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From foe to friend: using animal toxins to investigate ion channel function

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Abstract

Ion channels are vital contributors to cellular communication in a wide range of organisms, a distinct feature that renders this ubiquitous family of membrane-spanning proteins a prime target for toxins found in animal venom. For many years, the unique properties of these naturally-occurring molecules have enabled researchers to probe the structural and functional features of ion channels and to define their physiological roles in normal and diseased tissues. To illustrate their considerable impact on the ion channel field, this review will highlight fundamental insights into toxin-channel interactions as well as recently developed toxin screening methods and practical applications of engineered toxins.

Keywords

Animal toxin; Voltage-gated ion channel; Transient receptor potential channel; Toxin engineering; Screening approaches

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1. Introduction

Voltage-gated ion channels regulate the ion permeability of the cell membrane and as a result, generate electrical signals that disseminate vital information across the human body[1]. Evolution has endowed venomous animals and poisonous plants with the ability to exploit this excitatory role by producing toxins that modify ion channel opening or closing (*i.e.* gating) with the goal of incapacitating prey or defending against predators[2]. Historically, toxins from scorpion, spider, sea anemone, cone snail, snake, frog, puffer fish, and insect venoms have been used to gain insights into the function, structure, and pharmacological sensitivities of various members of the voltage-gated ion channel family[3] including potassium (K_v), sodium (Na_v), and calcium (Ca_v) channels which constitute the main topic of this review. In addition, recent structural advances in the Transient Receptor Potential (TRP) channel field were made possible, in part, by the availability of a unique peptide isolated from tarantula venom that traps the channel in a distinct conformation[4; 5; 6]. Animal toxins have also contributed to the generation of essential insights into membrane proteins other than voltage-gated ion channels such as acid-sensing[7; 8], mechanosensitive[9], and chloride ion channels[10]; acetylcholine[11], NMDA[12], and G-protein coupled receptors[13]; and Na^+/K^+ ATPase[14].

In general, toxins that interfere with voltage-gated ion channel function do so through two mechanisms: pore-blocking toxins inhibit ion flow by binding to the outer vestibule or within the ion conduction pore[15; 16] whereas gating-modifier toxins interact with a channel region that alters conformation during opening or inactivation to influence the gating mechanism[17; 18; 19]. As such, gating-modifier toxins constitute powerful tools for researchers seeking to address the unique challenges associated with voltage-gated ion channel voltage sensors as they undergo complex conformational changes during channel activation and inactivation. As illustrated in the next sections, knowledge on the precise working mechanism of toxins is crucial to help elucidate ion channel function. Since many reviews have already summarized a large body of toxin work, this review will illustrate the considerable impact of toxins on the ion channel field by highlighting pioneering experiments that resulted in fundamental insights into toxin-channel interactions as well as potential applications of toxins or toxin-derived compounds. All toxins mentioned in this review are summarized in Table 1.

2. Voltage-gated potassium channel toxins

Most voltage-gated potassium (K_v) channels are homotetrameric in nature, with each subunit containing six transmembrane helices (S1-S6): the S1-S4 helices form the voltage-sensing domain whereas the S5-S6 helices of four subunits come together in a circular arrangement to form the potassium ion-selective pore[20; 21; 22; 23; 24]. Toxins that target K_v channels can do so by interacting with the pore region or particular regions within the voltage sensors[25]. Pore-blocking toxins have greatly facilitated K_v channel research by enabling purification of novel channels and by providing insights into channel subunit stoichiometry as well as the shape of the extracellular pore region[26; 27; 28; 29; 30; 31; 32]. A particularly well-studied example is charybdotoxin (CTX), a 37-residue peptide isolated from the venom of the deathstalker scorpion *Leiurus quinquestriatus* (Fig. 1a)[33]. CTX

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exhibits a simple, bimolecular binding mechanism, in which a single toxin molecule inhibits the channel by physically plugging the pore (Fig. 1a)[34]. Early observations led to the hypothesis that CTX approximates a “tethered potassium ion” by bringing a positive charge close to a potassium ion-binding site near the extracellular side within the pore[35]. This hypothesis was later proven correct when a lysine was identified as the most important residue for CTX function[36]. This residue is conserved in all members of the CTX-like toxin family (*e.g.* agitoxin2) that bind with a similar orientation on the K_v channel and inhibit ion flux through a common mechanism[37; 38]. Recently, the crystal structure of CTX bound to a K_v channel was elucidated (Fig. 3a), a remarkable achievement that required many hurdles to be overcome[39]. Similar to what was observed with the solid-state NMR structure of the KcsAK_v1.3-kaliotoxin complex[40], the structure of the CTX-K_v channel complex revealed that the 27th residue of the toxin, a lysine, indeed makes its way into the pore and ends up close to the outermost of the four binding sites for potassium ions that are responsible for the ion selectivity of the channel. This observation confirmed a previously postulated hypothesis as to how intracellular potassium ions can permeate along the pore and influence the dissociation of toxin bound to the external end of the pore[35]. The structure also explains why mutant toxins without a lysine at this position are less effective at blocking K_v channels.

Unlike pore blockers, gating-modifier toxins interact with the voltage-sensing domain to influence K_v channel opening. An early clue that toxins can inhibit K_v channels through a mechanism other than pore occlusion came from experiments with hanatoxin (Fig. 1b), a 35-residue peptide isolated from the venom of the Chilean rose-hair tarantula *Grammostola spatulata*, where transfer of the outer vestibule (S5–S6 linker) of K_v2.1 into a toxin-insensitive channel failed to confer toxin sensitivity[41; 42]. Moreover, when co-applied with a pore blocker such as agitoxin2, hanatoxin displayed the ability to bind concurrently, thereby supporting the notion of a binding site outside of the pore region. Ensuing evidence demonstrating that this class of toxins indeed modifies K_v channel gating by influencing their voltage sensors came from three observations. First, toxin-bound channels still open and conduct ions, but the energy (or voltage-step magnitude) required to open toxin-bound channels is substantially increased (Fig. 1b)[41; 42; 43]. Second, these toxins have distinct effects on voltage sensor movements, as was observed in gating current measurements[44; 45]. Third, classic as well as more recent mutagenesis experiments suggest that these toxins interact with defined regions within the voltage sensors (Fig. 1c)[20; 46; 47; 48]. In particular, gating-modifier toxins helped identify the S3b-S4 helix-turn-helix motif, or voltage-sensor “paddle”, which moves at the protein-lipid interface to drive activation of the voltage sensors and opening of the pore[47]. Strikingly, this motif can be transplanted into different voltage-gated ion channel isoforms without losing their capacity to interact with toxins[20]. One intriguing aspect of gating-modifier toxins is their ability to interact with the paddle motif in the resting conformation in which case the voltage sensor is buried within the lipid membrane. However, the amphipathic character observed in many toxin structures (Fig. 1b) is consistent with the notion that membrane partitioning may be required for the toxin to reach the channel[49; 50; 51; 52; 53]. It will be interesting to determine the docking site of gating-modifier toxins with the paddle motif using mutant cycle approaches or by obtaining high-resolution structures of a gating-modifier toxin bound to a K_v or Na_v channel. Still,

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toxin binding affinities and large-scale production are two important limitations to overcome.

