Multitarget nociceptor sensitization by a promiscuous peptide from the venom of the King Baboon spider

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The King Baboon spider, Pelinobius muticus, is a burrowing African tarantula. Its impressive size and appealing coloration are tempered by reports describing severe localized pain, swelling, itchiness, and muscle cramping after accidental envenomation. Hyperalgesia is the most prominent symptom after bites from P. muticus, but the molecular basis by which the venom induces pain is unknown. Proteotoxocrampic analysis of P. muticus venom uncovered a cysteine-rich peptide, δ/κ-therapotoxin-Pm1a (δ/κ-TRTX-Pm1a), that elicited nocifensive behavior when injected into mice. In small dorsal root ganglion neurons, synthetic δ/κ-TRTX-Pm1a (Pm1a) induced hyperexcitability by enhancing tetrodotoxin-resistant sodium currents, impairing repolarization and lowering the threshold of action potential firing, consistent with the severe pain associated with envenomation. The molecular mechanism of nociceptor sensitization by Pm1a involves multimodal actions over several ion channel targets, including NaV1.8, Kv2.1, and tetrodotoxin-sensitive NaV channels. The promiscuous targeting of peptides like δ/κ-TRTX-Pm1a may be an evolutionary adaptation in pain-inducing defensive venoms.

hyperexcitability | Kv2.1 | NaV1.8 | pain | target promiscuity

The King Baboon spider, Pelinobius muticus (formerly Citharischus crawshayi) (1, 2), is a large, fawn-colored tarantula found in Kenya and Tanzania that feeds on beetles, cockroaches, and other spiders, but will occasionally hunt vertebrates, such as mice, lizards, snakes, and birds. Larger species of Old World tarantulas like P. muticus spend most of their time in burrows and, unless provoked, pose no immediate risk to humans. Little is known about P. muticus envenomations, but like other tarantulas, they have evolved formidable chelicerae along with a venom that delivers mechanically and chemically painful bites to defend against perceived threats (3).

The triggering of unpleasant sensory experiences using pain-producing toxins would likely discourage most potential aggressors (4). Pain development and discomfort are universal features of spider envenomation (5). Spider venoms contain neurotoxins that produce paralysis and toxicity to humans through their ability to modulate ion channels and receptors. For example, mature male Australian funnel-web spiders use alogentic δ-hexa peptides in their venom to deter vertebrate predators after they leave their burrows in search of females (6).

The study of spider-venom toxin pharmacology is exploring potential pain physiology. Examples of such venom-derived peptides include Hm1a from the Togo starburst tarantula Heteroscodra maculata, which was used to show for the first time that the voltage-gated sodium (NaV) channel NaV1.1, is involved in the transduction of mechanical pain (7), whereas Tsp1a from a Peruvian Thrixopelma tarantula was used to identify a key role for NaV1.7 in the chronic visceral pain associated with irritable bowel syndrome (8).

Despite suggestive evidence that African tarantulas produce potent neurotoxic venoms, their effects and molecular composition have not been thoroughly explored (9). Understanding the study of spider-venom toxin pharmacology has uncovered a cysteine-rich peptide, δ/κ-therapotoxin-Pm1a (δ/κ-TRTX-Pm1a), which was used to show for the first time that the voltage-gated sodium (NaV) channel NaV1.1, is involved in the transduction of mechanical pain (7), whereas Tsp1a from a Peruvian Thrixopelma tarantula was used to identify a key role for NaV1.7 in the chronic visceral pain associated with irritable bowel syndrome (8).

Significance

Pain development and discomfort are universal features of spider envenomation, yet severe pain arising from bites by Old World spiders is poorly understood. Molecular analyses of the venom of the King Baboon spider revealed abundant expression of the inhibitory cystine knot peptide Pm1a. Synthetic Pm1a induces pain in mice while simultaneously enhancing pro excitatory sodium currents and decreasing inhibitory potassium currents. These concomitant effects promote hyperexcitability in pain-sensing neurons that can be reversed by pharmacological inhibition of voltage-gated sodium channels. The coordinated modulation of excitatory and inhibitory ion channels involved in pain propagation may represent an economical and effective defense strategy in pain-inducing defensive venoms.

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that form three disulfide bridges, with a theoretical molecular mass toxin (Fig. 1). A 24-residue propeptide and the 42-residue mature peptide comprised of a 19-residue signal peptide, followed by a transcripts per million (Fig. 1). Of 20 structures (Fig. 1), was determined using NMR spectroscopy. The final ensemble of sPm1a structures is of high stereochemical quality well defined when the flexible regions are excluded. The backbone RMSDs (residues 3 to 27 and 31 to 35), suggesting that the structure is well conserved N-terminal “GVDK motif” (1).

To enable full structural and functional characterization, δ/κ-TRTX-Pm1a was synthesized in two fragments using Fmoc solid-phase peptide synthesis. Reduced, full-length toxin was generated by native chemical ligation (details in SI Appendix, Materials and Methods and Fig. S1). After folding and purification, the obtained synthetic product (hereafter sPm1a) was indistinguishable by HPLC and mass spectrometry from δ/κ-TRTX-Pm1a isolated from venom (Fig. 1D).

Three-Dimensional Structure of sPm1a. The 3D structure of sPm1a was determined using NMR spectroscopy. The final ensemble of 20 structures (Fig. 1F) revealed a highly flexible C-terminal region and a moderately dynamic β-hairpin loop, which together lead to a high overall backbone atom root-mean-square deviation (RMSD) of 0.78 ± 0.20 Å. The backbone RMSD is much lower (0.23 ± 0.03 Å) for the relatively rigid regions (residues 3 to 27 and 31 to 35), suggesting that the structure is well defined when the flexible regions are excluded. The ensemble of sPm1a structures is of high stereochemical quality as judged by a MolProbity score of 1.7 ± 0.1 (SI Appendix, Table S1).

The disulfide-bond connectivity of sPm1a was unequivocally determined from preliminary structure calculations performed without disulfide-bond restraints (10). The dipolar connectivities in the NOESY spectrum are consistent with the pattern Cys7–Cys21, Cys14–Cys26, and Cys20–Cys34 (red tubes in Fig. 1F). The three disulfide bonds form a cystine knot in which the Cys7–Cys21 and Cys14–Cys26 disulfides and the intervening section of the polypeptide chain form a closed loop that is pierced by the Cys20–Cys34 disulfide bond. Together with a canonical β-hairpin that is supported by two of the disulfide bonds, the overall structure of sPm1a conforms to the ICK motif commonly adopted by spider toxins known to modulate voltage-gated ion channels (11).

