



# Australian funnel-web spiders evolved human-lethal $\delta$ -hexatoxins for defense against vertebrate predators

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Australian funnel-web spiders are infamous for causing human fatalities, which are induced by venom peptides known as  $\delta$ -hexatoxins ( $\delta$ -HXTXs). Humans and other primates did not feature in the prey or predator spectrum during evolution of these spiders, and consequently the primate lethality of  $\delta$ -HXTXs remains enigmatic. Funnel-web envenomations are mostly inflicted by male spiders that wander from their burrow in search of females during the mating season, which suggests a role for  $\delta$ -HXTXs in self-defense since male spiders rarely feed during this period. Although 35 species of Australian funnel-web spiders have been described, only nine  $\delta$ -HXTXs from four species have been characterized, resulting in a lack of understanding of the ecological roles and molecular evolution of  $\delta$ -HXTXs. Here, by profiling venom-gland transcriptomes of 10 funnel-web species, we report 22  $\delta$ -HXTXs. Phylogenetic and evolutionary assessments reveal a remarkable sequence conservation of  $\delta$ -HXTXs despite their deep evolutionary origin within funnel-web spiders, consistent with a defensive role. We demonstrate that  $\delta$ -HXTX-Ar1a, the lethal toxin from the Sydney funnel-web spider *Atrax robustus*, induces pain in mice by inhibiting inactivation of voltage-gated sodium ( $\text{Na}_v$ ) channels involved in nociceptive signaling.  $\delta$ -HXTX-Ar1a also inhibited inactivation of cockroach  $\text{Na}_v$  channels and was insecticidal to sheep blowflies. Considering their algogenic effects in mice, potent insecticidal effects, and high levels of sequence conservation, we propose that the  $\delta$ -HXTXs were repurposed from an initial insecticidal predatory function to a role in defending against nonhuman vertebrate predators by male spiders, with their lethal effects on humans being an unfortunate evolutionary coincidence.

venom | evolution | spider

Despite their fearsome reputation, only a few species of spiders can cause death or serious harm to humans (1). An infamous exception is the Australian funnel-web spider, arguably the world's deadliest spider (2). These spiders produce extraordinarily complex venoms, with each venom containing up to several thousand peptide toxins (3, 4). Despite this chemical complexity, a single family of toxins known as the  $\delta$ -hexatoxins ( $\delta$ -HXTXs) is responsible for the human envenomation syndrome (5). There are currently 35 described species of Australian funnel-web spiders and 38 species of related non-Australian funnel-web spiders in the genus *Macrothele*, but to date only 12  $\delta$ -HXTX sequences have been reported from six species within this broad clade. A homologous  $\delta$ -actinopodotoxin ( $\delta$ -AOTX) is present in the venom of the related Australian mouse spider *Misulena bradleyi* (6), which can cause serious human envenomations with symptoms resembling those from funnel-web spider bites (7).

$\delta$ -HXTXs and  $\delta$ -AOTX comprise 42 to 44 residues and contain four disulfide bonds, three of which are arranged in an inhibitor cystine knot (ICK) motif (8, 9).  $\delta$ -HXTXs slow the inactivation of vertebrate tetrodotoxin-sensitive voltage-gated sodium ( $\text{Na}_v$ ) channels and insect  $\text{Na}_v$  channels by binding to the voltage sensor in channel domain IV (10, 11). In human bite victims,  $\delta$ -HXTXs cause disturbances in respiration, blood pressure, and heart rate, followed by severe hypotension. Without treatment with commercial antivenom (5), fatalities can occur by respiratory and circulatory failure within a few hours of the bite (12). Interestingly, in striking contrast to humans and other primates, some vertebrates such as dogs and cats are insensitive to funnel-web envenomation (13).

Humans did not feature in the prey or predator spectrum during evolution of funnel-web spiders, as primates were not

## Significance

The venom of Australian funnel-web spiders contains  $\delta$ -hexatoxins ( $\delta$ -HXTXs) that exert fatal neurotoxic effects in humans by inhibiting inactivation of voltage-gated sodium channels, but their precise ecological role remains unclear. Sequencing of venom-gland transcriptomes from 10 funnel-web species uncovered 22  $\delta$ -HXTXs. Evolutionary analysis revealed extreme conservation of these toxins, despite their ancient origin. We isolated the lethal  $\delta$ -HXTX from venom of the Sydney funnel-web spider and showed that it induces pain in mice, suggesting a role in predator deterrence. Although humans are not the target of  $\delta$ -HXTXs, these toxins likely evolved to deter vertebrate predators commonly encountered by these spiders, such as bandicoots, birds, and lizards. Thus, the lethal potency of  $\delta$ -HXTXs against humans is an unfortunate evolutionary coincidence.

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The authors declare no competing interest.

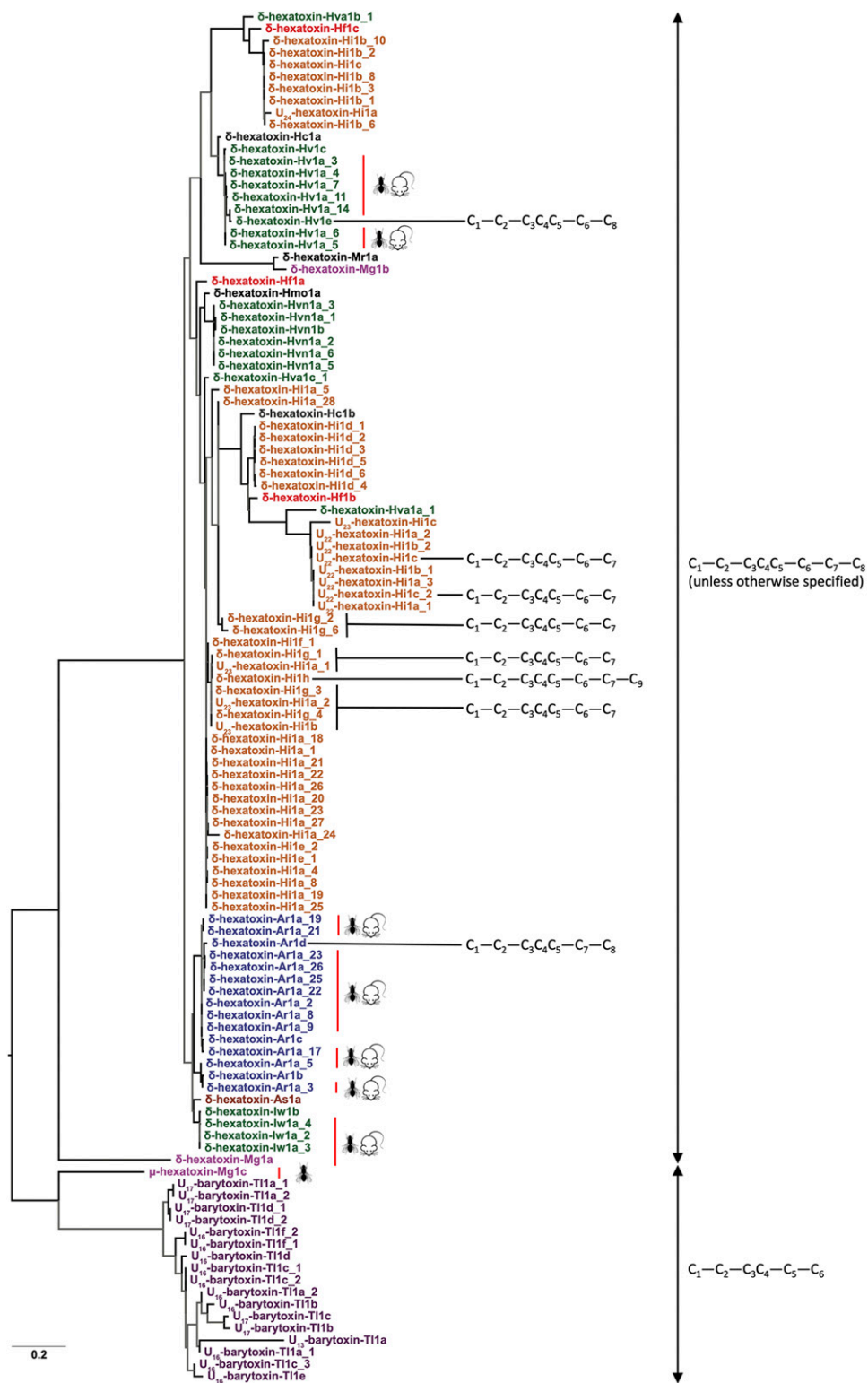
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**Fig. 1.** Phylogenetic reconstruction of  $\delta$ -HXTXs. Tree representing the phylogenetic history of  $\delta$ -HXTXs as estimated by maximum-likelihood inference. The thickness of branches corresponds to node supports (thick branch, bootstrap  $\geq 75$ ; thin branch, bootstrap  $< 75$ ), and various species are presented in distinct colors. The cysteine pattern (black lines) and activities (red lines) are annotated (*Right*). Fly and mouse icons indicate insecticidal and vertebrate activity, respectively. The underscore sequence numbers refer to the unique preprotoxin nomenclature used in the ArchnoServer database (23).

