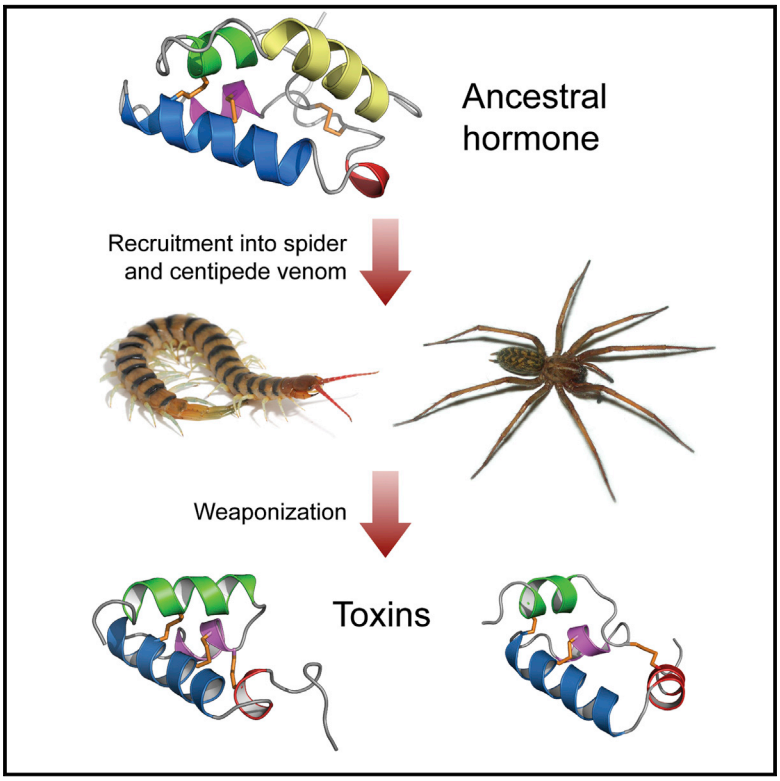
Article

Structure

Weaponization of a Hormone: Convergent Recruitment of Hyperglycemic Hormone into the Venom of Arthropod Predators

Graphical Abstract



Highlights

- ITP/CHH hormones were convergently recruited into the venom of spiders and centipedes
- Venom ITP/CHH peptides were weaponized via key structural adaptations to form toxins
- ITP/CHH-derived toxins are defined by a helical fold that is unique for venom proteins
- We report the first three-dimensional structure of any centipede protein

Authors

Eivind A.B. Undheim, Lena L. Grimm, Chek-Fong Low, ..., Greta J. Binford, Mehdi Mobli, Glenn F. King

Correspondence

glenn.king@imb.uq.edu.au (G.F.K.), m.mobli@uq.edu.au (M.M.)

In Brief

Undheim et al. introduce a new class of venom peptides and show that they are derived from an ancient family of hormones. These hormones were convergently recruited into spider and centipede venom and subsequently weaponized for life as a toxin via key structural adaptations.

Undheim et al., 2015, Structure *23*, 1283–1292
July 7, 2015
http://dx.doi.org/10.1016/j.str.2015.05.003

CellPress

Structure
Article

Weaponization of a Hormone: Convergent Recruitment of Hyperglycemic Hormone into the Venom of Arthropod Predators

Eivind A.B. Undheim,[1,2] Lena L. Grimm,[1] Chek-Fong Low,[1] David Morgenstern,[1] Volker Herzig,[1] Pamela Zobel-Thropp,[4] Sandy Steffany Pineda,[1] Rosaline Habib,[5] Slawomir Dziemborowicz,[5] Bryan G. Fry,[3] Graham M. Nicholson,[5] Greta J. Binford,[4] Mehdi Mobli,[1,2,*] and Glenn F. King[1,*]

[1]Institute for Molecular Bioscience, The University of Queensland, St Lucia, QLD 4072, Australia
[2]Centre for Advanced Imaging, The University of Queensland, St Lucia, QLD 4072, Australia
[3]School of Biological Sciences, The University of Queensland, St Lucia, QLD 4072, Australia
[4]Department of Biology, Lewis & Clark College, Portland, OR 97219, USA
[5]School of Medical & Molecular Biosciences, University of Technology Sydney, Broadway, NSW 2007, Australia
*Correspondence: glenn.king@imb.uq.edu.au (G.F.K.), m.mobli@uq.edu.au (M.M.)
http://dx.doi.org/10.1016/j.str.2015.05.003

SUMMARY

Arthropod venoms consist primarily of peptide toxins that are injected into their prey with devastating consequences. Venom proteins are thought to be recruited from endogenous body proteins and mutated to yield neofunctionalized toxins with remarkable affinity for specific subtypes of ion channels and receptors. However, the evolutionary history of venom peptides remains poorly understood. Here we show that a neuropeptide hormone has been convergently recruited into the venom of spiders and centipedes and evolved into a highly stable toxin through divergent modification of the ancestral gene. High-resolution structures of representative hormone-derived toxins revealed they possess a unique structure and disulfide framework and that the key structural adaptation in weaponization of the ancestral hormone was loss of a C-terminal α helix, an adaptation that occurred independently in spiders and centipedes. Our results raise a new paradigm for toxin evolution and highlight the value of structural information in providing insight into protein evolution.

INTRODUCTION

Arthropod venoms are complex chemical cocktails that are used for predation, defense, and competitor deterrence. Most of these venoms comprise a complex mixture of inorganic salts, biogenic amines, peptides, and proteins, but small peptides (<10 kDa) account for most of their molecular and functional diversity (King and Hardy, 2013; Liu et al., 2012; Rodriguez de la Vega et al., 2010; Yang et al., 2012). Consistent with their primary role in rapid immobilization of prey, most venom peptides target ion channels and receptors in the central or peripheral nervous system with an exceptionally high degree of potency and selectivity (King and Hardy, 2013; Terlau and Olivera, 2004). These peptides are generally stabilized by multiple intramolecular disulfide bonds, which impart high levels of chemical, thermal, and biological stability (Fry et al., 2009; Saez et al., 2010; Yang et al., 2013). As a result of their potency, selectivity, and stability, arthropod venom peptides have proved to be a rich source of pharmacological tools as well as potential therapeutics and insecticides (King, 2011, 2015; Klint et al., 2012; Smith et al., 2013). Surprisingly, however, very little is known about the evolutionary origin of arthropod venom peptides and, in particular, the structural adaptations that underlie their unique biophysical properties.

Toxin evolution is best understood in snakes, in which a variety of body proteins have been recruited into the venom, duplicated to produce multigene families, and then often subjected to extensive functional diversification (Fry et al., 2008, 2009). However, a major problem in reconstructing molecular evolutionary histories for cysteine-rich arthropod venom peptides is that the conserved disulfide framework directs the protein fold to such an extent that virtually all non-cysteine residues can be mutated during the process of neofunctionalization without perturbing the protein structure (Sollod et al., 2005). As a result, the sequences of toxins that have undergone extensive functional derivation during the course of hundreds of millions of years of evolution tend to contain little or no information that allows for identification of ancestral or convergently recruited proteins. In some cases this has led to divergent views on the molecular history of certain toxin classes (Smith et al., 2011; Sunagar et al., 2013). Moreover, this problem hampers our understanding of the unique structural adaptations in these toxins, and our ability to recapitulate these adaptations in vitro.

Recently, a family of spider-venom peptides were shown to belong to a ubiquitous ecdysozoan neuropeptide hormone family known as the ion transport peptide/crustacean hyperglycemic hormone (ITP/CHH) family (Gasparini et al., 1994; McCowan and Garb, 2013). ITP/CHH peptides have also been recruited into the venoms of wasps and ticks (McCowan and Garb, 2013). These venom peptides, the so-called latrodectins and homologs, are widespread in modern spiders (infraorder Araneomorphae), and it was proposed that they were

