Discovery of a selective Na\textsubscript{v}1.7 inhibitor from centipede venom with analgesic efficacy exceeding morphine in rodent pain models

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Loss-of-function mutations in the human voltage-gated sodium channel Na\textsubscript{v}1.7 result in a congenital indifference to pain. Selective inhibitors of Na\textsubscript{v}1.7 are therefore likely to be powerful analgesics for treating a broad range of pain conditions. Herein we describe the identification of \textmu-SLPTX-Ssm6a, a unique 46-residue peptide from centipede venom that potently inhibits Na\textsubscript{v}1.7 with an IC\textsubscript{50} of \(-25\) nM. \textmu-SLPTX-Ssm6a has more than 150-fold selectivity for Na\textsubscript{v}1.7 over all other human Na\textsubscript{v} subtypes, with the exception of Na\textsubscript{v}1.2, for which the selectivity is 32-fold. \textmu-SLPTX-Ssm6a contains three disulfide bonds with a unique connectivity pattern, and it has no significant sequence homology with any previously characterized peptide or protein. \textmu-SLPTX-Ssm6a proved to be a more potent analgesic than morphine in a rodent model of chemical-induced pain, and it was equipotent with morphine in rodent models of thermal and acid-induced pain. This study establishes \textmu-SLPTX-Ssm6a as a promising lead molecule for the development of novel analgesics targeting Na\textsubscript{v}1.7, which might be suitable for treating a wide range of human pain pathologies.

Normal pain is a key adaptive response that serves to limit our exposure to potentially damaging or life-threatening events. In contrast, aberrant long-lasting pain transforms this adaptive response into a debilitating and often poorly managed disease. Chronic pain affects \(-20\%\) of the population, with the incidence rising significantly in elderly cohorts (1). The economic burden of chronic pain in the United States was recently estimated to be \(-$600\) billion per annum, which exceeds the combined annual cost of cancer, heart disease, and diabetes (2). There are few drugs available for treatment of chronic pain, and many of these have limited efficacy and dose-limiting side-effects.

Voltage-gated sodium (Na\textsubscript{v}) channels are integral transmembrane proteins that provide a current pathway for the rapid depolarization of excitable cells (1, 3), and they play a key role in conveying nociceptor responses to synapses in the dorsal horn (4). Humans contain nine different Na\textsubscript{v} channel subtypes, denoted Na\textsubscript{v}1.1 to Na\textsubscript{v}1.9 (5, 6). In recent years, Na\textsubscript{v}1.7 has emerged as a promising analgesic target based on several remarkable human genetic studies. Gain-of-function mutations in the SCN9A gene encoding the pore-forming \(\alpha\)-subunit of Na\textsubscript{v}1.7 cause severe episodic pain in inherited neuropathies, such as erythromelalgia and paroxysmal extreme pain disorder (7), whereas loss-of-function mutations in SCN9A result in a congenital indifference to pain (CIP) (8). The latter phenotype can be recapitulated in rodents via complete knockout of Na\textsubscript{v}1.7 in all sensory and sympathetic neurons (9). Moreover, certain polymorphisms in SCN9A correlate with sensitivity to nociceptive inputs (10). Remarkably, apart from their inability to sense pain, loss of smell (anosmia) is the only other sensory impairment in individuals with CIP (11, 12). Thus, the combined genetic data suggest that subtype-selective blockers of Na\textsubscript{v}1.7 are likely to be useful analgesics for treating a broad range of pain conditions.

Centipedes are one of the oldest extant arthropods, with the fossil record dating back 430 million yr (13). Centipedes were one of the first terrestrial taxa to use venom as a predation strategy, and they have adapted to capture a wide variety of prey, including insects, fish, molluscs, amphibians, reptiles, and even mammals (13, 14). The centipede venom apparatus, which is unique and bears little resemblance to that of other arthropods, evolved by modification of the first pair of walking legs into a set of pincer-like claws (forcipules) (13). Venom is secreted via a pore located near the tip of each forcipule. There are \(-3,300\) extant species of centipedes, yet the venom of only a handful has been studied in any detail. We recently demonstrated that the venom of the Chinese red-headed centipede Scolopendra subspinipes mutilans is replete with unique, disulfide-rich peptides that potently modulate the activity of mammalian voltage-gated ion channels (14), and therefore we decided to explore this venom as a potential source of Na\textsubscript{v}1.7 inhibitors. We describe the purification from this venom of a highly selective inhibitor of Na\textsubscript{v}1.7 that is more effective than morphine in rodent pain models.