present 150 to 200 million y ago (MYA) when these spiders originated (14). Thus, the underlying reason for the peculiar susceptibility of humans to  $\delta$ -HXTXs and the ecological role of these toxins remain enigmatic. The  $\delta$ -HXTXs are insecticidal

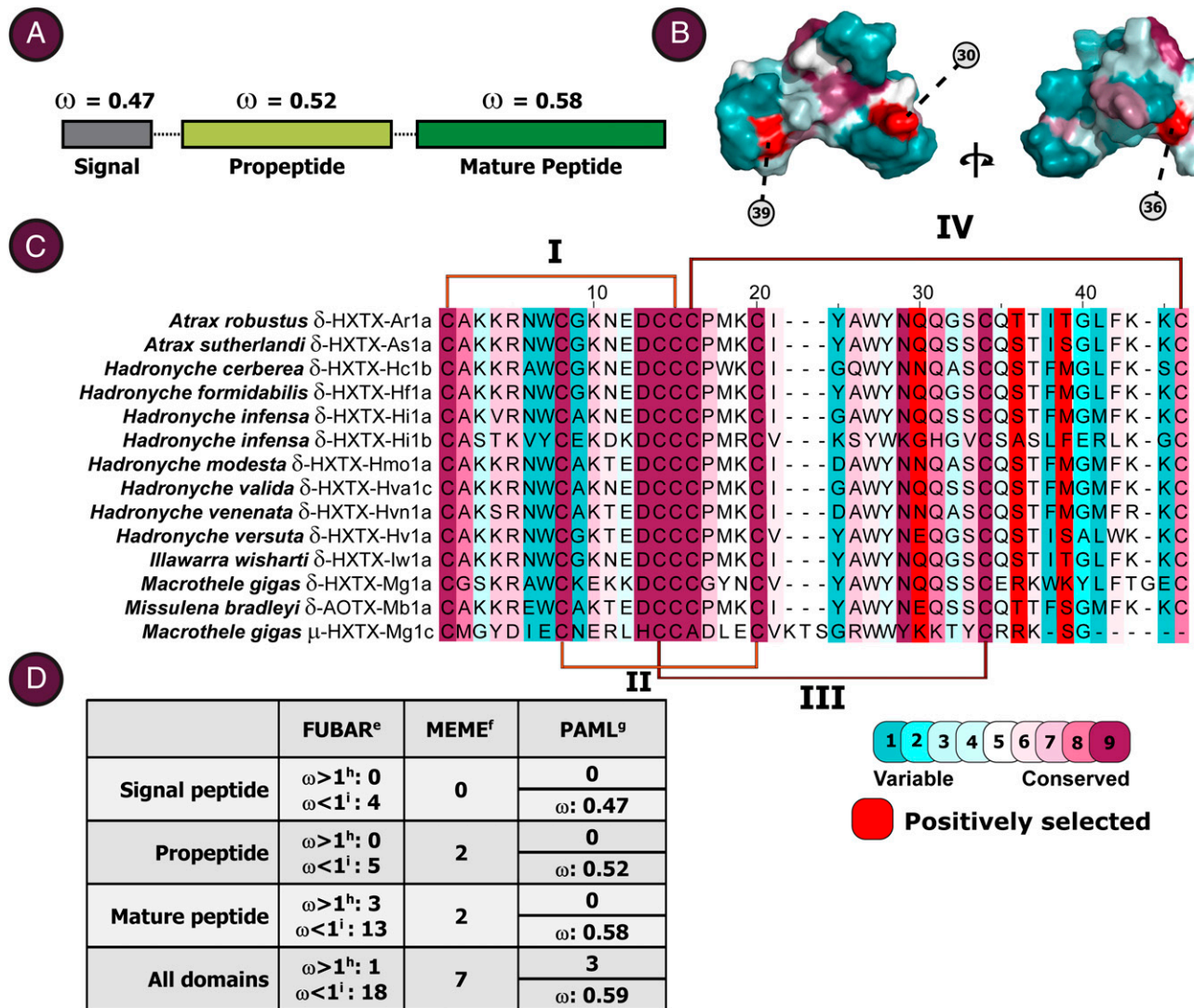
(15, 16), which might suggest a role in prey capture. However, in some species, these toxins are secreted in very low abundance in the venoms of female spiders and immature males, consistent with the fact that only sexually mature male spiders cause severe

or lethal human envenomations (17). Moreover, it is hard to reconcile a role for these toxins in predation given that sexually mature males, in whose venom the toxins are most abundant, rarely feed during the mating season. Rather, the fact that adult males leave the safety of their burrows to search for female spiders (2), making them more susceptible to predators, suggests a role for the  $\delta$ -HXTXs in predator deterrence. A well-documented strategy for defensive toxins is to induce pain (18–20), and pain is a common symptom following funnel-web envenomation (2, 17). Consistent with the idea that the  $\delta$ -HXTXs serve a defensive role by inducing pain in vertebrate predators, Magi 4 from the venom of a Japanese funnel-web spider potentiates the activity of  $\text{Na}_V1.1$  and  $\text{Na}_V1.6$  (21), which are involved in pain signaling (19, 22).

In the current study, we identified 22  $\delta$ -HXTX sequences from 10 species of Australian funnel-web spiders, and evaluated their molecular evolution, phylogenetic histories, insecticidal activity, and potency against human  $\text{Na}_V$  channels involved in pain signaling. Taken together, our data provide strong evidence that the  $\delta$ -HXTXs were recruited by funnel-web spiders as a weapon to deter vertebrate predators, and that their lethal effects on humans is an unfortunate evolutionary coincidence.