A unique feature of voltage-sensor targeting toxins is that they can interact with different families of voltage-activated ion channels. Such promiscuous behavior has been observed for toxins such as hanatoxin (see Figs. 1 and 2), ω-grammotoxin SIA, ProTx-I, ProTx-II, BDS-I, and SGTx1[42; 46; 54; 55; 56; 57]. The widespread targeting of paddle motifs by animal toxins highlights the pharmacological importance of this part of the voltage sensor.

3. Voltage-gated sodium channel toxins

In contrast to K_v channels, the channel-forming component of the voltage-gated sodium (Na_v) channel complex consists of four domains (DI-IV), each with six transmembrane segments (S1-S6), connected by intracellular linkers[58]. These similar, but non-identical domains each consist of a voltage-sensing region (S1-S4), while the S5-S6 helices from each domain come together to form a sodium ion-selective pore in the membrane. The pore can open when all four voltage sensors move in response to changes in membrane voltage. In general, all four sensors activate following membrane depolarization; however, those in domains I-III are most important for channel opening, whereas the one in domain IV plays a distinctive role in fast inactivation[54; 59; 60; 61; 62; 63; 64]. Recently, the first crystal structures of bacterial Na_v channels were solved[65; 66; 67; 68]. Although seminal observations were reported, prokaryotic Na_v channels are homotetramers whereas their mammalian counterparts are multi-domain monomers. Moreover, key characteristics of mammalian Na_v channels such as the amino acid composition of the selectivity filter, fast inactivation gate, and presence of auxiliary subunits differ substantially from prokaryotic variants[65; 66; 67; 68].

A few years after Hodgkin and Huxley discovered the fundamental role of Na_v channels in electrical signaling[69; 70], one of the historically most important ion channel toxins was isolated from the Japanese puffer-fish[71]. This naturally-occurring marine toxin, tetrodotoxin (TTX), interacts strongly with the Na_v channel pore region to occlude the sodium ion permeation pathway (Fig. 2a)[72; 73]. TTX has been widely used to study the structural and functional properties of Na_v channels. For example, after the TTX structure was solved in 1964[74], Hille exploited this information to predict the diameter of the Na_v channel pore, thereby providing unique insights into the molecular structure of this ion channel family[15]. Later, the low-nanomolar affinity of [^{3}H]-TTX contributed to the isolation of the Na_v channel pore-forming subunit from the electric eel[75; 76]. Likewise, [^{3}H]-saxitoxin (STX) isolated from marine dinoflagellates played a vital role in the purification of a rat brain and skeletal muscle Na_v channel isoform, thereby also demonstrating the existence of auxiliary β-subunits[77; 78; 79]. Moreover, recently synthesized STX variants show promise as a tool to elucidate the role of Na_v channels in signal conduction and their dysregulation in specific disease states[80; 81]. Nowadays, TTX-sensitivity is used to classify the nine mammalian Na_v channel isoforms into two groups: TTX-sensitive channels ($Na_v1.1$-$Na_v1.4$, $Na_v1.6$-$Na_v1.7$) are blocked by nanomolar concentrations of TTX whereas $Na_v1.8$ and $Na_v1.9$ are inhibited by millimolar concentrations[1]. Although inhibition of $Na_v1.5$ requires micromolar concentrations of TTX, the response can be substantially increased by substituting a cysteine in the S5-S6 loop of domain I with tryptophan or phenylalanine[82; 83; 84]. These aromatic

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residues support high affinity blockade through the formation of a cation-π interaction with TTX[83; 85]. The cationic groups also interact with anionic amino acids within the pore of the channel to prevent Na^+ flux. Experiments designed to pinpoint residues that reduced the susceptibility of Na_v channels to TTX led to the discovery of the DEKA motif which consists of four residues that make up the Na_v channel selectivity filter: aspartate (DI S5-S6 loop), glutamate (DII S5-S6 loop), lysine (DIII S5-S6 loop), and alanine (DIV S5-S6 loop)[86; 87].

Similar to K_v channels, animal venoms also contain toxins that target Na_v channel voltage sensors to disrupt channel function. Well-studied examples include members of the long-chain scorpion toxin family which, in general, interact with the extracellular loops between S3 and S4 to stabilize the Na_v channel voltage sensors in the activated or resting state[17; 18; 19; 88; 89]. Based on their functional effects, two classes of Na_v channel scorpion toxins have been defined[90]. First, early antibody and photo-affinity labeling studies indicated that the α-scorpion toxin LqTX interacts with the S5-S6 loops of domains I and IV in rat neuronal Na_v channels[91; 92]. Later, mutagenesis experiments revealed a primary interaction with the S3-S4 paddle motif within domain IV, as well as a secondary binding site within the domain I S5-S6 loop and domain IV S1-S2 loop[93; 94]. Because of their binding locus, α-scorpion toxins as well as the functionally similar sea anemone toxins have been used extensively to elucidate the role of the domain IV voltage sensor in Na_v channel gating (Fig. 2b). For example, a recent study using Ts3 from the Brazilian yellow scorpion *Tityus serrulatus* on native Na_v channels within GH3 cells in concert with fluorescently labeled voltage-sensing domains, demonstrated that the toxin's inhibitory effect on movement of the domain IV voltage-sensing domain results in inhibition of fast inactivation and facilitation of recovery from fast inactivation[95].

A second class of Na_v channel gating-modifier toxins found in scorpion venom are the long-chain β-scorpion toxins which induce sub-threshold channel opening by shifting the threshold for channel activation to more negative membrane potentials (Fig. 2b)[54; 96; 97]. Distinct from α-scorpion and sea anemone toxins, β-scorpion toxins primarily target the paddle motif within the domain II voltage sensor and stabilize it in an activated state[98; 99]. As a result, subsequent depolarizations require the transition of fewer voltage-sensing domains, resulting in the hyperpolarized voltage-dependent activation observed in toxin-bound channels.

In contrast with scorpion and sea anemone toxins, exploration of the mechanism through which spider toxins belonging to the inhibitor cysteine knot (ICK) family interact with mammalian Na_v channels is a more recent phenomenon. Depending on which voltage sensors are targeted and how they couple to the Na_v channel gating process, spider toxins can have three diverse effects on ion channel function[100]. Most commonly, these toxins inhibit channel opening in response to membrane depolarization (e.g. hanatoxin (Fig. 2b), ProTx-I, ProTx-II)[42; 55]. A second possibility for these toxins is to prevent fast inactivation by impairing movement of the domain IV voltage sensor (*e.g.* SGTx1, JZTX-I)[54; 101]. Finally, and similar to β-scorpion toxins, spider ICK toxins can facilitate channel opening by shifting the activation voltage to more hyperpolarized membrane potentials (*e.g.* Magi5)[102].