In contrast to the highly disordered C terminus (residues 36 to 42), the N terminus of sPm1a is relatively inflexible. A hydrogen bond is formed between the backbone amide of Val2 and the carbonyl oxygen of Cys20 (Fig. 1F), stabilizing the N-terminal tail in a position that is in close proximity to the core of the peptide. Hydrophobic patches are commonly found on the surface of gating-modifier peptides (GMPs) from tarantulas, such as SGTX1 and GoxTX-1E, and they are proposed to interact with both the hydrophobic membrane lipids and the voltage-sensor domain on target ion channels (12, 13). Accordingly, sPm1a has most of its hydrophobic residues (Try9, Leu-10, Phe11, Leu24, Try33, and Thr36) on one face of the peptide, and they form a distinctive hydrophobic patch on the opposite side to the N terminus (Fig. 1G and H).

sPm1a Elicits Nocifensive Behaviors in Mice. To determine whether sPm1a can recapitulate the pain experienced after a P. muticus bite, we delivered the synthetic peptide directly to primary afferent terminals in the hindpaw of adult mice in vivo and evaluated the resulting behavioral responses. Intraplantar injection of sPm1a resulted in immediate and dose-dependent emergence of spontaneous nocifensive behaviors, including paw flinching and flinching (bites per 15 min). Control (Ctr): 0.7 ± 0.2; 0.06 nmol sPm1a = 27.8 ± 3.5, P < 0.05; 0.6 nmol sPm1a = 187.0 ± 16.5, P < 0.0001; n = 4 to 8 per condition (Fig. 2A) that persisted for ~15 min (SI Appendix, Fig. S2). These sPm1a-induced nocifensive behaviors included a considerable TTX-resistant component, with intraplantar administration of TTX partially reversing paw flinching (flinches per 15 min: 0.6 nmol sPm1a + 0.02 nmol TTX = 74.4 ± 5.4; P < 0.001) (Fig. 2A and SI Appendix, Fig. S2). Consistent with a major contribution of TTX-R channels to sPm1a-induced effects, coadministration of the selective NaV1.8 inhibitor A803467 (14) significantly reversed spontaneous pain behaviors (flinches per 15 min: 0.6 nmol sPm1a + 0.2 nmol A803467 = 93.8 ± 8.8; P < 0.001) (Fig. 2A and SI Appendix, Fig. S2). Substantial changes in mechanical paw withdrawal threshold (PWT; Ctr: 3.7 ± 0.1 g; 0.6 nmol sPm1a = 1.3 ± 0.2 g; n = 6; P < 0.001) (Fig. 2B) and thermal thresholds (withdrawal temperature: Ctr: 49.8 ± 0.3 °C; 60 pmol sPm1a = 46.0 ± 0.4 °C; n = 6, P > 0.001) (Fig. 2C) were also observed following sPm1a injection (Fig. 2C), indicating mechanical and thermal hypersensitivity. Both NaV channel blockers, TTX and A803467, partially and significantly reduced mechanical allodynia (paw withdrawal force: 600 pmol sPm1a = 1.0 ± 0.1 g; 600 pmol sPm1a + 20 pmol TTX = 2.4 ± 0.3 g; 600 pmol sPm1a + 200 pmol A803467 = 2.5 ± 0.2 g; n = 5 to 8; P < 0.001) (Fig. 2B), suggesting the TTX-R component is not limited to the spontaneous nocifensive behavior but also plays a role in mechanical alldynia induced by sPm1a.

sPm1a Induces Hyperexcitability in Small Dorsal Root Ganglion Neurons. Nociceptors are sensory neurons with crucial roles in the generation and conduction of pain signals and sensitization following injury or inflammation underlying hyperalgesia. The cell bodies of most sensory neurons reside in the dorsal root ganglion (DRG) in which nociceptors are typically recognized by their small cell size relative to other cell types (15). DRG neuron subtypes display different action potential (AP) waveform and electrophysiological signatures that are common to major sub-classes (16). For practical reasons, the overriding DRG neuron distinction used here was based on cell size estimated by membrane capacitance measurements, which is directly proportional to cell membrane area. Relatively broad APs with a “hump” in the falling phase made evident by two local minima in the AP first derivative (dv/dt) (see Insets in Fig. 2D and E) are considered a distinctive feature of nociceptors. We performed whole-cell patch-clamp recordings from dissociated small DRG neurons (C ≤ 25 pF, equivalent to cell diameter ≤ 30 μm) from adult mice in the presence and absence of sPm1a. Sensitization,
manifested by increased neuroexcitability, is characteristic of nociceptors (17), whereas sPm1a exposure enhances the excitability of small DRG neurons. The current-clamp recording shown in Fig. 2 was obtained from a DRG neuron that fired spontaneously in control conditions (Fig. 2D, black), while the addition of 1 μM sPm1a (Fig. 2D, red) resulted in a two- to fourfold increase in firing frequency, AP peak amplitude, and AP half-width (quantification for this cell is included in SI Appendix, Fig. S3). Note that the nociceptor AP hump evidenced by the first derivative of representative voltage responses (Fig. 2D, Inset, dV/dt) in control conditions (Fig. 2D, black) displays the stereotypical two local minima that spread further apart in the presence of 1 μM sPm1a (Fig. 2D, red), suggestive of a repolarization delay in the presence of the venom peptide.

To compare excitability changes in small DRG neurons in the absence and presence of sPm1a, we used ramp current injections of 1 s duration (from 0 to 250 pA) (i.e., Fig. 2E, Inset), which allows simultaneous assessment of the number of APs and firing latency. The recordings shown in Fig. 2E display the firing responses of a 15 pF neuron stimulated by a subthreshold somatic ramp current injection (0 to 250 pA) in which exposure to sPm1a caused concentration-dependent increases in excitability. Under control conditions (Fig. 2E, black trace), no APs were observed, yet application of 0.1 μM sPm1a caused this cell to fire two APs (Fig. 2E, yellow), with progressively higher sPm1a concentrations increasing the number of APs fired (0.3 μM, Fig. 2E, green: 6 APs; 1 μM, Fig. 2E, red: 9 APs). Accordingly, the first AP fired appeared earlier (first latency) during the stimulation as the sPm1a concentration bathing this neuron was increased. Superimposed first derivative traces for the first AP fired in each condition are displayed in Fig. 2E, Center Inset, showing a concentration-dependent rightwards shift in the second local minima.