## Results

**$\delta$ -HXTX Sequences.**  $\delta$ -HXTXs from seven funnel-web spider species (*Hadronyche infensa*, *Hadronyche valida*, *Hadronyche venenata*, *Hadronyche versuta*, *Atrax robustus*, *Atrax sutherlandi*, and *Illawarra wisharti*) were sequenced via rapid amplification of



**Fig. 2.** Molecular evolution of  $\delta$ -HXTXs. (A) Toxin precursor domains and their rate of evolution, indicated as  $\omega$  values (i.e., nonsynonymous-to-synonymous substitution rate ratio). (B) Structure of  $\delta$ -HXTX-Hv1a (Protein Data Bank ID code 1VTX) (8), depicting the locations of positively selected sites. (C) Sequence alignment of  $\delta$ -HXTXs from representative species. The positions of the four disulfide bonds are indicated by lines above and below the sequence alignment, while the extent of evolutionary conservation of amino acids (calculated from the complete alignment of 114 sequences) is denoted by the illustrated color code. Note: Loop IV is not present in the plesiotypic *T. loki* sequence and is instead characteristic of the apotypic  $\delta$ -HXTX sequences. (D) Statistics associated with selection analyses. <sup>e</sup>Fast unconstrained Bayesian approximation (FUBAR); <sup>f</sup>sites experiencing episodic diversifying selection (0.05 significance) by the mixed-effects model evolution (MEME); <sup>g</sup>positively selected sites detected by the Bayes empirical Bayes approach implemented in M8 of Phylogenetic Analysis by Maximum Likelihood (PAML); <sup>h</sup>number of sites under pervasive diversifying selection at posterior probability  $\geq 0.95$  (FUBAR); <sup>i</sup>number of sites under pervasive purifying selection at posterior probability  $\geq 0.95$  (FUBAR).

cDNA (complementary DNA) ends (RACE) (*SI Appendix*). In addition, we generated cDNA libraries for *H. infensa*, *Hadronyche modesta*, *Hadronyche cerbera*, and *Hadronyche formidabilis*. Our complete dataset (*Dataset S1*), based on our RACE and transcriptomic data plus sequences available from the literature, comprised 169 mature peptide sequences and 167 corresponding nucleotide sequences (the latter were only missing for  $\delta$ -HXTX-Hv1b and  $\delta$ -AOTX-Mb1a). Our dataset contained 132  $\delta$ -HXTXs, 1  $\delta$ -AOTX, and 18 homologous U-HXTXs from *H. infensa*, 17 related barytoxins (U-BATXs) from *Tritame loki*, and 1  $\mu$ -HXTX from *Macrothele gigas*. Removal of 53 duplicate or incomplete sequences and the two peptide sequences that lacked a corresponding nucleotide sequence yielded a total of 114 nucleotide sequences for phylogenetic analysis. The data revealed 22 mature  $\delta$ -HXTX sequences to complement the 12 published  $\delta$ -HXTX sequences.

**Phylogenetic History and Molecular Evolution of  $\delta$ -HXTXs.** The identification of homologous sequences from the barychelid spider *T. loki* in this study reveals that these U-BATXs,  $\mu$ -HXTX-Mg1c, and the  $\delta$ -HXTXs were probably derived from a common ancestral toxin. However, there are also important differences between the U-BATXs and  $\mu$ -HXTX-Mg1c in comparison with all  $\delta$ -HXTXs from atracrid and macrothelid funnel-web spiders. First, U-BATXs and  $\mu$ -HXTX-Mg1c have only six Cys residues that form an ICK motif, whereas the  $\delta$ -HXTXs usually contain eight Cys residues, with one additional disulfide bond. Second, the  $\delta$ -HXTXs comprise a rare triplet of Cys residues in positions 14 to 16 (based on  $\delta$ -HXTX-Ar1a), which is not present in  $\mu$ -HXTX-Mg1c or any of the U-BATXs. Moreover, in the Bayesian phylogenetic analyses, all funnel-web spider sequences clustered together and were well-separated from the *T. loki* sequences (Fig. 1). All of these considerations taken together highlight the divergent evolution of  $\delta$ -HXTXs in atracrid and macrothelid funnel-web spiders following their phylogenetic separation from other mygalomorph lineages (Fig. 1).

In contrast to the massive sequence variations observed in most venom proteins, the  $\delta$ -HXTXs are surprisingly well-conserved (Fig. 2). The signal peptide, propeptide, and mature peptide coding regions in the  $\delta$ -HXTXs were characterized by extremely low omega ( $\omega$ ) values (i.e., the ratio of non-synonymous to synonymous substitutions) (Fig. 2*A* and Table 1), indicative of strong sequence conservation despite being recruited into the venoms of funnel-web spiders around 150 to 200 MYA (14). Consistent with the findings of the maximum-likelihood method, the Bayesian approaches identified only three positively selected sites (2.6% of sites)—positions that rapidly diversify across time—while many sites (15.6%) were found to be evolving under the pervasive influence of negative selection, which ensures sequence conservation across time. A

few amino acid sites ( $n = 7$ ) were found to be experiencing diversifying selection, albeit in an episodic fashion (i.e., in short bursts across time) (Fig. 2*B–D*).

**Cys Derivations and Codon Usage.** The three-dimensional structure of many venom peptides, including the  $\delta$ -HXTXs, relies heavily on the formation of disulfide bonds. Analysis of the arrangement of eight Cys residues in the  $\delta$ -HXTXs (Fig. 3*A*) indicates that Cys residues 1 to 5 are extremely well conserved in all funnel-web spiders. However, many subtypes were discovered with either missing or novel cysteines, indicating the probable evolution of novel forms and functions. For instance, while one derivation each was found with a missing cysteine at positions 6 and 7,  $\delta$ -HXTXs lacking Cys-8 were more common with 11 derivations in  $\delta$ -HXTXs from *H. infensa*, with one of them even possessing a novel cysteine residue (which we refer to as position 9). The precursor peptides with the eighth Cys residue missing correspond to four mature toxin sequences, and comparison with combined matrix-assisted laser desorption/ionization (MALDI) and Orbitrap mass spectrometry (MS) data for *H. infensa* venom (4) reveals closely matching molecular masses for three of the monomers, one homodimer, and four heterodimers (Fig. 3*B*). Analysis of the codons used for Cys residues also revealed some genus-specific variations (Fig. 3*C*). In all funnel-web  $\delta$ -HXTXs, Cys-2 is encoded by TGT, whereas Cys-3 is encoded by TGC. In *Atrax* and *Illawarra*, all other Cys residues are encoded only by TGT (except for Cys-8 in *Illawarra* being solely encoded by TGC). In *Hadronyche*, Cys-1, 5, 6, 7, and 8 are dominated by TGT and Cys-4 is preferentially encoded by TGC. In contrast to all other funnel-web spider genera, in *Macrothele* all Cys residues (except for Cys-2) are dominantly encoded by TGC. The observed codon bias could be important for the rapid expression of toxins in the venom gland.

