Structure of Membrane-active Toxin from Crab Spider *Heriaeus melloteei* Suggests Parallel Evolution of Sodium Channel Gating Modifiers in Araneomorphae and Mygalomorphae*

Antonia A. Berkut,1,2, Steve Peigneur,1 Mikhail Yu. Myshkin,1,5 Alexander S. Paramonov,1 Ekaterina N. Lyukmanova,1 Alexander S. Arseniev,1,5 Eugene V. Grishin,1 Jan Tytgat,5 Zakhar O. Shenkarev,1 and Alexander A. Vassilevski1,4

From the 1M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia, 2Moscow Institute of Physics and Technology (State University), 117303 Moscow, Russia, and 4Taxicology and Pharmacology, University of Leuven, 3000 Leuven, Belgium

**Background:** Several toxins from mygalomorph spiders are known to inhibit sodium channel activation. Hm-3 toxin from the araneomorph spider *Heriaeus melloteei* (Thomisidae) was recombinantly expressed in *Escherichia coli* and determined its structure by NMR spectroscopy. Typical for spider toxins, Hm-3 was found to adopt the so-called “inhibitor cystine knot” or “knottin” fold stabilized by three disulfide bonds. Its molecule is amphiphilic with a hydrophobic ridge on the surface enriched in aromatic residues and surrounded by positive charges. Correspondingly, Hm-3 binds to both neutral and negatively charged lipid vesicles. Electrophysiological studies showed that at a concentration of 1 μM Hm-3 effectively inhibited a number of mammalian and insect sodium channels. Importantly, Hm-3 shifted the dependence of channel activation to more positive voltages. Moreover, the inhibition was voltage-dependent, and strong depolarizing prepulses attenuated Hm-3 activity. The toxin is therefore concluded to represent the first sodium channel gating modifier from an araneomorph spider and features a “membrane access” mechanism of action. Its amino acid sequence and position of the hydrophobic cluster are notably different from other known gating modifiers from spider venom, all of which are described from mygalomorph species. We hypothesize parallel evolution of inhibitor cystine knot toxins from Araneomorphae and Mygalomorphae suborders.

We present a structural and functional study of a sodium channel activation inhibitor from crab spider venom. Hm-3 is an insecticidal peptide toxin consisting of 35 amino acid residues from the spider *Heriaeus melloteei* (Thomisidae). We produced Hm-3 recombinantly in *Escherichia coli* and determined its structure by NMR spectroscopy. Typical for spider toxins, Hm-3 was found to adopt the so-called “inhibitor cystine knot” or “knottin” fold stabilized by three disulfide bonds. Its molecule is amphiphilic with a hydrophobic ridge on the surface enriched in aromatic residues and surrounded by positive charges. Correspondingly, Hm-3 binds to both neutral and negatively charged lipid vesicles. Electrophysiological studies showed that at a concentration of 1 μM Hm-3 effectively inhibited a number of mammalian and insect sodium channels. Importantly, Hm-3 shifted the dependence of channel activation to more positive voltages. Moreover, the inhibition was voltage-dependent, and strong depolarizing prepulses attenuated Hm-3 activity. The toxin is therefore concluded to represent the first sodium channel gating modifier from an araneomorph spider and features a “membrane access” mechanism of action. Its amino acid sequence and position of the hydrophobic cluster are notably different from other known gating modifiers from spider venom, all of which are described from mygalomorph species. We hypothesize parallel evolution of inhibitor cystine knot toxins from Araneomorphae and Mygalomorphae suborders.

Spider venoms are multicomponent mixtures of biologically active molecules, the main functions of which are to subdue prey and deter predators (1, 2). These venoms usually contain low molecular weight compounds (3, 4), proteins (5, 6), and peptides (7, 8) with the latter group most often prevailing over the other two in terms of content and/or functional importance. Based on the target and structural features, most venom peptides can be classified into two large groups: linear cytolytic peptides (2, 9) and disulfide-containing neurotoxins (1, 2, 7, 8). The latter usually adopt the inhibitor cystine knot (ICK) or “knottin” fold and target ion channels and receptors of the prey or predator nervous system. Numerous studies have shown that currents through voltage-gated ion channels can be affected either by pore block or modification of the gating mechanisms.

In the case of neurotoxins acting on voltage-gated sodium channels (Na$_v$s), a number of peptide pore blockers were identified. They were shown to bind to the so-called receptor site 1 (as well as the classic low molecular weight blockers tetrodotoxin and saxitoxin) and inhibit ion conductivity due to the direct blockade of the pore. The majority of well described peptide pore blockers were isolated from cone snail venoms (the so-called μ-conotoxins) (10). So far there is only one known

**Results:** Hm-3 toxin from the araneomorph spider *Heriaeus melloteei* inhibits mammalian and insect sodium channel activation and possesses membrane activity.

**Conclusion:** Hm-3 binds to sodium channel voltage sensors through membrane access.

**Significance:** The first sodium channel activation inhibitor from Araneomorphae points to parallel evolution of *H. melloteei* and its distant mygalomorph relatives.

---

1 Both authors contributed equally to this work.
2 Supported by Russian Science Foundation Grant 14-14-01180 to perform large scale production of Hm-3 and physiological studies.
3 Supported by Fonds Wetenschappelijk Onderzoek Vlaanderen Grants G.0433.12, G.071.10N, and G.0257.08; Inter-University Attraction Poles Program Grant IUAP 7/10 from Belgian State, Belgian Science Policy; and KU Leuven Grant OT/12/081.
4 Recipient of a stipend of the President of Russian Federation. To whom correspondence should be addressed. E-mail: avas@ibch.ru.
5 The abbreviations used are: ICK, inhibitor cystine knot; CaV, voltage-gated calcium channel; K$_v$, voltage-gated potassium channel; Na$_v$, voltage-gated sodium channel; SUV, small unilamellar vesicle; Trx, thioredoxin; POPC, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; DOPG, 1,2-dioleoyl-sn-glycerol-3-phosphoglycerol; I-V, current-voltage; POPG, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phospho-racemic-(1-glycerol).
spider venom peptide that acts as a sodium channel pore blocker: PnTx1 from *Phoneutria nigriventer*. This peptide toxin was shown to compete with \( \mu \)-conotoxins but not with tetrodotoxin for channel binding (11).

To modify sodium channel gating, neurotoxins can use different strategies. They can retard or inhibit inactivation and either facilitate or inhibit activation of a channel (12). These types of action are usually associated with binding of the peptides to so-called receptor sites 3 and 4 of the channels. The major parts of the channels contributing to these sites are extracellular loops connecting the S3 and S4 segments in domains IV and II correspondingly. The mode of action of the gating modifier toxins is usually explained by a voltage sensor trapping mechanism (13).

A delay of inactivation is characteristic of such classic site 3 ligands as scorpion \( \alpha \)-toxins (14) and certain sea anemone toxins (15). Moreover, a similar effect is characteristic of some spider toxins binding to site 3 (16, 17) and, what is worthy of note, site 4 (18). Promotion of sodium channel activation is also quite widespread among toxins: scorpion \( \beta \)-toxins (19) and some spider toxins (20) binding to site 4 show this kind of effect. Importantantly, the channel voltage dependence of activation is shifted to more hyperpolarized voltages under the action of these toxins. Conversely, several spider peptide toxins are known to inhibit sodium channel activation: ProTx-I and ProTx-II from *Thrixopelma pruriens* (21), JZTX-III from *Chilobrachys jingzhao* (22), and HWTX-IV from *Haplopelma schmidti*, all produced by mygalomorph species. The data on the exact receptor site for these toxins presented in the literature are sometimes ambiguous. For instance, HWTX-IV was first misclassified as a site 1 toxin (23), which was further corrected to site 4 when new data were acquired (24). In part, this is due to the apparent lack of a shift in the channel voltage dependence of activation, which is more often the case, as with ProTx-I, ProTx-II, and JZTX-III that are also believed to bind to site 4 (25, 26).

Yet another sodium channel activation inhibitor is the peptide Hm-3 from the spider *Heriaeus melloteei* (crab spider, Thomisidae, Araneomorphae), which was isolated from the venom by our group earlier (27). The goal of the present study was to characterize Hm-3 in detail by investigating its three-dimensional structure and running electrophysiological measurements and thus gain some insight into its mechanism of action.