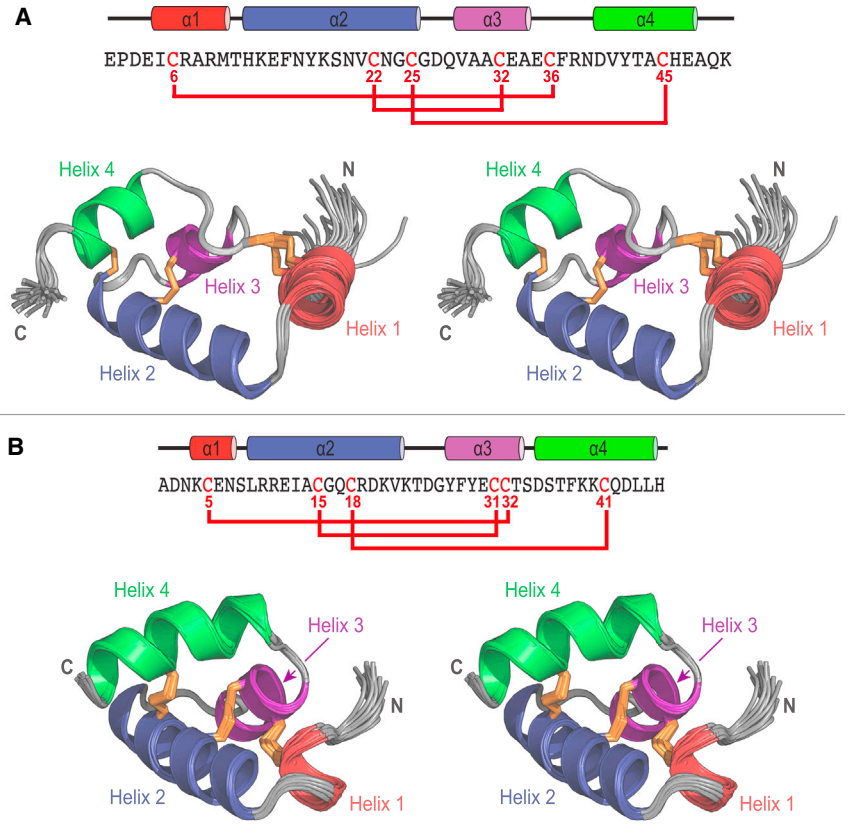


Figure 1. Structures of Ta1a and Ssm6a

Stereoview of the ensembles of Ta1a (PDB: 2KSL) and Ssm6a (PDB: 2MUN) structures overlaid over residues 4–49 and 4–45 are shown in the bottom panels of (A) and (B), respectively. Disulfide bonds are highlighted in orange and the four helices and N and C termini are labeled. The primary structures of Ta1a and Ssm6a illustrating the disulfide framework (red) and secondary structure elements (cylinders) are shown in the top panels of (A) and (B), respectively. See also Table S1 for structural statistics for both ensembles; Figures S1 and S2 for production, activity and stability of recombinant Ta1a; and Figures S3 and S4 for fully assigned 2D ^{1}H-^{15}N HSQC spectra of Ta1a and Ssm6a, respectively.

ancestrally recruited into araneomorph spider venom ~375 Ma (McCowan and Garb, 2013). The ITP/CHH family is functionally diverse and includes developmental hormones as well as peptides involved in osmoregulation and regulation of carbohydrate metabolism (Montagné et al., 2010). However, the functional role of latrodectins remains unclear as they are not insecticidal or toxic to mice (Gasparini et al., 1994; Grishin et al., 1993), nor do they produce hyperglycemic effects when injected into crab, crayfish, or shrimps (Soyez, 1997). Instead, it has been proposed that spider-venom ITP/CHH peptides serve as co-factors for larger, pore-forming proteins known as latrotoxins (Grishin et al., 1993).

The one exception among the apparently non-toxic spider-venom ITP/CHH peptides is TaITX-1, an insecticidal toxin produced in the venom of the common agelenid spider *Tegenaria agrestis* (Araneomorphae: Agelenidae) (Johnson et al., 1998). The recommended name for the toxin based on the rational nomenclature devised for venom peptides (King et al., 2008) is U_1-agatoxin-Ta1a (henceforth Ta1a). The toxin is remarkable for its high level of activity against lepidopteran crop pests and its direct effect on the insect CNS (Johnson et al., 1998). Although the molecular mechanism of the toxin is unknown, it induces a slow-onset excitatory phenotype that ultimately leads to an irreversible spastic paralysis (Johnson et al., 1998), suggesting that the target is likely to be an ion channel found exclusively, or at least primarily, in the CNS.

Here we show that Ta1a and a neurotoxin family found in venoms from members of the centipede genus *Scolopendra* represent convergent recruitments of ITP/CHH peptides into the toxin arsenals of these predatory arthropods. Furthermore, we show that these venom peptides have evolved remarkably convergent structural adaptations to attain exceptional stability and solubility, two properties characteristic of toxins. The hormone-derived centipede-venom peptides, in particular, are among the most stable peptides ever described, and hence they provide a novel three-dimensional scaffold for engineering drugs and insecticides and for understanding the atomic interactions underlying protein stability.

RESULTS

HAND Toxins: A New Class of Neuropeptide-Derived Venom Peptides

We produced recombinant versions of Ta1a and the recently described centipede-venom peptide μ-SLPTX$_3$-Ssm6a (henceforth Ssm6a; Undheim et al., 2014) using an *E. coli* periplasmic expression system optimized for production of disulfide-rich peptides (Klint et al., 2013) (Figure S1). Recombinant Ssm6a co-eluted with native Ssm6a as reported previously (Yang et al., 2013). Moreover, homonuclear nuclear magnetic resonance (NMR) data confirmed that native and recombinant Ssm6a have identical folds. For production of recombinant Ta1a, an additional N-terminal serine residue was added to improve TEV-protease cleavage efficiency (Kapust et al., 2002), and this prevented the use of co-elution by high-performance liquid chromatography (HPLC) to assess correct folding. The authenticity of the recombinant Ta1a was therefore verified by demonstrating that it is highly insecticidal as reported previously for the native toxin (Johnson et al., 1998) (Figure S2A).

The structures of Ta1a and Ssm6a were determined using heteronuclear NMR (Figure 1). Statistics highlighting the high precision and stereochemical quality of the structures are shown in Table S1, while fully assigned 2D ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectra for Ta1a and Ssm6a are shown in Figures S3 and S4, respectively. The high

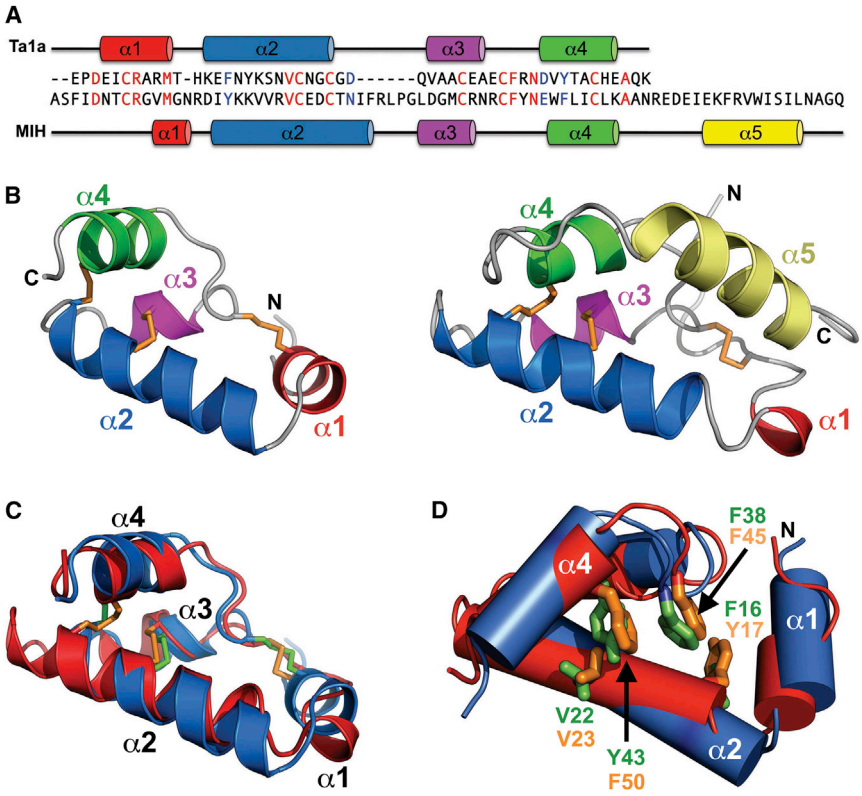


Figure 2. Comparison of the Structures of MIH and Ta1a

(A) Alignment of the amino acid sequences of Ta1a and MIH showing the location of α helices. Sequence identities are highlighted in red and conservative substitutions are shown in blue.

(B) Comparison of the 3D structures of Ta1a (left) and MIH (right), with topologically equivalent helices shown in the same color. Note the additional α5 helix in MIH.

(C) Overlay of the structures of MIH (red, with orange disulfides) and Ta1a (blue, with green disulfides). Note the topological equivalence of the four helices and the three disulfide bridges. The α5 helix in MIH has been removed for clarity.

(D) Overlay of the structures of MIH (red, with orange side chains) and Ta1a (blue, with green side chains) highlighting topologically equivalent hydrophobic residues that facilitate close packing of helices α1–α4. The structures have been rotated toward the viewer compared with the overlay shown in (C). See also Table S2 for a summary of the DALI statistics for the top five structural matches with Ta1a.

stereochemical quality of both ensembles stems from a complete absence of bad close contacts, high Ramachandran plot quality (>97% of residues in the most favored region), and very few unfavorable side-chain rotamers. The average MolProbity scores of 0.79 and 0.84 for Ta1a and Ssm6a, respectively, place both ensembles in the 100th percentile relative to other structures ranked by MolProbity.