Sensitization of nociceptors develops as a consequence of tissue insult or inflammation and it is manifested as a reduction in the firing threshold or an increased response to noxious stimulation (17). Under control conditions, the firing characteristics of dissociated DRG neurons are notoriously heterogeneous. Paired responses in the absence (Ctr) and presence of 1 μM sPm1a from 36 small DRG neurons (obtained from seven independent isolations) are summarized in Fig. 2F–H. First, we observed
Fig. 2. sPm1a elicits nocifensive behavior in mice, induces hyperexcitability in small DRG neurons, and enhances the activity of TTX-R NaV channels. sPm1a injection induces (A) dose-dependent spontaneous pain behavior (paw flinches/time), (B) mechanical allodynia (decreased PWT in g), and (C) thermal hypersensitivity (decreased paw withdrawal temperature thresholds) (doses are expressed in nmol/paw) (see SI Appendix, Materials and Methods for details). TTX and A803467 reverse spontaneous pain (A) and mechanical allodynia induced by sPm1a (B). (B) Image credit: Shutterstock/Eric Isselee. One-way ANOVA with Tukey’s multiple comparison test: *P ≤ 0.05; ‡P ≤ 0.0001. (D) Spontaneous firing in a small DRG neuron (black, Upper) was enhanced by exposure to 1 μM sPm1a (red, Lower). The Inset shows the first derivative (dV/dt) of representative APs in the absence and presence of sPm1a (black and red, respectively). (E) sPm1a-evoked concentration-dependent increase in AP firing in a small DRG neuron. The ramp stimulation protocol (Inset, 1 s, 0 to 250 pA) applied in control conditions (black trace) did not elicit AP firing, whereas increasing concentrations of sPm1a caused a concentration-dependent increase in the number of APs fired upon the same stimulus (yellow: 0.1 μM; green: 0.3 μM; red: 1 μM sPm1a). The first derivative (dV/dt) of the first AP from the recordings on top is shown in the Center Inset. (F-H) Paired responses of mouse small DRG neurons before (Ctr) and after exposure to sPm1a (1 μM) and mean ± SEM (F) Individual and number of APs fired (n = 36), (G) first latency (n = 24; **P ≤ 0.001) and (H) membrane potential at the end of the ramp protocol (all neurons n = 36, P = 0.9563; responsive neurons n = 11, P < 0.0001). Solid symbols joined by solid lines and empty symbols joined by dotted lines correspond to sPm1a-responsive and nonresponsive DRG neurons, respectively. ns, not significant.
that sPm1a caused a significant increase in the number of APs fired (Fig. 2F) in 83% (30 of 36; number of APsCtr, 1.3 ± 0.3 vs. number of APs sPm1a, 7.1 ± 1.2; n = 36, paired t test P < 0.0001). Second, sPm1a application significantly decreased the time to fire the first AP (Fig. 2G) in 89% (24 of 27) of the neurons in which a first latency could be measured in control conditions (during 1 s ramps) (Ctr: 257.1 ± 53.1 ms vs. sPm1a: 195.5 ± 34.8 ms; n = 27, paired t test P = 0.0095). Third, sPm1a had variable effects on the resting membrane potential, with depolarization observed in some neurons as well as either hyperpolarization or no change in others (n = 36, paired t test P = 0.09563) (Fig. 2H), likely reflecting the heterogeneity of the small DRG neurons tested.

From these observations, we infer that the function of δs-TRTX-Pm1a within P. muticus venom involves sensitization of afferent neurons to maximize nociceptive signal generation upon the mechanical and chemical insults caused by bite puncture wounds and venom injection.

sPm1a-Enhanced Firing Involves Neuronal TTX-R NaV and Delayed Rectifier Kv Conductances. AP firing in sensory neurons is largely determined by the voltage-gated sodium conductance carried by TTX-S and TTX-R NaV channels. Small DRG neurons bathed in TTX at a concentration sufficient to block TTX-S NaV channels were exposed to sPm1a. The firing responses of a small DRG neuron to ramp current injection (0 to 250 pA) bathed in TTX (0.5 μM) (Fig. 3A, Left, dark gray), combined TTX and sPm1a (1 μM) (Fig. 3A, Center, red) and after removal of sPm1a (Fig. 3A, Right, light gray) are shown in Fig. 3A. The additive exposure of sPm1a (Fig. 3A, red) decreased latency to discharge, increased discharge activity, and impaired repolarization (right-shift in dV/dt local minima) (Fig. 3A, Inset TTX; gray, sPm1a/TTX; red). Removal of sPm1a from the bath was washed out over 6 min with continuous superfusion (Fig. 2H), likely reflecting the heterogeneity of the small DRG neurons tested. When assessed under voltage clamp, whole-cell NaV currents from small sensory neurons in the presence of TTX (0.5 μM) were augmented significantly by sPm1a as can be observed from the recordings shown in Fig. 3E (stimulus: 25 ms, −10 mV, Vh −80 mV, 0.1 Hz). The dark gray current traces in Fig. 3E correspond to the total TTX-R component of the DRG neuron NaV current. In the presence of TTX (0.5 μM), exposure to sPm1a (1 μM) resulted in a large increase in inward current (Fig. 3E, red) that was reversed by removing sPm1a from the bath containing TTX. This can be readily seen in the diary plot of the accumulated charge during the stimulus estimated by integrating the area under the current trace (Fig. 3E, Right).

In small DRG neurons, application of sPm1a (1 μM) (Fig. 3E, red) caused an average 6.1 ± 2.8-fold increase in charge mediated by TTX-R NaV channels (n = 6). Removal of sPm1a from the bath solution allowed TTX-R NaV currents to return to control magnitude whereas application of A803467 (1 μM) reduced the sPm1a-sensitive TTX-R NaV component of small mouse DRG neurons by 87.3 ± 11.1% (n = 5)

The suppression of sPm1a-enhanced firing and TTX-R NaV current reduction by the Nav1.8-selective inhibitor A803467 suggests that the increase in AP firing is largely driven by enhancement of Nav1.8-mediated NaV currents. However, TTX-R NaV1.9 channels are also expressed in DRG neurons. The use of a pulse protocol favoring Nav1.9 activation did not reveal a substantial contribution of this isoform in the population analyzed here (SI Appendix, Fig. S4), hampering our ability to assess the effects of sPm1a on Nav1.9 mediated currents.