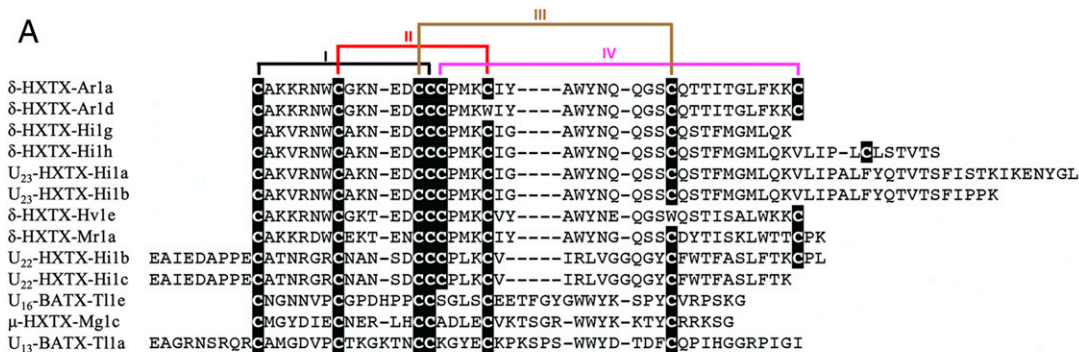
**Na<sub>v</sub> Channel Subtype Selectivity of  $\delta$ -HXTX-Ar1a.** In order to examine the biological role of the  $\delta$ -HXTXs, we used reversed-phase high-performance liquid chromatography (RP-HPLC) to isolate  $\delta$ -HXTX-Ar1a (hereafter Ar1a) from the venom of *A. robustus* (Fig. 4). We then used a Fluorescent Imaging Plate Reader (FLIPR)-based fluorescence assay to assess the ability of Ar1a to potentiate currents from human Na<sub>v</sub>1.1 to Na<sub>v</sub>1.8 channels stably expressed in HEK293 cells. This assay was previously used to determine the pharmacological activity of the scorpion venom peptide OD1. OD1 activates several Na<sub>v</sub> channel subtypes and the potency and subtype selectivity of OD1 as determined by FLIPR was found to be comparable to data obtained using electrophysiological assays (24).

Ar1a had no effect on Na<sub>v</sub>1.4, Na<sub>v</sub>1.5, Na<sub>v</sub>1.7, and Na<sub>v</sub>1.8 at concentrations up to 150 nM, whereas a concentration-dependent

**Table 1. Collection sites and species used for venom-gland library construction**

Species	Collection site	Number and sex of specimens	Collector
<i>Atrax robustus</i>	Sydney, NSW	1 M/1 F	Graham Nicholson
<i>Atrax sutherlandi</i>	Gerringong, NSW	1 M	D.T.R.W.
<i>Hadronyche infensa</i>	Fraser Island and Toowoomba, QLD	5 F (RACE)	D.T.R.W.
<i>Hadronyche infensa</i>	Fraser Island and Toowoomba, QLD	3 F (454)	S.S.P.
<i>Hadronyche modesta</i>	Kalorama, Dandenong Ranges, VIC	4 F	B.G.F.
<i>Hadronyche valida</i>	Binna Burra, QLD	1 juvenile M	D.T.R.W.
<i>Hadronyche venenata</i>	Tooms Lake, TAS	1 F	Glenn Gregg
<i>Hadronyche versuta</i>	Blue Mountains, NSW	1 F	Graham Nicholson
<i>Hadronyche formidabilis</i>	South Tamborine, QLD	1 F/4 juveniles	S.D.
<i>Hadronyche cerbera</i>	Blue Mountains, NSW	2 F	S.D.
<i>Illawarra wisharti</i>	Gerringong, NSW	1 M	D.T.R.W.

F, female; M, male.



**B**

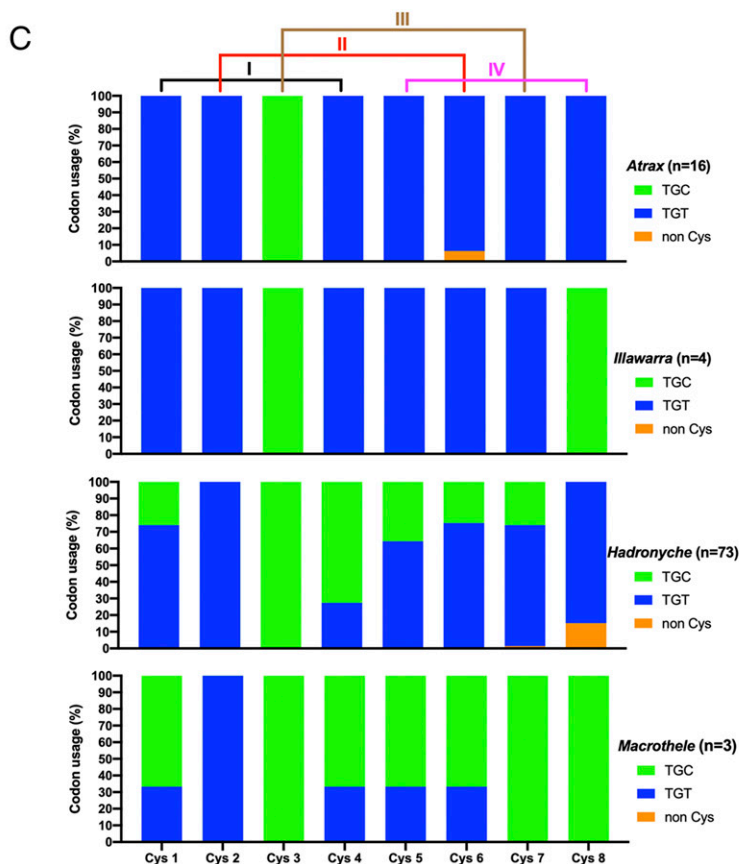
Monomers	Calculated (Da)	Observed (Da)	
U <sub>22</sub> -HXTX-Hi1c	5450.468	5451.09	
U <sub>23</sub> -HXTX-Hi1a	7513.534	7512.077	7515.85
delta-HXTX-Hi1g	4686.977	4685.82	4687.941

Homodimers	Calculated (Da)	Observed (Da)
U <sub>23</sub> -HXTX-Hi1a x U <sub>23</sub> -HXTX-Hi1a	15025.068	15025.29