Results

Purification of \textmu-SLPTX-Ssm6a. A unique peptide denoted \textmu-SLPTX-Ssm6a (hereafter Ssm6a) was purified from venom of the centipede S. subspinipes mutilans using a combination of Sephadex G-50 gel-
Cells were held at −80 mV for over 5 min to allow adequate equilibration, then current traces were evoked using a 50-ms step depolarization to −10 mV every second. TTX (100 nM) was added to the bathing solution to separate TTX-resistant (TTX-r) currents from other sodium currents in DRG neurons (17). Nav1.1, Nav1.8, and Nav1.9 are TTX-r, whereas all other subtypes are TTX-sensitive (TTX-s). TTX-s currents were completely inhibited by 1 μM Ssm6a, whereas 10 μM toxin had no effect on TTX-r currents (Fig. 2A). Inhibition of TTX-s currents was dose-dependent with an IC_{50} of 23 nM (Fig. 2B). The action of Ssm6a on TTX-s currents was fast, and toxin dissociation was rapid after washing with extracellular solution. Time constants governing block (τ_{on}) and unblock (τ_{off}) following exposure to 1 μM Ssm6a were 8.0 s and 10.2 s, respectively.

In the presence of 20 nM Ssm6a, the current–voltage relationship for TTX-s Na_v channel currents was shifted ~15 mV in a depolarizing direction (Fig. 2D), and consequently the channel conductance–voltage relationship was positively shifted by ~18 mV (Fig. 2E). In contrast, Ssm6a did not induce a shift in steady-state inactivation of TTX-s Na_v channel currents in DRG neurons (Fig. 2F). At a saturating concentration of Ssm6a (1 μM), the inhibition of TTX-s currents in rat DRG neurons and HEK293 cells expressing human Na_v 1.7 was partly overcome by depolarizations to large positive test potentials (>60 mV) (Fig. S4). This partial reversal of channel inhibition, along with the depolarizing shift in the voltage dependence of activation, is characteristic of gating modifiers that interact with the voltage-sensing domains of Na_v channels (18). In contrast, as expected, inhibition of Na_v 1.7 currents by the pore-blocker TTX was largely voltage-independent (Fig. S4).

**Selectivity of Ssm6a for Na_v Channel Subtypes.** We examined the effect of Ssm6a on human (h) Na_v channel subtypes 1.1–1.8 expressed in HEK293 cells. Currents were elicited by a 20-ms filtration chromatography and reverse-phase (RP) HPLC (Fig. L4). The primary structure of Ssm6a was determined via Edman degradation in combination with analysis of a venom-gland transcriptome (Fig. 1B). The 46-residue mature toxin is produced by posttranslational processing of a 111-residue prepropeptide (Fig. S1). The sequence of transcript encoding Ssm6a. The signal peptide is shown in gray, the pro-peptide region is underlined, and the mature peptide is shown with white text on a black background. The 3′-UTR including the poly(A) tail is also shown. (C) Comparison of the primary structure of Ssm6a with other venom peptides reported to act on Na_v, including proteoin-1 (27), ATX-II (39), and μ-conotoxin KIIIA (40).

**Effect of μ-SLPTX-Ssm1a on Na_v Channels.** Because Na_v 1.7 is preferentially expressed in dorsal root ganglia (DRG) and sympathetic neurons (16), we investigated the ability of Ssm6a to block Nav1.7 channel currents in adult rat DRG neurons using whole-cell patch-clamp electrophysiology. Although Nav1.7 is the most highly expressed Nav channel in DRG neurons, they also express other subtypes, including Nav1.8 and Nav1.9.

![Fig. 1. Purification of Ssm6a from venom of the centipede S. subspinipes mutilans. (A) Lyophilized venom (2.0 mg) was dissolved in 0.1 M phosphate, pH 6.0 then fractionated on a C_18 RP-HPLC column (Left). Elution was performed at a flow rate of 1.5 mL/min using a gradient of acetonitrile in 0.1% trifluoroacetic acid. The peak indicated by an arrow was purified further by analytical C_18 RP-HPLC (Right). (Inset) A photo by Yasunori Koide of S. subspinipes mutilans. (B) Sequence of transcript encoding Ssm6a. The signal peptide is shown in gray, the pro-peptide region is underlined, and the mature peptide is shown with white text on a black background. The 3′-UTR including the poly(A) tail is also shown. (C) Comparison of the primary structure of Ssm6a with other venom peptides reported to act on Na_v, including proteoin-1 (27), ATX-II (39), and μ-conotoxin KIIIA (40).](image1)