The first derivative of the voltage response to current injection in small DRG neurons exposed to sPm1a was typically right-shifted in the time axis indicating impairment in AP repolarization ( Insets in Figs. 2D and E and 3A and C). Pharmacological inhibition of TTX-R currents suppressed AP firing but the membrane potential remained depolarized in about a third of the small DRG neurons recorded (Fig. 2H, solid circles joined by solid lines; i.e., arrow in Fig. 3C). Therefore, we assessed whether the Kv conductance of mouse small DRG neurons was modulated by sPm1a. Fig. 3F portrays a representative family of outward Kv current traces obtained from a standard current–voltage (I–V) protocol in control (Fig. 3F, black, Kv total) and after the addition of 1 μM sPm1a, thereby observing a sPm1a-resistant Kv current (sPm1a-R) (Fig. 3F, red, dotted traces). Point-to-point subtraction revealed the sPm1a-sensitive (sPm1a-S) Kv component as a slow-activating outward current (Fig. 3F, red), with voltage dependence of activation (V0.5: 5.8 ± 2.2 mV, n = 9) (SI Appendix, Fig. S5A) similar to that of recombinant Kv2.1 channels (V0.5: 6.0 ± 0.8 mV, n = 7) (Fig. 4). We also used a double-pulse protocol in which 5-s duration depolarizing prepulses (from −90 to +40 mV) maximally inactivated A-type small DRG Kv channels to better observe the delayed rectifier (KDR) component of neuronal Kv (SI Appendix, Fig. S5B). Application of sPm1a (1 μM) under these conditions caused a significant 30 to 40% reduction of KDR at all prepulse potentials tested (n = 5; Ctr vs. sPm1a at each prepulse potential paired t test P < 0.05) (Fig. 3F and SI Appendix, Fig. S5).

sPm1a Enhances Nav1.8 and Inhibits Kv2.1 Channels. In nociceptors, Nav1.8 and the KDR are the dominating conductances of the AP (20). Kv2.1 homo- and heteromeric channels mediate the majority of the delayed rectifier Kv current in small DRG neurons (21), and because of their slow activation and inactivation kinetics the activity of Kv2 channels is expected to contribute significantly to neuronal repolarization/after-hyperpolarization (22). We performed double immunostaining for Nav1.8 and Kv2.1 channels in our primary cultures of dissociated small DRG neurons from adult mice. Fig. 4A shows confocal images of adult mouse DRG neurons in which Nav1.8 (Fig. 4A, red) and Kv2.1 (Fig. 4A, green) are abundant and recognized within the same neurons as
sPm1a-enhanced firing is reversible and involves neuronal TTX-R NaV, and delayed rectifier Kv conductances. (A) Current-clamp recordings of a small DRG neuron stimulated with a ramp current injection (Right Inset) in the presence of TTX (0.5 μM; Left, dark gray), in a combination of TTX and sPm1a (1 μM; Center, red) and after washout of sPm1a (wo, Right, light gray). Center Inset: dV/dt of first AP before (dark gray) and after exposure to sPm1a (red, respectively). (B) Paired number of APs from neurons in (black circle: TTX), in sPm1a (red circle: TTX+sPm1a), and after washout (gray circle: wo). One-way ANOVA with Tukey’s multiple comparisons test: **P < 0.01. (C) Firing responses of an adapting small DRG neuron. Control (left; black); exposure to sPm1a (0.3 μM; Center, green) and complete inhibition of firing upon exposure to A803467 (1 μM; Right, blue). Depolarized membrane potential at the end of the ramp in the A803467-inhibited condition is marked by the red arrow. Center Inset: dV/dt plot of first AP in absence and presence sPm1a (black and green, respectively). Right Inset: stimulation protocol. (D) Paired number of APs from neurons in control (black circle), in sPm1a (1 μM, red circle), and after addition of A803467 (1 μM, blue circle). One-way ANOVA with Tukey’s multiple comparisons test: **P < 0.01; *P ≤ 0.001. (E) Left) Voltage-clamp recording of a small DRG neuron TTX-R NaV* current elicited by a depolarizing pulse (Inset, 25 ms, –10 mV, Vh = –80 mV) in the presence of TTX (0.5 μM, dark gray). Exposure to sPm1a (1 μM, red) and A803467 (1 μM, blue). (Right) Diary plot showing reversible TTX-R enhancement by sPm1a and complete inhibition of the sPm1a-sensitive TTX-R component by A803467. (F, Left) DRG Kv currents in the absence (black, Kv tot) and presence of 1 μM sPm1a (gray dotted, sPm1a-R). sPm1a-sensitive Kv currents are evidenced by point-to-point subtraction (red, sPm1a-S). (Right) Small DRG neuron hNav measured after 5-s prepulses to progressively depolarized potentials (SI Appendix, Fig. S5B) in the absence and presence of 1 μM sPm1a (n = 5; paired t test for control vs. sPm1a at each prepulse potential, *P < 0.05).

sPm1a action on human NaV1.8-mediated Na+ currents in HEK293 cells mirrored its effect on mouse small DRG neurons (Fig. 3E), with NaV1.8 currents enhanced by sPm1a in a concentration-dependent manner (Fig. 3B). Peak current amplitude, like other macroscopic current metrics, is a complex variable reflecting net changes in gating and conductance, therefore we tracked concentration-dependent effects of sPm1a by measuring the absolute charge (area under the curve) flowing during the stimulation pulse (25 ms, –10 mV, Vh = –80 mV) at different concentrations of sPm1a. The concentration–response relationship shown in Fig. 4C was normalized to the absolute charge increase registered upon exposure to 10 μM sPm1a, giving an estimated EC50 of 1.1 ± 0.1 μM (nH1 = 1.5 ± 0.1, n = 3 to 9 per concentration) for enhancement of hNav1.8 currents.

