to the decrease in the current threshold for AP generation by promoting the opening of other ion channels (i.e., Na<sub>1.8</sub> and voltage-gated calcium channels)<sup>15,46</sup>. Our data demonstrated that HpTx1 depolarized RMP by 2.0 mV and 3.2 mV in WT and Na<sub>1.7</sub>-KO mice, respectively, leading to a reduced current threshold and increased AP and finally enhancing the membrane excitability of some small DRG neurons (Supplementary Table 1). Some studies have shown that histamine induces short-range AP propagation in sensory terminals and evokes a local neurogenic flare in individuals with CIP<sup>6,47</sup>, which suggests that nociceptive afferents lacking Na<sub>1.7</sub> are not entirely nonfunctional. Histamine has been found to improve the activity of Na<sub>1.9</sub> and enhance AP firing in DRG neurons<sup>37,48,49</sup>. Studies examining the mechanism by which a gain-of-function mutation of Na<sub>1.9</sub> causes painful disorders have shown depolarized RMP in DRG neurons expressing some of these mutants (for example, a significant 3.5-mV depolarization of RMP was reported for the L1158P mutant)<sup>50–52</sup>. On the other hand, Na<sub>1.9</sub> does not contribute much to the amplitude of APs<sup>44</sup>, suggesting that enhancing Na<sub>1.9</sub> activity is unable to compensate for the loss of Na<sub>1.8</sub>, which contributes to the majority (58–90%) of the inward current during the rising phase of an all-or-none AP in nociceptive sensory neurons<sup>19,47,53</sup>. Indeed, our data showing that HpTx1 failed to affect AP firing in Na<sub>1.8</sub>-KO DRG neurons and pain sensitivity in Na<sub>1.8</sub>-KO mice are consistent with this
Fig. 6 The molecular mechanism of HpTx1 action on Na\textsubscript{v}1.9 and Na\textsubscript{v}1.7 channels. a Sequence alignments corresponding to the DIV s3b-s4 region of Na\textsubscript{v}1.8 subtypes. The highlighted sequences show the regions swapped between Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9. b Representative current traces from Na\textsubscript{v}1.9/1.8 DIV s3b-s4 P1 (top) and Na\textsubscript{v}1.8/1.9 DIV s3b-s4 P1 (bottom) chimera channels in the absence (black) and presence (red) of HpTx1. c Effects of HpTx1 on WT and mutant hNa\textsubscript{v}1.9 channels. Dot plots display the effect of the 0.75 μM HpTx1 on the peak current (top, n = 14 for WT; n = 4 for T1444L, M1445L, I4466F, and T1448A; n = 5 for L1449I and E1450L; n = 3 for N1451K) and the persistent current (bottom, n = 14 for WT; n = 4 for T1444L, I4466F, T1448A, and N1451K; n = 5 for M1445L, L1449I, and E1450L). Key residues involved in the interaction between HpTx1 and hNa\textsubscript{v}1.9 are labeled (one-way ANOVA with Dunnett’s multiple comparison test, Log_{10}(I/Imax) = 17.72, P < 0.0001; Log_{10}(I/Imax) = 8.157, P < 0.0001). d (top) Sequence alignments corresponding to the DII s3b-s4 region of Na\textsubscript{v}1.8 subtypes. The highlighted sequences show the regions swapped between Na\textsubscript{v}1.7 and Na\textsubscript{v}1.8. Representative current traces from Na\textsubscript{v}1.7/1.8 DII s3b-s4 (bottom left) and Na\textsubscript{v}1.8/1.7 DII s3b-s4 (bottom right) chimera channels in the absence (black) or presence of 5 μM HpTx1 (red). e Dose-dependent inhibitory curves show the effect of HpTx1 on WT (n = 7) and mutant hNa\textsubscript{v}1.7 channels (n = 4 for FB13S, n = 6 for L814A and A815S, n = 3 for D816K, n = 6 for V817K, n = 7 for E818G, n = 4 for E818R, n = 5 for G819R, and n = 3 for Na\textsubscript{v}1.7/1.8 DII s3b-s4) and the Na\textsubscript{v}1.8/1.7 DII s3b-s4 chimera channel (n = 5). f Bars show the fold changes in IC\textsubscript{50} values of HpTx1 for mutant channels compared with that for the WT hNa\textsubscript{v}1.7 channel. Data are presented as the mean ± S.E.M. Exact P values are presented in Supplementary Data 1. Source data are provided as a Source Data file.