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Recently, the identification of an S3b–S4 paddle motif within each of the four Na_v channel voltage sensors significantly advanced our insights into the multifaceted working mechanism of spider toxins[54]. In this study, it was demonstrated that each of the four paddle motifs can interact with toxins from spiders, scorpions, and sea anemones, and that multiple paddle motifs can be targeted by a single toxin. These novel insights also led to the identification of the first tarantula toxin (ProTx-I) active on $Na_v1.9$, an enigmatic Na_v channel isoform predominantly expressed in nociceptive dorsal root ganglion neurons[103]. The taxonomically related tarantula toxin ProTx-II targets $Na_v1.7$, a Na_v channel isoform implicated in various debilitating pain syndromes, with an affinity that is >100-fold higher than other Na_v channel isoforms[104]. As is the case with HWTX-IV from the Chinese bird spider *Selenocosmia huwena*, ProTx-II preferentially targets the domain II voltage sensor in $Na_v1.7$[105; 106; 107]. However, ProTx-II also binds to the domain IV paddle motif in this channel subtype, thereby resulting in a slowing of fast inactivation. Overall, toxins isolated from spider venom have already proven to be valuable tools for probing the structure and functional mechanisms of Na_v channels. Moreover, future opportunities may arise for the use of these small and stable peptides as therapeutic drugs.

4. Voltage-gated calcium channel toxins

The channel-forming subunit (α1) of voltage-activated calcium channels (Ca_v) shares a similar architecture with the corresponding part in Na_v channels: four homologous domains (DI-DIV), each containing a voltage-sensor (S1-S4 segments) and two transmembrane segments (S5-S6) that contribute to the pore structure[108; 109]. Like Na_v channels, α1 can function as a standalone subunit that determines many of the biophysical and pharmacological properties of the channel. However, Ca_v channels are normally formed by association of α1 with three auxiliary subunits (β, α2δ, and γ) that regulate channel function and expression[108; 110]. In contrast to the other voltage-gated ion channels, Ca_v channels have the distinctive property of allowing voltage-controlled membrane passage of calcium ions, a key intracellular signaling factor. As the permeant ion has a positive reversal potential, the opening of Ca_v channels results in further membrane depolarization, which allows them to function as an action potential generating mechanism, and to control the firing activity of excitable cells[108]. Thus, Ca_v channels are involved in physiological processes where they couple calcium signaling with membrane excitability, such as hormone and neurotransmitter release, and initiation of electrical activity-dependent transcriptional events[111]. Historically, Ca_v channels were classified based on how calcium currents activated in response to membrane depolarization. Two large families were found: low-voltage (LVA: T-type) and high-voltage-activated (HVA: L-, N-, P/Q-, and R-types) channels[112]. Subsequent biochemical and pharmacological studies and molecular cloning identified the molecular identities that form Ca_v channels. Thus, the L-type corresponds to the Ca_v1 subfamily[113], the N-, P/Q-, and R-types correspond to Ca_v2[112], and the T-type corresponds to Ca_v3[114]. Extensive alternative splicing of the ten known α1 genes, together with combinations of several β-subunit types and posttranslational modifications, further enhance the molecular and functional diversity of Ca_v channels[115].

Organic molecules and especially toxins have been instrumental in discovering the many members of the Ca_v family as well as in understanding their molecular structure and

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function. For example, the L-type channel was isolated and characterized on the basis of its sensitivity to dihydropyridine[116; 117; 118]. However, most subsequent discoveries were enabled by the identification of two groups of toxins: the ω-conotoxin family of pore blockers and the functionally heterogeneous ω-agatoxin family of pore blockers and gating modifiers. The first ω-conotoxin, GVIA, was discovered in the venom of the cone snail *Conus geographus* and is still one of the most widely used pharmacological agents in the study of Ca_v channels[119; 120; 121]. GVIA is selective for N-type ($Ca_v2.2$) channels and was initially employed to inhibit neurotransmitter release, thus functionally linking N-type channels to synaptic transmission[120; 122]. Later, GVIA was used to determine the molecular identity of the human N-type channel, by showing that a heterologously expressed channel consisting of α1b, β2, and α2δ clones was able to generate a current with kinetic properties similar to the N-type current endogenously expressed in neurons[123; 124]. Evidence supporting a pore-blocking mechanism for GIVA comes from several observations[119; 121; 125; 126]. First, early structure-function studies relied on GVIA[125; 127] to determine the architecture of the outer vestibule of the N-type channel. Consequently, chimeric constructs between the GVIA-sensitive (N-type) and GVIA-insensitive (P/Q-type, $Ca_v2.1$) channels showed that the toxin binds to the S5–S6 linker region of domain III of the α1 subunit[125] suggesting that GVIA likely acts via physical occlusion of the pore. Second, increasing the external concentration of the permeant ion greatly decreased the efficiency of toxin block, indicating that the toxin competes with divalent cations bound at the locus of selectivity[128; 129]. Third, residues in close proximity to the region identified by Ellinor et al.[125] also contribute to block of N-type Ca_v channels by GVIA since a single amino acid substitution in this region is sufficient to alter the reversibility of toxin block[126]. Fourth, GVIA block is prevented by ω-agatoxin IIIA binding, which partially occludes the pore to reduce channel conductance[130]. Of note is that the toxin binds to the channel with very high affinity; unless the channel has been inactivated, in which case the interaction is much weaker[127]. The ability of GVIA to discriminate between activated and inactivated states of the channel suggests that the process of inactivation is accompanied by a conformational change in the outer vestibule[127]. Evidence that GVIA may also alter the gating properties of N-type channels stems from work by Jones et al.[131] in which they report that the toxin affects inactivation-induced immobilization of N-type channel gating currents at depolarized voltages. This hypothesis was substantiated by Yarotskyy and colleagues who showed that GVIA induced a 10mV depolarizing shift in the gating charge versus voltage relationship as well as a decreased off-gating current time constant and a smaller on-gating current[132].

The most relevant member of the ω-agatoxin family[133] is IVA, a gating modifier toxin isolated from the funnel-web spider *Agelenopsis aperta*. IVA is specific for P/Q-type channels and it has been used to isolate P-currents in Purkinje neurons[134; 135] and Q-currents (also with ω-conotoxin MVIIC[136]) in granular cerebellar neurons[137; 138; 139]. The subunit composition of P/Q channels was determined by immunoprecipitation of the channel complex labeled with IVA, which suggested that α1, α2δ, and β subunits but not γ subunits form the channel[140; 141; 142]. IVA has 10 times higher affinity for P-channels, and thus it can distinguish between P- and Q-subtypes in mammalian tissues. It is known now that the P- and Q-subtypes are the result of alternative splicing in the S3-S4 linker of domain IV[143], which has also been identified as a toxin binding site[144; 145; 146]. However, recent evidence

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suggests that this alternative splicing cannot fully explain the difference in affinity for IVA[147]. Other factors, such as expression system-dependent toxin responses, the identity of the auxiliary subunit(s), and post-translational channel modifications may also be involved in generating the complete P- and Q-channel phenotypes[143; 148].

The HVA channels are targeted by multiple members of the ω-conotoxin and ω-agatoxin families and by related toxins, such as ω-grammotoxin SIA[149] and SNX482[150]. In contrast, there are fewer known toxins that target LVA channels. The first discovered toxin that acts with high-affinity against T-type channels is kurtoxin, isolated from the venom of the scorpion *Parabuthus transvaalicus*. Kurtoxin is a gating modifier that affects the kinetics of activation and inactivation of the channel. It has been proposed that the toxin interacts with the domain IV voltage-sensor of the T-type channel[151; 152]. Recent studies identified other toxins (*e.g.*, ProTx-I and ProTx-II) that inhibit T-type channels[153; 154]. However, their affinity and effects on channel gating are different compared with those of kurtoxin. For example, ProTx-II modifies both the activation and deactivation kinetics but has no effect on inactivation[153]. Determining the binding sites of these toxins will be useful to dissect out the role of individual voltage sensors in the gating mechanism and the structural aspects of inactivation in T-type channels.