![Fig. 2. Effect of Ssm6a on Na_v channel currents in rat DRG neurons. All current traces were evoked by a 50-ms step depolarization to −10 mV from a holding potential of −80 mV every 5 s. (A) Inhibition of TTX-s Na_v channel currents by 1 μM Ssm6a. (B) Concentration–response curve for block of TTX-s Na_v currents in DRG neurons by Ssm6a (n = 5). (C) Time course for block of TTX-s currents by Ssm6a and reversal of block by washing with external solution. (D) Current–voltage (I–V) relationship for TTX-s currents before and after application of 100 nM Ssm6a. (E) Ssm6a shifts the conductance–voltage relationships to more positive potentials (n = 3). (F) Ssm6a had no effect on the voltage-dependence of steady-state inactivation, which was estimated using a standard double-pulse protocol (n = 5). Data points are expressed as mean ± SE and curves are fits to either the Hill (B, D, E) or Boltzmann (F) equation.](image2)
depolarizing potential of −10 mV from a holding potential of −80 mV every 5 s. At a concentration of 50 nM, Ssm6α potently inhibited hNaV,1.7, decreasing current amplitude by ∼63% (Fig. 3D). The peptide was a much weaker inhibitor of hNaV,1.1, hNaV,1.2, and hNaV,1.6; at 2 μM, Ssm6α depressed hNaV,1.1 currents by 25% (Fig. 3A), whereas 1 μM Ssm6α reduced hNaV,1.2 and hNaV,1.6 currents by 31% (Fig. 3B) and 25% (Fig. 3C), respectively. The calculated IC50 values were 4.1 μM, 813 nM, 15.2 μM, and 25.4 nM for hNaV,1.1, hNaV,1.2, hNaV,1.6, and hNaV,1.7, respectively (Fig. 3E). Ssm6α had no effect on hNaV,1.3, hNaV,1.4, hNaV,1.5, and hNaV,1.8 (Fig. S5).

Similar to the effect of proton-2α on rat DRG neurons, Ssm6α shifted the conductance–voltage relationship in a depolarizing direction (19). Ssm6α shifted the conductance–voltage relationship about +10.7, +12.9, +9.55, and +13.5 mV for hNaV,1.1, hNaV,1.2, hNaV,1.6, and hNaV,1.7, respectively (Fig. 4). Ssm6α did not induce a shift in steady-state inactivation for these NaV channel subtypes (Fig. 4).

Notably, the concentration–response curves for Ssm6α inhibition of TTX-s NaV currents in DRG neurons (Fig. 2B) and hNaV,1.7 currents in HEK293 cells (Fig. 3E) were rather shallow (Hill coefficient ≈0.5), suggesting that the peptide might interact with multiple sites on NaV,1.7 that exhibit negative cooperativity. Such a mechanism of action would not be unprecedented, because it has been demonstrated that several NaV channel toxins derived from arachnid venoms bind to more than one of the voltage-sensor paddles in NaV,1.2 (19).

Effects of Ssm6α on Pain. Because NaV,1.7 plays a key role in nociception in humans, the analgesic effect of Ssm6α was tested in several rodent pain models in which pain was induced by noxious chemicals, acid, or heat.

Intraplantar injection of formalin leads to a biphasic pain response in mice: an early nociceptive response (phase I, 0–5 min) caused by direct stimulation of TRPA1 in a subpopulation of C-fiber nociceptors is followed by a quiescent period that precedes a second phase of nociceptive behavior (phase II, 15–30 min) because of peripheral inflammation together with central sensitization (20). Intraperitoneal injection of Ssm6α drastically decreased both phase I and phase II responses (Fig. 5A). Control mice (saline injection) licked their paws an average of 212 times during phase I. Ssm6α was highly effective at attenuating phase I pain, with the number of paw licks reduced by 14%, 60%, and 92% at peptide concentrations of 1, 10, and 100 nmol/kg, respectively (Fig. 5A). On a molar basis, Ssm6α was significantly more effective than morphine, which reduced the number of paw licks during phase I by 3%, 19%, and 69% at concentrations of 1, 10, and 100 nmol/kg, respectively (Fig. 5A).

Ssm6α was also highly effective at attenuating the second phase of nociceptive behavior following formalin injection, during which control mice licked their paws an average of 694 times. At concentrations of 1, 10, and 100 nmol/kg, Ssm6α decreased the number of paw licks during phase II by 25%, 54%, and 80%, respectively (Fig. 5B). On a molar basis, Ssm6α was significantly more effective than morphine, which reduced the number of paw licks during phase II by 3%, 16%, and 57% at concentrations of 1, 10, and 100 nmol/kg, respectively (Fig. 5B).

Ssm6α and morphine were similarly effective at reducing thermal pain (Fig. 5D). In mice subjected to photothermal heat, tail withdrawal latency was increased from 5 s in the saline-treated control group to 6.3 s, 9.2 s, and 13.4 s in mice treated with 1, 10, and 100 nmol/kg Ssm6α, respectively. Similar increases in paw withdrawal latency were observed for mice treated with the same concentrations of morphine (Fig. 5D).

Side-Effect Profile. At doses up to 10-fold higher than those for which we observed robust analgesic effects, Ssm6α had no evident side effects. At a dose of 1 μmol/kg, Ssm6α had no effect on blood pressure (Fig. S6A), heart rate (Fig. S6B), or motor function (Fig. S6C).