Multiple studies have indicated that Na\textsubscript{v}1.8 may serve as a major contributor to the rising phase of the AP\textsuperscript{19,53}. Recently, Bennett et al. further refined the contribution of Na\textsubscript{v}1.7 to AP generation\textsuperscript{47}, proposing that Na\textsubscript{v}1.7 also contributes to the rising phase, consistent with our finding of a slight decrease in AP amplitude when Na\textsubscript{v}1.7 is knocked out (Supplementary Table 1). To date, no evidence has shown that Na\textsubscript{v}1.9 contributes to the rising phase. These findings suggest that the roles of the three channels in AP firing overlap to some extent. Under normal conditions, because of the low amount of Na\textsubscript{v}1.9 expressed in DRG neurons, its contribution to AP firing related to pain signaling may not be prominent\textsuperscript{55}. Unlike for Na\textsubscript{v}1.7 and Na\textsubscript{v}1.8, no loss-of-function mutants for Na\textsubscript{v}1.9 have been found in the clinic, and Na\textsubscript{v}1.9-KO mice do not show evident changes in physiological nociception. On the other hand, in hyperalgesic conditions, for example, with HpTx1 treatment, inflammatory factors\textsuperscript{56}, cold\textsuperscript{51,57} and gain-of-function mutations\textsuperscript{56,50-52}, the contribution of Na\textsubscript{v}1.9 can be enhanced and even compensate for the loss of Na\textsubscript{v}1.7. Therefore, enhanced activation of Na\textsubscript{v}1.9 may recover the pain responses in Na\textsubscript{v}1.7-related CIP, as revealed in our studies.

Potentiated pain sensitivity should be beneficial for human Na\textsubscript{v}1.7-null CIP individuals. Minett et al.\textsuperscript{39} observed that upregulated endogenous opioids contribute to the analgesia phenotype in Na\textsubscript{v}1.7-null mutant mice and that the analgesia associated
with the loss of Na$_v$1,7 in both mice and humans is substantially reversed by the opioid antagonist naloxone. Our study also revealed that pain sensitivity in Na$_v$1,7-related CIP can be evoked by activating Na$_v$1,9. These studies confirm that a practical treatment strategy for Na$_v$1,7-related CIP may be through targeting the different pain signaling pathways other than Na$_v$1,7 itself. In addition to the ability of agonists such as the peptide toxin HpTx1 to enhance Na$_v$1,9 activity, various other factors have been shown to affect Na$_v$1,9 activity as well. For example, glial cell-derived neurotrophic factor (GDNF) significantly increases Na$_v$1,9 mRNA and current density,$^{40,62}$ and some inflammatory mediators, such as pros-taglandin E2 (PGE2), GTP, and histamine, can markedly increase the current density of Na$_v$1,9 currents and AP firing.$^{37,48,49,63,64}$ Therefore, enhancing the activation of Na$_v$1,9 by regulating these endogenous molecules may be a feasible strategy for treating CIP. However, the possible risks caused by activating Na$_v$1,9 should be noted. Similar to some gain-of-function mutations of Na$_v$1,9, activating Na$_v$1,9 might result in spontaneous pain in humans.$^{16,50}$ while hyperactive Na$_v$1,9 similar to the L811P and L1302F mutants, may lead to pain insensitivity.$^{46}$

Methods

Venom collection and toxin purification. The venom was obtained by electrical stimulation of female spiders of H. venatoria, and the freeze-dried crude venom was stored at −20°C prior to analysis. Lyophilized venom was dissolved in double-distilled water. Every time, 10 mg dried venom was purified by semipreparative reverse-phase HPLC using an Ultimate$^®$ XB-C18 column (300 Å, 10 mm × 250 mm, Welch Materials Inc., Shanghai, China) on the Hanbon HPLC system (Hanbon SciTech, Jiangsu, China). The following linear gradient of solvent A (0.1% formic acid in acetonitrile) in solvent B (0.1% formic acid in water) was used at a flow rate of 3 mL min$^{-1}$: 15% A for 5 min, then 15−60% A over 45 min. Absorbance was measured at 215 nm, and fractions were collected and lyophilized before storage at −20°C.