The primary and tertiary structure of sPm1a resembles those of venom-derived GMPs. In order to evaluate the effect of sPm1a on recombinant NaV1.8 channels, we compared Na+ currents in the absence and presence of 1 μM sPm1a (Fig. 4D, black and red, respectively) that were elicited by 100 ms depolarizing pulses from –80 mV to 50 mV in 5-mV steps, from a holding potential of –80 mV. Moreover, a significant decrease in the current–voltage relationship (SII, V0.5 = –38.7 ± 1.3 mV, n = 8; vs. –29.9 ± 1.3 mV, n = 8; P = 0.0003) (Fig. 4E, closed symbols) and delayed open-state inactivation kinetics (τinact at –10 mV: Ctr: 7.0 ± 1.0 ms, n = 8; sPm1a: 26.2 ± 3.6 ms, n = 8; P = 0.0002), resulting in a 16.7 ± 5.4% (n = 16) increase in the persistent currents recorded at the end of a 100 ms pulse to –10 mV (Fig. 4D and E). An ~8 mV hyperpolarizing shift in the voltage dependence of activation (V0.5 = –6.2 ± 1.1 mV, n = 7; vs. –14.5 ± 0.8 mV, n = 16; P < 0.0001) (Fig. 4E, open symbols) and a delay in activation kinetics (τact at –10 mV: Ctr: 0.5 ± 0.3 ms, n = 5; sPm1a: 1.6 ± 0.1 ms, n = 5; P = 0.0083) were detected in

Fig. 3. sPm1a-enhanced firing is reversible and involves neuronal TTX-R NaV, and delayed rectifier Kv conductances. (A) Current-clamp recordings of a small DRG neuron stimulated with a ramp current injection (Right Inset) in the presence of TTX (0.5 μM; Left, dark gray), in a combination of TTX and sPm1a (1 μM; Center, red) and after washout of sPm1a (wo, Right, light gray). Center Inset: dV/dt of first AP before (dark gray) and after exposure to sPm1a (red, respectively). (B) Paired number of APs from neurons in (black circle: TTX), in sPm1a (red circle: TTX+sPm1a), and after washout (gray circle: wo). One-way ANOVA with Tukey’s multiple comparisons test: **P < 0.01. (C) Firing responses of an adapting small DRG neuron. Control (left; black); exposure to sPm1a (0.3 μM; Center, green) and complete inhibition of firing upon exposure to A803467 (1 μM; Right, blue). Depolarized membrane potential at the end of the ramp in the A803467-inhibited condition is marked by the red arrow. Center Inset: dV/dt plot of first AP in absence and presence sPm1a (black and green, respectively). Right Inset: stimulation protocol. (D) Paired number of APs from neurons in control (black circle), in sPm1a (1 μM, red circle), and after addition of A803467 (1 μM, blue circle). One-way ANOVA with Tukey’s multiple comparisons test: **P < 0.01; *P ≤ 0.001. (E) Left) Voltage-clamp recording of a small DRG neuron TTX-R NaV* current elicited by a depolarizing pulse (Inset, 25 ms, –10 mV, Vh = –80 mV) in the presence of TTX (0.5 μM, dark gray). Exposure to sPm1a (1 μM, red) and A803467 (1 μM, blue). (Right) Diary plot showing reversible TTX-R enhancement by sPm1a and complete inhibition of the sPm1a-sensitive TTX-R component by A803467. (F, Left) DRG Kv currents in the absence (black, Kv tot) and presence of 1 μM sPm1a (gray dotted, sPm1a-R). sPm1a-sensitive Kv currents are evidenced by point-to-point subtraction (red, sPm1a-S). (Right) Small DRG neuron hNav measured after 5-s prepulses to progressively depolarized potentials (SI Appendix, Fig. S5B) in the absence and presence of 1 μM sPm1a (n = 5; paired t test for control vs. sPm1a at each prepulse potential, *P < 0.05).

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the presence of sPm1a. Hence, our data provide compelling evidence that sPm1a is a gating-modifier peptide that strongly potentiates rodent and human NaV1.8-mediated currents.

The similarities in current kinetics, voltage dependence of activation, strong Kv2.1 immunoreactivity in mouse DRG neuron primary cultures and its frequent targeting by spider NaV GMPs (23), prompted us to investigate the effects of sPm1a on human Kv2.1 heterologously expressed in mammalian cells using automated patch-clamp electrophysiology. We found Kv2.1 channels to be sensitive to sPm1a (Fig. 4F), with an IC50 of 0.4 ± 0.01 μM (nH = 1.4, n = 7) (Fig. 4G). Similar to other spider-venom peptides like hanatoxin, sPm1a caused a ∼27-mV depolarizing shift in the voltage dependence of Kv2.1 activation (V0.5: 32.9 ± 0.4 mV, n = 7; P = 0.0001) without significant changes in SSI (Ctr V0.5: -24.6 ± 3.5 mV, n = 5, vs. sPm1a V0.5: -18.2 ± 4.9 mV, n = 5; unpaired t test P = 0.3189) (Fig. 4H). Functional enhancement of repolarizing Kv currents depresses neuronal excitability in primary sensory neurons; thus, inhibition of KDR by a large shift in the activation voltage as verified for Kv2.1 channels suggests that sPm1a may also enhance excitability.

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by altering the neuronal refractory period. These results are consistent with a role for sPm1α inhibition of KDR currents in neuronal sensitization and hyperexcitability leading to pain perception upon *P. muticus* envenomation.

**sPm1α Modulates Na\(_V\) Channels, But Not Nicotinic Acetylcholine Receptors.** Gating modifier spider-venom peptides often target multiple Na\(_V\) channels. Automated patch-clamp screening of human 
Na\(_V\)1.1–Na\(_V\)1.7 isoforms revealed that sPm1α nonselectively inhibits peak currents mediated by all these channels with IC\(_{50}\) values of 0.38 to 2.3 μM (*SI Appendix*, Table S2). In the presence of 1 μM sPm1α, depolarization-triggered fast inactivation was substantially delayed three- to fourfold for currents elicited by the TTX-S channels Na\(_V\)1.1, Na\(_V\)1.3, Na\(_V\)1.6, and Na\(_V\)1.7 (τ\(_{sPm1α}/\tau_{CTr} = 3.5\) to 4.5) (*SI Appendix*, Table S3), which are abundantly expressed in peripheral neurons. In contrast, the inactivation kinetics of Na\(_V\)1.2 (brain), Na\(_V\)1.4 (skeletal muscle), and Na\(_V\)1.5 (cardiac muscle) were not significantly modified (τ\(_{sPm1α}/\tau_{CTr} \sim 1\)) (*SI Appendix*, Table S3).