5. Transient Receptor Potential channel toxins

Transient Receptor Potential (TRP) ion channels are tetrameric non-selective cation channels that are architecturally similar to the members of the voltage-gated ion channel family[155; 156]. Although some TRP channels display modest voltage-activation[157; 158], they are functionally quite distinct from voltage-gated ion channels. One defining characteristic of TRP channels is that they are activated by a diverse range of stimuli including temperature, small organic compounds, and mechanical stress[159; 160]. The mechanistic underpinnings of this polymodal gating are poorly understood and the lack of selective pharmacological tools has greatly hampered mechanistic studies on TRP channels. Therefore, recent reports on the discovery of animal toxins that selectively modulate the activity of TRP channel subtypes have given a fresh lease of life to efforts focused on understanding the mechanism of action of these enigmatic ion channels. Additionally, TRP channels are believed to play important roles in pain signalling[161; 162] giving rise to the intriguing possibility that selective toxin modulators of these channels may have therapeutic potential. Indeed, reports on modulation of TRPV1 and TRPA1 channels by animal toxins have energized the field tremendously and the major findings of these studies are summarized below.

Also known as the vanilloid receptor, TRPV1 is the most extensively studied TRP channel[163; 164]. It is activated by a diverse range of small molecules including capsaicin, the active ingredient of chilli peppers that is responsible for their “hot” sensation, and also by temperatures >42°C. The first report on the activation of TRPV1 by animal toxins was published in 2006 by Julius and co-workers who reported the isolation of three toxins from the venom of the Trinidad chevron tarantula *Psalmopoeus cambridgei* and demonstrated that these toxins activated rat TRPV1[165]. Furthermore, these “vanillotoxins” had no effect on the activity of other tested TRP channels. The most potent of these toxins, VaTx3, activated

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TRPV1 with an EC_{50} of 0.32 μM. The least potent TRPV1 activator among these three vanillotoxins, VaTx1, was found to also inhibit $K_v2.1$. This inhibition was characterized by a rightward shift in the conductance-voltage relationship of $K_v2.1$, an effect that mirrors that of voltage sensor-targeting toxins such as hanatoxin that bind to voltage-gated channel paddle motifs[25; 42].

In 2010, another landmark study reported the discovery of a so-called double-knot toxin (DkTx) which was isolated from the venom of the Chinese bird spider, *Haplopelma huwena* and demonstrated to be a potent TRPV1 agonist[4]. This toxin consists of two ICK domains attached via a linker. Each ICK domain has the ability to individually activate TRPV1, albeit with much lower potency compared to DkTx[4; 166]. Moreover, channel activation by the separated domains is readily reversible whereas unbinding of DkTx is extremely slow, resulting in almost irreversible activation of the channel. The concentration-response curve for single unit-activation of TRPV1 yields a Hill coefficient of >1 suggesting that several DkTx molecules bind to the channel in a cooperative fashion. A high-yielding *E. coli* expression system for the heterologous production of DkTx has been developed and has provided access to milligram quantities of the toxin, thereby facilitating structural and functional studies[4; 5; 166]. Recent single-particle electron cryo-microscopy studies provided a 3.8 Å resolution structure of the DkTx-TRPV1 channel complex which also has a small molecule agonist, resiniferatoxin, bound to the channel (Fig. 3b)[6]. This structure revealed that two molecules of DkTx bind to one molecule of TRPV1 at the subunit interface in the pore domain (the S5-S6 region). Comparison of this structure with that of the channel without bound ligand[167] provides valuable insights into the conformational changes that are involved in channel opening upon toxin binding. It appears that the S1-S4 domain of TRPV1 remains relatively static during gating whereas the mobile regions in TRPV1 are the pore helix and the outer loop connecting the S5 and S6 helices. These observations paint a fascinating picture of the conformational changes involved in TRPV1 gating which appear to be in sharp contrast to those involved in gating of voltage-activated ion channels where the S1-S4 domain is believed to undergo large conformational changes upon application of voltage stimuli and the S5-S6 pore domain remains relatively static[24; 168]. At this stage, however, these ideas are mere hypotheses that will no doubt be tested thoroughly in the near future by detailed biophysical studies.

The TRPA1 channel is characterized by the presence of seventeen repeats of the ankyrin protein motif[169; 170]. It is activated by plant products such as icilin, allicin, and isothiocyanates and is believed to be activated by noxious cold and mechanical stress[171; 172; 173; 174; 175]. In 2007, Schaefer and co-workers reported that an ICK toxin isolated from *G. spatulata*, GsMTx-4, activates human TRPA1 expressed heterologously in HEK293 cells[176]. This toxin is also a blocker of mechanosensitive ion channels[9]. Recently, ProTx-I, a promiscuous inhibitor of voltage-gated ion channels[54], was shown to be an antagonist of TRPA1[177]. In this work, the authors employed the recently developed tethered-toxin approach[178; 179] to screen ~100 peptide toxins as potential modulators of TRPA1, which led to the discovery of ProTx-I as a TRPA1 inhibitor.

The discovery of animal toxins that selectively modulate TRP channel function has tremendous implications in TRP channel biology. They are likely to be powerful

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mechanistic tools for understanding the molecular details of TRP channel gating, in much the same way that both pore-blocking and paddle-targeting ICK toxins have contributed to our understanding of gating in voltage-activated channels. Moreover, the use of these toxins as therapeutic molecules targeting TRP channels remains an unexplored area of research and may be worth pursuing given the success of a cone snail toxin (ω-conotoxin MVIIA) targeting spinal populations of $Ca_v2.2$ that obtained FDA-clearance for treatment of intractable chronic pain[180].

6. High-throughput screening for novel animal toxins targeting ion channels

Most ion channel toxins reported to date, including those described in this review, were discovered by serendipity or low-throughput characterization of individual venom components. Conversely, most venom peptides are now discovered through holistic analysis of venoms using a combination of advanced proteomic methods and sequencing of venom-gland transcriptomes[181]. The ability to produce these peptides via recombinant expression[182] or chemical synthesis[183] means that a much larger number of animal toxins are now available for drug discovery efforts and, moreover, it provides access to toxins from miniature venomous animals that would otherwise be inaccessible using conventional bioassay-guided venom fractionation. However, the growing number of available toxins combined with an increasing interest in venom-based ion channel drug discovery has created a need for higher-throughput discovery pipelines[181].