Mass spectrometry and sequencing. The molecular weight of a peptide was analysed by MALDI-TOF MS spectrum (AB SCIEX TOF/TOF$^®$ 5800 system, Applied Biosystems, USA). The cleaved N-terminal amino acid sequence of a peptide was obtained by automated Edman degradation using an Applied Biosystems 491 pulsed-liquid-phase sequencer from Applied Biosystem Inc.

Plasmid constructs and mutagenesis. Human Na$_v$1,7 (hNa$_v$1,7) and rat Na$_v$1,8 (rNa$_v$1,8) clones and beta subunit (β1 and β2) clones were kindly gift from Dr. Theodore R.Cummings (Department of Pharmacology and Toxicology, Stark Neurosciences Research Institute, Indiana University School of Medicine, USA). hNa$_v$1,7 and rNa$_v$1,8 were subcloned into the vectors pDNA3.1 and pCMV-blank vectors, respectively. hNa$_v$1,9 was subcloned into the pEGFP-N1 vector. The C-terminal of hNa$_v$1,9 was linked a GFP to construct a fusion protein channel (hNa$_v$1,9-GFP). The detail methods of mutagenesis are provided in the Supplementary Methods.

Cell culture and transfection. ND7/23 and HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U ml$^{-1}$ penicillin and 100 µg ml$^{-1}$ streptomycin in a 5% CO$_2$ incubator at 37°C. Cells were trypsinized, diluted with culture medium, and grown in 35-mm dishes. When grown to 90% confluence, ND7/23 cells were transfected with hNa$_v$1,9-GFP or hNa$_v$1,9-GFP mutants using Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer’s instructions. Transfected cells were first maintained at 37°C with 5% CO$_2$ for 24 h, and then incubated at 29°C with 5% CO$_2$ for 20 h before use in electrophysiology experiments. Transfections of hNa$_v$1,7 and hNa$_v$1,8 with β2-eGFP and other ion channels (K$_v$1.2, TRPV1 and ASICs with eGFP) into HEK293T cells, and rNa$_v$1,8 and rNa$_v$1,8 mutants together with eGFP into ND7/23 cells were performed by using LipoLectamine 2000 (Thermo Fisher Scientific), according to the manufacturer’s instruction. Six hours after transfection, the cells were seeded onto poly-D-lysine-coated coverslips (Thermo Fisher Scientific), and grown in 35-mm dishes. When grown to 90% confluence, 5% CO$_2$ for 24 h before whole-cell patch-clamp recording. The green fluorescent was used for visual identification of individual transfected cells.

Dorsal root ganglion neuron isolation and culture. Six to eight-week-old C57Bl/6 WT, rNa$_v$1,7, rNa$_v$1,7-KO, rNa$_v$1,8-KO, rNa$_v$1,7LNa$_v$1,8-DKO, or rNa$_v$1,9-KO mice were euthanized via cervical dislocation under anesthesia. Dorsal root ganglion (DRG) neurons were collected from the lumbar spinal cord L4−L5. DRG neurons were dissociated by enzymatic treatment with collagenase (1 mg ml$^{-1}$) and trypsin (0.3 mg ml$^{-1}$) at 37°C for 30 min. cells were seeded onto poly-L-lysine coated coverslips and cultured in DMEM (Gibco) containing 10% heat-inactivated fetal bovine serum (Gibco) and at 37°C in a humidified incubator with 5% CO$_2$ for 3 h before whole-cell patch-clamp recording. Note that male and female mice in half were used in each experiment.

Electrophysiology. Whole-cell patch-clamp recordings were performed at room temperature (23 ± 2°C) using an EPC-10 USB patch-clamp amplifier operated by PatchMaster software (HEKA Elektronik, Lambrecht, Germany) or Axopatch 200B amplifier (Molecular Devices). Fire-polished electrodes (2.0−2.5 MΩ) were fabricated in borosilicate glass using a P-97 puller (Sutter, Novato, CA). Capacity transients were canceled; voltage errors were minimized with 80% series resistance compensation. The liquid junction potential was corrected using Axopatch 200B series amplifier, the Henderson equation was used to calculate the junction potential based on the ionic strength of bath and pipette solution. During the whole-cell recording performed in the EPC-10 USB amplifier, the liquid junction potential was not corrected. Voltage-dependent currents were noted with Patchmaster at 5 min after establishing a whole-cell configuration, sampled at 30 kHz, and filtered at 2.9 kHz. The configuration of electrophysiology recording buffer and stimulation pulse are provided in the Supplementary Information.