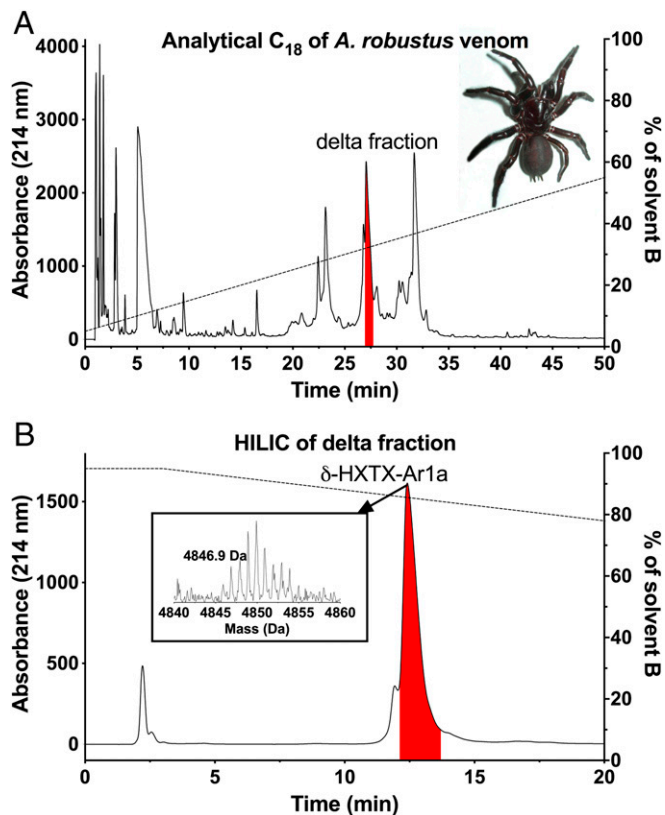
Heterodimers	Calculated (Da)	Observed (Da)
U <sub>23</sub> -HXTX-Hi1b x U <sub>22</sub> -HXTX-Hi1c	12150.595	12149.65
delta-HXTX-Hi1g x U <sub>22</sub> -HXTX-Hi1c	10135.445	10134.3
delta-HXTX-Hi1g x U <sub>23</sub> -HXTX-Hi1a	12198.511	12197.7
delta-HXTX-Hi1g x U <sub>23</sub> -HXTX-Hi1b	11387.104	11384.13



**Fig. 3.** Cys derivations and codon usage in delta-HXTXs. (A) Alignment of delta-HXTXs representing major changes in the Cys framework. (B) Expected and experimentally determined molecular masses for the seven Cys residue derivations of delta-HXTX homologs from *H. infensa*. Several masses closely matching either monomers or homo/heterodimers were observed using MALDI or Orbitrap mass spectrometry. (C) Codon usage for Cys residues in delta-HXTXs from different funnel-web genera. Lines at the top represent the four disulfide bonds in the prototypical Ar1a toxin.

potentiation of veratridine-evoked responses was observed in cells expressing Na<sub>V</sub>1.1, Na<sub>V</sub>1.2, Na<sub>V</sub>1.3, and Na<sub>V</sub>1.6 (Fig. 5A). Ar1a was an equipotent potentiator of Na<sub>V</sub>1.1 (half-maximum effective concentration (EC<sub>50</sub>) of 30.2 nM), Na<sub>V</sub>1.2 (EC<sub>50</sub> 38.9 nM),

Na<sub>V</sub>1.3 (EC<sub>50</sub> 38.9 nM), and Na<sub>V</sub>1.6 (EC<sub>50</sub> 91.2 nM) with comparable mean pEC<sub>50</sub> (i.e., negative logarithm of the EC<sub>50</sub>) values of 7.52 ± 0.24, 7.41 ± 0.09, 7.41 ± 0.15, and 7.04 ± 0.08, respectively.



**Fig. 4.** Isolation and purification of Ar1a from *A. robustus* venom. (A) Chromatogram from  $C_{18}$  RP-HPLC fractionation of *A. robustus* venom with the peak containing Ar1a highlighted in red. (A, Inset) Photo of a female *A. robustus*. (B) Chromatogram from hydrophobic interaction liquid chromatography (HILIC) of the Ar1a-containing peak from *A.* The dashed line indicates the solvent gradient, with the percentage of solvent B (90% acetonitrile/0.045% trifluoroacetic acid) indicated on the right ordinate axis. (B, Inset) MALDI-MS spectrum of purified Ar1a.

**Mammalian Nocifensive Responses.** Administration of Ar1a (100 nM, 20  $\mu$ L) by shallow subcutaneous (intraplantar) injection into the foot pad of male C57BL/6 mice elicited mild, transient nocifensive responses consisting of flinching, lifting, licking, and shaking of the hind paw. This nocifensive behavior was characterized by relatively slow onset (5 min postinjection) to a peak at 15 min postinjection with  $10.5 \pm 1.5$  flinches per 5 min. No systemic effects, such as the muscle fasciculations, salivation, or other effects associated with the human envenomation syndrome, were observed at this dose.

**Toxicity of Ar1a in Blowflies.** Injection of Ar1a into sheep blowflies (*Lucilia cuprina*) caused contractile paralysis with a median paralytic dose (PD<sub>50</sub>) of  $319 \pm 42$  pmol/g at 1 h postinjection (Fig. 5B). However, even at the highest dose tested (which was limited by the amount of native  $\delta$ -HXTX-Ar1a available), all flies fully recovered within 24 h, indicating that the toxin's insecticidal effects in blowflies are reversible.

**Effect of Ar1a on BgNa<sub>v</sub>1.** Given its activity on human Na<sub>v</sub> channels, we decided to examine if the insecticidal effects of Ar1a are due to potentiation of the activity of insect Na<sub>v</sub> channels. For this purpose, we examined the effects of Ar1a on the BgNa<sub>v</sub>1 channel from the German cockroach *Blattella germanica* expressed in *Xenopus* oocytes. At 1 nM, Ar1a had no effect on BgNa<sub>v</sub>1 currents whereas 10 nM Ar1a induced a substantial persistent current (Fig. 5C). BgNa<sub>v</sub>1 fast inactivation was

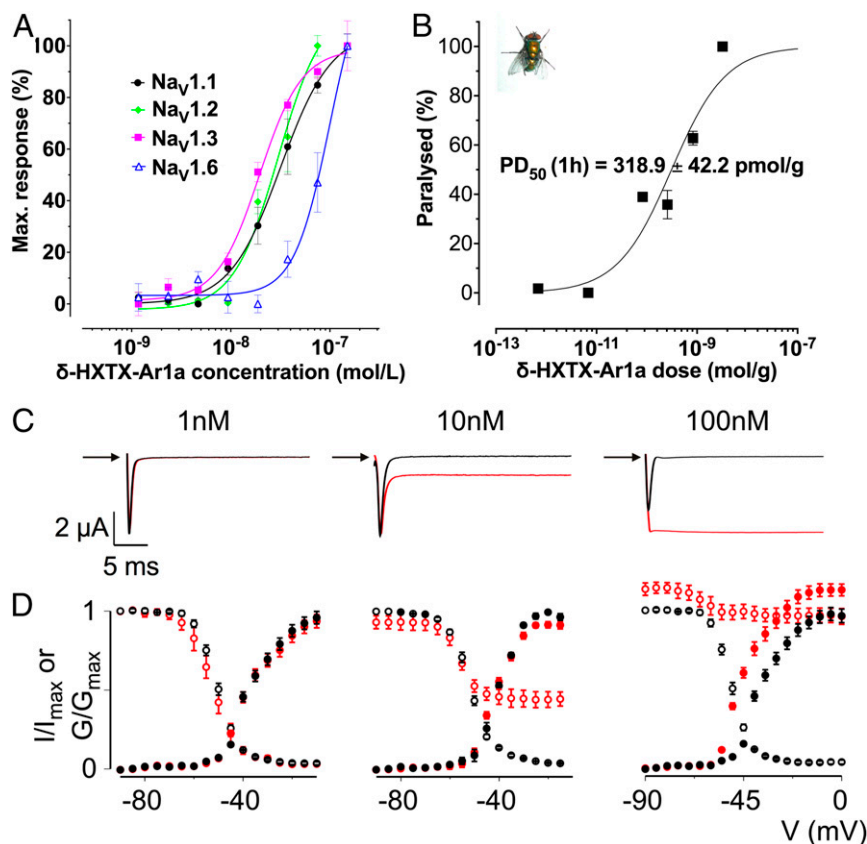
completely inhibited by 100 nM Ar1a. Boltzmann fits of the normalized conductance–voltage relationships and steady-state inactivation relationships revealed no significant difference in  $V_{1/2}$  before and after addition of 1 or 10 nM Ar1a ( $t$  test,  $P \geq 0.01$ ) but 100 nM Ar1a caused substantial hyperpolarizing shifts in the  $V_{1/2}$  of both channel activation (from  $-37.2 \pm 0.6$  to  $-44.9 \pm 0.7$  mV) and steady-state inactivation (from  $-50.3 \pm 0.1$  to  $-63.3 \pm 0.7$  mV) (Fig. 5D).