Subexcitability is commonly attributed to inactivation of Na\(_V\) channels, and in small DRG neurons the TTX-S channel Na\(_V\)1.7 is important in setting the threshold of AP firing, ultimately regulating neuronal excitability (24–26). A closer examination of Na\(_V\)1.7 modification by sPm1α (1 μM) by manual patch-clamp of HEK293 cells revealed that this peptide slows Na\(_V\)1.7 fast inactivation by approximately threefold (τ\(_{\text{fast}}\) CTr: 0.8 ± 0.2 ms, n = 6; vs. sPm1α: 2.7 ± 0.4 ms, n = 6; unpaired t test P = 0.0017) and shifts the voltage dependence of activation to more positive potentials (V\(_{1/2}\) CTr: −25.4 ± 1.8 mV, n = 5; vs. sPm1α: −10.6 ± 1.7 mV, n = 6; P = 0.0002) without evident changes to SSI (SSI V\(_{1/2}\) CTr: −75.5 ± 0.5 mV, n = 5; vs. sPm1α: −76.5 ± 0.4 mV, n = 7; P = 0.1458) (*SI Appendix*, Fig. S7).

We also examined whether the effects induced by *P. muticus* bites may be due in part to modulation of the nicotinic acetylcholine receptors (nACHRs) at the neuromuscular junction. We used two-electrode voltage-clamp electrophysiology to examine the effect of sPm1α on human (h) neuronal and rodent (r) muscle nACHR subtypes expressed in *Xenopus laevis* oocytes. The amplitude of acetylcholine-evoked currents mediated by hα3β2, hα4β2, hα7, rat1β1γ2, and rat1β1δ6 nACHRs were unchanged in the presence of 1 μM sPm1α (*SI Appendix*, Table S4), suggesting that cholinergic neurotransmission is not directly affected by sPm1α.

sPm1α modulation of TTX-S Na\(_V\) channels in nociceptive neurons, such as Na\(_V\)1.7, may contribute to the pain-sensitization evoked by sPm1α. However, the multimodal effects of sPm1α and compositional variability and amplitude of the TTX-S component in DRG neurons challenge our ability to rigorously elucidate the contribution of this conductance to modified excitability within the scope of this study. We have therefore attempted to address this question from a theoretical standpoint in the following section.

**Modeling sPm1α-Induced Hyperexcitability of Small DRG Neurons.** sPm1α modulates TTX-R Na\(_V\)1.8, delayed rectifier K\(_{\text{r}}\) currents, and TTX-S Na\(_V\) channels in small DRG neurons and their recombinant molecular counterparts in heterologous expression systems. The overall effects of sPm1α on a variety of small DRG neurons are consistent with sensitization through facilitation of repetitive firing, lower AP thresholds, and decreased firing latency. We, therefore, employed a mathematical model of nociceptor excitability to “dissect” the contribution of selected sPm1α-sensitive conductances in isolation and their concerted actions.

Hodgkin–Huxley formulations of Na\(_V\)1.8 and TTX-S Na\(_V\) channels, based on those published by Verma et al. (27), were implemented in the Brain-Dynamics Toolbox (28) and Na\(_V\)1.8 currents were simulated in response to standard voltage-clamp protocols. Model parameters describing voltage dependence and rates of channel activation and inactivation (details in *SI Appendix*, Tables S5 and S6) (29) were adjusted to recapitulate Na\(_V\)1.8 behavior both at baseline and in the presence of sPm1α (Fig. 5A). This model of the Na\(_V\)1.8/sPm1α molecular interaction was then incorporated into a single-compartment model of small DRG neuron soma (26) to predict emergent effects on neuronal electrophysiology. In addition to modification of Na\(_V\)1.8 by sPm1α, the effects on KDR were modeled as a 30% decrease in activation and inactivation without TTX-S's kinetic parameters as indicated by our experimental data (Fig. 3D). The TTX-S Na\(_V\) voltage dependence and rate parameters of activation and inactivation in control and in the presence of sPm1α are provided in *SI Appendix*, Table S7. Simulated effects on neuronal excitability of individual molecular targets of sPm1α (Na\(_V\)1.8/TTX-S/KDR) are presented in Fig. 5 and *SI Appendix*, Fig. S8. Under control conditions, current injection generates a single AP (Fig. 5 B, Top), consistent with our data from mouse DRG neurons. Inclusion of sPm1α-modified Na\(_V\)1.8 (in the absence of any sPm1α-dependent changes in KDR or TTX-S) increased the number of APs fired in the modeled neuron (Fig. 5B, red dotted trace, 2 APs), while inclusion of either reduced KDR conductance or altered TTX-S Na\(_V\) gating/conductance alone did not cause an increase in firing (Fig. 5 B, Bottom; dotted orange and blue traces, respectively, and Fig. 5 D, Left) and likely counteract each other (Fig. 5 D, Center, first latency for KDR and TTX-S). However, inclusion of the effects of all sPm1α targets (Na\(_V\)1.8, KDR, TTX-S) in the simulated neuron resulted in substantially enhanced excitability (Fig. 5 C and D), consistent with a typical mouse DRG neuron stimulated with an equivalent current injection (Fig. 5E), while the simulated number of APs and latency (Fig. 5 D and E) qualitatively reproduced our in vitro data (Fig. 2 F and G). Thus, the modifications that we implemented to the Mandge and Manchanda model (30) closely reflect the effects of sPm1α on small DRG neurons reported here.