For electrophysiology experiments, the stock solution of HpTx1 was diluted with fresh bath solution to a concentration of tenfold of the interested concentration, 30 µl of the concentrated peptide was diluted into the recording chamber (containing 270 µl bath solution) far from the recording pipet (the recording cell), and was mixed by repeatedly pipetting to achieve the specified final concentration. TTX and capsaicin were dissolved in DMSO to make 1 mM stock solutions. The final concentration of DMSO did not exceed 0.1%, which was found to have no significant effect on Na$_v$ currents.

Animal experiments. Na$_v$1,9-KO mice have been described earlier.$^{55}$ The floxed Na$_v$1,7 mice and Na$_v$1,8-Ros are described by Nasser et al.$^{57}$ These mice were housed at the constant temperature of 24°C and 50−60% humidity under controlled conditions of 12 h light/dark cycles and provided with free access to laboratory-standard food and water.

Seven to eight-week-old male (n = 3) and female (n = 3) mice were used on behavioral testing. Before testing, mice were habituated on an elevated platform of the mesh floor and plastic testing chambers for 30 min. HpTx1 was dissolved in saline and administered at doses of 1 µl and 10 µl, respectively. HpTx1 was injected into the hind paws of mice (10 µl saline, 1 µl or 10 µl HpTx1), and the seconds of licking/biting behavior were immediately recorded during a 20 min period following the injection. Nocifensive responses were assessed by the total seconds of licking/biting behavior during 20 min.

The latency to respond to heat was measured to assess the thermal pain threshold after 30 min of injection (Plantar Test Analgesia Meter, IITC Inc. Life Science). It was recorded three times by using radiant heat light onto the plantar side of the paw with an interval of at least 5 min. In order to avoid injury to the mice, a cutoff of 20 s was set.

The mechanical withdrawal threshold was assessed by recording the max force that was continuously applied until paw withdrawal. Electronic von Frey (Anesthesimeter, IITC Inc. Life Science), and recorded three times with an interval of at least 5 min. Note that all of the behavioral tests were double blind.

Immunofluorescence. DRG neurons were collected from the lumbar spinal cord (L4−L5). The DRG were fixed in 4% PFA in PBS for 2 h 30 min at 4°C then cryoprotected in PBS containing 30% sucrose overnight at 4°C. The DRG were frozen in OCT, and cryosectioned at 12 µm. The sections were permeabilized in PBS with 0.5% TritonX-100 for 10 min, and mounted with a goat serum for 1 h. The sections were incubated for 24 h at 4°C with polyconal rabbit anti-Na$_v$1,9 (1:200; alomone labs). Then the sections were incubated with biotinylated griffonia simplicifolia Lectin I isolectin B4 (10 µg ml$^{-1}$, Vector Laboratories, California, USA) for 30 min at room temperature. After incubation, sections were acquired three times with PBS (0.05% Tween 20, 5 min). Finally, incubated with Alexa-Fluor 488-150 (1:500, Invitrogen) and Alexa-Fluor 594 Streptavidin (1:200, Yeasen Biotechnology, Shanghai, China). After being washed three times for 5 min in PBST, sections were mounted with coverslips. Fluorescence images were acquired with the FV1000 confocal microscope (Olympus, Tokyo, Japan).

Study approval. All of the animal experiments were performed in accordance with the Guidelines for Laboratory Animal Research set by Hunan Normal University and Huazhong University of Science and Technology. The experiments were approved by the Institutional Animal Use and Care Committee of the College of Medicine, Hunan Normal University and Ethics Committee of Huazhong University of Science and Technology.
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Author contributions

X.Z. and T.B.M. conducted most of the experiments and analyzed the data, including peptide toxin purification, patch-clamp recording, mutagenesis, immunofluorescence, animal behavior tests, and data analysis; L.Y.Y. also conducted patch-clamp recording and animal behavior tests. L.L.L. conducted animal behavior tests. S.J.P., Z.X., Z.Q.W., Z.X., Q.F.Z., L.W., and Y.Z.H. also conducted peptide toxin purification and patch-clamp recordings. M.Z.C. conducted MALDI–TOF mass spectrometry and data analysis; X.W.Z. designed the animal behavior tests. X.Z. and T.B.M. prepared the paper; X.Z., T.R.M., S.P.L., J.Y.L., and Z.H.L. designed the study and reviewed the paper.

Competing interests

The authors declare no competing interests.

Additional information

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