Ta1a has an exclusively α-helical structure comprised of four helices, which we designated, from N to C terminus, helix 1 (Glu4–Met10), helix 2 (His12–Cys25), helix 3 (Val29–Ala34), and helix 4 (Asp40–Glu47). The four helices in Ta1a toxins are cross-braced by three disulfide bonds with C_1–C_5, C_2–C_4, C_3–C_6 disulfide connectivity (Figure 1A), which was unequivocally determined from NMR nuclear Overhauser effect spectroscopy (NOESY) data (Mobli and King, 2010). This disulfide framework is unique for spider toxins and it distinguishes the Ta1a fold from both the inhibitor cystine knot (ICK) and Kunitz-type venom peptides, which have C_1–C_4, C_2–C_5, C_3–C_6 and C_1–C_6, C_2–C_4, C_3–C_5 disulfide architectures, respectively (Saez et al., 2011; Yuan et al., 2008).

A search for structural homologs of Ta1a using DALI (Holm and Rosenstrom, 2010) yielded a close match to the structure of an ITP/CHH family member, namely molt-inhibiting hormone (MIH) from the Kuruma prawn *Marsupenaeus japonicus*. Despite an overall sequence identity of only 26% (Figure 2A), MIH can be aligned over the entire length of Ta1a with a backbone root-mean-square deviation (RMSD) of 2.2 Å and a statistically significant *Z* score of 3.8 (Figures 2B and 2C). There were no other statistically significant structural matches with Ta1a (Table S2). The structural homology between MIH and Ta1a confirms previous molecular evolutionary analyses indicating that Ta1a is a highly derived member of the ITP/CHH family (McCowan and Garb, 2013).

Ssm6a shares only 22% sequence identity with Ta1a (Figure 3A), but the two toxins are clearly structural homologs (Figures 1; Figures 3B and 3C). Optimal superposition of the two toxins yields a backbone RMSD of ~3.6 Å, and the four helices in Ssm6a, namely helix 1 (Lys4–Asn7), helix 2 (Leu9–Val22), helix 3 (Tyr27–Thr33), and helix 4 (Asp35–Leu45), align well with the corresponding helices in Ta1a. Moreover, the structure of Ssm6a aligns well that of MIH, with a backbone RMSD of ~2.3 Å, despite the low sequence identity of 22%.

The structural homology between Ssm6a, Ta1a, and MIH indicates that ITP/CHH family peptides have been convergently recruited into the venom of both spiders and centipedes. These strikingly helical peptides constitute a new structural class of venom toxins, which we refer to as helical arthropod-neuropeptide-derived (HAND) toxins. Structural comparisons (Figure 2) reveal that the four helices present in Ta1a and Ssm6a are conserved in almost identical locations in MIH. Strikingly, however, MIH has a C-terminal helix (α5), which is not found in either Ta1a or Ssm6a, making this a key structural difference between HAND toxins and the ancestral ITP/CHH peptides.

Phylogenetic Relationship between HAND Toxins and ITP/CHH Peptides

To date, ITP/CHH peptides have been described in the venoms of only a few araneomorph spider families (Sicariidae, Theridiidae, and Agelenidae) (McCowan and Garb, 2013). In order to investigate whether ITP/CHH peptides represent an ancestral recruitment into spider venoms and to determine when HAND toxins arose in spiders, we sampled venoms from a taxonomically broader range of araneomorph and mygalomorph spiders. We identified ITP/CHH peptides in venom-gland transcriptomes from four additional species of araneomorph spiders, namely the

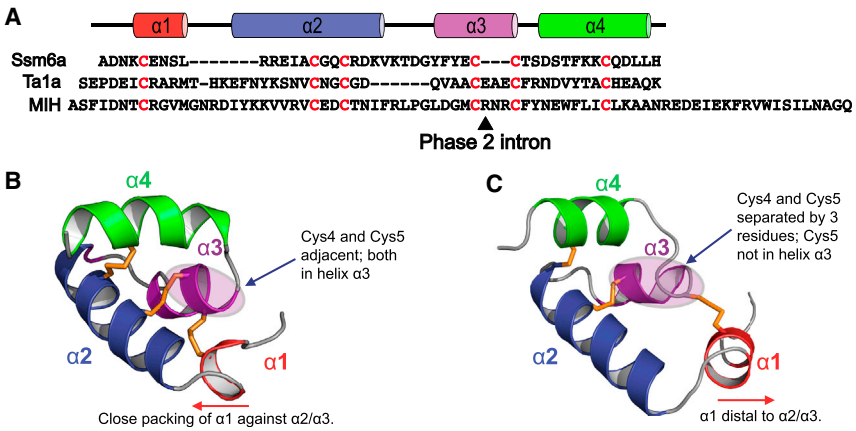


Figure 3. Comparison of the Structures of Ta1a and Ssm6a

(A) Alignment of the amino acid sequences of Ssm6a, Ta1a, and MIH, with conserved cysteine residues highlighted in red. The secondary structure of Ssm6a is shown above the alignment. Also shown is the location of the phase 2 intron in CHH/ITP genes, which interrupts the codon that encodes the residue immediately following Cys_4.

(B and C) Schematic of the 3D structures of (B) Ssm6a (PDB: 2MUN) and (C) Ta1a (PDB: 2KSL). Topologically equivalent helices are shown in the same color. In contrast with Ta1a and ancestral CHH/ITP peptides such as MIH, residues Cys_4 and Cys_5 are adjacent in the sequence of Ssm6a due to a deletion of three residues bordering the phase 2 intron. The resultant shift of Cys_5 to the C terminus of α3 enables the α1 helix to pack more closely against α3 in the structure of Ssm6a.

African six-eyed sand spider *Sicarius dolichocephalus* (Sicariidae), the brown huntsman *Heteropoda jugulans* (Sparassidae), the giant water spider *Megadolomedes australianus* (Pisauridae), and the golden orb-web weaver *Nephila pilipes* (Nephilidae); however, none of these sequences correspond to HAND toxins as they all contain the ancestral α5 helix. Furthermore, no ITP/CHH peptides or HAND toxins were identified in the venom-gland transcriptomes of the three species of mygalomorph spiders examined, namely the Australian funnel-web spider *Hadronyche infensa* (Hexathelidae), the Australian tarantula *Coremiocnemis tropix* (Theraphosidae), or the brush-foot trapdoor spider *Trittame loki* (Barychelidae). Together, these latter three spider families encompass more than 50% of all known mygalomorph species, which suggests that ITP/CHH peptides were recruited into spider venoms following the split between araneomorph and mygalomorph spiders ~375 Ma (Figure 5) (McCowan and Garb, 2013).

Local BLAST searches against venom-gland transcriptomes from the scolopendromorph centipedes *Scolopendra alternans* (Undheim et al., 2014), *S. subspinipes dehaani* (Liu et al., 2012), and *S. morsitans* (Undheim et al., 2014) revealed two CHH-like sequences in *S. morsitans* but only HAND-type sequences in *S. alternans* and *S. subspinipes* (Figure S5). In contrast, while a significant diversity of HAND toxins were found in the venom-gland transcriptomes of *S. alternans* and *S. subspinipes* (Figure S5), these were conspicuously absent in *S. morsitans*. In addition, a BLAST search of MIH against the entire UniProt database revealed the presence of an ITP/CHH peptide in the geophilomorph centipede *Strigamia maritima* (UniProt: T1JMX7).

Consistent with previous findings (McCowan and Garb, 2013), all venom-gland ITP/CHH sequences formed a single clade with respect to previously reported non-venom ITP/CHH family peptides (Figure 4). Within the venom clade, arachnids and mandibulate arthropods formed well-supported reciprocally monophyletic clades. In contrast, HAND toxins are clearly non-monophyletic, with both *Tegenaria* and *Scolopendra* HAND toxins nested within the taxonomical lineages of their respective CHH/ITP-clades. Thus, spider and centipede HAND toxins represent independent, convergent derivations of non-toxic CHH/ITP venom peptides. Based on estimates of time of most recent common ancestor, these derivations occurred in both centipede and spider HAND toxins within the last 140 million years (Figure 5). In centipedes this is the estimated time of divergence between *Scolopendra* and the closely related genus *Asanada* (Joshi and Karanth, 2011), while in spiders it is the estimated time of divergence between the families Agelenidae (*Tegenaria*) and Pisauridae (*Megadolomedes*) (Selden and Penney, 2010).