### EXPERIMENTAL PROCEDURES

#### Hm-3 Isolation—Native Hm-3 was isolated from the venom of *H. melloteei* following a technique described previously (27).

#### Recombinant Peptide Production—To produce recombinant Hm-3, a procedure similar to the one developed earlier was used (28). Hm-3-encoding gene was assembled from a number of synthetic oligonucleotides (Table 1) using a combination of PCR and ligation techniques. In part, it was amplified using a forward primer containing a BamHI restriction site and a methionine codon and a reverse primer containing a SalI restriction site and a stop codon. The PCR fragment was cloned into the expression vector pET-32b (Novagen) using restriction with BamHI and SalI enzymes followed by ligation. As a result, the plasmid pET-32b-Hm-3 was produced and was then used to transform *Escherichia coli* BL21(DE3) and Origami B cells.

The bacteria were cultured at 37 °C in Luria-Bertani medium with the appropriate antibiotics as a selective factor to the mid-log phase. Expression was then induced by 0.2 mM isopropyl \( \beta \)-D-thiogalactopyranoside, and bacteria were cultured at room temperature (24 °C) overnight (16 h). Then the cells were harvested by centrifugation, and the pellet was resuspended in 300 mM NaCl, 50 mM Tris-Cl buffer (pH 6.8), and 5% glycerol and ultrasonicated.

The hybrid protein Trx-Hm-3 with thioredoxin (Trx) as a carrier was purified by affinity chromatography using TALON Superflow resin (Clontech) following the manufacturer’s protocol. Finally, the chimeric protein was cleaved at methionine residues by cyanogen bromide (CNBr) using a procedure described previously (29). HCl was added to the protein solution (−1 mg/ml) to the concentration of 0.5 M, and CNBr was then added to the concentration of 100 mM. The probe was then incubated at room temperature (24 °C) overnight (16 h) in the dark. Recombinant Hm-3 was purified by reversed-phase HPLC on a Jupiter C8 column (250 × 10 mm; Phenomenex) using a linear gradient of acetonitrile concentration (0–60% in 60 min) in the presence of 0.1% trifluoroacetic acid. The purity of the target peptide was checked by MS, N-terminal sequencing, and analytical chromatography on a Vydac 218TP54 C18 column (4.6 × 250 mm; Separations Group) in a shallow acetonitrile gradient (25–50% in 50 min).

#### NMR Experiments and Spatial Structure Calculation—NMR experiments were performed using a 0.5 mM Hm-3 solution in 0.5% D$_2$O or 100% D$_2$O at pH 5.2. All NMR spectra were acquired on a Bruker Avance 800 spectrometer equipped with a cryoprobe at 35 °C. $^1$H and $^{13}$C resonance assignments were obtained by a standard procedure using a combination of two-dimensional total correlation spectroscopy, NOESY, and $^{13}$C heteronuclear single quantum correlation spectra at natural $^{13}$C abundance (30). $^1$H chemical shifts were measured relative to the residual protons of H$_2$O; the chemical shift of the signal was arbitrarily chosen as 4.70 ppm at 35 °C. $^{13}$C chemical shifts

### Table 1

| Name | Sequence | Length |
|------|----------|--------|
| f1   | ATGCCGAA/CCATGGGATGTAGCCACAAAGAT  | 31    |
| f2   | ACGAAGTG/CGCTGTGTTCCAGAGCTGTGAG  | 39    |
| f3   | CCCTATCA/CGATAATCCACAAAGGAACT  | 45    |
| rev1/2 | A/ACAGCGACACTCTTTATCTGGCTATACCT | 36    |
| rev2/3 | GATACTT/ACATGATAGGGCTCACCACACACCT | 36    |
| rev  | GCTCTCG/ACCACAGCACTATCTTTAGATTCC | 35    |
Sodium Channel Toxin from Crab Spider

were referenced indirectly. The $^{3}J_{\alpha^{N}-H_{\beta}}$ coupling constants were determined from line shape analysis of NOESY cross-peaks. The $^{3}J_{\alpha^{N}-H_{\beta}}$ coupling constants were measured using ACME (31) in the two-dimensional double quantum-filtered COSY spectrum acquired in 100% D$_{2}$O. Temperature coefficients of amide protons ($\Delta^{3}J_{H^{N}/H^{\beta}}/\Delta T$) were measured in a temperature range from 25 to 45 °C using two-dimensional total correlation spectroscopy spectra. To identify the slowly exchanging amide protons, the Hm-3 sample was lyophilized and redissolved in 100% D$_{2}$O. The hydrogen-deuterium exchange kinetics was measured using one-dimensional $^{1}$H spectra.

Spatial structure calculation was performed in the CYANA 3.0 program (32). Upper interproton distance constraints were derived from the intensities of cross-peaks in two-dimensional NOESY spectra ($r_{m} = 100$ ms) via a $1/r^{6}$ calibration. Torsion angle restraints and stereospecific assignments were obtained from $J$ coupling constants and NOE intensities. Hydrogen bonds were introduced based on temperature gradient and deuterium exchange rates of H$_{3}$I protons. The disulfide bond connectivity pattern was established on the basis of the observed NOE contacts and verified on the preliminary stages of the spatial structure calculation.

Hm-3 Binding to Lipid Vesicles—Small unilamellar vesicles (SUVs) were prepared by sonication using POPC or a POPC/DOPG (3:1) mixture (Avanti Polar Lipids) in 10 mM Tris acetate buffer (pH 7.0) with or without 150 mM NaCl. The final lipid concentrations were measured by one-dimensional $^{1}$H NMR spectroscopy by dissolving small fractions of the SUV preparation in a CDCl$_{3}$/CD$_{3}$OD/D$_{2}$O (15:10:3) mixture. Titration of an ACME (31) in the two-dimensional double quantum-filtered exchange kinetics was measured using one-dimensional $^{1}$H NMR spectrum was measured, and the equilibrium constant of the site on the vesicle surface formed by the degree of cooperativity and eventually the number of molecules necessary to modulate one channel) was obtained.

Electrophysiology—Genes of the following channels were used to prepare mRNA and inject Xenopus oocytes as described previously (33, 34): mammalian Na$_{v}$1.1–Na$_{v}$1.6 and Na$_{v}$1.8; DmNa$_{v}$1 from Drosophila; mammalian voltage-gated potassium channels (K$_{s}$K$_{v}$1.1, K$_{v}$1.3, K$_{v}$2.1, K$_{v}$A2.2, and K$_{v}$10.1; and mammalian voltage-gated calcium channel (Ca$_{v}$)Ca$_{v}$3.3. Two-electrode voltage clamp recordings were performed at room temperature (18–22 °C) using a Geneclamp 500 amplifier (Molecular Devices) controlled by a pClamp data acquisition system (Axon Instruments). Whole-cell currents from oocytes were recorded 1–4 days after injection. Bath solution composition was: 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_{2}$, 2 mM MgCl$_{2}$, and 5 mM HEPES (pH 7.4). Voltage and current electrodes were filled with 3 mM KCl. Resistances of both electrodes were kept between 0.7 and 1.5 megaohms. The elicited currents were filtered at 1 kHz and sampled at 20 kHz using a four-pole low pass Bessel filter. Leak subtraction was performed using a $-P/4$ protocol. To avoid overestimation of a potential toxin-induced shift in the current-voltage relationships of inadequate voltage control when measuring large sodium currents in oocytes, only data obtained from cells exhibiting currents with peak amplitude below 2 $\mu$A were considered for analysis.

For the electrophysiological analysis of toxins, a number of protocols were applied from a holding potential of −90 or −100 mV with a start-to-start interval of 0.2 Hz. Sodium current traces were evoked by 50-ms or 100-ms depolarizations to $V_{\text{max}}$ (the voltage corresponding to maximal sodium current in control conditions).