A key motivation for computationally modeling biological observations is that it allows the study of variables in isolation that would otherwise not be possible, as it is the case of promiscuous (Na\(_V\)1.8, KDR, Na\(_V\) TTX-S, and so forth) and multimodally (gating and kinetics) active peptides like sPm1α. Of particular interest is how modification of TTX-Na\(_V\) conductance (TTX-S\(^{\text{Pm1α}}\)) and kinetics (TTX-S\(^{\text{KDR}}\)) contribute to the effects of sPm1α on neuronal excitability. Modeled traces resulting from independent incorporation of changes to TTX-S\(^{\text{KDR}}\) and TTX-S\(^{\text{Pm1α}}\) into Na\(_V\)1.8/KDR models (*SI Appendix*, Fig. S8) show that neither of these parameters in isolation (or combined) affects firing responses at the beginning of the current stimulus (*SI Appendix*, Fig. S8). For the neuron in our reductionist model, inhibition of the TTX-S conductance progressively decreases the time between AP bursts which, in combination with an enhancement of Na\(_V\)1.8 and impairment of KDR, decreases the time between burst for later time points (*SI Appendix*, Fig. S8). Notwithstanding, our model suggests that a sPm1α-like delay in TTX-S Na\(_V\) inhibition kinetics (TTX-S\(^{\text{KDR}}\)) appears to dominate over its inhibitory effects on this conductance (*SI Appendix*, Fig. S8 C–E).

It has been shown that TTX-S Na\(_V\)1.7 activation amplifies small depolarizations that bring peripheral neurons within firing threshold, with the Na\(_V\)1.8 conductance contributing the bulk of the inward current underlying the AP upstroke during repetitive firing of pain-sensing neurons (31). Experimentally, sPm1α inhibits peak currents and delays fast inactivation of TTX-S Na\(_V\) channels (SI Appendix, Fig. S7, Tables S2 and S3). Overall, our computational approach lends support to the notion that modification of the Na\(_V\)1.8 current alone increases neuronal excitability, yet it is insufficient to fully recapitulate our experimental observations and therefore the additional inhibition of KDR is required for the enhancement of excitability observed in small DRG neurons. Furthermore, multimodal modification of TTX-S Na\(_V\) channels may also contribute to sPm1α’s actions depending on the complement and relative contribution of TTX-S Na\(_V\) channels present in individual DRG neurons.
Discussion

Spontaneous activity and increased firing frequency of nociceptors are the key sources of pain in the peripheral nervous system. Although many venom-derived peptides promiscuously modulate the activity of ion channels involved in nociception, their combined actions on neuronal excitability have not been thoroughly elucidated (32). In this study, we identified a peptide from *P. muticus* venom, δ/κ-TRTX-Pm1a, chemically synthesized sPm1a, determined its 3D structure, and functionally characterized its actions, in vivo, ex vivo, and heterologous expression systems. Mathematical modeling supported our functional findings whereby sPm1a enhances nociceptor excitability mainly by the simultaneous stimulation of repetitive firing (through TTX-R NaV1.8 current enhancement) and impairment of repolarization (by inhibiting KDR, KV2.1), with a potential contribution from TTX-S NaV modified excitability, leading to manifestations of
pain behavior in mice and their reversal through inhibition of Na\textsubscript{V}1.8 and TTX-S Na\textsubscript{V} channels (Fig. S5).

sPm1a modulates the availability of the quintessentially nociceptive channels Na\textsubscript{V}1.8 and Na\textsubscript{V}1.7, as well as a major contributor to membrane repolarization, KV\textsubscript{2.1}, to produce severe pain. We observed overlapping expression of these Na\textsubscript{V} channels with KV\textsubscript{2.1} in small DRG neurons (Fig. 4 and SI Appendix, Fig. S8), in agreement with previous analyses of unmyelinated C-fiber low-threshold mechanoreceptors, MrG\textsuperscript{d} nonpeptidergic nociceptors, and CGRP\textsuperscript{+} peptidergic nociceptors (16). Na\textsubscript{V} channel activation mediates the positive feedback mechanisms necessary to initiate an AP, and therefore changes in the availability or distribution of these channels can have a profound effect on cell excitation. AP-firing thresholds are primarily determined by Na\textsuperscript{+} current density and the voltage dependence of activation and inactivation but are also fine-tuned by other neuronal conductances, in particular by delayed rectifier KV\textsubscript{V} channels.

The TTX-R conductance in DRG neurons is enhanced through the action of sPm1a on the voltage dependence of Na\textsubscript{V}1.8 activation and inactivation, as well as its current amplitude and kinetics. In parallel, sPm1a induces a depolarizing shift in the voltage dependence of activation of the most abundant low-threshold TTX-S channel in nociceptors, Na\textsubscript{V}1.7, which results in an apparent loss of current availability at hyperpolarized potentials. Substantially delayed TTX-S Na\textsubscript{V} channel inactivation kinetics but also that depolarized potentials is also observed. Inactivation of the TTX-R Na\textsuperscript{+} current is known to limit the duration of small DRG neuron firing in response to maintained stimuli, hence inactivation of Na\textsubscript{V}1.8 is believed to underlie adaptation in capsacin-sensitive small DRG neurons (33), whereas inhibition of inactivation results in sensitization to painful stimuli. Patients suffering from small fiber neuropathies carry gain-of-function mutations in Na\textsubscript{V}1.8 that lower the threshold for channel activation, accelerate recovery from inactivation, impair inactivation (34), and increase resurgent currents (35). In these patients, DRG neuronal hyperexcitability is characterized by lower AP generation thresholds, increased firing rates in response to suprathreshold stimulation, and spontaneous firing, thus providing a pathophysiological basis for the reported allodynia and hyperalgesia. This neurophysiological behavior is in agreement with the observed actions of sPm1a on endogenous Na\textsubscript{V}1.8 and recombinant Na\textsubscript{V}1.8 currents, small DRG neuron sensitization, and pain behavior observed in mice.

Together with modification of Na\textsubscript{V}1.8, the multimodal effects of sPm1a on DRG neuron TTX-S Na\textsubscript{V} isofoms (Na\textsubscript{V}1.7, Na\textsubscript{V}1.1, and Na\textsubscript{V}1.6) can perhaps contribute to the increased excitability of DRG neurons. For example, the delay in inactivation caused by sPm1a mimics gain-of-function mutations of Na\textsubscript{V}1.7 that lead to increased excitability and spontaneous activity in nociceptive neurons in congenital chronic pain patients (36). Nevertheless, in any given nociceptor, the relative contribution of particular TTX-S isofoms and the extent to which their conductance and inactivation kinetics are modulated by sPm1a hinder accurate prediction.