Stability of HAND Toxins

Venom peptides are generally reticulated with multiple disulfide bonds, which often provides them with high levels of thermal, chemical, and biological stability (Lavergne et al., 2015). The most robust disulfide-rich venom-peptide scaffold described to date is the ICK motif found in venom peptides from spiders, cone snails, and scorpions. Although these peptides can generally be unfolded in the absence of chaotropic agents, the midpoint of the thermal unfolding transition (T_m) often exceeds 100°C (Heitz et al., 2008). The HAND toxin Ssm6a was recently demonstrated to have even higher thermal stability than ICK toxins, making it perhaps the most stable venom peptide studied to date (Yang et al., 2013). It was not possible to obtain a complete thermal unfolding curve for Ssm6a in the absence of a chemical denaturant, and even in the presence of 8 M urea it had a very high T_m of 70.6 ± 0.1°C (Figure S2B) (Yang et al., 2013). Thus, we decided to examine the thermal denaturation of Ta1a to determine if extreme thermostability is a common property of HAND toxins. Ta1a was found to have only moderate thermal stability, with an estimated T_m of 51.0 ± 0.4°C in 0 M urea and less than 30°C in 8 M urea. Nevertheless, like Ssm6a (Yang et al., 2013), the thermal unfolding of Ta1a was completely reversible upon cooling (Figure S2), suggesting that HAND toxins might be good model systems for studying folding and unfolding transitions in fully helical proteins.

DISCUSSION

The structure of Ta1a is remarkable for a spider-venom peptide. All 44 structures of disulfide-rich spider toxins determined to date are dominated by β sheet secondary structure; helices are extremely rare, with a single short helix present in only six

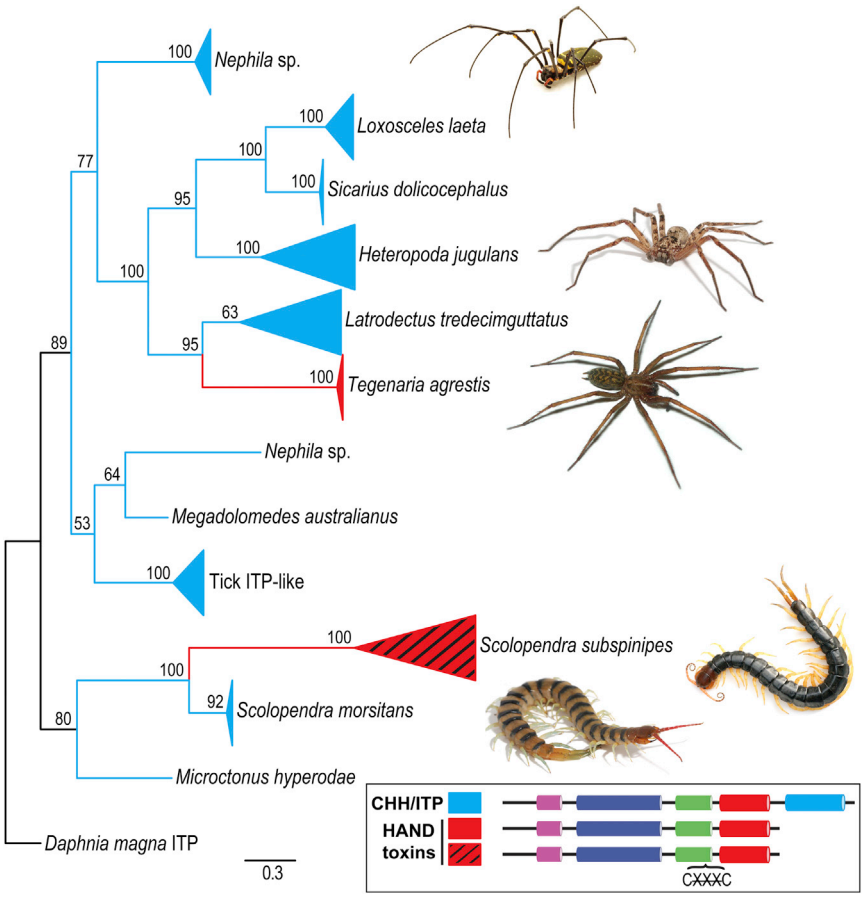


Figure 4. Bayesian Phylogenetic Reconstruction of Arthropod Venom CHH/ITP Peptides

Clades of venom and putative venom ITP/CHH peptides containing the ancestral fifth α helix are colored blue, while HAND toxin clades lacking helix α5 are shown in red. Cross-hatching indicates sequences with a truncation between Cys_4 and Cys_5 resulting in these cysteines being adjacent in the sequence. Posterior probabilities are shown above each node.

toxins (Fletcher et al., 1997a; Lee et al., 2004; Liao et al., 2006; Takahashi et al., 2000; Wang et al., 2001; Yuan et al., 2008). In contrast, the structure of Ta1a is comprised exclusively of four tightly packed α helices, with β strands being conspicuously absent (Figure 1). Although a few exclusively α-helical disulfide-rich toxins have been described (Srinivasan et al., 2002; Tudor et al., 1996) (Figure 6), their disulfide connectivity and overall fold are distinctly different from HAND toxins, which appear to be unlike any other characterized disulfide-rich toxin family (Lavergne et al., 2015; Mouhat et al., 2004). The unique helical structure and disulfide connectivity of Ta1a and Ssm6a indicate that HAND toxins constitute a new class of arthropod venom peptides.

There are several conserved structural elements of Ta1a, Ssm6a, and MIH that point to a shared evolutionary origin for HAND toxins and ITP/CHH peptides. First, the six cysteine residues are conserved, leading to three topologically equivalent disulfide bonds. These cysteine residues are major contributors to the internal hydrophobic core of these small proteins and they direct the 3D fold. The remaining critical elements of the hydrophobic core are also conserved, including three sets of buried aromatic residues in Ta1a and MIH (Figure 2D). In Ta1a, residues Phe16, Phe38, and Tyr43 play a key role in the packing of α1 against α2, α2 against α3, and α2 against α4, respectively. The same role is played by the topologically equivalent residues Tyr17, Phe45, and Phe50 in MIH. Ssm6a lacks the equivalent of the first two structural aromatic residues due to the tighter overall packing of α1 against α2 and α3 (Figure 3B), but it does contain the latter topologically equivalent aromatic residue in Phe38. Val22 in Ta1a and the topological equivalent Ala22 in Ssm6a and Val23 in MIH also contribute to the packing of α2 against α4.

Although they share a number of conserved structural features, a striking difference between the 3D architecture of HAND and ITP/CHH peptides is loss of the ancestral α5 helix in HAND toxins (Figure 1). This modification implies significant functional differences between these two classes of peptides, as α5 contains residues that are critical for the activity of non-venom ITP/CHH peptides (Katayama et al., 2003). Furthermore, α5 is the only helix in the ancestral fold that is not stabilized by a disulfide bond, and therefore loss of this helix might impart increased stability and resistance to proteases. This is consistent with the extraordinary thermal stability of the centipede HAND toxins and the observation that Ssm6a is stable in human plasma for at least 1 week (Yang et al., 2013). Moreover, HAND toxins appear to be significantly more soluble than ITP/CHH peptides; while MIH could not be dissolved in water at concentrations >1 mM (Katayama et al., 2003), we could dissolve Ta1a and Ssm6a in water at concentrations >5 mM. Thus, we hypothesize that loss of the ancestral α5 helix improved both the solubility and stability of HAND toxins, making them more suitable as venom peptides. Moreover, these adaptations are consistent with newly derived functions for HAND toxins as residues critical for hormone function (Katayama et al., 2003) were removed with the excision of α5.

Although loss of α5 appears to be a key step in weaponization of ITP/CHH peptides, comparative structure/function studies of HAND and ITP/CHH peptides will be required to determine the functional significance of this structural modification. Examination of venom forms of ITP/CHH peptides should provide important insights into the functional and structural transitions experienced by ITP/CHH peptides as they transform into HAND toxins. ITP/CHH peptides, which all contain the ancestral α5, have been found in a taxonomically diverse range of arthropod venoms (Figures 4 and 5) but with the exception of the latrodectins they remain functionally uncharacterized. Latrodectins are inactive in both insects and mammals (Gasparini