Plasma Stability and Duration of Action of Ssm6α. Ssm6α must be highly stable in vivo because it drastically reduced nociceptive behavior in phase II of the formalin-induced pain model, the onset of which occurred ∼45 min after intraperitoneal injection of the peptide. Consistent with this hypothesis, Ssm6α was found to be extraordinarily stable in isolated human plasma, with no significant degradation over 1 wk, compared with a half-life of 64 min for rat atrial natriuretic peptide under the same conditions (Fig. 6A). However, the analgesic effect of Ssm6α declined monotonically over a period of 4 h in rodent models of acid- and heat-induced pain, as well as phase I of the formalin pain model. Interestingly, at the highest doses tested, Ssm6α still produced robust analgesic effects after 4 h in phase II of the formalin pain model. The major factor determining systemic concentrations of Ssm6α, and thus the time course of its analgesic efficacy, is likely to be the rate at which it is cleared by the liver and kidney (21).

Because of its unique amino acid sequence and disulfide framework, we propose that Ssm6α has a unique 3D structure. Consistent with this hypothesis, the far-UV CD spectrum of Ssm6α (Fig. 6B) revealed that it is predominantly α-helical, in striking contrast to all other venom peptides that target NaV channels whose primary secondary structure elements are two to three β-strands. This unusual 3D-fold not only provides Ssm6α with a high level of resistance to proteases, as evidenced by its stability in human plasma, but it also provides it with unusually high thermal stability. We were unable to obtain complete thermal unfolding curves for Ssm6α in the absence of a chemical denaturant; even in 4 M urea, the peptide was only ~50% unfolded at 90 °C (Fig. 6C). The estimated midpoint (Tm) of the
thromal unfolding transition for Ssm6a in 8 M urea was 70.6 ± 0.1 °C (Fig. 6C).

**Discussion**

Clinical genetic studies have identified hNav1.7 as a critical mediator of pain sensitization (7). Loss-of-function mutations in hNav1.7 cause a congenital indifference to pain with no other sensory impairments except anosmia (8, 22, 23), whereas gain-of-function mutations are associated with painful neuropathies (7, 24). Ablation of Nav1.7 in all mouse sensory neurons abolishes mechanical pain, inflammatory pain, and reflex withdrawal responses to heat, without affecting neuropathic pain. However, ablation of Nav1.7 in both sensory and sympathetic neurons abolishes all pain sensations and recapitulates the pain-free phenotype seen in CIP patients (9). Thus, Nav1.7 appears to be an attractive target for the development of novel analgesics for treating a wide range of pain pathologies.

Development of Nav1.7-based analgesics has proved difficult, as it is essential to avoid off-target effects on closely related NavV channels with critical physiological roles. In particular, it is essential to avoid off-target effects on hNav1.5, which is responsible for the rising phase of the cardiac action potential (25), the muscle-specific hNav1.4, and hNav1.6, the primary NavV channel at nodes of Ranvier (26).

Spider venoms have proved to be a rich source of Nav1.7 channel inhibitors (27), but most spider-venom peptides isolated to date are not sufficiently selective to be therapeutically useful. For example, although pro toxin-II blocks hNav1.7 with extremely high affinity (IC50 ~300 pM), it is also a high-affinity inhibitor of hNav1.2 (IC50 ~41 nM), hNav1.5 (IC50 ~79 nM), and hNav1.6 (IC50 ~26 nM) (27). Because of its lack of selectivity, pro toxin-II proved lethal to rats when administered intravenously at a dose of 1.0 mg/kg or intrathecally at 0.1 mg/kg (28). Although several small-molecule blockers of hNav1.7 have been described, they generally suffer from a similar lack of subtype selectivity (25, 29).

In this study we examined centipede venom as a potential source of selective Nav1.7 inhibitors because we previously demonstrated that these venoms are replete with ion channel modulators (14). We described the purification and functional characterization of a unique peptide (Ssm6a) with potent analgesic properties from venom of the centipede S. subspinipes mutillans. Ssm6a contains 46-residues with three disulfide bonds and it has no significant homology with any previously described protein or peptide. The disulfide linkage pattern (C1–C4, C2–C4, C3–C6) is notably different to that of the inhibitor cystine knot peptides (30).

Ssm6a is the most subtype-selective inhibitor of Nav1.7 reported to date. Ssm6a selectively inhibits hNav1.7 with an IC50 of 25 nM (Fig. 3D and E) by shifting the voltage-dependence of activation to more depolarized potentials (Fig. 4D), but it has no effect on hNav1.3, hNav1.4, hNav1.5, and hNav1.8, and only inhibits hNav1.1, hNav1.2, and hNav1.6 at very high concentrations. Most importantly from a therapeutic perspective, Ssm6a has 600-fold or higher selectivity for Nav1.7 over the key off-target subtypes Nav1.4, Nav1.5, and Nav1.6, and it has no effect on hERG (Kv11.1) at concentrations up to 10 μM (Fig. S7).