The current-voltage ($I$-$V$) relationships were determined by 100-ms step depolarizations between −90 and 70 mV using 5-mV increments. Na$^{+}$ conductance ($g_{\text{Na}}$) was calculated from the currents according to Ohm’s law: $g_{\text{Na}} = I_{\text{Na}}/(V - V_{\text{rev}})$, where $I_{\text{Na}}$ represents the sodium current peak amplitude at a given test potential $V$ and $V_{\text{rev}}$ is the reversal potential. The values of $g_{\text{Na}}$ were plotted as a function of voltage and fitted using the Boltzmann equation: $g_{\text{Na}}/g_{\text{max}} = [1 + \exp((V - V_{1/2})/k)^{-1}$, where $g_{\text{max}}$ represents maximal current and $k$ is the slope factor.

To assess the toxin-induced effects on the steady-state inactivation, a standard two-step protocol was applied. In this protocol, 100-ms conditioning 5-mV step predepolarizations ranging from −90 to 60 mV were followed by a 50-ms test pulse to $V_{\text{max}}$. Data were normalized to the maximal sodium current amplitude, plotted against prepulse potential, and fitted using the Boltzmann equation: $I_{\text{Na}}/I_{\text{max}} = 1/(1 + \exp((V - V_{1/2})/k))$, where $V_{1/2}$ is the voltage corresponding to half-maximal inactivation and $k$ is the slope factor.

To assess the concentration-response relationships of the toxin-induced inhibitory effects, dose-response curves were constructed. Current traces were evoked as described above in control and in the presence of a range of toxin concentrations. The percentage of toxin-induced inhibition of the sodium current peak amplitudes was plotted against the logarithm of applied concentrations and fitted with the Hill equation to obtain the EC$_{50}$ value (i.e. the toxin concentration that produces 50% of the maximal effect) of the effects of Hm-3. From the Hill equation, the Hill coefficient (i.e. the degree of cooperativity and eventually the number of molecules necessary to modulate one channel) was obtained.

To investigate the voltage-dependent reversal of Hm-3 inhibition, a depolarizing pulse of increasing amplitude (up to +100 mV) and/or duration (up to 800 ms) was followed by a 20-ms repolarization to the holding potential of −100 mV and then a test pulse to 0 mV. The exponential time course of relief of toxin inhibition was determined by plotting the normalized current.
as a function of time. The normalization was performed as follows. The steady-state level of $I_{Na}$ peak current obtained after application of 1 μM Hm-3 was set to 0. The level of $I_{Na}$ peak current obtained after a 700-ms prepulse duration was set to 1. These normalized current values were plotted as a function of time: $f(t) = (I_t - I_0)/(I_{700 ms} - I_0)$. The time values of depolarizing prepulse required to recover 60% of the current from inhibition ($\tau$) were calculated to express the different kinetics of reversal of inhibition for different channels.

Comparison of two sample means was made using a paired Student’s $t$ test ($p < 0.05$). All data are presented as means ± S.E. of at least five independent experiments ($n \geq 5$). All data were analyzed using pClamp Clampfit 10.0 (Molecular Devices) and Origin 7.5 software (Originlab).

RESULTS

Hm-3 is a peptide toxin isolated previously from *H. melloteei* venom (27) with the following amino acid sequence (UniProt accession number COHJK3): GCIAKNKECAWFSGEWCCG-ALSCKYSIKNLKICV. Because only minute amounts of toxin may be purified from the natural source, we decided to produce Hm-3 recombinantly.

Recombinant Hm-3 Production—To provide enough material for NMR and electrophysiological studies, an *E. coli* expression system for Hm-3 was developed. An Hm-3-encoding gene was produced from a number of synthetic oligonucleotides and cloned into the pET-32b expression vector. *E. coli* BL21(DE3) and Origami B cells were then transformed by the recombinant plasmid pET-32b-Hm-3. The target peptide was purified after cleanup of the chimeric protein Trx-Hm-3 by affinity chromatography, its cleavage by CNBr, and separation of the hydrolysate by reversed-phase HPLC. We failed to acquire a correctly folded product in *E. coli* BL21(DE3), but utilization of the *E. coli* Origami B strain was successful (Fig. 1A). The average molecular mass of the resulting recombinant Hm-3 was measured by MALDI MS (3907.8 Da) and was found to be equal to the mass of native Hm-3 (3907.7 Da) (27) and the calculated mass (3907.7 Da). To prove recombinant and native Hm-3 equivalency, they were co-eluted in analytical reversed-phase HPLC and shown to have the same retention time (Fig. 1B). The yield of recombinant Hm-3 was 0.7 mg/liter of bacterial culture.

Spatial Structure of Hm-3—Spatial structure of Hm-3 was studied by NMR spectroscopy in aqueous solution at pH 5.2 and 35 °C (Fig. 2A). The set of 20 structures (Fig. 3A) was calculated in CYANA from 200 random starts using the following experimental data: upper NOE-based distance restraints, $J$-coupling-based torsion angle restraints, and hydrogen bond restraints (Table 2). The Hm-3 structure (Fig. 3B) involves a $\beta$-hairpin formed by two strands (Cys23–Ser26 and Leu31–Cys34) connected by a turn in the 310-helix conformation (Ile27–Arg29). The $\beta$-hairpin protrudes from a “globular core” cross-linked by three disulfide bonds (Cys2–Cys18, Cys9–Cys23, and Cys17–Cys34) presenting the “cystine knot” arrangement (I-IV, II-V, and III-VI). Other possible disulfide bond connectivity patterns were tested in the course of spatial structure calculations, but they did not correspond to the experimental NMR data. The toxin is therefore concluded to assume the conventional ICK (knottin) fold. The Hm-3 “core” accommodates several tight $\beta$-turns (Fig. 3A). In addition to disulfide bonds,
Overview of NMR data collected for Hm-3 in aqueous solution and titration of the toxin with SUVs composed of POPC. A, NMR data were measured for 0.5 mM Hm-3 at pH 5.2 and 35 °C. 1H chemical shift indices (CSI), 1H/C CSI, coupling constants, temperature coefficients of amide protons (ΔT ΔH/ΔT), hydrogen-deuterium exchange rates for HN protons (Kp), and NOE connectivities are shown versus the peptide sequence. The positive and negative values of chemical shift indices denote β-strand and α-helical propensity, respectively. The large (>8.5 Hz), small (<5.5 Hz), and medium (others) 1H/H 1H/H couplings are designated by filled triangles, open squares, and stars, respectively. The crosses denote amide protons with Δ1H/ΔT less than 4.3 ppm/K. The filled and half-filled circles denote HN protons with hydrogen-deuterium half-exchange times >30 and >10 min, respectively. The NOE connectivities correspond to the relative intensity of the cross-peak in the 100-ms NOESY spectrum. Secondary structure is shown on a separate line: β-strands are designated by arrows, 310-helix turn by a box, and tight β-turns by wavy lines. B, the amide-aromatic regions of one-dimensional 1H NMR spectra (20 µM Hm-3, 150 mM NaCl, and 10 mM Tris acetate, pH 7.0 at 35 °C) measured at different lipid concentrations are shown. The intensity of NMR signals is proportional to the equilibrium concentration of the free peptide in solution (Cj). The signal of impurity is marked by an asterisk.

interaction of Hm-3 with lipid vesicles. The amphiphilic properties of the Hm-3 surface pointed to the ability of the peptide to partition into lipid membranes. To investigate the membrane-binding propensity of Hm-3, SUVs consisting of either zwitterionic (POPC) or a mixture of zwitterionic and anionic (POPC/DOPG, 3:1) lipids were used. Titration of the peptide sample with liposomes led to a gradual decrease of the Hm-3 NMR signal intensity (Fig. 2B). The observed attenuation could be explained by a tight association of the peptide molecules with the vesicle surface. In this case, because of a very slow reorientation of SUVs in solution, the bound peptide molecules become unobservable by high resolution NMR spectroscopy, and the intensity of the NMR signal is directly proportional to the equilibrium concentration of the free peptide in solution (Cj). The measured binding curves (Fig. 3D) revealed an effective partitioning of Hm-3 into the zwitterionic and partially anionic SUVs at close to physiological ionic strength conditions (150 mM NaCl). Comparison of these data with the results obtained in low-salt conditions indicated that addition of 150 mM NaCl into the binding buffer diminished the peptide affinity to the POPC/DOPG SUVs only slightly (Fig. 3D).