The basic requirements of high-throughput screening (HTS) include high-assay sensitivity and accuracy as well as high-assay robustness and reproducibility. This is particularly important with respect to the screening of crude venoms or partially purified venom fractions. In contrast to combinatorial chemical libraries, which often comprise many thousands if not millions of compounds, natural product libraries frequently consist of crude or partially purified mixtures of compounds with diverse biological effects. This reduces the resource requirements for screening, but at the same time introduces the potential for interference from non-target-specific effects from other components of the venom. Thus, while the traditional goal for HTS in the pharmaceutical industry has been to increase screening capacity through automation and miniaturization of assays, HTS in the context of venom-based drug discovery arguably requires greater emphasis on data quality[181].

Fluorescence-based assays are commonly used for high-throughput ion channel screens[184; 185]. In these assays, the transmembrane flux of ions resulting from channel activation is detected by changes in fluorescence using either ion-specific or membrane-potential-sensitive dyes. The change in fluorescence is detected using specialized plate readers (*e.g.*, $FLIPR^{tetra}$). Calcium-sensitive dyes[186] have been used most frequently in high-throughput ion channel screens[184]. A significant limitation with these and other non-electrophysiology-based HTSs is the lack of voltage control, which is important when screening for toxins that interact with voltage-gated ion channels. As discussed above, many ion channel toxins are gating modifiers that have highest affinity for specific states of the channel. Without voltage control, the conductance state of the channel cannot be controlled, making it difficult to detect toxins with certain modes of action, such as those that cause use-

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dependent inhibition. Use-dependent toxins are of particular interest for modulating channels where subtype-selective binding is difficult to achieve but mechanistic selectivity yields an acceptable therapeutic window (*e.g.*, use-dependent Na_v channel inhibitors that target rapidly firing sensory neurons might be useful analgesics).

The 'gold standard' for determination of the mechanism of action of ion channel modulators is electrophysiology, and recent advances include the development of automated platforms for single-cell electrophysiology studies, thus increasing throughput[184]. Several planar array-based automated electrophysiology systems have been developed to incorporate the precision and accuracy of manual patch-clamp experiments[184]. Of these systems, the PatchXpress® (Axon Instruments), QPatch™ (Sophion) and Patchliner® (Nanion) platforms have gained acceptance as ion channel drug-discovery platforms in the pharmaceutical industry[184]. These automated patch-clamp instruments allow screening for state-dependent inhibitors[187].

In order to rationally improve the potency and selectivity of ion channel toxins, it is essential to map the epitopes that mediate their interaction with the channel of interest (*i.e.*, the toxin pharmacophore). Structure-activity relationships for peptides are traditionally developed in the first instance via alanine scanning mutagenesis, where a panel of toxin mutants is produced in which each residue is individually replaced with Ala and the effect on toxin function is examined[188]. Although this approach has been used to map the pharmacophore of several ion channel toxins[51; 53; 189; 190; 191], it is laborious and time consuming as a full panel of alanine mutants must be produced by chemical or recombinant methods. A much faster and cheaper alternative is to perform the entire alanine scan using the recently reported tetheredtoxin approach[177]. In this method, oocytes are injected with mRNA encoding the channel of interest *plus* mRNA encoding native toxin or one of the alanine mutants. The toxin construct encodes an N-terminal signal sequence to ensure that the toxin is exported from the cell and a C-terminal glycosylphosphatidylinositol (GPI) recognition sequence that directs the oocyte to covalently attach a GPI anchor to the toxin[178; 179]. Thus, after production and export, the peptide toxin is tethered to the extracellular surface of the oocyte via the C-terminal GPI anchor[177]. Inclusion of a long hydrophilic linker between the C-terminus of the peptide and the GPI anchor provides sufficient flexibility for the toxin to access the co-expressed channel, while tandem Myc epitopes incorporated into the linker region facilitate quantification of toxin expression[177]. Because this approach obviates the need for production and purification of toxin mutants, it reduces the time period for performing an alanine scan from months to as little as one week. As for any alanine scanning mutagenesis approach, false positives will be obtained if the tethered toxin is not correctly folded. Thus, it is important to subsequently produce and test the function and folding of the small number of mutants identified as hits in the tethered-toxin screen. Despite this requirement, the greatly improved throughput of this approach facilitates the mapping of pharmacophores across multiple channels if a toxin has polymodal pharmacology, thereby enabling the rational engineering of toxin selectivity. For example, this approach was recently used to map the pharmacophores on ProTx-I that mediate its interaction with TRPA1 and $Na_V1.2$. These pharmacophores were found to be distinct but

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overlapping, which allowed rational design of analogues that are selective for TRPA1 or $Na_v1.2$[177].

7. Toxin engineering: a rationale

The functional properties of toxins found in animal venoms are generally suitable for use as ligands for ion channels, since these have been honed by evolution to act effectively in a native physiological context. Although many toxins can be utilized in their unaltered state, there are a number of reasons for modifying them. To provide an intellectual framework for this section, we discuss the rationale for "toxin engineering".

One reason for initiating a toxin-engineering project is to alter toxin selectivity. For example, it may be desirable to shift toxin specificity from its preferred molecular target to another one. Alternatively, a native toxin may not act with sufficient selectivity or potency and peptide engineering is required to enhance specificity for the particular channel isoform being investigated. For biomedical applications or for diagnostic and therapeutic purposes, a wide variety of toxin engineering protocols can be envisioned that would alter *1)* the binding properties of the peptide, *2)* its ability to cross membranes, *3)* its stability under physiological conditions, or *4)* its pharmacodynamic properties[192]. Since this is a vast and rapidly moving field[193; 194; 195], it falls beyond the scope of this review. Instead, we concentrate on toxin engineering for improving molecular specificity, an important issue for those toxins to be used as tools for investigating basic ion channel function.

Most animal toxins have been optimized by natural selection to efficiently target a single physiologically-relevant protein target present in a specific animal (*e.g.*, the prey, predators, or competitors of the venomous species that evolved the toxin). If selection has indeed occurred to target a specific ion channel, would it really be productive to further modify a toxin? An example of a specific scenario that would require toxin engineering is as follows. Researchers employ specific toxins to investigate circuits present in mammalian systems (and less frequently, other vertebrates, or in non-vertebrate model systems such as *Drosophila*). However, the actual molecular target of a venom peptide may be an ion channel in specific phylogenetically-distant insects. Nonetheless, this target may be a homolog of an ion channel of interest in a specific mammalian circuit. Because of the evolutionary distance, there is not necessarily a 1:1 correspondence. For example, there may be a single physiologically-relevant molecular target in the insect, yet in mammalian systems that ion channel may have diverged into two closely-related homologous ion channel isoforms. Consequently, this venom peptide may target both mammalian isoforms with equal affinity. To be a suitable research tool, this toxin would need to be engineered to be specific for only one of the two derivative isoforms in the mammalian circuitry. In the sections that follow, we examine two actual cases of toxin engineering that were carried out with the goal of developing powerful tools for studying ion channel function. In both cases, the molecular targeting specificity of a native toxin was altered to be appropriate for mammalian applications.