Consistent with its potent and highly selective block of Nav1.7, Ssm6a proved to be an effective analgesic in rodent pain models (Fig. 5). On a molar basis, Ssm6a was several-fold more effective than morphine in a rodent model of formalin-induced pain (Fig. 5A and B) and it was equipotent with morphine in its ability to reduce thermal and acid-induced pain (Fig. 5C and D). Ssm6a is highly stable in human plasma, and intraperitoneal administration of the peptide at doses up to 1 μmol/kg produced no adverse effects on blood pressure, heart rate, or motor function. Thus, Ssm6a appears to be an excellent lead molecule for development of analgesics targeted against hNav1.7. However, future experiments should be directed toward examination of the analgesic activity of Ssm6a in more complex rodent pain models that better resemble human pain phenotypes, as well as a more detailed assessment of its selectivity for the nociceptive circuitry.

Finally, we speculate that the biological role of Ssm6a in centipede venom is likely to be block of NavV channels in insect
prey. In contrast to humans, insects express only a single Na\textsubscript{v} channel that is a common target of peptides in the venom of arthropod predators, such as spiders and scorpions (6). Ssm6a blocks hNav.1.7 within seconds of administration (Fig. 2C), and a similarly rapid block of insect Na\textsubscript{v} channels would induce rapid paralysis and ultimately death of insect prey. The species from which Ssm6a was purified belongs to Scolopendromorpha, one of the five extant orders of centipedes (13), and thus it will be interesting from a toxinological perspective to determine whether this unusual class of toxins is unique to this order or represents a more basal recruitment within the centipede phylogenetic tree.

Although it might seem counterintuitive that a venom peptide used for predation could be a useful therapeutic, the significant differences in primary structure and tissue distribution between insect and human ion channels makes this possible. There are now six Food and Drug Administration-approved drugs derived from venom peptides or proteins, with many more in clinical trials or various stages of preclinical development (21). The approved group includes Prialt, a Ca\textsubscript{2+} blocker from the venom of a marine cone snail that is used for treatment of severe intractable pain (21). The present study suggests that centipede venoms, which to date have been largely neglected, might provide a novel source of lead molecules for drug development.

Materials and Methods

Assignment of the Disulfide Bonds. Ssm6a (0.1 mg) was partially reduced in 10 \textmu L of citrate buffer (1 M, pH 3.0) containing 6 M guanidine HCl and 0.05 M TCEP for 10 min at 40 °C. The partially reduced sample was fractionated via C\textsubscript{4} FP-HPLC using a linear acetonitrile gradient (0–60% over 40 min). Intermediates with free thiols (as determined using MALDI-TOF mass spectrometry) were lyophilized and alkylated with iodoacetamide (0.5 M, pH 8.3). Alkylated peptides were desalted using C\textsubscript{18} RP-HPLC then subjected to Edman degradation on a Shimadzu protein sequencer (PPSQ-31A; Shimadzu).

Patch-Clamp Recording on Rat DRG Neurons. Rat DRG neurons were acutely dissociated and maintained in short-term primary culture, as previously described (31). Ca\textsuperscript{2+}, Na\textsuperscript{+}, and K\textsuperscript{+} currents were recorded using the whole-cell patch-clamp technique with an Axon Multiclamp 700B amplifier (Molecular Devices). The P/4 protocol was used to subtract linear capacitative and leakage currents. Series resistance was typically 6–8 M\textohm and was compensated to 80% (32). Data were acquired and analyzed using Clampfit 10.0 (Molecular Devices) and SigmaPlot (Systat Software). All animal experiments described in this work were approved by the Animal Care and Use Committee at Kunming Institute of Zoology, Chinese Academy of Sciences (2011-162).

Patch-Clamp Recordings on Human Na\textsubscript{v} Channels. Human Na\textsubscript{v}1.1 subunits, the human Na\textsubscript{v}1.8 subunit, and eGFP were transiently transfected into HEK293T cells and whole-cell patch-clamp recordings performed as previously described (33). The standard pipette solution contained: 140 mM CsF, 1 mM EGTA, 10 mM NaCl, 3 mM KCl, and 10 mM MgCl\textsubscript{2}, pH 7.3. The standard bath solution was: 140 mM NaCl, 3 mM KCl, 1 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, and 10 mM Hepes, pH 7.3. Data were acquired and analyzed using Clampfit 10.0 and SigmaPlot. All datapoints are shown as mean ± SE (n = number of separate experimental cells examined). Dose–response curves were fitted using the following Hill logistic equation: 

\[
y = 1 - \left(1 - f_{\text{max}}\right)/\left(1 + \left(\frac{[\text{Tx}]}{I_{\text{C50}}}\right)^n\right)
\]

where n is an empirical Hill coefficient and \(f_{\text{max}}\) is the fraction of current resistant to inhibition at high toxin ([Tx]) concentration. Steady-state activation and inactivation curves were fitted using the Boltzmann equation: 

\[
y = \frac{1}{1 + \exp\left[\left(V_{m} - V_{1/2}\right)/V_{\text{k}}\right]}$

where \(V_{1/2}\) and \(V_{k}\) represented midpoint voltage of kinetics, test potential and slope factor, respectively. \(t_{\text{on}}\) and \(t_{\text{off}}\) values were obtained from single exponential fits using the equations \(f(t) = a_0 + a_1\exp(-t\cdot t_{\text{on}})\) and \(f(t) = a_0 + a_1\exp(-t\cdot t_{\text{off}})\), respectively.