The partition equilibrium equation (Equation 1) is usually used for the analysis of peptide interactions with lipid membranes. Interestingly, this simple isotherm containing only one variable parameter (partition coefficient Kp) did not fit satisfactorily the measured Hm-3 binding curves (Fig. 3D, dashed lines). The obtained Kp values are collected in Table 3 for comparison with previous studies. Conversely, the usage of a more complex Langmuir isotherm (Equation 2) having two variable parameters (Kv and N) provided a reliable approximation of the measured data (Fig. 3D, solid lines). The results (Table 3) revealed that irrespectively of the lipid composition of SUVs and salt concentration the site of the peptide binding on the vesicle surface is formed by ~10 lipid molecules (N). The observed changes in Hm-3 affinity toward charged SUVs upon variation of salt concentration are mostly induced by the changes in the peptide affinity to this site (parameter Kv) (Table 3). The absence of a pronounced dependence in the efficiency of Hm-3 membrane binding upon variation of the charge of lipid headgroups and salt concentration indicates that hydrophobic interactions play a central role in the formation of the peptide-membrane complex.

Electrophysiological Characterization—At a concentration of 1 µM, recombinant Hm-3 was tested against a panel of NaV α-s (Fig. 4A). The toxin was found to inhibit sodium current
through mammalian channels $Na_v_{1.2}$ and $Na_v_{1.4-1.6}$ and the insect channel Dm$Na_v_{1.1}$, $Na_v_{1.3}$, and $Na_v_{1.8}$ channels were not affected by 1 mM Hm-3. A clear rightward shift of the I-V curve was observed for $Na_v_{1.4}$, $Na_v_{1.5}$, and Dm$Na_v_{1.1}$ channels, whereas this was less pronounced for $Na_v_{1.2}$ and $Na_v_{1.6}$ channels (Fig. 4A, right panels). It should be noted that when higher concentrations of the toxin were applied a shift of the activation dependence of the $Na_v_{1.2}$ and $Na_v_{1.6}$ channels

**FIGURE 3.** Spatial structure of Hm-3 in aqueous solution, comparison of Hm-3 spatial structure with other ion channel activation inhibitors from spider venom, and isotherms of Hm-3 binding to lipid vesicles. A, a set of 20 “best” Hm-3 structures superimposed over the backbone atoms. The disulfide bonds are shown in orange. B, ribbon representation of Hm-3 spatial structure. Hydrophobic, positively charged, and negatively charged residues are colored in green, blue, and red, respectively. (C) Ribbon representation and two-sided view of Hm-3, HWTX-IV, JZTX-III, ProTx-I, and SGTrx1 surfaces (Protein Data Bank codes are 2MQU, 2MQX, 2ITL, 2M9L, and 1LAA, respectively). The molecules are superimposed over the heavy atoms (carbon, nitrogen, and sulfur) of six conserved Cys residues. On the surfaces, the hydrophobic (Ala, Met, Ile, Leu, Val, Phe, Trp, Tyr, and Pro), polar (Asn, Gln, Gly, His, Ser, and Thr), positively charged (Arg and Lys), and negatively charged (Asp and Glu) residues are colored in green, magenta, blue, and red, respectively. Cys residues are in yellow. D, the binding curves describing Hm-3 interactions with POPC and POPC/DOPG (3:1) SUVs are approximated by the partition equilibrium equation (Equation 1; dashed lines) and by the Langmuir isotherm (Equation 2; solid lines). Fitted parameters are summarized in Table 3. The dilution curve is shown by a dotted line.
did occur (data not shown). The native venom-extracted toxin showed identical activity to the recombinant Hm-3 against NaV1.4 (data not presented). Because it was established that voltage clamp errors might occur in two-electrode experiments with heterologously expressed channels in oocytes, we verified whether the IC50 concentration of tetrodotoxin (1 nM for NaV1.2 and 4 nM for NaV1.4) alters the voltage dependence of activation and found no alteration of the activation and steady-state inactivation curves (data not shown).

To assess the concentration dependence of the Hm-3-induced inhibitory effects, dose-response curves were constructed in which the percentage of current inhibition was plotted as a function of toxin concentration (Fig. 4B). The EC50 values yielded 1447 ± 462 nM (h = 0.8 ± 0.1) for NaV1.2, 103 ± 15 nM (h = 0.7 ± 0.1) for NaV1.4, 268 ± 23 nm (h = 0.7 ± 0.1) for NaV1.5, 205 ± 80 nm (h = 0.6 ± 0.1) for NaV1.6, and 553 ± 13 nM for DmNaV1 channels. These results are comparable with other characterized toxins exhibiting similar effects, such as ProTx-II (EC50 of 540 nM against NaV1.2; Ref. 25), HWTX-IV (EC50 of 150, 338, and 400 nM for NaV1.2, NaV1.3, and NaV1.4; Ref. 24), and IZTX-III (EC50 of 348 nM against NaV1.5; Ref. 26). Hill coefficient values in all cases approximated 1, suggesting a necessity of one toxin molecule to modulate a channel.

To investigate whether Hm-3 acts in a voltage-dependent manner, the sodium peak current in toxin conditions was calculated as a fraction of the corresponding peak current in control using the I-V relationship data. The obtained data were fitted with a linear regression (Fig. 4C). It was concluded that the degree of Hm-3-induced inhibition is voltage-dependent.

The characteristics of Hm-3 modulation of NaV1.4 and DmNaV1 channel kinetics were investigated further. For DmNaV1, a significant shift of the midpoint of activation is clearly observed in the normalized activation curves (Fig. 5A): the V1/2 shifted from −21.7 ± 0.1 mV in control conditions to −41.1 ± 0.4 mV after toxin application. For NaV1.4, on the contrary, no significant shift was seen (Fig. 5C).

A reversed situation was observed in the steady-state inactivation curves. For DmNaV1, little change in the voltage dependence of inactivation was seen: the V1/2 values were −49.8 ± 2.2 mV in control and −45.2 ± 1.6 mV in toxin conditions (Fig. 5B). But for NaV1.4, a shift was observed: V1/2 values were −56.0 ± 0.3 mV and −62.1 ± 0.7 mV in control and toxin situations, respectively (Fig. 5D).

Different sensitive channels exhibit strikingly different patterns of changes in the I-V curves. These differences could be attributed to the different binding affinity of the Hm-3 toxin to specific channels. We investigated whether this was due to different toxin binding off-rates among the sensitive channels. A strong depolarizing pulse to +100 mV of increasing duration was followed by 20 ms at the holding potential of −100 mV, which was subsequently followed by a test pulse to 0 mV (Fig. 6A). It was found for both tested channels that the conditioning pulse caused a reversal of the Hm-3-induced inhibition of the sodium current (Fig. 6B). The strongest conditioning depolarization pulse did not result in a complete reversal of Hm-3-induced inhibition. Maximum values yielded 68 (NaV1.4) and 90% (DmNaV1) of control at +100 mV and 635-ms pulse duration ($p < 0.05, n = 5$). The inhibition was reversed in an exponential time course. However, different kinetics was observed in NaV1.4 and DmNaV1. To quantify these differences in the kinetics of reversal of inhibition, the above protocol was applied with conditioning pulses ranging from +40 to +100 mV (Fig. 6C). For both channels, the relief of inhibition was the fastest at +100 mV and slowest at +40 mV (Fig. 6D and E). However, at both voltages, the off-rate was much higher for DmNaV1 compared with NaV1.4. At +100 mV, $\tau_{+100\text{ mV}} = 227\text{ ms}$ for NaV1.4, and $\tau_{+100\text{ mV}} = 114\text{ ms}$ for DmNaV1. Accordingly, at +40 mV, $\tau_{+40\text{ mV}} = 910\text{ ms}$ for NaV1.4, and $\tau_{+40\text{ mV}} = 370\text{ ms}$ for DmNaV1. Isochronal activation curves for toxin-bound channels can be obtained by plotting the normalized percent

### Table 2

Statistics for the best CYANA structures of Hm-3

| r.m.s.d. (Å) overall (Gly1–Val35) | Total NOE contacts |
|---------------------------------|--------------------|
| 263                             | 10.5 ± 0.04        |