The three main Na\textsubscript{V} isoforms expressed in nociceptive neurons are TTX-R Na\textsubscript{V}1.8 and Na\textsubscript{V}1.9 and TTX-S Na\textsubscript{V}1.7 (36). The major Na\textsuperscript{+} current during an AP in small DRG somata is mediated by TTX-R Na\textsubscript{V}1.8 and Na\textsubscript{V}1.9 channels (37). The pulse protocol used in this study to assess small DRG neuron Na\textsuperscript{+} currents (Fig. 4 and SI Appendix, Table S4) failed to identify consistent modulatory effects of sPm1a. However, given the vast array of molecular players involved in neuronal excitability, other sPm1a targets may be identified in future studies.

Pain is a complex process that involves the detection of noxious stimuli in the peripheral nervous system and processing of the nociceptive input in the central nervous system (48). Spontaneous activity in nociceptors has been described for patients with painful polyneuropathy (49). Mechanically insensitive or silent nociceptors comprise ∼30% of all C-fibers in vertebrates, with activation thresholds that are typically very high and thus are thought not to contribute to acute pain signaling (50). However, inflammation and nerve injury may lower AP-firing thresholds, thereby rendering silent nociceptors active, which might be important in mechanical hyperalgesia and central sensitization (51). Increased Na\textsuperscript{+} influx per AP via TTX-R Na\textsubscript{V}1.8 is proposed to underlie the characteristically pronounced activity-dependent slowing of conduction in mechano-insensitive, "silent" nociceptors (52). The effects of sPm1a on each nociceptive neuron would rely predominantly on the relative expression of Na\textsubscript{V}1.8 and the abundance of (sensitive) delayed rectifier K\textsubscript{V} channels, but also on other targets such as TTX-S Na\textsubscript{V} channels, including Na\textsubscript{V}1.7. High levels of Na\textsubscript{V}1.8 expression have been reported in mechano-insensitive CHRNA3\textsuperscript{+} murine nociceptors and in functionally silent porcine nociceptors (53). Unsilencing by exposure to δ\textgreek{c}-TRTX-Pm1a/sPm1a upon envenomation would recruit this population of C-fibers to contribute to hyperalgesia. We propose that the lowered AP threshold and
decreased repolarization in small DRG neurons observed in the presence of sPm1a enhance neuronal excitability and is likely a key contributor to the hyperalgesic symptoms reported after *P. mutilus* envenomations.

Pain caused by bites from Australian funnel-web spiders (genera *Atrax*, *Hadronyche*, and *Illawarra*) and Brazilian armed spiders (genus *Phoneutria*) is due, at least in part, to enhancement of neuronal NaV channel activity by δ-hexatoxins and δ-phonetoxins, respectively (4, 54). Treatments for envenomation by these spiders typically involve antivenom (where available), muscle relaxants, narcotics, analgesics, and intravenous calcium (54). We showed that coadministration of A803467 (inhibiting NaV1.1, 1.8), or TTX (blocking NaV1.1-NaV1.4, NaV1.6, and NaV1.7), reduces sPm1a-evoked nocifensive behavior in mice, while inhibition of NaV1.8 reversed sPm1a-induced hyperexcitability of DRG neurons. Hence, our results are consistent with the use of currently approved and widely available local anesthetics to relieve pain elicited by *P. mutilus* envenomations.

Landmark structure–function studies have shed light on the molecular mechanism of actions of prototypical gating-modifier spider-venom peptides (55, 56). Amphipathic tarantula toxins partially partition into the membrane and interact with extracellular protein–lipid interfaces of voltage-sensor domains binding to the paddle-like S3-S4 linkers and S4 helices, and hence are also called voltage-sensor toxins (12, 46, 55–58). Pm1a’s sequence, structure, and functional modification of NaV and Kβ channel gating are consistent with the action of voltage-sensor toxins. Future research aimed at uncovering the molecular details of the interaction between Pm1a and the channel targets identified herein is warranted as it may contribute important insights into the molecular mechanism of action of GMPs.

In summary, we show here that a single venom peptide can modulate three major determinants of neuronal excitability. Target promiscuity, defined as the ability to modulate the function of more than one type of receptor, is a common feature of spider toxins (23, 59). Many gating-modifier toxins are highly promiscuous and target a wide range of ion channels and receptors (23, 59). For example, Protoxin-I (βδ/TTX-Tp1a) from venom of the tarantula *Thrixopelma piruviens* targets a wide range of NaV channels, Cav-3.1, and TRPA1 (transient receptor potential mucolysin 1) (60). Hm1a was originally identified as a Kβ channel inhibitor (61) but later recognized to elicit robust pain behaviors in mice and hypersensitivity to mechanical stimuli via potent enhancement of NaV1.1 mediated currents in colonic afferents (7). Hence, Hm1a is another example of a toxin with cross-target promiscuity, ultimately resulting in the generation of pain such as we describe here for Pm1a.

Recognition of common structures in different receptors has been proposed to contribute to target promiscuity (46, 56, 62). Trading selectivity for functionality through the deployment of a single allogenic peptide-like δ/TTX-Pm1a to simultaneously modulate multiple receptors involved in AP generation in sensory neurons is an economic and effective tactic to evoke pain in predators as a defense strategy. Targeting a physiological function (excitability) rather than a specific molecular target (i.e., membrane protein) may represent an evolutionary adaptation of pain-producing defensive venoms. By extension, depressing neuronal excitability by targeting complementary players with “multimodal analgesics” may provide novel therapeutic alternatives for the treatment of chronic pain.

**Materials and Methods**

Animal experiments were conducted in accordance with the Australian National Health and Medical Research Council Code for the Care and Use of Animals for Scientific Purposes (63) and were approved by the local University of Queensland Animal Ethics Committee. Details of the *P. mutilus* venom biochemistry and bioinformatics analysis, δ/TTX-Pm1a purification, sPm1a synthesis, NMR spectroscopy, in vivo and in vitro functional characterization of sPm1a, and all materials used in this study are listed in detail in SI Appendix, Materials and Methods.

**Data Availability.** Atomic coordinates for the structure of sPm1a have been deposited in the Protein Data Bank (ID code 7JPM) (64). The chemical shifts of sPm1a were deposited in the BioMagResBank (accession code 30785) (65). Source code is deposited in Zenodo: https://doi.org/10.5281/zenodo.5009443 (29). All other study data are included in the article and/or SI Appendix.

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