A first example of toxin engineering concerns ShK[196], a sea anemone toxin from *Stichodactyla helianthus*, that has been used to investigate the molecular mechanisms

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underlying autoimmunity. Early investigations by Chandy and coworkers established the expression of specific K_v channel isoforms in T-cells. The specific T-cell subclass implicated in the pathology of autoimmune disease requires the expression of $K_V1.3$, a member of the Shaker subfamily of K_v channels[197]. This switch in gene expression is essential for amplification of the T-cell population responsible for diverse types of autoimmune disease. In the search for ligands specific for $K_v1.3$, ShK was found to block this particular K_v channel isoform with high affinity. However, the native toxin also inhibited the closely related $K_V1.1$ isoform. Subsequently, Chandy and coworkers carried out a toxin-engineering program in which they synthesized 38 analogs to improve ShK selectivity towards $K_V1.3$ (see Chi et al., 2012 for a review[198]). As a result of these efforts, several analogs were identified that showed much improved selectivity for $K_V1.3$. It is worth mentioning that in this particular case, the toxin engineering strategy did not involve substitution of the normal complement of amino acids. Instead, ShK was chemically derivatized at the N-terminus with an L-phosphotyrosine attached to an aminoethyloxy-acetyl linker and was also amidated at the C-terminus. Next, the $K_v1.3$ selective analogs were shown to be efficacious in various animal models of autoimmune disease. One of these analogs, ShK-186, is currently under drug development by Kineta Inc.; ShK-186 recently completed Phase Ia clinical trials and it will soon enter Phase 1b trials for treatment of psoriatic arthritis. Preliminary work using a variety of animal models indicates the potential of ShK derivatives for treating diverse types of autoimmune disease such as type I diabetes, rheumatoid arthritis, and multiple sclerosis.

Given that the $K_V1.3$ isoform is expressed in many tissues including neurons, it was surprising to learn that the engineered sea anemone peptide is clinically effective without having a serious side-affect profile. Chandy and coworkers have a potential explanation for this phenomenon: there is a metalloproteinase expressed in neurons, which has a domain that binds to the homomeric $K_V1.3$ channel but not to heteromeric combinations[199; 200]. The implication is that in neurons, $K_V1.3$ channel homomers cannot be expressed on the plasma membrane; however, immune cells lack the metalloproteinase and as such the T-cells implicated in autoimmunity may be the only significant cell population with the homomeric $K_V1.3$ channel expressed on the membrane surface. As a result, the engineered ShK variant has much more specificity than would have been anticipated purely from $K_V1.3$ gene expression patterns. Overall, this case nicely illustrates the advantages of having a highly-specific ligand for understanding the role of a molecular target in normal physiology and in pathophysiology.

A second example of toxin engineering relates to nicotinic acetylcholine receptors (nAChRs) present in the dopaminergic circuitry (for a review, see Quik and McIntosh, 2006[201]; Olivera et al., 2008[202]; Azam and McIntosh, 2009[203]). Although diverging from the focus of the review on voltage-gated ion channels, we present this example to illustrate how toxin engineering can lead to surprising insights into ion channel function. The biomedical rationale of this study is the observation that heavy smokers appear to be protected from Parkinson's disease, perhaps because the nicotine in tobacco provides a protective effect[204]. In order to understand the molecular machinery underlying this phenomenon, it is desirable to define which nicotinic receptor isoforms might mediate the protective effect of nicotine.

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The initial data that triggered this investigation is shown in Figure 4. A radiolabeled *Conus* peptide, α-conotoxin MII from the fish-hunting cone snail *Conus magus* (the Magician's cone), was shown to label the same region of the striatum as a ligand that binds the dopamine transporter. Since α-conotoxins are nAChR antagonists, this observation suggested that the target of the peptide was present in the dopaminergic circuitry. In order to determine the selectivity of α-conotoxin MII, cloned nAChR subunits were expressed and assayed in Xenopus oocytes and ensuing experiments revealed that α-conotoxin MII inhibits nAChRs containing α6 or α3 subunits. This is not entirely unexpected, since these two α-subunits are the most closely related of all mammalian nAChR subunits. However, employing the toxin to identify the precise composition of the nAChRs present in the striatal dopaminergic circuitry would be impossible. An additional complication is that functional nAChRs are pentameric, with most being heteromeric combinations of two α- and three non-α-subunits. Thus, not only was there a problem in differentiating between α3 and α6-containing nAChRs, but also in determining the identity of the other subunits. One approach to remedy this limitation was to engineer an α-conotoxin MII variant with greater subunit selectivity. This was accomplished by synthesizing a series of single-substitution analogs, followed by combining those substitutions that had higher affinity for α6-containing nAChRs compared to those containing α3[205]. Using an engineered peptide with two amino acid substitutions, it was readily established that the dopaminergic circuitry in the striatum had α6-containing (and not α3) nAChRs. In combination with knockout mice studies[193], the receptor was defined as having a $(\alpha 6)_2(\beta 2)_2\beta 3$ composition.

In the course of these studies, an unexpected observation was made: when radiolabeled α-conotoxin MII was displaced by either the native variant or by the analog that could distinguish between α6 and α3, a single displacement curve was obtained. However, one analog of α-conotoxin MII (shown in Fig. 4) had a biphasic displacement, suggesting that there were two isoforms of the α6-containing nAChR present in the striatum. Follow-up experiments indeed established that the two α6-containing isoforms in the striatum are likely to be those shown in the figure, *i.e.*, one isoform containing $(\alpha 6)_2(\beta 2)_2\beta 3$ and a second isoform containing $\alpha 6\alpha 4(\beta 2)_2\beta 3$. These studies also indicated that the high-affinity isoform that contains an α6 subunit disappears early in the progression to a disease state in a primate model of Parkinson's disease[206]. Thus, engineering native α-conotoxin MII enabled the identification of the nAChR subtype in the dopaminergic circuitry as well as the possible connection of these nicotinic receptors with the progression of the disease state.

4. Conclusion

Animal toxins have been successfully employed for many years to investigate ion channel function. The ability to construct cDNA libraries from animal venom glands together with recombinant/synthetic production methods and high-throughput screening techniques will allow researchers to better exploit animal peptides to investigate the functional aspects of various ion channel isoforms and receptors as well as examine their role in normal and disease states. Moreover, synthetic methods such as cyclization, minimization, and the use of diselenide bridges[192; 195] may overcome the challenges that exist between the initial drug discovery stage and the clinical application of these toxins, which will enable their use as therapeutics.

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Highlights

- Ion channels are vital contributors to cellular communication and are targeted by toxins found in animal venoms.
- For many years, the unique properties of toxins have enabled researchers to probe the structural and functional features of ion channels.
- This review highlights fundamental insights into toxin-channel interactions by focusing on representative examples from the voltage-gated sodium, voltage-gated potassium, voltage-gated calcium, and transient receptor potential channel field.
- Recent developments in toxin screening methods are discussed.
- Practical examples of cases in which toxin engineering was successfully used to address specific ion channel questions are also provided.