### Statistics for calculated structures

| Structures calculated/selected | 200/20 |
|--------------------------------|--------|
| Violations of restraints       | 2      |
| Distance (>0.2 Å)              | 2      |
| Distance (>0.4 Å)              | 0      |
| Dihedral angles (>1°)          | 0      |
| r.m.s.d. (Å) overall           | 0.22 ± 0.07 |
| Backbone                       | 0.62 ± 0.14 |
| Heavy atoms                    | 0.62 ± 0.14 |

### Table 3

Energetic and stoichiometric parameters of Hm-3 interactions with SUVs obtained using partition equilibrium equation (Equation 1) and Langmuir isotherm (Equation 2)

| Lipids             | Partition equilibrium*, $K_p$ | $K_N$ | $N$ |
|--------------------|------------------------------|-------|-----|
| POPC/DOPG (3:1)    | $12.5 ± 3.0$                | $1.09 ± 0.57$ | $9.7 ± 0.8$ |
| POPC/DOPG (3:1), 150 mM NaCl | $8.6 ± 1.3$                | $0.29 ± 0.09$ | $10.6 ± 0.8$ |
| POPC, 150 mM NaCl  | $9.5 ± 1.4$                 | $0.27 ± 0.02$ | $9.4 ± 0.7$ |

* $K_p$ is the partition coefficient. The concentration of the “non-aqueous” phase was taken to be equal to lipid concentration in the outer leaflet of the vesicles (60% of total lipid).

* $K_N$ is the affinity constant of the peptide to the site on the vesicle surface formed by $N$ lipid molecules.
increase in test pulse peak amplitude after 700-ms conditioning depolarizations against the range of potentials used in the conditioning pulses. A shift toward more positive potentials was observed for toxin-modulated NaV1.4 and DmNaV1 channels. However, a more pronounced shift was seen for the NaV1.4 channels because the $V_{1/2}$ shifted 30 mV toward more positive potential values compared with a moderate 10-mV shift of the midpoint of activation for DmNaV channels (Fig. 6, F and G).

Finally, to check the toxin selectivity to sodium channels, Hm-3 was investigated for its activity against NaVs. No activity was observed for KV1.1, KV1.3, KV2.1, KV4.2, KV10.1, or CaV3.3 at 1 μM Hm-3 (data not shown).
DISCUSSION

Hm-3 Is a Sodium Channel Gating Modifier—Electrophysiological studies demonstrate that Hm-3 effectively inhibits both mammalian and insect NaVs (Fig. 4A, left panels). Moreover, the toxin shifts the dependence of channel activation to more depolarized voltages (Fig. 4A, right panels). Several spider toxins were shown to exhibit a similar effect on sodium channels, such as ProTx-I and -II from T. pruriens (21), HWTX-IV from H. schmidti (24), IZTX-III from C. jingzhao (26), CcoTx1 and CcoTx2 from Ceratogyrus marshalli, and PaurTx3 from Paraphysa scrofa (35) (see Fig. 7 for amino acid sequences). All these toxins are believed to represent gating modifiers; i.e., their blocking effect on the channel is associated with the inhibition of the gating mechanism as opposed to a direct pore block.

The following observations justify allocation of Hm-3 to gating modifiers. (i) Our results indicate that the Hm-3-induced inhibition of the sodium peak current is voltage-dependent (Fig. 4C). It was shown in previous studies that voltage-dependent enhancement or reversal of toxin activity can be considered as a hallmark of gating modifier toxins (25, 36, 37). (ii) Strong depolarizing prepulses provide relief from Hm-3-induced inhibition (Fig. 6, A and B), also characteristic of gating modifiers (24, 25). (iii) Furthermore, the reversal of the inhibition is voltage-dependent (Fig. 6, C–G), a phenomenon described previously for other gating modifiers from spider venoms such as ProTx-I and -II (25) and HWTX-IV (38). We therefore conclude that Hm-3 represents the first sodium channel gating modifier from an araneomorph spider.

The observed differences in channel affinity as characterized by the off-rates of toxin binding to NaV1.4 (Fig. 6, B and D) and DmNaV1 (Fig. 6, B and E) provide a possible explanation for the striking difference of change in the I-V curves between these NaVs (Fig. 4A). According to the voltage sensor trapping model (24, 25), gating modifiers such as Hm-3 bind to a voltage sensor in its inward position and are thrown off by the voltage-driven outward movement of the sensor upon depolarization. The difference in binding affinity may result in different I-V curves. It was found that strong positive conditioning pulses provide the necessary energy to force the outward movement of the sensor into the activated position and thus provide the necessary energy to remove Hm-3 from its receptor site. However, the energy required to remove Hm-3 from NaV1.4 is much greater than from DmNaV1. We conclude that both Hm-3-bound channels are activated, but the activation is greatly retarded and positively shifted because of the energy required to dissociate Hm-3.

The observed differential activity of Hm-3 against NaV1.4 and DmNaV1 activation and steady-state inactivation (Fig. 5) may be interpreted accordingly. Hm-3 is hardly removed upon depolarization from NaV1.4, and a preconditioning pulse is required. The Hm-3-bound channels are not activated, and this is observed by the strong reduction in Na+ conductance. The activation curve is basically formed by non-bound channels showing control characteristics (Fig. 5C). Moreover, the bound Hm-3 interferes with the inactivation process and hampers the steady-state inactivation (Fig. 5D). On the contrary, Hm-3 is
more easily removed upon depolarization from DmNa\textsubscript{v}1. The activation curve is therefore a superposition of bound and unbound channels (Fig. 5A). Similarly, the steady-state inactivation curve is less affected due to the easy dissociation of the toxin (Fig. 5B).

It has been demonstrated for ProTx-II (25, 38), HWTX-IV (24), and JZTX-III (26) that they bind to receptor site 4. Judging by its activity, we suggest that Hm-3 might also bind to site 4. To unambiguously identify the potential site of interaction, structure-function experiments such as alanine scanning of Hm-3 together with site-directed mutagenesis of the expected site are needed.

One important issue about gating modifiers is their usually low specificity or promiscuity (39). As such, HWTX-IV not only affects Na\textsubscript{v} activation by binding to site 4 but also influences the inactivation by interacting with site 3 (40). In addition to Na\textsubscript{v}s, ProTx-I and -II also block T-type calcium channels (41), ProTx-I blocks TRPA1 (42), and JZTX-III was found to block voltage-gated potassium K\textsubscript{v}2.1 channels (43). It is therefore not surprising that Hm-3 shows quite a broad activity spectrum with respect to different Na\textsubscript{v}s.

### Hm-3 Shares Low Homology but Similar Fold with Other Spider Neurotoxins

We previously isolated three peptides from the crab spider 	extit{H. melloteei} called Hm-1, Hm-2, and Hm-3 (27, 44). The first two toxins were shown not to affect the activation or inactivation properties of sodium channels, thus presumably acting on receptor site 1. On the contrary, Hm-3, the subject of this study, shifted the voltage dependence of activation of Na\textsubscript{v}1.4 to more depolarized voltages (27). A search for potential homologs revealed that a number of toxins show only limited similarity to Hm-3. Among them are Hm-2 (44), \(\beta/\delta\)-agatoxins from Agelena orientalis (45), \(\mu\)-agatoxins from 	extit{Agelenopsis aperta} (46), and CSTX-17 from 	extit{Cupiennius salei} (47), and the level of their sequence similarity with Hm-3 is \(50-60\%\). Interestingly, \(\beta/\delta\)- and \(\mu\)-agatoxins also affect sodium channel activation albeit in a reversed manner: they promote channel activation similarly to scorpion \(\beta\)-toxins that bind to receptor site 4 (45).