Formalin-Induced Paw Licking. Pain was induced in mice by intraplantar injection of formalin, and pain attenuation was compared in mice injected intraperitoneally with either morphine or Ssm6a dissolved in 100 \textmu L saline. Control mice received the same intraperitoneal volume of saline. After 30-min pretreatment, animals were injected with 20 \textmu L 0.92% (vol/vol) formalin at the plantar surface of right hind paw. Mice were then placed individually into open polyvinyl cages (20 × 40 × 15 cm). The time spent licking the injected paw was recorded by digital video camera during phase I (0–5 min postinjection) and phase II (15–30 min postinjection).

Thermal Pain Test. A photothermal pain detector (YLS-12A; Jianan) was used to measure the pain threshold of mice subjected to intense heat (34). The light beam of the detector was focused on the middle portion of the tail, and mice measured to have a tail withdrawal latency of 4–6 s were selected for...
the tail-flick test. Test animals were injected intraperitoneally with 100 μL saline containing Smș6a or morphine 30 min before photothermal heating of the tail. The control group received the same volume of saline. Tail withdrawal latency was measured as the time taken to withdraw the tail from the light beam.

Abdominal Writhing Induced by Acetic Acid. Mice were injected intraperitoneally with 100 μL saline containing Smș6a or morphine 30 min before intraperitoneal injection of 200 μL 0.6% (vol/vol) acetic acid, which induces abdominal contractions and hind limb stretching (25). The control group received the same volume of saline. Mice were placed into open polyvinyl cages (20 × 40 × 15 cm) immediately after acid challenge, and abdominal contractions were counted cumulatively over a period of 30 min.

Recombinant Peptide Production. Recombinant Smș6a was produced via expression in the periplasm of Escherichia coli as described for the spider-venom peptide PCtx1 (36). Further details are provided in SI Materials and Methods (Fig. S8). Note that native toxin was used for all electrophysiological and animal studies.

Plasma Stability. Lyophilized human plasma (Sigma-Aldrich, batch 101M7025) was resuspended in an equivalent volume of ultrapure water, then lyophilized recombinant Smș6a or rat atrial natriuretic peptide (American Peptide Company) was added to a final concentration of 20 μM and samples were incubated at 37 °C for 7 d. Triplicate samples were taken at selected time points, quenched by addition of urea, then plasma proteins were precipitated with 20% (vol/vol) trichloroacetic acid. Samples were centrifuged at 14,900 × g for 15 min, then supernatants were fractionated by gel permeation chromatography (37). The peak corresponding to intact Smș6a was identified by coelution with native toxin and mass determination via MALDI-TOF mass spectrometry using α-cyano-4-hydroxycinnamic acid matrix on a 4700 Proteomics Bioanalyzer (Applied Biosystems). Smș6a levels were then quantified from peak absorbance at 214 nm.

CD Spectropolarimetry. CD spectra of Smș6a (20 μM in 10 mM KH2PO4, pH 7.2) were acquired at 20 °C under constant N2 flush using a Jasco J-810 spectropolarimeter. Spectra were the sum of eight scans acquired over the region 260–190 nm at 20 nm/mm. Percent helicity was determined from the mean residue ellipticity at 222 nm (θ222), as described previously (37).

Thermal denaturation profiles were obtained by monitoring θ222, as the temperature was increased from 20 to 95 °C at a rate of 2 °C/min. Denaturation curves were fitted with a six parameter sigmoidal function (38) to obtain Tm values. The thermal denaturation of Smș6a was completely reversible upon cooling.

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Supporting Information

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SI Materials and Methods

Venom Collection and Neurotoxin Purification. Adult *Scolopendra subspinipes mutilans* L. Koch (both sexes, *n* = 3,000) were purchased from Jiangsu Province, China. Venom was collected manually by stimulating the venom glands, which are located within the first pair of modified legs, known as forcipules or maxillipeds (1), with a 3-V alternating current. Each milking occurred 1 wk after the previous milking. The unique peptide toxin was purified from the venom using a combination of gel filtration and reverse-phase (RP) HPLC, and stored at −20 °C until further use.

Mass Spectrometry. The μ-SLPTX-Ssm6α (hereafter Ssm6α) was dissolved in 0.1% (vol/vol) trifluoroacetic acid (TFA)/water and 0.5 μL was spotted onto a MALDI-TOF plate with 0.5 μL α-cyano-4-hydroxycinnamic acid (CHCA) matrix [10 mg/mL in 60% (vol/vol) acetonitrile]. Spots were analyzed using an UltraFlex I mass spectrometer (Bruker Daltonics) in positive ion mode.