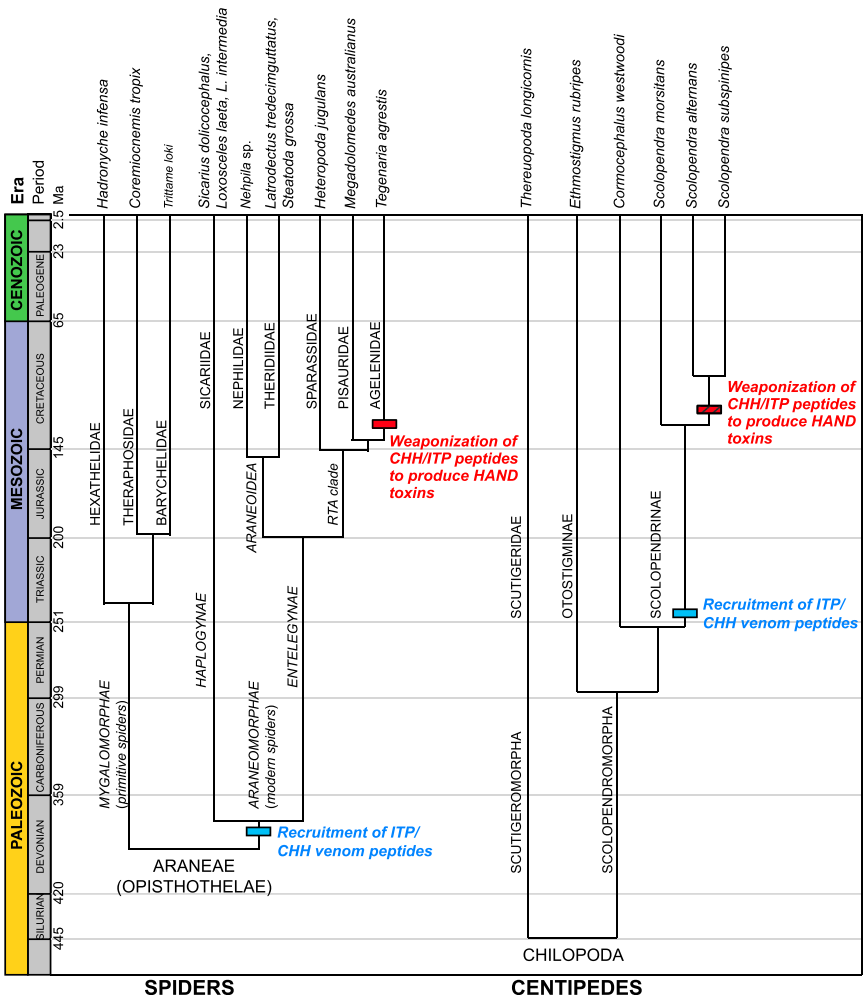


Figure 5. Timeline of CHH/ITP and HAND Toxin Recruitments

Simplified phylogenetic tree of spiders (Araneae, left) and centipedes (Chilopoda, right) showing the approximate earliest timing of the recruitment of CHH/ITP peptides into their venom and their weaponization to produce HAND toxins. The chronogram of spider evolution is derived from Ayoub et al. (2007) and Selden and Penney (2010), while the chronogram for centipedes is derived from Murienne et al. (2010) and Joshi and Karanth (2011).

et al., 1994; Grishin et al., 1993) and they do not produce hyperglycemic effects when injected into crab, crayfish, or shrimps (Soyez, 1997). Rather, they appear to serve as co-factors that enhance the activity of larger, pore-forming proteins known as latrotoxins (Grishin et al., 1993). It is not known which residues mediate this protein-protein interaction and whether α5 is involved. Thus, it would be interesting to examine the function of a wider range of venom ITP/CHH peptides in order to examine whether the apparently non-toxic chaperone-like role of latrodectins represents an intermediate step in the transition from hormone to neurotoxin.

Phylogenetic analysis revealed that spider and centipede HAND toxins are independent structural derivations from an ancestral venom ITP/CHH peptide (Figure 2). It was previously suggested that ITP/CHH peptides were basally recruited into the venom of the common ancestor of araneomorph spiders ~375 Ma (McCowan and Garb, 2013). In support of this, the complete absence of ITP/CHH peptides in the venom-gland transcriptomes of three mygalomorph families as reported here suggests that these peptides were first recruited into spider venoms after the split between the infraorders Araneomorphae and Mygalomorphae (Figure 5). However, the much narrower taxonomic distribution of HAND toxins indicates that the structural derivations required to weaponize ITP/CHH peptides occurred relatively recently (~140 Ma) in both spiders and centipedes (Figure 5). In particular, the recent recruitment and narrow taxonomic distribution of HAND toxins in spiders (*T. agrestis* only) strongly contrasts with ICK-type toxins, which are ubiquitous in spider venoms and were probably first recruited in a basal spider prior to the split between Mygalomorphae and Araneomorphae over 390 Ma (Ayoub et al., 2007). HAND toxin genes do, however, show signs of duplication and diversification, with three orthologs identified in *T. agrestis* and 21 in *S. alternans* and *S. subspinipes* (Figure S5). Moreover, their convergently evolved structural properties suggest that HAND toxins play an important functional role in the venoms of these predatory arthropods.

Arthropod ITP/CHH peptides are expressed in the form of three exons that often produce multiple products through alternative splicing (Montagné et al., 2010). Interestingly, the two exons encoding the ITP/CHH peptide in the genome of the geophilomorph centipede *Strigamia maritima* are interrupted by a phase-two intron two base pairs after the codon encoding Cys_4 (Figure 3A) (Chipman et al., 2014). This intron position also appears to be conserved across the venom clade of the ITP/CHH family (McCowan and Garb, 2013; Sanggaard et al., 2014). We therefore hypothesize that truncation of α3 between Cys_4 and Cys_5 occurred after a mutation that resulted in an intron extension in an ancestral centipede HAND toxin.

The presence of introns in ITP/CHH genes may also explain the conspicuous lack of ITP/CHH peptides in mygalomorph spider venom. A search of the recently published genome of the velvet spider *Stegodyphus mimosarum* (Araneomorphae) (Sanggaard et al., 2014), revealed an ITP/CHH-like gene (Genbank: AZAQ01021694.1) containing an unusually large intron (~9.7 kb) in the same position within the mature peptide as in *S. maritima*. Moreover, unlike araneomorph spiders (Krapcho et al., 1995), mygalomorphs appear to express intronless toxin genes (Jiang et al., 2008; Pineda et al., 2012; Tang et al., 2010), possibly indicating an inability to splice transcripts expressed in the toxin-producing regions of the venom gland.

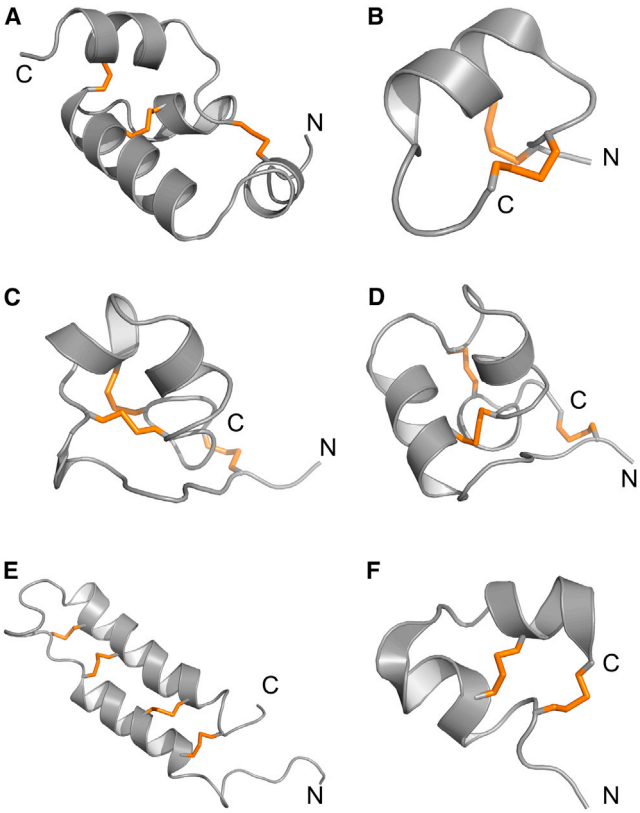


Figure 6. Comparison of the HAND-Toxin Fold with Other Exclusively α-Helical Toxin Classes

(A–F) Schematic of the 3D structures of (A) HAND-toxin fold represented by Ta1a (PDB: 2KSL); (B) α-conotoxin fold represented by the α-conotoxin Vc1.1 (PDB: 2H8S) (Clark et al., 2006); (C) ShKT motif represented by the sea anemone toxins ShK (PDB: 1ROO) (Tudor et al., 1996) and (D) its structural homolog BgK (PDB: 1BGK) (Dauplais et al., 1997; Srinivasan et al., 2002); (E) helical hairpin fold represented by B-IV (PDB: 1VIB) from ribbon worm venom (Barnham et al., 1997); (F) κ-Hefutoxin1-like fold represented by the scorpion toxin κ-Hefutoxin1 (PDB: 1HP9) (Srinivasan et al., 2002).

Disulfide bonds are highlighted in orange and the N and C termini are labeled.

Thus, even if ITP/CHH peptides were to be expressed in a mygalomorph venom gland, the presence of introns in the mature region of the peptide might prevent their recruitment into venom. This might underlie the absence of HAND toxins in mygalomorph spiders and perhaps explain why this remarkable scaffold has not seen the same functional radiation in spiders as the intronless ICK-encoding genes.

In summary, we identified a new venom-peptide family in arthropods, which we named HAND toxins, that are characterized by a unique disulfide-stabilized helical scaffold. These toxins arose independently in centipedes and spiders via modification of an ancestral hormone. The remarkable structural convergence seen in spider and centipede HAND toxins illustrates that stability and solubility are important structural adaptations for venom proteins and it lends support to the notion that recruitment and adaptive radiation of toxins is dictated by a handful of key biophysical properties (Fry et al., 2009). We also identified a plausible genetic mechanism that may both form a barrier to toxin recruitment and enable structural adaptations to take place. As more spider and centipede genomes are sequenced it will be interesting to examine the role of introns and alternative splicing in structural evolution of arthropod toxins, and in particular whether introns are retained in HAND toxins or secondarily lost in order to facilitate successful gene duplication events.