**FIGURE 6. Voltage dependence of reversal of Hm-3-induced inhibition.** 
A and C, protocols for investigation of the voltage-dependent kinetics of reversal of toxin inhibition. B, the kinetics of sodium current increase as a result of a prolonged conditioning pulse at +100 mV as shown in A. Normalized current is plotted as a function of time. Data are presented for Na\textsubscript{v}1.4 (\(\Delta\)) and DmNa\textsubscript{v}1 (\(\triangledown\)). D and E, voltage dependence of reversal at +100 (\(\square\)), +80 (\(\bullet\)), and +40 mV (\(\triangleleft\)) for Na\textsubscript{v}1.4 (D) and DmNa\textsubscript{v}1 (E); the protocol is shown in C. Normalized current is plotted against conditioning pulse duration. F and G, voltage dependence of activation of toxin-modified Na\textsubscript{v}1.4 (F) and DmNa\textsubscript{v}1 (G) channels. Peak test pulse currents after 700-ms prepulses to various potentials from the applied protocol as described in C were measured and normalized to the sodium peak current achieved at +100 mV. These normalized peak currents are plotted as a function of prepulse potential (\(\square\)) and fitted with a Boltzmann relationship. Control activation curves (\(\square\)) are shown for comparison. Voltages of half-maximal activation (\(V_{1/2}\)) are shown in the panels. Error bars represent S.E.
**Sodium Channel Toxin from Crab Spider**

Hm-3 --GIAKNKEDSNGKKKDAG--SKEKRKLIV-------

Classical potassium channel activation inhibitors

VsTx1 --EEREGKKN-SNDK-DVSSR--WSDAEPS--

HaTx1 --EERLEFGKDR--TTSSDHKRLR-GKFRD--FRRWDDFTPS

SGTix1 --TERELFGKDR--TATTDKHLLR-AGKSDG--FWAVDDG--

Sodium channel activation inhibitors

ProTx-4 --EERLEFGKDR--AGQGRK--KSESRH--WYMDGTFTS

ProTx-II --YKQKWTDK--SDEKSRGEL--VRQ--WEKKLW--

HWTx-IV --ELRLEKSN--SDKNDKSKSRLVSTKRT--KWRV--

JZTX-III --DGELGKFRDK--KPRGKPKGKGY--ASKTSLAWVEP--

CooTx-1 --DGELGKFRDK--KNKDKKNY--TSKRDR--KYVDK--

CooTx-2 --DGELGKFRDK--KNKDKKNY--TSKRDR--KRYYLY--

PauTx3 --DGELGKFRDK--SNKDNKRPN--LVSSRD--KWKYQI--

**FIGURE 7.** Amino acid sequence alignment of ion channel activation inhibitors from spider venom. Cysteine residues are shaded in gray. Residues constituting the hydrophobic clusters on the surface of toxins with known spatial structures are shaded in black.

Despite low sequence similarity to other known peptides, our NMR studies demonstrate that Hm-3 adopts the ICK (or knot-tin) fold (Fig. 3A), the most widespread fold in spider toxins (2, 48). Other sodium channel activation inhibitors as well as potassium channel activation inhibitors known from spiders (Figs. 3C and 7) also form the ICK fold. As a result of fold conservation, the disulfide-containing core of the Hm-3 molecule could be relatively well superimposed with ICK motifs from other spider toxins (Fig. 3C, upper row; root mean square deviation values calculated over the heavy atoms of six conserved Cys residues are in the range from 0.8 to 1.5 Å). Despite the Hm-3 overall fold being similar to that of other gating modifiers, notable differences can be spotted. Some loops that connect the cysteine residues contributing to the conventional ICK signature are of different length in Hm-3 and other ion channel activation inhibitors from spiders. (i) The most remarkable difference is loop 4 (between the last two cysteine residues, CysV and CysX), which is at least four residues longer in Hm-3. Consequently, the β-hairpin is larger in Hm-3 compared with other gating modifiers. (ii) The C terminus, on the contrary, is very short in Hm-3, containing only one residue following the last cysteine. Moreover, the distribution of physicochemical properties on the surface of Hm-3 is different from that in other toxins (see below).

**Hm-3 Molecule Is Amphiphilic**—All sodium channel activation inhibitors from spiders with a known three-dimensional structure present a Janus-faced molecular surface (23, 42, 49) (Fig. 3C), and analysis of Hm-3 spatial structure has shown that it is not an exception. One Hm-3 face contains a ridge of hydrophobic residues formed by Trp11, Phe12, Trp16, Tyr25, Ile27, and Leu31, whereas the opposite face comprises charged residues Lys5, Lys7, Glu8, Glu15, Lys28, Arg29, and Lys32 (Fig. 3C). The hydrophobic residues forming the ridge are located in loops 2 (CysII-CysIII) and 4 (CysIV-CysV) (Fig. 7). Other well studied ion channel activation inhibitors from spiders (Fig. 7) present a conserved pattern containing hydrophobic residues in loops 1 (CysI-CysII) and 4 (CysIV-CysV) and the C terminus that donate to the hydrophobic cluster (Fig. 3C). This pattern is also found in potassium channel activation inhibitors from spiders (50–52) (Fig. 7) but differs from the distribution of key hydrophobic residues in the sequence of Hm-3. This leads to a completely different localization of the hydrophobic clusters on the Hm-3 surface as compared with other spider toxins. Indeed, superimposition of the toxin molecules by the conserved ICK motif revealed that the hydrophobic face of Hm-3 lies on the opposite side to the hydrophobic faces of “classical” gating modifiers (Fig. 3C).

**Hm-3 Action Is Membrane-mediated**—Experiments on Hm-3 partitioning into lipid membranes showed the toxin affinity for liposomes, which is characteristic of ion channel activation inhibitors from spiders (Figs. 2B and 3D). Hm-3 bound to both neutral and negatively charged lipid vesicles, and the affinity was high (Table 3). To compare, (i) VsTx1 does not bind to POPC in 150 mM NaCl (Kp > 0.002 × 10^3 M⁻¹) (52), although it binds to 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine/POPG (3:1) in 150 mM KCl (Kp ~ 2 × 10^3 M⁻¹) (53) and POPG in 150 mM NaCl (Kp ~ 2 × 10^3 M⁻¹) (52). (ii) SGTx1 binds to POPC (Kp ~ 0.1 × 10^3 M⁻¹), POPG/POPC (1:1) (Kp ~ 62 × 10^3 M⁻¹), and POPG/POPC (1:1) in 100 mM KCl (Kp ~ 2 × 10^3 M⁻¹) and POPG/POPC (1:1) in 100 mM KCl and 1 mM Ca²⁺ (Kp ~ 0.35 × 10^3 M⁻¹) (54). (iii) HaTx1 binds to POPG/POPC (1:1) (Kp ~ 87 × 10^3 M⁻¹), POPG/POPC (1:1) in 100 mM KCl and 1 mM Ca²⁺ (Kp ~ 0.87 × 10^3 M⁻¹) (54). (iv) ProTx-II and scorpion site 4 toxin CssII also bind to lipid giant unilamellar vesicles (phosphatidylcholine/phosphatidylserine, 3:1), although the Kp was not measured (55). (v) In this work, Hm-3 was shown to bind to POPC/DOPG (3:1) (Kp ~ 12.5 × 10^3 M⁻¹), POPC/DOPG (3:1) in 150 mM NaCl (Kp ~ 8.6 × 10^3 M⁻¹), and POPC in 150 mM NaCl (Kp ~ 9.5 × 10^3 M⁻¹).

The fact that Hm-3 binding to charged liposomes moderately depends on salt concentration indicates that electrostatic interactions are involved in the formation of the Hm-3-membrane complex only partially. At the same time, a comparatively high Hm-3 affinity to both charged and neutral liposomes under physiologically relevant conditions (in salt solution) means that hydrophobic interactions play the major role in the binding.

It is commonly hypothesized that the observed affinity of ion channel activation inhibitors to lipids underlies their mode of action, the so-called “membrane access” mechanism (53). According to this hypothesis, the toxin first binds to the cell membrane with its hydrophobic patch, then drifts to its target (voltage-gated ion channel), and finally binds to a voltage sensor, thereby freezing the channel in the resting state. For a long time, the S3–S4 linker of domain II (site 4) was thought to represent the sole binding site for activation inhibitors, but new data demonstrate that the situation is more complicated and that voltage sensors from other domains could also be involved (56, 57). Taking all the presented data on Hm-3 activity into consideration, we believe that it adds to the diversity of spider venom peptides inhibiting sodium channels and featuring the membrane access mode of action.