## Discussion

In the present study, we employed a multipronged approach involving venom-gland transcriptomics, molecular and phylogenetic analyses, and functional assays to determine the role of the lethal  $\delta$ -HXTXs in the ecology of funnel-web spiders.

**Enigmatic Evolutionary Conservation of  $\delta$ -HXTXs.** Molecular evolutionary assessments revealed that the genes encoding for  $\delta$ -HXTXs have remained nearly unchanged despite originating in the common ancestor of atracid and macrothelid funnel-web spiders 150 to 200 MYA (14). Together with our phylogenetic analysis, this indicates that despite the single early origin of  $\delta$ -HXTXs in funnel-web spiders, they have diversified at a much slower evolutionary rate than many other spider toxins (25–29). The increased level of sequence conservation is consistent with a role for the  $\delta$ -HXTXs in defense. Due to their relatively limited use and consequent exclusion from the typical Red Queen mode of competitive evolution, defensive toxins are theorized to evolve slower than their predatory counterparts (30).

Our phylogenetic analysis demonstrates that all  $\delta$ -HXTX sequences evolved from a common ancestral toxin scaffold, with early gene duplications and diversification present before the recently proposed split into the families Macrothelidae and Atracidae (31). Further support of a split between these families is provided by the nucleotide sequences used to encode the Cys residues, with Atracidae being dominated by TGT and Macrothelidae being dominated by TGC. Our phylogeny also provides evidence for at least two neofunctionalization events. The first event occurred during the early evolution of  $\delta$ -HXTXs.  $\mu$ -HXTX-Mg1c, the most basal of the funnel-web spider sequences included in our analysis, has an ICK motif but lacks the two Cys residues involved in the formation of the fourth disulfide bond (32).  $\mu$ -HXTX-Mg1c is a homolog of  $\mu$ -HXTX-Mg1a and  $\mu$ -HXTX-Mg1b, which are both known to be insecticidal but not active against vertebrates (33). On the other hand,  $\delta$ -HXTX-Mg1a, which is sister to the remaining  $\delta$ -HXTX sequences, has both vertebrate and insecticidal activities (33). These activities would be consistent with a repurposing of  $\delta$ -HXTXs from the purely insecticidal activity of their ancestral plesiotypic form to dual activity against both mammals and insects, which occurred around 150 to 200 MYA (14). Given the absence of primates in Australia at the time when  $\delta$ -HXTXs originated [humans first populated Australia 65,000 y ago (34), and Australia lacks indigenous nonhuman primates], the primate toxicity of  $\delta$ -HXTXs can only be regarded as coincidental. The second neofunctionalization event is apparent in the genus *Hadronyche* with multiple convergent losses of the last Cys residue. Such convergence points toward a strong selection pressure, possibly resulting in a change in selectivity or potency. The fact that an odd number of Cys residues is energetically unfavored led us to investigate whether these seven Cys residue derivations of  $\delta$ -HXTXs form dimers. Mass spectrometry analysis of *H. infensa* venom (4) revealed the presence of masses corresponding to monomers and homo- and heterodimers. Unfortunately, nothing is yet known about the activities of these  $\delta$ -HXTX derivations or the dimers that are formed, which provides an exciting area for future investigations.



**Fig. 5.** Biological characterization of Ar1a. (A) Na<sub>v</sub> subtype selectivity of Ar1a. The effect of Ar1a on Na<sub>v</sub>1.1 to Na<sub>v</sub>1.8 heterologously expressed in HEK293 cells in combination with the human b1 subunit was assessed using a membrane potential assay. Ar1a elicited a concentration-dependent increase in membrane potential in the presence of veratridine (5 μM) in cells expressing Na<sub>v</sub>1.1 (pEC<sub>50</sub> 7.52 ± 0.24), Na<sub>v</sub>1.2 (pEC<sub>50</sub> 7.41 ± 0.09), Na<sub>v</sub>1.3 (pEC<sub>50</sub> 7.41 ± 0.15), and Na<sub>v</sub>1.6 (pEC<sub>50</sub> 7.04 ± 0.08). Data are presented as mean ± SEM (*n* = 3). (B) Dose–response curve for paralysis of *L. cuprina* blowflies (shown in *Inset*) injected with Ar1a. Paralysis was assessed at 1 h postinjection. Error bars indicate SEM. The PD<sub>50</sub> was determined as the mean ± SEM of three independent experiments. All paralytic effects were reversible within 24 h and no lethal effects were observed. (C) Dose-dependent inhibition of BgNa<sub>v</sub>1 fast inactivation by Ar1a. Representative sodium currents were elicited by a depolarization to –20 mV before (black) and after (red) addition of toxin from a holding potential of –90 mV. (D) Normalized conductance–voltage relationships ( $G/G_{max}$ ; black filled circles) and steady-state inactivation relationships ( $I/I_{max}$ ; black open circles) of BgNa<sub>v</sub>1 before (black circles) and after (red circles) Ar1a application. Channel-expressing oocytes were depolarized in 5-mV steps from a holding potential of –90 mV. Error bars represent SEM; *n* = 3 to 5.

**Clues from the Activity of Ar1a.** δ-HXTXs were previously demonstrated to inflict potent but reversible paralysis in blowfly larvae and crickets (16). We found that Ar1a potently inhibits fast inactivation of the cockroach BgNa<sub>v</sub> channel, which is consistent with the contractile paralysis induced in blowflies and reminiscent of the effects of other toxins that target insect Na<sub>v</sub> channels (reviewed in ref. 35).

Employing venom components to cause pain is a common evolutionary strategy for self-defense in venomous animals (19, 30, 36–38). Strong support for a defensive role of δ-HXTXs is therefore provided by the nocifensive response that Ar1a induced following intraplantar injections in mice. Although the lethal dose of δ-HXTXs varies considerably within vertebrates (13), we presume that high local tissue concentrations of δ-HXTXs resulting from a funnel-web spider bite will induce algogenic effects in a much wider range of vertebrates than half-maximum lethal dose (LD<sub>50</sub>) experiments might indicate.