EXPERIMENTAL PROCEDURES

Production of Recombinant HAND Toxins

Synthetic genes for Ta1a and Ssm6a, with codons optimized for high-level expression in *E. coli*, were produced and inserted into the pLicC vector by GeneArt (Life Technologies). Ta1a and Ssm6a were then expressed and purified using the approaches described previously (Klint et al., 2013; Yang et al., 2013). Uniformly $^{13}C/^{15}N$-labeled Ta1a and Ssm6a were produced by growing cells in a defined minimal media supplemented with $^{13}C_6$-glucose and $^{15}NH_4Cl$ as the sole carbon and nitrogen sources, respectively. Purification was performed in the same way for unlabeled and $^{13}C/^{15}N$-labeled toxin.

Final purification using reverse phase HPLC yielded a single peak for both Ta1a and Ssm6a (Figure S1). The expected mass of recombinant Ta1a and Ssm6a were confirmed using MALDI-TOF mass spectrometry on a Model 4700 Proteomics Bioanalyser (Applied Biosystems) with α-cyano-4-hydroxycinnamic acid as matrix.

Insecticidal Assays

The insecticidal activity of recombinant Ta1a was tested by injecting the toxin, dissolved in insect saline, into the ventrolateral thoracic region of sheep blowflies (*Lucilia cuprina*, mass 18–24 mg, 10 flies per dose) using a maximum volume of 2 μl per fly. Lethality was measured after 24 hr and LD_{50} (median lethal dose) values were calculated as described previously (Herzig and Hodgson, 2008).

Structure Determination

NMR data were acquired at 298 K using a 900-MHz AVANCE spectrometer (Bruker) equipped with a cryoprobe. Data used for resonance assignment were acquired using non-uniform sampling (NUS); sampling schedules that approximated the rate of signal decay along the indirect dimensions were generated using sched3D (Mobli et al., 2010). The decay rates used were 1 Hz for all constant time ^{15}N dimensions, 30 Hz for all ^{13}C dimensions, and 15 Hz for the semi-constant indirect ^{1}H dimension of the H(CC)(CO)NH-TOCSY experiment. Pulse programs were modified from the standard BRUKER library for NUS mode and are available upon request. ^{13}C- and ^{15}N-edited HSQC-NOESY experiments were acquired using linear sampling. All NUS data were processed using maximum entropy reconstruction as described (Mobli et al., 2007). All experiments were acquired in H_2O except for the ^{13}C NOESY, which was acquired in D_2O.

Chemical shift assignments for Ta1a and Ssm6a have been deposited in BioMagResBank (BMRB) under accession numbers BMRB: 16667 and 25223, respectively. Distance restraints for structure calculations were derived from 3D ^{13}C- and ^{15}N-edited NOESY-HSQC spectra acquired with a mixing time of 200 ms. NOESY spectra were manually peak picked and integrated, then peaklists were assigned and an ensemble of structures calculated automatically using CYANA (Güntert, 2004). For Ta1a, the chemical shift tolerances used for auto-assignment in CYANA were 0.025 ppm in both ^{1}H dimensions and 0.25 ppm for the heteronucleus ($^{13}C/^{15}N$). For Ssm6a we used 0.03 ppm in the direct ^{1}H dimension, 0.01 ppm in the indirect ^{1}H dimension, and 0.4 ppm for heteronuclei.

Backbone dihedral-angle restraints were derived from TALOS chemical shift analysis (Cornilescu et al., 1999); the restraint range was set to twice the estimated SD. The Glu2-Pro3 peptide bond in Ta1a was shown to adopt the *trans* conformation based on characteristic nuclear Overhauser effects (NOEs) and Cα/Cβ chemical shifts of Pro3. Initial structure calculations in combination with analysis of NOE networks allowed assignment of χ_1 restraints for 20 residues as well as stereospecific assignment of the β/γ-methylene protons of Met11 and methyl groups of Val22/Val30.

Slowly exchanging amide protons in Ta1a were identified by their presence in a 2D ^{15}N-^{1}H HSQC spectrum acquired 24 hr after reconstituting lyophilized protein in D_2O; for most of these amide protons, the corresponding hydrogen-bond acceptors were identified from preliminary structure calculations.

Hydrogen-bond restraints of 1.7–2.2 Å and 2.7–3.2 Å were employed for H_N–O and N–O distances, respectively, in structure calculations (Fletcher et al., 1997b; Wang et al., 2000). The disulfide bond configuration was unambiguously identified in the first round of structure calculations and disulfide bond restraints were applied in subsequent calculations (Fletcher et al., 1997a).

CYANA was used to calculate 200 structures from random starting conformations, then the 25 conformers with highest stereochemical quality as judged by MolProbity (Davis et al., 2007) were selected to represent the solution structure of Ta1a or Ssm6a. During the automated NOESY assignment/structure calculation process the CANDID module of CYANA assigned 93.5% (1,430/1,529) of all NOESY crosspeaks for Ta1a and 88.0% (1,931/2,194) for Ssm6a. Atomic coordinates for the Ta1a and Ssm6a ensembles are available from the PDB (accession numbers PDB: 2KS1 and 2MUN, respectively).

Preparation of Venom-Gland cDNA Libraries

cDNA libraries were prepared and sequenced from the venom glands of the araneomorph spiders *Sicarius dolichocephalus*, *Heteropoda jugulans*, *Megadolomedes australianus*, and *Nephila pilipes*, as well as the mygalomorph spiders *Hadronyche infensa* and *Coremiocnemis tropix*. Three *H. infensa* specimens were aggravated to deplete their venom-gland contents and encourage production of toxin transcripts as previously described (Binford et al., 2009). Similarly, the venom glands of four *Nephila pilipes*, two *Megadolomedes australianus*, four *Heteropoda jugulans*, and four *Coremiocnemis tropix* were depleted by electrostimulation. Four days later, the venom glands were removed and total RNA was isolated from fresh homogenized tissue using TRIzol (Invitrogen). mRNA enrichment was performed using an Oligotex direct mRNA kit (Qiagen) with 30 μg of total RNA as starting material.

mRNA (100 ng) was supplied to the Australian Genome Research Facility for cDNA library preparation followed by 454 sequencing using the Roche GS FLX platform. After data filtering, the remaining sequences were assembled using MIRA (Chevreux et al., 2004). Contig and singlet files were BLASTed against the NCBI database using the BLAST2GO software suite (http://www.blast2go.com) using an e-value cutoff of 1×10^{-6}. Toxin transcripts and putative toxins were extracted as FASTA files and manually inspected.

For *S. dolicocephalus*, electrical stimulation was used to extract venom from a single specimen, then 3 days later the venom glands were removed and flash frozen as described (Binford et al., 2009). RNA was immediately isolated from the homogenized venom glands using the ChargeSwitch Total RNA Cell Kit (Invitrogen) and used to construct a cDNA library using SMART cDNA Technology (Clontech) with synthesis by long distance PCR. The manufacturer's protocols were followed with only one deviation; we size fractionated using a Nuclear TraPCR filter (Clontech), which retains fragments $\geq$100 bp, in order to capture transcripts encoding small neurotoxins. Phagemids were packaged into MaxPlax packaging extracts (Epicentre). We isolated clones from phagemids, which resulted in amplicons from PCR screening with primer sequences on the TriplEx2 vector (Clontech). The phagemid clones were converted to double-stranded DNA plasmids (pTriplEx2) using Cre recombinase site-specific recombination at *loxP* sites flanking the inserts and sequenced using conventional Sanger sequencing at the Arizona Genomics Analysis Core. Isolates S57 and S79 encoded orthologs of U_1-TRTX-Ta1a.

Phylogenetic Analysis

The sequences of MIH, Ta1a, and Ssm6a were searched against UniProt protein database and local BLAST searches were performed against all predicted open reading frames from the transcriptomic data using CLC Main Workbench (CLC-Bio) using a significance threshold of 10^{-3}. The resulting sequences were aligned using CLC Main Workbench then adjusted manually for optimal alignment (Figure S5). Datasets were analyzed using Bayesian inference implemented in MrBayes version 3.2.1 (Ronquist and Huelsenbeck, 2003) as described previously (Undheim et al., 2014).

CD Spectropolarimetry

CD spectra of Ta1a (20 μM in 10 mM $KHPO_4$ (pH 7.2) ± 8 M urea) were acquired under constant N_2 flush using a Jasco J-810 spectropolarimeter. Thermal denaturation profiles were obtained by monitoring the ellipticity at 222 nm (θ_{222}) as the temperature was increased from 20°C to 95°C at a rate of 2°C/min. Denaturation curves were fitted with a six-parameter sigmoidal function to obtain T_m values (Bains et al., 1999).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.str.2015.05.003.