**Evolutionary Considerations**—We note that whereas H. meloloei belongs to the Araneomorphae suborder all other spider toxins with a known spatial structure that inhibit sodium channel activation (see Fig. 7) were purified from species belonging to the Mygalomorphae suborder. The following possibilities may be considered. (i) “Pure” divergence may have occurred, i.e.
mutation of a primordial gating modifier toxin gene that originated in a common ancestor of mygalo- and araneomorphs. In this case, it is hard to explain the marked difference in the amino acid sequence and the location of the hydrophobic cluster in Hm-3 and gating modifiers from mygalomorphs. (ii) “Pure” convergence may have occurred, i.e. independent evolution of ICK toxins targeting Na\textsubscript{v}s in mygalo- and araneomorphs. However, spider ICK toxins are currently believed to have a common ancestor. (iii) We therefore propose that parallel evolution took place. Based on our current grasp of spider toxin diversity, we assume the following evolutionary scenario: common ancestor of ICK toxins → divergence of ICK toxins in Mygalomorphae and Araneomorphae → parallel evolution of ion channel gating modifiers featuring the membrane access mechanism.

Acknowledgments—We thank Drs. Bert Billen (KU Leuven) and Anton Nikolsky (Abbott Laboratories) for kind help to start the work on Hm peptides.

REFERENCES

1. Estrada, G., Villegas, E., and Corzo, G. (2007) Spider venoms: a rich source of acrypolymaines and peptides as new leads for CNS drugs. Nat. Prod. Rep. 24, 145–161
2. Vassilevski, A. A., Kozlov, S. A., and Grishin, E. V. (2009) Molecular diversity of spider venom. Biochemistry 74, 1505–1534
3. Grishin, E. V., Volkova, T. M., Arsen’ev, A. S., Reshetova, O. S., and Onoprienko, V. V. (1986) Structural-functional characteristics of argiope— the ion channel blockers from the spider Arigope lobata venom. Bioorg. Khim. 12, 1121–1124
4. Schroeder, F. C., Taggi, A. E., Gronquist, M., Malik, R. U., Grant, J. B., Eisner, T., and Meinwald, J. (2008) NMR-spectroscopic screening of spider venom reveals sulfated nucleosides as major components for the brown recluse and related species. Proc. Natl. Acad. Sci. U.S.A. 105, 14283–14287
5. Grishin, E. V. (1998) Black widow spider toxins: the present and the future. Toxicon 36, 1693–1701
6. da Silva, P. H., da Silveira, R. B., Appel, M. H., Mangilli, O. C., Gremski, W., and Veiga, S. S. (2004) Brown spiders and loxoscelism. Toxicon 44, 693–709
7. Escobas, P. (2006) Molecular diversification in spider venoms: a web of combinatorial peptide libraries. Mol. Divers. 10, 545–554
8. King, G. F. and Hardy, M. C. (2013) Spider-venom peptides: structure, pharmacology, and potential for control of insect pests. Annu. Rev. Entomol. 58, 475–496
9. Kuhn-Nentwig, L. (2003) Antimicrobial and cytolytic peptides of venomous arthropods. Cell. Mol. Life Sci. 60, 2651–2668
10. French, R. J., Yoshikami, D., Sheets, M. F., and Olivera, B. M. (2010) The tetrodotoxin receptor of voltage-gated sodium channels—perspectives from interactions with micro-conotoxins. Mar. Drugs 8, 2153–2161
11. Silva, A. O., Peigneur, S., Diniz, M. R., Tytgat, J., and Beira˜o, P. S. (2012) Inhibitory effect of the recombinant Phormia nigriclava Tx1 toxin on voltage-gated sodium channels. Biochimie 94, 2756–2763
12. Catterall, W. A., Castéle, S., Yarov-Yarovov, V., Yu, F. H., Konoki, K., and Scheuer, T. (2007) Voltage-gated ion channels and gating modifier toxins. Toxicon 49, 124–141
13. Castéle, S., Qu, Y., Rogers, J. C., Rochat, H., Scheuer, T., and Catterall, W. A. (1998) Voltage sensor-trapping: enhanced activation of sodium channels by β-scorpion toxin bound to the S3-S4 loop in domain II. Neuron 21, 919–931
14. Bosmans, F., and Tytgat, J. (2007) Voltage-gated sodium channel modulation by scorpion a-toxins. Toxicon 49, 142–158
15. Moran, Y., Gordon, D., and Gurevitz, M. (2009) Sea anemone toxins affecting voltage-gated sodium channels—molecular and evolutionary features. Toxicon 54, 1089–1101
16. de Lima, M. E., Stankiewicz, M., Hamon, A., de Figueiredo, S. G., Cordeiro, M. N., Diniz, C. R., Martin-Eauclaire, M., and Pelhate, M. (2002) The toxin TX4(6-1) from the spider Phoneutria nigriventer slows down Na\textsubscript{v} current inactivation in insect CNS via binding to receptor site 3. J. Insect. Physiol. 48, 53–61
17. Little, M. J., Wilson, H., Zappa, C., Castéle, S., Tyler, M. I., Martin-Eauclaire, M. F., Gordon, D., and Nicholson, G. M. (1998) A-Tetractoxotins from Australian funnel-web spiders compete with scorpion α-toxin binding on both rat brain and insect sodium channels. FEBS Lett. 439, 246–252
18. Corzo, G., Escobas, P., Villegas, E., Karbat, I., Gordon, D., Gurevitz, M., Nakajima, T., and Gilles, N. (2005) A spider toxin that induces a typical effect of scorpion α-toxins but competes with β-toxins on binding to insect sodium channels. Biochemistry 44, 1542–1549
19. Pedraza Escalona, M., and Possani, L. D. (2013) Scorpion β-toxins and voltage-gated sodium channels: interactions and effects. Front. Biosci. 18, 572–587
20. Corzo, G., Gilles, N., Satake, H., Villegas, E., Dai, L., Nakajima, T., and Haupt, J. (2003) Distinct primary structures of the major peptide toxins from the venom of the spider Macrotelutes gigas that bind to sites 3 and 4 in the sodium channel. FEBS Lett. 547, 43–50
21. Middleton, R. E., Warren, V. A., Kraus, R. L., Hwang, J. C., Liu, C. J., Dai, G., Brochu, R. M., Kohler, M. G., Gao, Y. D., Garsky, V. M., Boguski, M. J., Mehl, J. T., Cohen, C. J., and Smith, M. M. (2002) Two tarantula peptides inhibit activation of multiple sodium channels. Biochemistry 41, 14734–14747
22. Xiao, Y., Tang, J., Yang, Y., Wang, M., Hu, W., Xie, J., Zeng, X., and Liang, S. (2004) Jingzhaoxin-III, a novel spider toxin inhibiting activation of voltage-gated sodium channel in rat cardiac myocytes. J. Biol. Chem. 279, 26220–26226
23. Peng, K., Shu, Q., Liu, Z., and Liang, S. (2002) Function and solution structure of huwentoxin-IV, a potent neuronal tetrodotoxin (TTX)-sensitive sodium channel antagonist from Chinese bird spider Selenocosmia huwena. J. Biol. Chem. 277, 47564–47571
24. Xiao, Y., Bingham, J. P., Zhu, W., Moczylidowski, E., Liang, S., and Cummins, T. R. (2008) Tarantula huwentoxin-IV inhibits neuronal sodium channels by binding to receptor site 4 and trapping the domain II voltage sensor in the closed configuration. J. Biol. Chem. 283, 27300–27313
25. Sokolov, S., Kraus, R. L., Scheuer, T., and Catterall, W. A. (2008) Inhibition of sodium channel gating by trapping the domain II voltage sensor with protoxin II. Mol. Pharmacol. 73, 1020–1028
26. Rong, M., Chen, J., Tao, H., Wu, Y., Jiang, P., Lu, M., Su, H., Chi, Y., Cai, T., Zhao, L., Zeng, X., Yao, X., and Liang, S. (2011) Molecular basis of the tarantula toxin jingzhaoxin-III (β-TTX-Cj1α) interacting with voltage sensors in sodium channel subtype Nav1.5. FASEB J. 25, 3177–3185
27. Nikolsky, A. S., Billen, B., Vassilevski, A. A., Filkin, S. Y., Tytgat, J., and Grishin, E. V. (2009) Voltage-gated sodium channels are targets for toxins from the venom of the spider Heriaeus melloteei. Biochem. (Mosk.) Suppl. Ser. A Membr. Cell Biol. 3, 245–253
28. Shlyapnikov, Y. M., Andreev, Y. A., Kozlov, S. A., Vassilevski, A. A., and Grishin, E. V. (2008) Bacterial production of latacin 2a, a potent anti-microbial peptide from spider venom. Protein Expr. Purif. 60, 89–95
29. Andreev, Y. A., Kozlov, S. A., Vassilevski, A. A., and Grishin, E. V. (2010) Cyanogen bromide cleavage of proteins in salt and buffer solutions. Anal. Biochem. 407, 144–146
30. Cavanagh, J. (2007) Protein NMR Spectroscopy: Principles and Practice, 2nd Ed., Academic Press, Amsterdam
31. Delaglio, F., Wu, Z., and Bax, A. (2001) Measurement of homonuclear proton couplings from regular 2D COSY spectra. J. Magn. Reson. 149, 276–281
32. Güntert, P. (2004) Automated NMR structure calculation with CYANA. Methods Mol. Biol. 278, 353–378
33. Liman, E. R., Tytgat, J., and Hess, P. (1992) Subunit stoichiometry of a mammalian K+ channel determined by construction of multimeric cDNAs. Neuron 9, 861–871
34. Peigneur, S., Billen, B., Derua, R., Waelkens, E., Debaveye, S., Béress, L., and Tytgat, J. (2011) A bifunctional sea anemone peptide with Kunitz type protease and potassium channel inhibiting properties. Biochem. Pharma-
Sodium Channel Toxin from Crab Spider