With regard to the subtype selectivity of δ-HXTXs, δ-HXTX-Mg1a (Magi 4) from the Japanese funnel-web spider preferentially activated rat Na<sub>v</sub>1.1 and Na<sub>v</sub>1.3 and mouse Na<sub>v</sub>1.6 while also showing weak activity on rat Na<sub>v</sub>1.2 channels (3). For Ar1a, we observed equipotent activity across Na<sub>v</sub>1.1, Na<sub>v</sub>1.2, Na<sub>v</sub>1.3, and Na<sub>v</sub>1.6. Overall, this is consistent with a defensive role, as both Na<sub>v</sub>1.1 and Na<sub>v</sub>1.6 are known to be involved in pain

signaling (19, 22, 39). The activity of Ar1a at Na<sub>v</sub>1.6 is further consistent with the observed effects of Australian funnel-web venoms in the chick biventer assay (*SI Appendix, Fig. S1*) (40), as Na<sub>v</sub>1.6 is the predominant isoform at the nodes of Ranvier in motor neurons. Thus, inhibition of the inactivation of this Na<sub>v</sub> channel isoform could contribute to both sensory and motor effects in envenomed individuals, making δ-HXTXs a powerful weapon to deter predators.

**Differential Expression of δ-HXTXs.** The clinical syndrome resulting from funnel-web spider envenomation of vertebrates is driven by the δ-HXTXs (5). Male *A. robustus* venom was reported to be at least six times more potent than the female venom (41). In addition, the venoms of six male funnel-web species were found to be more potent than females' in inducing toxic effects in the chick biventer nerve-muscle preparation (40), consistent with increased expression of δ-HXTXs in male venoms. Male funnel-web spiders are more exposed to vertebrate predation once they leave the safety of their burrows to search for female mates, so increased expression of a defensive toxin would make ecological sense to allow adult males to defend against these predators. Moreover, since adult male mygalomorph spiders consume less food than females (42), the increased expression of δ-HXTXs in

mature male spiders is inconsistent with a role for these toxins in prey capture.

## Conclusion

In summary, our data suggest that the  $\delta$ -HXTXs likely evolved from having an ancestral role in predation to a primary role in defense against ecologically important vertebrate predators, with their lethal potency against humans being an unfortunate evolutionary coincidence.

## Materials and Methods

Australian funnel-web spiders were collected from various locations and states across Australia, as summarized in Table 1. The spiders were individually housed at  $\sim 23$  to  $25^\circ\text{C}$  in dark cabinets until venom and venom glands were dissected.

**Nomenclature.** Toxins were named according to the rational nomenclature described previously (43). Spider taxonomy was taken from World Spider Catalog version 21.0 (44).

**Messenger RNA Isolation and cDNA Library Construction.** Messenger RNA and cDNA libraries were isolated and constructed using the protocols summarized in *SI Appendix*. For details of RACE, Sanger, and next-generation sequencing, see *SI Appendix*.

**Phylogenetics and Selection Analyses.** Reconstruction of the phylogenetic history and molecular evolution of  $\delta$ -HXTXs was performed as detailed in *SI Appendix*.

**RP-HPLC Purification of Ar1a.** Milked lyophilized venom from male *A. robustus* specimens was supplied by the Australian Reptile Park. The venom was reconstituted in MilliQ water to a concentration of  $\sim 5$  mg/mL and Ar1a was purified using RP-HPLC as outlined in *SI Appendix*.

**Determination of the  $\text{Na}_v$  Subtype Selectivity of Ar1a.** The activity of Ar1a on human  $\text{Na}_v$  channels stably expressed in HEK293 cells was determined using a FLIPR<sup>Tetra</sup> assay (24) as described in *SI Appendix*.

**Allogenic Effects of Ar1a.** Ethical approval for in vivo experiments was obtained from The University of Queensland Animal Ethics Committee

(PHARM/512/12/RAMACIOTTI) and they were conducted in accordance with the Queensland Animal Care and Protection Act (2002), the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (eighth edition, 2013), and the International Association for the Study of Pain Guidelines for the Use of Animals in Research. Details of intraplantar administration of Ar1a and assessment of induced nociceptive behavior are detailed in *SI Appendix*.

**Insecticidal Effects of Ar1a.** Native Ar1a was tested for insecticidal toxicity by injection into sheep blowflies (45) as described in *SI Appendix*.

**Activity of Ar1a on  $\text{BgNa}_v1$ .** Two-electrode voltage-clamp electrophysiology was used to determine the activity of Ar1a on  $\text{BgNa}_v1$  heterologously expressed in *Xenopus* oocytes, as outlined in *SI Appendix*.

**Data Availability.** Metadata and annotated nucleotide sequences generated in this paper were deposited in the European Nucleotide Archive under project accession nos. [PRJEB6062](#) for *H. infensa*, [PRJEB14734](#) for *H. cerberaea*, and [PRJEB14965](#) for *H. formidabilis*. All UniProt and ArachnoServer accessions for sequences used for phylogenetic analysis are listed in *SI Appendix*. Raw data have also been deposited in Figshare, 10.6084/m9.figshare.12798617.

All study data are included in the article and *SI Appendix*.