Peptide Sequencing. The complete amino acid sequence of purified neurotoxin was determined by Edman degradation using a pulsed liquid-phase Shimadzu protein sequencer (PPSQ-31A, Shimadzu) according to the manufacturer’s instructions.

cDNA Library and Cloning. Total RNA was extracted from the venom glands of 20 centipedes using TRIZol (Life Technologies) and used to prepare cDNA using a SMART PCR cDNA synthesis kit (Clontech). The first strand was synthesized using the 3' SMART CDS Primer II A [5' AAGCAGTGGTATCAACGCAGAGT 3'], where *n* = A, C, G, or T and *N*1 = A, G, or C] and SMART II A oligonucleotide, (5' AAAAGCTTGTTATCAACGCAGATGTTCAACGAGCTACGAGG 3'). The 5' PCR primer II A (5' AAGCAGTGGTATCAACGCAGAGT 3') provided in the kit was used to synthesize the second strand using Advantage polymerase (Clontech). A directional cDNA library was then constructed with a plasmid cloning kit (SuperScriptTM Plasmid System, GIBCO/BRL) following the manufacturer’s instructions. The resultant cDNA library comprised 2.2 × 105 independent colonies.

A PCR-based method for high stringency screening of DNA libraries was used for screening and isolating cDNA clones. The sense-direction primer was designed according to the amino acid sequence determined by Edman degradation 5'-GC(T/C/G)GA(T/C)AA(A/G)TG- (T/C)GA(A/G)AA(T/C)TC(A/T/C/G)T-3'. The primer was used in conjunction with an antisense SMART II A primer II in PCR to screen for transcripts encoding the neurotoxin. PCR was performed using Advantage polymerase (Clontech) using the following conditions: 2 min at 94 °C, followed by 30 cycles of 10 s at 92 °C, 30 s at 50 °C, 40 s at 72 °C. Finally, the PCR products were cloned into the pGEM-T Easy vector (Promega). DNA sequencing was performed on an ABI PRISM 377 DNA sequencer (Applied Biosystems).

hERG Assays. *Xenopus laevis* oocytes were injected with cRNA encoding the hERG channel then incubated at 17 °C in 96 mM NaCl, 2 mM KCl, 5 mM Heps, 1 mM MgCl2, and 1.8 mM CaCl2, 50 μg/mL gentamycin, pH 7.6 with NaOH. hERG currents were examined at room temperature (−22 °C) 1–2 d after cRNA injection via two-electrode voltage-clamp techniques (Axoclamp 900A; Molecular Devices) using a 90-μL recording chamber. Data were low pass filtered at 2 kHz and digitized at 10 kHz using pCLAMP 10 (Molecular Devices). Microelectrode resistances were 0.1–1 MΩ when filled with 3 M KCl. The external recording solution comprised 96 mM NaCl, 2 mM KCl, 5 mM Heps, 2 mM MgCl2, 1 mM CaCl2, pH 7.6. Oocytes were voltage clamped at a holding potential of −80 mV, and 1-s pulses to a test potential of 0 mV were applied every 12.5 s until the current magnitude reached a steady-state level. After baseline recordings, the bath solution was displaced with 100 μL of test solution and recording continued until a new steady-state level was achieved. The spider-venom peptide VSTX1, a known inhibitor of hERG, was used as a positive control (2).

Recombiant Production of μ-SLPTX-Ssm6α. Recombinant Ssm6α was produced via expression in the periplasm of *Escherichia coli*, as reported previously for the spider-venom peptide PcTx1 (3), with minor modifications. A synthetic gene encoding μ-SLPTX-Ssm6α, with codons optimized for high-level expression *E. coli*, was synthesized and inserted into the pLicC-MBP vector (4) by GeneArt (Life Technologies). This vector (pLicC-EU2) encodes a MalE signal sequence for periplasmic export, a His6 affinity tag for protein purification, a maltose binding protein (MBP) fusion tag for increased solubility (5), and a tobacco etch virus (TEV) protease recognition site preceding the Ssm6α gene. The last residue of the TEV cleavage site was altered to match the first residue of Ssm6α so that cleavage of the fusion protein by TEV yields the native sequence.