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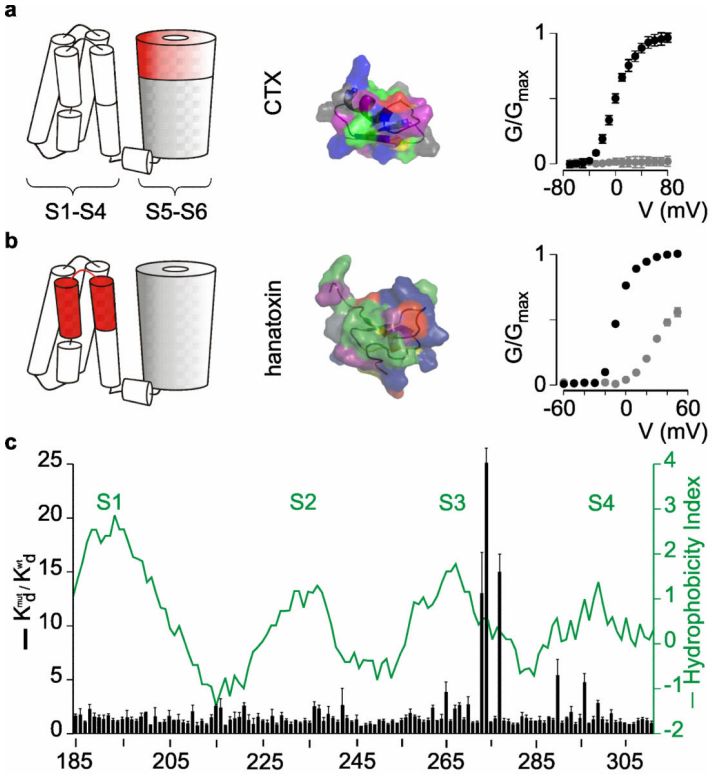

Figure 1. K_V channel toxins

a, Left: ion channel cartoon showing one voltage-sensing domain consisting of S1-S4 segments connected to the S5-S6 segments which make up the pore of the channel. CTX binds to the pore of a K_V channel (indicated in red). Middle: Representative NMR structure of CTX (PDB code 2CRD) including both a transparent surface representation as well as the protein backbone with blue = Lys, Arg, His; red = Glu, Asp; purple = Ser, Thr, Tyr; green = Ala, Ile, Leu, Val, Phe, Trp, Met; and yellow = Cys. The same color coding is used in Figure 2. The toxin is oriented with the functionally important Lys27 residue pointing down. Right: Effect of CTX on the conductance (G)-voltage (V) relationship of $K_V2.1$ $\Delta7$[46] (control: black circles) expressed in *X. laevis* oocytes showing that the toxin (grey circles) completely blocks potassium currents over a wide voltage range. Error bars represent SEM. **b**, Left: hanatoxin binds to the S3b-S4 paddle motif within K_V channel voltage sensors (indicated in red). Middle: Representative NMR structure of hanatoxin (PDB code 1D1H_A). Right: Effect of hanatoxin on the G-V relationship of $K_V2.1$ $\Delta7$ expressed in *X. laevis* oocytes demonstrating that the toxin inhibits potassium currents at relatively mild depolarizations whereas the channel can activate with hanatoxin bound at more depolarized voltages[43]. Hanatoxin is promiscuous and also inhibits Na_V channel activation as shown in Fig. 2. Structure images were created using DSViewer Pro. **c**, Bar graph plot of normalized hanatoxin apparent affinity (K_d) values for alanine mutations spanning from S1 to S4 in $K_V2.1$[47]. Results revealed the molecular determinants of the hanatoxin receptor site within the $K_V2.1$ S3b-S4 paddle motif. The solid line superimposed on the bar graph is a 17-residue window analysis of the Kyte-Doolittle hydrophobicity index.

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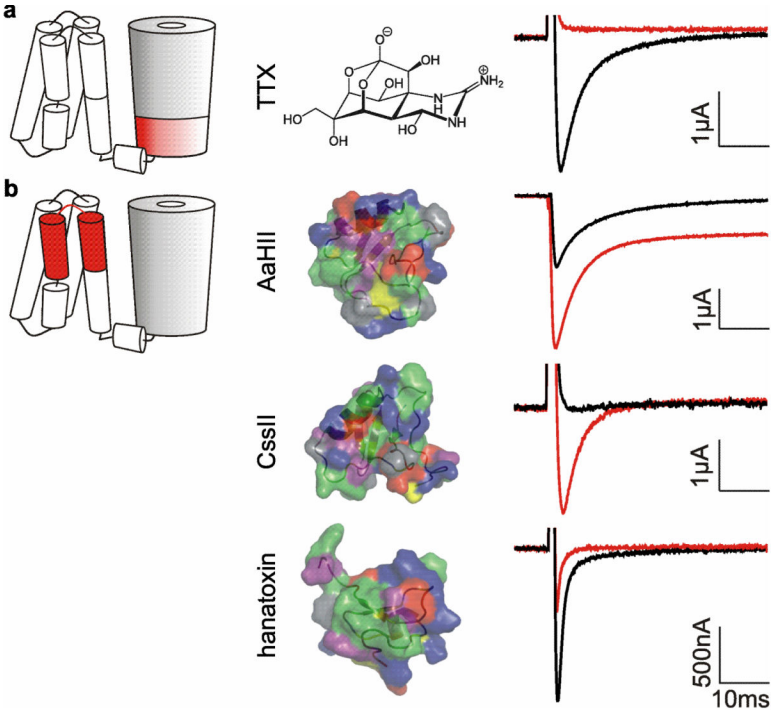

Figure 2. Na_v channel toxins

Effects of 10nM TTX, 100nM AaHII (an α-scorpion toxin from *Androctonus australis* Hector)[207], 1μM CssII (a β-scorpion toxin from *Centruroides suffusus suffusus*)[208], and 100nM hanatoxin on $rNa_v1.2a$ channels expressed in *X. laevis* oocytes. Hanatoxin is promiscuous and also inhibits K_v channel activation as shown in Fig. 1. Left column shows the location within the Na_v channel where these toxins bind (red) which is either the pore region for TTX (**a**) or the voltage-sensing domain for AaHII, CssII, and hanatoxin (**b**). Middle column displays representative toxin structures (PDB codes for AaHII and CssII are 1PTX and 2LJM, respectively) including both a transparent surface representation as well as the protein backbone with the same residue color coding as in Figure 1. Right column shows $rNa_v1.2a$ sodium currents elicited by a depolarization to a suitable membrane voltage before (black) and after toxin addition (red). TTX potently blocks currents (**a**) whereas depending on which voltage sensors are targeted and how they couple to Na_v channel gating, scorpion and spider toxins (**b**) can inhibit channel fast inactivation (domain IV: AaHII), facilitate channel opening by shifting the activation voltage to more hyperpolarized membrane potentials (domain II: CssII), or inhibit channel opening in response to membrane depolarization (domain I/II/IV: hanatoxin).