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1. T. J. Hauke, V. Herzig, Dangerous arachnids—Fake news or reality? *Toxicon* **138**, 173–183 (2017).
2. G. K. Isbister, H. W. Fan, Spider bite. *Lancet* **378**, 2039–2047 (2011).
3. P. Escoubas, B. Sollod, G. F. King, Venom landscapes: Mining the complexity of spider venoms via a combined cDNA and mass spectrometric approach. *Toxicon* **47**, 650–663 (2006).
4. S. S. Pineda *et al.*, Structural venomomics reveals evolution of a complex venom by duplication and diversification of an ancient peptide-encoding gene. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 11399–11408 (2020).
5. G. M. Nicholson, M. J. Little, M. Tyler, T. Narahashi, Selective alteration of sodium channel gating by Australian funnel-web spider toxins. *Toxicon* **34**, 1443–1453 (1996).
6. J. E. Bond, B. E. Hendrixson, C. A. Hamilton, M. Hedin, A reconsideration of the classification of the spider infraorder Mygalomorphae (Arachnida: Araneae) based on three nuclear genes and morphology. *PLoS One* **7**, e38753 (2012).
7. G. K. Isbister, Mouse spider bites (*Missulena* spp.) and their medical importance. A systematic review. *Med. J. Aust.* **180**, 225–227 (2004).
8. J. I. Fletcher, B. E. Chapman, J. P. Mackay, M. E. Howden, G. F. King, The structure of versutoxin ( $\delta$ -atractotoxin-Hv1) provides insights into the binding of site 3 neurotoxins to the voltage-gated sodium channel. *Structure* **5**, 1525–1535 (1997).
9. P. K. Pallaghy, K. J. Nielsen, D. J. Craik, R. S. Norton, A common structural motif incorporating a cystine knot and a triple-stranded  $\beta$ -sheet in toxic and inhibitory polypeptides. *Protein Sci.* **3**, 1833–1839 (1994).
10. S. J. Gunning *et al.*, Isolation of delta-missulenatoxin-Mb1a, the major vertebrate-acting spider delta-toxin from the venom of *Missulena bradleyi* (Actinopodidae). *FEBS Lett.* **554**, 211–218 (2003).
11. G. M. Nicholson, M. J. Little, L. C. Birinyi-Strachan, Structure and function of  $\delta$ -atractotoxins: Lethal neurotoxins targeting the voltage-gated sodium channel. *Toxicon* **43**, 587–599 (2004).
12. E. J. Mylecharane, I. Spence, D. D. Sheumack, R. Claessens, M. E. Howden, Actions of robustoxin, a neurotoxic polypeptide from the venom of the male funnel-web spider (*Atrax robustus*), in anaesthetized monkeys. *Toxicon* **27**, 481–492 (1989).
13. G. M. Nicholson, A. Graudins, Spiders of medical importance in the Asia-Pacific: Atractotoxin, latrotoxin and related spider neurotoxins. *Clin. Exp. Pharmacol. Physiol.* **29**, 785–794 (2002).
14. N. A. Ayoub, C. Y. Hayashi, “Spiders (Araneae)” in *The Timetree of Life*, S. B. Hedges, S. Kumar, Eds. (Oxford University Press, Oxford, UK, 2009), pp. 255–259.
15. F. Grolleau *et al.*, Electrophysiological analysis of the neurotoxic action of a funnel-web spider toxin,  $\delta$ -atractotoxin-HV1a, on insect voltage-gated  $\text{Na}^+$  channels. *J. Exp. Biol.* **204**, 711–721 (2001).
16. M. J. Little *et al.*,  $\delta$ -Atractotoxins from Australian funnel-web spiders compete with scorpion  $\alpha$ -toxin binding on both rat brain and insect sodium channels. *FEBS Lett.* **439**, 246–252 (1998).
17. G. K. Isbister *et al.*, Funnel-web spider bite: A systematic review of recorded clinical cases. *Med. J. Aust.* **182**, 407–411 (2005).
18. C. J. Bohlen, D. Julius, Receptor-targeting mechanisms of pain-causing toxins: How ow? *Toxicon* **60**, 254–264 (2012).
19. J. D. Osteen *et al.*, Selective spider toxins reveal a role for the  $\text{Na}_v1.1$  channel in mechanical pain. *Nature* **534**, 494–499 (2016).
20. J. V. Lin King *et al.*, A cell-penetrating scorpion toxin enables mode-specific modulation of TRPA1 and pain. *Cell* **178**, 1362–1374.e16 (2019).
21. N. Yamaji *et al.*, Synthesis, solution structure, and phylum selectivity of a spider delta-toxin that slows inactivation of specific voltage-gated sodium channel subtypes. *J. Biol. Chem.* **284**, 24568–24582 (2009).
22. M. R. Israel *et al.*,  $\text{Na}_v$  1.6 regulates excitability of mechanosensitive sensory neurons. *J. Physiol.* **597**, 3751–3768 (2019).
23. S. S. Pineda *et al.*, ArachnoServer 3.0: An online resource for automated discovery, analysis and annotation of spider toxins. *Bioinformatics* **34**, 1074–1076 (2018).
24. T. Durek *et al.*, Chemical engineering and structural and pharmacological characterization of the  $\alpha$ -scorpion toxin OD1. *ACS Chem. Biol.* **8**, 1215–1222 (2013).
25. J. E. Garb, C. Y. Hayashi, Molecular evolution of  $\alpha$ -latrotoxin, the exceptionally potent vertebrate neurotoxin in black widow spider venom. *Mol. Biol. Evol.* **30**, 999–1014 (2013).
26. R. A. Haney *et al.*, Effects of gene duplication, positive selection, and shifts in gene expression on the evolution of the venom gland transcriptome in widow spiders. *Genome Biol. Evol.* **8**, 228–242 (2016).
27. C. McCowan, J. E. Garb, Recruitment and diversification of an ecdysozoan family of neuropeptide hormones for black widow spider venom expression. *Gene* **536**, 366–375 (2014).



28. S. S. Pineda *et al.*, Diversification of a single ancestral gene into a successful toxin superfamily in highly venomous Australian funnel-web spiders. *BMC Genomics* **15**, 177 (2014).
29. E. A. B. Undheim *et al.*, A proteomics and transcriptomics investigation of the venom from the barychelid spider *Trittame loki* (brush-foot trapdoor). *Toxins (Basel)* **5**, 2488–2503 (2013).
30. N. R. Casewell, W. Wüster, F. J. Vonk, R. A. Harrison, B. G. Fry, Complex cocktails: The evolutionary novelty of venoms. *Trends Ecol. Evol.* **28**, 219–229 (2013).
31. M. Hedin, S. Derkarabetian, M. J. Ramirez, C. Vink, J. E. Bond, Phylogenomic reclassification of the world's most venomous spiders (Mygalomorphae, Atracinae), with implications for venom evolution. *Sci. Rep.* **8**, 1636 (2018).
32. D. J. Craik, N. L. Daly, C. Waine, The cystine knot motif in toxins and implications for drug design. *Toxicon* **39**, 43–60 (2001).
33. G. Corzo *et al.*, Distinct primary structures of the major peptide toxins from the venom of the spider *Macrothele gigas* that bind to sites 3 and 4 in the sodium channel. *FEBS Lett.* **547**, 43–50 (2003).
34. C. Clarkson *et al.*, Human occupation of northern Australia by 65,000 years ago. *Nature* **547**, 306–310 (2017).
35. G. F. King, P. Escoubas, G. M. Nicholson, Peptide toxins that selectively target insect Na<sub>v</sub> and Ca<sub>v</sub> channels. *Channels (Austin)* **2**, 100–116 (2008).
36. C. J. Bohlen *et al.*, A bivalent tarantula toxin activates the capsaicin receptor, TRPV1, by targeting the outer pore domain. *Cell* **141**, 834–845 (2010).
37. C. J. Bohlen *et al.*, A heteromeric Texas coral snake toxin targets acid-sensing ion channels to produce pain. *Nature* **479**, 410–414 (2011).
38. S. D. Robinson *et al.*, A comprehensive portrait of the venom of the giant red bull ant, *Myrmecia gulosa*, reveals a hyperdiverse hymenopteran toxin gene family. *Sci. Adv.* **4**, eaau4640 (2018).
39. J. R. Deus *et al.*, An animal model of oxaliplatin-induced cold allodynia reveals a crucial role for Na<sub>v</sub>1.6 in peripheral pain pathways. *Pain* **154**, 1749–1757 (2013).
40. A. Gaudins, D. Wilson, P. F. Alewood, K. W. Broady, G. M. Nicholson, Cross-reactivity of Sydney funnel-web spider antivenom: Neutralization of the in vitro toxicity of other Australian funnel-web (*Atrax* and *Hadronyche*) spider venoms. *Toxicon* **40**, 259–266 (2002).
41. S. K. Sutherland, J. Tibballs, *The Genera Atrax and Hadronyche, the Funnel-Web Spiders. Australian Animal Toxins*, (Oxford University Press, Melbourne, 2001).
42. V. Herzig, Ontogenesis, gender, and molting influence the venom yield in the spider *Coremiocnemis tropix* (Araneae, Theraphosidae). *J. Venom Res.* **1**, 76–83 (2010).
43. G. F. King, M. C. Gentz, P. Escoubas, G. M. Nicholson, A rational nomenclature for naming peptide toxins from spiders and other venomous animals. *Toxicon* **52**, 264–276 (2008).
44. World Spider Catalog, version number 21.0. <https://wsc.nmbe.ch>, (Natural History Museum Bern, 2020).
45. N. S. Bende *et al.*, The insecticidal neurotoxin Aps III is an atypical knottin peptide that potently blocks insect voltage-gated sodium channels. *Biochem. Pharmacol.* **85**, 1542–1554 (2013).