AUTHOR CONTRIBUTIONS

E.A.B.U., M.M., and G.F.K. conceived and directed this program of research. E.A.B.U., M.M., G.F.K., G.M.N., G.J.B., and B.G.F. designed experiments and analyzed the data. E.A.B.U., M.M., V.H., L.L.G., C.F.L., D.M., P.Z.T., S.S.P., P.H., and S.D. performed the research. E.A.B.U., M.M., and G.F.K. wrote the manuscript with input from all authors.

ACKNOWLEDGMENTS

We thank Dr. Geoff Brown for supplying blowfly larvae and the Queensland NMR Network for access to the 900-MHz NMR spectrometer. We acknowledge financial support from the Australian Research Council (grants DP140101098 and Future Fellowship FTI10100925 to M.M.; grant DP1095728 to G.F.K.) and the Australian National Health & Medical Research Council (Principal Research Fellowship to G.F.K.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Received: February 15, 2015
Revised: April 28, 2015
Accepted: May 4, 2015
Published: June 11, 2015

REFERENCES

Ayoub, N.A., Garb, J.E., Hedin, M., and Hayashi, C.Y. (2007). Utility of the nuclear protein-coding gene, elongation factor-1 gamma (EF-1γ), for spider systematics, emphasizing family level relationships of tarantulas and their kin (Araneae: Mygalomorphae). Mol. Phylogenet. Evol. *42*, 394–409.

Bains, N.P.S., Wilce, J.A., Mackay, L.G., and King, G.F. (1999). Controlling leucine zipper specificity with interfacial hydrophobic residues. Lett. Pept. Sci. *6*, 381–390.

Barnham, K.J., Dyke, T.R., Kem, W.R., and Norton, R.S. (1997). Structure of neurotoxin B-IV from the marine worm *Cerebratulus lacteus*: a helical hairpin cross-linked by disulphide bonding. J. Mol. Biol. *268*, 886–902.

Binford, G.J., Bodner, M.R., Cordes, M.H., Baldwin, K.L., Rynerson, M.R., Burns, S.N., and Zobel-Thropp, P.A. (2009). Molecular evolution, functional variation, and proposed nomenclature of the gene family that includes sphingomyelinase D in sicariid spider venoms. Mol. Biol. Evol. *26*, 547–566.

Chevreux, B., Pfisterer, T., Drescher, B., Driesel, A.J., Müller, W.E.G., Wetter, T., and Suhai, S. (2004). Using the mira EST assembler for reliable and automated mRNA transcript assembly and SNP detection in sequenced ESTs. Genome Res. *14*, 1147–1159.

Chipman, A.D., Ferrier, D.E.K., Brena, C., Qu, J., Hughes, D.S.T., Schröder, R., Torres-Oliva, M., Znassi, N., Jiang, H., Almeida, F.C., et al. (2014). The first myriapod genome sequence reveals conservative arthropod gene content and genome organisation in the centipede *Strigamia maritima*. PLoS Biol. *12*, e1002005.

Clark, R.J., Fischer, H., Nevin, S.T., Adams, D.J., and Craik, D.J. (2006). The synthesis, structural characterization, and receptor specificity of the alpha-conotoxin Vc1.1. J. Biol. Chem. *281*, 23254–23263.

Cornilescu, G., Delaglio, F., and Bax, A. (1999). Protein backbone angle restraints from searching a database for chemical shift and sequence homology. J. Biomol. NMR *13*, 289–302.

Dauplais, M., Lecoq, A., Song, J., Cotton, J., Jamin, N., Gilquin, B., Roumestand, C., Vita, C., de Medeiros, C.L., Rowan, E.G., et al. (1997). On the convergent evolution of animal toxins. Conservation of a diad of functional residues in potassium channel-blocking toxins with unrelated structures. J. Biol. Chem. *272*, 4302–4309.

Davis, I.W., Leaver-Fay, A., Chen, V.B., Block, J.N., Kapral, G.J., Wang, X., Murray, L.W., Arendall, W.B., 3rd, Snoeyink, J., Richardson, J.S., and Richardson, D.C. (2007). MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. Nucleic Acids Res. *35*, 375–383.

Fletcher, J.I., Chapman, B.E., Mackay, J.P., Howden, M.E.H., and King, G.F. (1997a). The structure of versutoxin (δ-atracotoxin-Hv1) provides insights into the binding of site 3 neurotoxins to the voltage-gated sodium channel. Structure *5*, 1525–1535.

Fletcher, J.I., Smith, R., O'Donoghue, S.I., Nilges, M., Connor, M., Howden, M.E.H., Christie, M.J., and King, G.F. (1997b). The structure of a novel insecticidal neurotoxin, ω-atracotoxin-HV1, from the venom of an Australian funnel web spider. Nat. Struct. Biol. *4*, 559–566.

Fry, B.G., Scheib, H., van der Weerd, L., Young, B., McNaughtan, J., Ramjan, S.F., Vidal, N., Poelmann, R.E., and Norman, J.A. (2008). Evolution of an arsenal: structural and functional diversification of the venom system in the advanced snakes (Caenophidia). Mol. Cell. Proteomics *7*, 215–246.

Fry, B.G., Roelants, K., Champagne, D.E., Scheib, H., Tyndall, J.D., King, G.F., Nevalainen, T.J., Norman, J.A., Lewis, R.J., Norton, R.S., et al. (2009). The toxicogenomic multiverse: convergent recruitment of proteins into animal venoms. Annu. Rev. Genomics Hum. Genet. *10*, 483–511.

Gasparini, S., Kiyatkin, N., Drevet, P., Boulain, J.C., Tacnet, F., Ripoche, P., Forest, E., Grishin, E., and Ménez, A. (1994). The low molecular weight protein which co-purifies with α-latrotoxin is structurally related to crustacean hyperglycemic hormones. J. Biol. Chem. *269*, 19803–19809.

Grishin, E.V., Himmelreich, N.H., Pluzhnikov, K.A., Pozdnyakova, N.G., Storchak, L.G., Volkova, T.M., and Woll, P.G. (1993). Modulation of functional activities of the neurotoxin from black widow spider venom. FEBS Lett. *336*, 205–207.

Güntert, P. (2004). Automated NMR structure calculation with CYANA. Methods Mol. Biol. *278*, 353–378.

Heitz, A., Avrutina, O., Le-Nguyen, D., Diederichsen, U., Hernandez, J.F., Gracy, J., Kolmar, H., and Chiche, L. (2008). Knottin cyclization: impact on structure and dynamics. BMC Struct. Biol. *8*, 54.

Herzig, V., and Hodgson, W.C. (2008). Neurotoxic and insecticidal properties of venom from the Australian theraphosid spider *Selenotholus foelschei*. Neurotoxicology *29*, 471–475.

Holm, L., and Rosenstrom, P. (2010). Dali server: conservation mapping in 3D. Nucleic Acids Res. *38*, W545–W549.

Jiang, L., Chen, J., Peng, L., Zhang, Y., Xiong, X., and Liang, S. (2008). Genomic organization and cloning of novel genes encoding toxin-like peptides of three superfamilies from the spider *Orinithoctonus huwena*. Peptides *29*, 1679–1684.

Johnson, J.H., Bloomquist, J.R., Krapcho, K.J., Kral, R.M., Trovalto, R., Eppler, K.G., Morgan, T.K., and DelMar, E.G. (1998). Novel insecticidal peptides from *Tegenaria agrestis* spider venom may have a direct effect on the insect central nervous system. Arch. Insect Biochem. Physiol. *38*, 19–31.

Joshi, J., and Karanth, K.P. (2011). Cretaceous-tertiary diversification among select scolopendrid centipedes of South India. Mol. Phylogenet. Evol. *60*, 287–294.

Kapust, R.B., Tözser, J., Copeland, T.D., and Waugh, D.S. (2002). The P1′ specificity of tobacco etch virus protease. Biochem. Biophys. Res. Commun. *294*, 949–955.

Katayama, H., Nagata, K., Ohira, T., Yumoto, F., Tanokura, M., and Nagasawa, H. (2003). The solution structure of molt-inhibiting hormone from the Kuruma prawn *Marsupenaeus japonicus*. J. Biol. Chem. *278*, 9620–9623.

King, G.F. (2011). Venoms as a platform for human drugs: translating toxins into therapeutics. Expert Opin. Biol. Ther. *11*, 1469–1484.

King, G.F. (2015). Venoms to Drugs: Venoms as a Source for the Development of Human Therapeutics (Royal Society of Chemistry).

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.

King, G.F., Gentz, M.C., Escoubas, P., and Nicholson, G.M. (2008). A rational nomenclature for naming peptide toxins from spiders and other venomous animals. Toxicon *52*, 264–276.

Klint, J.K., Senff, S., Rupasinghe, D.B., Er, S.Y., Herzig, V., Nicholson, G.M., and King, G.F. (2012). Spider-venom peptides that target voltage-gated sodium channels: pharmacological tools and potential therapeutic leads. Toxicon *60*, 478–491.