504 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 290 • NUMBER 1 • JANUARY 2, 2015

35. Bosmans, F., Rash, L., Zhu, S., Dicobot, S., Lazdunski, M., Escoubas, P., and Tytgat, J. (2006) Four novel tarantula toxins as selective modulators of voltage-gated sodium channel subtypes. *Mol. Pharmacol.* 69, 419–429

36. Catterall, W. A. (1977) Membrane potential-dependent binding of scorpion toxin to the action potential Na⁺ ionophore. Studies with a toxin derivative prepared by lactoperoxidase-catalyzed iodination. *J. Biol. Chem.* 252, 8660–8668

37. Phillips, L. R., Milescu, M., Li-Smerin, Y., Mindell, J. A., Kim, J. I., and Zamponi, G. W. (2014) Block of voltage-gated sodium channel subtypes. *J. Biol. Chem.* 289, 36129–36139

38. McIntosh, L. A., Dicobot, S., Lazdunski, M., and Zamponi, G. W. (2007) Voltage-gated sodium channel subtypes. *Mol. Pharmacol.* 72, 714–721

39. Redaelli, E., Cassulini, R. R., Silva, D. F., Clement, H., Schiavon, E., Zamuolle, S., and Schaller, J. (2005) Two novel sodium channel inhibitors from *Heriaeus melloteei* venom. *FEBS J.* 272, 4130–4142

40. Xiao, Y., Jackson, J. O., 2nd, Liang, S., and Cummins, T. R. (2011) Common molecular determinants of tarantula huwentoxin-IV inhibition of Na⁺ channel voltage sensors in domains II and IV. *J. Biol. Chem.* 286, 27301–27310

41. Bladen, C., Hamid, J., Souza, I. A., and Zamponi, G. W. (2014) Block of T-type calcium channels by protoxins I and II. *Mol. Brain* 7, 36

42. Gui, J., Liu, B., Cao, G., Lipchik, A. M., Perez, M., Dekan, Z., Mobli, M., Daly, N. L., Alewood, P. F., Parker, L. L., King, G. F., Zhou, Y., Jortd, S. E., and Nitabach, M. N. (2014) A tarantula-venom peptide antagonizes the TRPA1 nociceptor ion channel by binding to the S1-S4 gating domain. *Curr. Biol.* 24, 473–483

43. Tao, H., Chen, J. J., Xiao, Y. C., Wu, Y. Y., Su, H. B., Li, D., Wang, H. Y., Deng, M. C., Wang, M. C., Liu, Z. H., and Liang, S. P. (2013) Analysis of the interaction of tarantula toxinjingzhaotoxin-III (β-TnTX-Cj1α) with the voltage sensor of Kv2.1 uncovers the molecular basis for cross-activities on Kv2.1 and Nav1.5 channels. *Biochemistry* 52, 7439–7448

44. Billen, B., Vossilevski, A., Nikolsky, A., Tytgat, J., and Grishin, E. (2008) Two novel sodium channel inhibitors from *Heriaeus melloteei* spider venom differentially interacting with mammalian channel's isoforms. *Toxicon* 52, 309–317

45. Billen, B., Vossilevski, A., Nikolsky, A., Dehaveye, S., Tytgat, J., and Grishin, E. (2010) Unique bell-shaped voltage-dependent modulation of Na⁺ channel gating by novel insect-selective toxins from the spider *Agelena orientalis*. *J. Biol. Chem.* 285, 18545–18554

46. Skinner, W. S., Adams, M. E., Quistad, G. B., Kataoka, H., Cesarin, B. J., Enderlin, F. E., and Schooley, D. A. (1989) Purification and characterization of two classes of neurotoxins from the funnel web spider, *Agelenopsis aperta*. *J. Biol. Chem.* 264, 2150–2155

47. Trachsel, C., Siegemund, D., Kämpfer, U., Kopp, L. S., Bähr, C., Grossmann, J., Lüthi, C., Cunningham, M., Nentwig, W., Kuhn-Nentwig, L., Schürch, S., and Schaller, I. (2012) Multicomponent venom of the spider *Cupiennius salei*: a bioanalytical investigation applying different strategies. *FEBS J.* 279, 2683–2694

48. Escoubas, P., and Rash, L. (2004) Tarantulas: eight-legged pharmacists and combinatorial chemists. *Toxicon* 43, 555–574

49. Liao, Z., Yuan, C., Peng, K., Xiao, Y., and Liang, S. (2007) Solution structure of jingzhaotoxin-III, a peptide toxin inhibiting both Nav1.5 and Kv2.1 channels. *Toxicon* 50, 135–143

50. Takahashi, H., Kim, J. I., Min, H. J., Sato, K., Swartz, K. J., and Shimada, I. (2000) Solution structure of hanatoxin1, a gating modifier of voltage-dependent K⁺ channels: common surface features of gating modifier toxins. *J. Mol. Biol.* 297, 771–780

51. Lee, C. W., Kim, S., Roh, S. H., Endoh, H., Kodera, Y., Maeda, T., Kohno, T., Wang, J. M., Swartz, K. J., and Kim, J. I. (2004) Solution structure and functional characterization of SGTx1, a modifier of Kv2.1 channel gating. *Biochemistry* 43, 890–897

52. Jung, H. J., Lee, J. Y., Kim, S. H., Eu, Y. J., Shin, S. Y., Milescu, M., Swartz, K. J., and Kim, J. I. (2005) Solution structure and lipid membrane partitioning of VSTx1, an inhibitor of the KvAP potassium channel. *Biochemistry* 44, 6015–6023

53. Lee, S. Y., and MacKinnon, R. (2004) A membrane-access mechanism of ion channel inhibition by voltage sensor toxins from spider venom. *Nature* 430, 232–235

54. Milescu, M., Vobecký, J., Roh, S. H., Kim, S. H., Jung, H. J., Kim, J. I., and Swartz, K. J. (2007) Tarantula toxins interact with voltage sensors within lipid membranes. *J. Gen. Physiol.* 130, 497–511

55. Smith, J. J., Alphy, S., Seibert, A. L., and Blumenthal, K. M. (2005) Differential phospholipid binding by site 3 and site 4 toxins. Implications for structural variability between voltage-sensitive sodium channel domains. *J. Biol. Chem.* 280, 11127–11133

56. Smith, J. J., Cummins, T. R., Alphy, S., and Blumenthal, K. M. (2007) Molecular interactions of the gating modifier toxin ProTx-II with NaV 1.5: implied existence of a novel toxin binding site coupled to activation. *J. Biol. Chem.* 282, 12687–12697

57. Bosmans, F., Martin-Eauclaire, M. F., and Swartz, K. J. (2008) Deconstructing voltage sensor function and pharmacology in sodium channels. *Nature* 456, 202–208