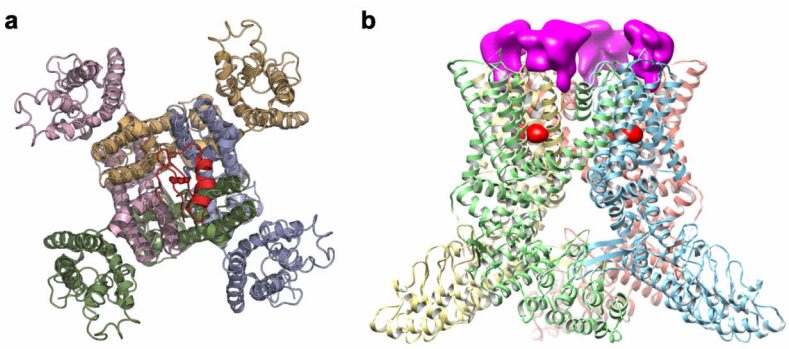

Figure 3. Structures of animal toxins bound to K_V and TRPV1 channels

a, X-ray crystal structure of CTX bound to $K_V1.2$ (PDB code 4JTC), viewed from the extracellular side of the membrane[39]. Each of the four $K_V1.2$ subunits is shown in a different color (green, pink, blue, and light orange). A single molecule of CTX (red) is bound to the extracellular surface of the channel. The sidechain of the critical Lys27 residue (red tube) protrudes into the channel pore to form hydrogen bonds with backbone carbonyl oxygens at the top of the selectivity filter, thereby blocking ion conduction. **b**, Side-on view of the structure of DkTx bound to TRPV1 determined using single particle electron cryomicroscopy (PDB code 3J5Q)[6]. The four TRPV1 subunits are shown in different colors (green, yellow, pink, and blue), and four bound molecules of resiniferatoxin, a TRPV1 agonist, are shown in red. Two DkTx molecules (pink surface representations) bind at the top of the extracellular face of the channel, with each ICK domain (two per DkTx molecule) nestled at a subunit interface, thereby locking the channel in an activated state.

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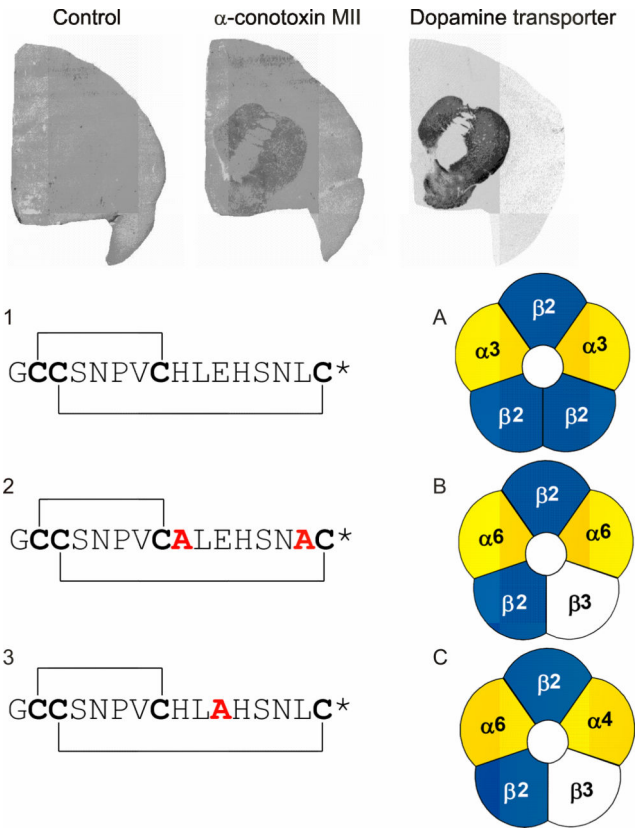

Figure 4. α-conotoxin MII interacts with nicotinic receptors

Top panel shows autoradiography carried out using radiolabeled α-conotoxin MII, as well as a radiolabeled ligand specific for the dopamine transporter. There is a striking overlap between labeling with the toxin and with the transporter ligand. The left panel shows (1) the sequences of native α-conotoxin MII that was characterized by Cartier et al.[209], from the venom of *Conus magus*, and (2, 3) two analogs developed through toxin engineering (the H9A, L15A analog and the E11A analog, respectively)[205]. The potential targets of α-conotoxin MII and its derivatives are shown on the right-hand panel. The native peptide (1) is an antagonist of all three molecular subtypes (A, B, C); the middle analog (2) differentiates between the α3 (A) and α6-containing (B, C) isoforms, and only antagonizes the latter. Analog 3 has the highest affinity for the C isoform, which has both an α6 and an α4 subunit. Thus, toxin engineering allowed identification of the α6-containing isoforms (B, C) in the dopaminergic circuitry.

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Table 1
Overview of toxins discussed in this review

Shown is the organism in which the toxin is found as well as its primary molecular target(s) and binding site(s). Putative binding sites are indicated in parentheses. Note that although tetrodotoxin is found in numerous venomous animals (e.g., pufferfish and blue-ringed octopus); it is actually produced by symbiotic bacteria (largely Pseudoalteromonas, Pseudomonas and Vibrio species). Spider names are from http://research.amnh.org/iz/spiders/catalog/.

Animal	Species	Toxin	Target	(Putative) binding site
Cone snail	*Conus geographus*	ω-conotoxin GVIA	Ca_v channels	Pore
	Conus magus	ω-conotoxin MVIIA	Ca_v channels	Pore
	Conus magus	α-conotoxin MII	nAChR	α3/α6 subunit
Dinoflagellate	Various dinoflagellates/cyanobacteria	Saxitoxin (STX)	Na_v/K_v channels[1]	Pore
Fish/octopus	Produced by symbiotic bacteria	Tetrodotoxin (TTX)	Na_v channels	Pore
Scorpion	*Leiurus quinquestriatus*	Charybdotoxin (CTX)	K_v channels	Pore
	Leiurus quinquestriatus	Agitoxin2	K_v channels	Pore
	Leiurus quinquestriatus	LqTX	Na_v channels	Voltage sensor
	Androctonus australis Hector	AaHII	Na_v channels	Voltage sensor
	Tityus serrulatus	Ts3	Na_v channels	Voltage sensor
	Centruroides suffusus suffusus	CssII	Na_v channels	Voltage sensor
	Parabuthus transvaalicus	Kurtoxin	Ca_v/Na_v channels	Voltage sensor
Sea anemone	*Anemonia sulcata*	BDS-I	Na_v/K_v channels	(Voltage sensor)
	Stichodactyla helianthus	ShK	K_v channels	Pore
Spider	*Grammostola rosea*	Hanatoxin (1/2)	$K_v/Na_v/Ca_v$ channels	Voltage sensor
	Grammostola rosea	ω-grammotoxin SIA	Ca_v/K_v channels	Voltage sensor
	Thrixopelma pruriens	ProTx-I	$Na_v/K_v/Ca_v$ channels/TRPA1	Voltage sensor
	Thrixopelma pruriens	ProTx-II	Na_v/Ca_v channels	Voltage sensor
	Stromatopelma calceatum griseipes	SGTx1	K_v/Na_v channels	Voltage sensor
	Chilobrachys guangxiensis	JZTX-I	Na_v/K_v channels	Voltage sensor
	Macrothele gigas spider	Magi5	Na_v channels	(Voltage sensor)
	Haplopelma huwenum	HWTX-IV	Na_v channels	Voltage sensor
	Agelenopsis aperta	ω-agatoxin IVA	Ca_v channels	Voltage sensor
	Hysterocrates gigas	SNX482	Ca_v channels	Voltage sensor
	Psalmopoeus cambridgei	Vanillotoxins	TRPV1	(Voltage sensor)
	Haplopelma huwenum	DkTx	TRPV1	Pore
	Grammostola rosea	GsMTx-4	TRPA1/Mechanosensitive channels	(Pore)

[1] See Ref. 210.