*E. coli* strain BL21 (DE3) was transformed with pLicC-EU2 and grown in Luria-Bertani medium at 37 °C with shaking at 160 rpm. Cultures were cooled to 16 °C and toxin expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an OD600 of 1.5–2.0 (Fig. S8, *Inset*). Cells were harvested 12 h later by centrifugation then lysed at 26 kPa using a constant-pressure cell disruptor (TS Series Benchtop Cell Disrupter, Constant Systems). The His6-MBP-toxin fusion protein was then captured by passing the soluble cell fraction over a Ni-NTA Superflow resin (Qiagen). Recombinant Ssm6α was obtained by cleaving the fusion protein with TEV protease at a concentration of 0.2 mg/mL in a redox buffer comprising 40 mM Tris, 400 mM NaCl, 0.6 mM reduced glutathione (GSH), 0.4 mM oxidized GSH, pH 8. Residual fusion protein was precipitated with 1% TFA before the liberated Ssm6α was purified to >95% purity via RP-HPLC (C18 column; Restek) using a gradient of 15–40% solvent B [0.043% TFA in 90% (vol/vol) acetonitrile] in solvent A (0.05% TFA in water) over 30 min (Fig. S8).

The identity of the recombinant Ssm6α was confirmed via MALDI-TOF mass spectrometry with CHCA matrix on a 4700 Proteomics Bioanalyzer (Applied Biosystems), and by coelution with native Ssm6α on RP-HPLC (Onyx Monolithic C18 column; Phenomenex). The final yield of Ssm6α was 2.5 mg/L of culture.

Ssm6α Safety Profiling. Groups of five mice were injected intraperitoneally with 1 μmol/kg Ssm6α or the same volume of saline. Blood pressure and heart rate were measured as we described previously (6). A swimming exercise performance test was performed as described previously (7) to examine the effect of Ssm6α on motor function. Mice were injected intraperitoneally with saline or Ssm6α (10, 100, 1,000, or 10,000 nmol/kg); the swim test was initiated 30 min after injection.

Fig. S1. MALDI-TOF mass spectral analysis of native Ssm6a purified from the venom of S. subspinipes mutilans revealed that it has a molecular mass of 5318.41 Da.

Fig. S2. Partial reduction of disulfide bonds in Ssm6a. (A) RP-HPLC chromatogram of Ssm6a after partial reduction with TCEP, which yielded four major species labeled I–IV. (B–D) MALDI-TOF mass spectral analysis of peaks I, II, and III indicated that they correspond to nonreduced Ssm6a (peak I), Ssm6a with one disulfide bond reduced (peak II), or Ssm6a with two reduced disulfide bridges (Peak III).
Fig. S3. Partial assignment of the disulfide-bond framework in Ssm6a. A partially reduced peptide containing two disulfide bridges was alkylated with iodoacetamide for 1 min and further purified using C18 RP-HPLC. Edman degradation analysis of the alkylated peptide yielded signals for alkylated Cys residues (Pth-CM-Cys) in cycles 5 and 32, indicative of a Cys5–Cys32 disulfide bond.

Fig. S4. Ssm6a is a gating modiﬁer. The toxin concentrations used in these experiments were 1 μM Ssm6a and 500 nM TTX. Current-voltage relationships for (A) rat dorsal root ganglia (DRG) neurons (n = 8 cells) and (B) hNa\textsubscript{1.7} currents in HEK293 cells (n = 12 cells). At a saturating concentration of Ssm6a (1 μM), the inhibition of TTX-s currents in DRG neurons and HEK293 cells expressing hNa\textsubscript{1.7} was partly overcome by depolarizations to large positive test potentials (>60 mV). In contrast, inhibition of Na\textsubscript{1.7} currents by the pore-blocker TTX was largely voltage-independent.
Fig. S5. Effect of Ssm6a on selected Na\textsubscript{v} channel subtypes. Ssm6a (10 μM, red traces) had no effect on currents mediated by (A) hNa\textsubscript{v}1.3, (B) hNa\textsubscript{v}1.4, (C) hNa\textsubscript{v}1.5, or (D) hNa\textsubscript{v}1.8.
Intraperitoneal injection of Ssm6a had no effect on (A) blood pressure, (B) heart rate, or (C) motor function (as judged by a swim test) at doses up to 1–10 μmol/kg. In each experiment, cohorts of five treated animals were compared with five saline-treated mice. Data are mean ± SE.
**Fig. S7.** Ssm6a has no effect on human ERG (Kv11.1) channels expressed in *Xenopus* oocytes. Representative K⁺ currents before (black) and after addition of Ssm6a (10 μM, red) or the positive control peptide VSTX1 (30 μM, gray). The voltage-clamp protocol is shown above the current traces. Ssm6a had no effect on the outward activating current or the deactivating tail current of hERG channels (*n* = 3).

**Fig. S8.** Expression and purification of recombinant Ssm6a. RP-HPLC chromatogram showing purification of recombinant Ssm6a after liberation from the His₆-MBP fusion tag by TEV protease. The peak corresponding to Ssm6a is highlighted with an asterisk. (Inset) A SDS/PAGE gel showing *E. coli* cells before (lane 1) and after (lane 2) induction of Ssm6a expression with IPTG. Lane 3 contains molecular mass standards, with masses indicated in kilodaltons on the right of the gel. The arrow indicates the running position of the MBP-toxin fusion protein.