Klint, J.K., Senff, S., Saez, N.J., Seshadri, R., Lau, H.Y., Bende, N.S., Undheim, E.A., Rash, L.D., Mobli, M., and King, G.F. (2013). Production of recombinant disulfide-rich venom peptides for structural and functional analysis via expression in the periplasm of *E. coli*. PLoS One *8*, e63865.

Krapcho, K.J., Kral, R.M., Jr., Vanwagenen, B.C., Eppler, K.G., and Morgan, T.K. (1995). Characterization and cloning of insecticidal peptides from the primitive weaving spider *Diguetia canities*. Insect Biochem. Mol. Biol. *25*, 991–1000.

Lavergne, V., Alewood, P.F., Mobli, M., and Alewood, P.F. (2015). The structural universe of disulfide-rich venom peptides. In Venoms to Drugs: Venoms as a Source for the Development of Human Therapeutics, G.F. King, ed. (Royal Society of Chemistry).

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.

Liao, Z., Yuan, C., Deng, M., Li, J., Chen, J., Yang, Y., Hu, W., and Liang, S. (2006). Solution structure and functional characterization of jingzhaotoxin-XI: a novel gating modifier of both potassium and sodium channels. Biochemistry *45*, 15591–15600.

Liu, Z.-C., Zhang, R., Zhao, F., Chen, Z.-M., Liu, H.-W., Wang, Y.-J., Jiang, P., Zhang, Y., Wu, Y., Ding, J.-P., et al. (2012). Venomic and transcriptomic analysis of centipede *Scolopendra subspinipes dehaani*. J. Proteome Res. *11*, 6197–6212.

McCowan, C., and Garb, J.E. (2013). Recruitment and diversification of an ecdysozoan family of neuropeptide hormones for black widow spider venom expression. Gene *536*, 366–375.

Mobli, M., and King, G.F. (2010). NMR methods for determining disulfide-bond connectivities. Toxicon *56*, 849–854.

Mobli, M., Maciejewski, M.W., Gryk, M.R., and Hoch, J.C. (2007). An automated tool for maximum entropy reconstruction of biomolecular NMR spectra. Nat. Methods *4*, 467–468.

Mobli, M., Stern, A.S., Bermel, W., King, G.F., and Hoch, J.C. (2010). A non-uniformly sampled 4D HCC(CO)NH-TOCSY experiment processed using maximum entropy for rapid protein sidechain assignment. J. Magn. Reson. *204*, 160–164.

Montagné, N., Desdevises, Y., Soyez, D., and Toullec, J.-Y. (2010). Molecular evolution of the crustacean hyperglycemic hormone family in ecdysozoans. BMC Evol. Biol. *10*, 62.

Mouhat, S., Jouirou, B., Mosbah, A., De Waard, M., and Sabatier, J.-M. (2004). Diversity of folds in animal toxins acting on ion channels. Biochem. J. *378*, 717–726.

Murienne, J., Edgecombe, G.D., and Giribet, G. (2010). Including secondary structure, fossils and molecular dating in the centipede tree of life. Mol. Phylogenet. Evol. *57*, 301–313.

Pineda, S.S., Wilson, D., Mattick, J.S., and King, G.F. (2012). The lethal toxin from Australian funnel-web spiders is encoded by an intronless gene. PLoS One *7*, e43699.

Rodriguez de la Vega, R.C., Schwartz, E.F., and Possani, L.D. (2010). Mining on scorpion venom biodiversity. Toxicon *56*, 1155–1161.

Ronquist, F., and Huelsenbeck, J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics *19*, 1572–1574.

Saez, N.J., Senff, S., Jensen, J.E., Er, S.Y., Herzig, V., Rash, L.D., and King, G.F. (2010). Spider-venom peptides as therapeutics. Toxins *2*, 2851–2871.

Saez, N.J., Mobli, M., Bieri, M., Chassagnon, I.R., Malde, A.K., Gamsjaeger, R., Mark, A.E., Gooley, P.R., Rash, L.D., and King, G.F. (2011). A dynamic

pharmacophore drives the interaction between psalmotoxin-1 and the putative drug target acid-sensing ion channel 1a. Mol. Pharmacol. *80*, 796–808.

Sanggaard, K.W., Bechsgaard, J.S., Fang, X., Duan, J., Dyrlund, T.F., Gupta, V., Jiang, X., Cheng, L., Fan, D., Feng, Y., et al. (2014). Spider genomes provide insight into composition and evolution of venom and silk. Nat. Commun. *5*, 3765.

Selden, P.A., and Penney, D. (2010). Fossil spiders. Biol. Rev. *85*, 171–206.

Smith, J.J., Hill, J.M., Little, M.J., Nicholson, G.M., King, G.F., and Alewood, P.F. (2011). Unique scorpion toxin with a putative ancestral fold provides insight into evolution of the inhibitor cystine knot motif. Proc. Natl. Acad. Sci. USA *108*, 10478–10483.

Smith, J.J., Herzig, V., King, G.F., and Alewood, P.F. (2013). The insecticidal potential of venom peptides. Cell. Mol. Life Sci. *70*, 3665–3693.

Sollod, B.L., Wilson, D., Zhaxybayeva, O., Gogarten, J.P., Drinkwater, R., and King, G.F. (2005). Were arachnids the first to use combinatorial peptide libraries? Peptides *26*, 131–139.

Soyez, D. (1997). Occurrence and diversity of neuropeptides from the crustacean hyperglycemic hormone family in arthropods. A short review. Ann. N. Y. Acad. Sci. *814*, 319–323.

Srinivasan, K.N., Sivaraja, V., Huys, I., Sasaki, T., Cheng, B., Kumar, T.K., Sato, K., Tytgat, J., Yu, C., San, B.C., et al. (2002). κ-Hefutoxin1, a novel toxin from the scorpion *Heterometrus fulvipes* with unique structure and function. Importance of the functional diad in potassium channel selectivity. J. Biol. Chem. *277*, 30040–30047.

Sunagar, K., Undheim, E.A.B., Chan, A., Koludarov, I., Muñoz-Gómez, S., Antunes, A., and Fry, B.G. (2013). Evolution stings: the origin and diversification of scorpion toxin peptide scaffolds. Toxins *5*, 2456–2487.

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.

Tang, X., Zhang, Y., Hu, W., Xu, D., Tao, H., Yang, X., Li, Y., Jiang, L., and Liang, S. (2010). Molecular diversification of peptide toxins from the tarantula *Haplopelma hainanum* (*Ornithoctonus hainana*) venom based on transcriptomic, peptidomic, and genomic analyses. J. Proteome Res. *9*, 2550–2564.

Terlau, H., and Olivera, B.M. (2004). *Conus* venoms: a rich source of novel ion channel-targeted peptides. Physiol. Rev. *84*, 41–68.

Tudor, J.E., Pallaghy, P.K., Pennington, M.W., and Norton, R.S. (1996). Solution structure of ShK toxin, a novel potassium channel inhibitor from a sea anemone. Nat. Struct. Biol. *3*, 317–320.

Undheim, E.A.B., Jones, A., Clauser, K.R., Holland, J.H., Pineda, S.S., King, G.F., and Fry, B.G. (2014). Clawing through evolution: toxin diversification and convergence in the ancient lineage Chilopoda (Centipedes). Mol. Biol. Evol. *31*, 2124–2148.

Wang, X.-H., Connor, M., Smith, R., Maciejewski, M.W., Howden, M.E.H., Nicholson, G.M., Christie, M.J., and King, G.F. (2000). Discovery and characterization of a family of insecticidal neurotoxins with a rare vicinal disulfide bond. Nat. Struct. Biol. *7*, 505–513.

Wang, X.-H., Connor, M., Wilson, D., Wilson, H.I., Nicholson, G.M., Smith, R., Shaw, D., Mackay, J.P., Alewood, P.F., Christie, M.J., and King, G.F. (2001). Discovery and structure of a potent and highly specific blocker of insect calcium channels. J. Biol. Chem. *276*, 40806–40812.

Yang, S., Liu, Z., Xiao, Y., Li, Y., Rong, M., Liang, S., Zhang, Z., Yu, H., King, G.F., and Lai, R. (2012). Chemical punch packed in venoms makes centipedes excellent predators. Mol. Cell. Proteomics *11*, 640–650.

Yang, S., Xiao, Y., Kang, D., Liu, J., Li, Y., Undheim, E.A., Klint, J.K., Rong, M., Lai, R., and King, G.F. (2013). Discovery of a selective $Na_V1.7$ inhibitor from centipede venom with analgesic efficacy exceeding morphine in rodent pain models. Proc. Natl. Acad. Sci. USA *110*, 17534–17539.

Yuan, C.H., He, Q.Y., Peng, K., Diao, J.B., Jiang, L.P., Tang, X., and Liang, S.P. (2008). Discovery of a distinct superfamily of Kunitz-type toxin (KTT) from tarantulas. PLoS One *3*, e3414.

