

Constraint shapes convergence in tetrodotoxin-resistant sodium channels of snakes

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Natural selection often produces convergent changes in unrelated lineages, but the degree to which such adaptations occur via predictable genetic paths is unknown. If only a limited subset of possible mutations is fixed in independent lineages, then it is clear that constraint in the production or function of molecular variants is an important determinant of adaptation. We demonstrate remarkably constrained convergence during the evolution of resistance to the lethal poison, tetrodotoxin, in six snake species representing three distinct lineages from around the globe. Resistance-conferring amino acid substitutions in a voltage-gated sodium channel, Na_v1.4, are clustered in only two regions of the protein, and a majority of the replacements are confined to the same three positions. The observed changes represent only a small fraction of the experimentally validated mutations known to increase Na_v1.4 resistance to tetrodotoxin. These results suggest that constraints resulting from functional tradeoffs between ion channel function and toxin resistance led to predictable patterns of evolutionary convergence at the molecular level. Our data are consistent with theoretical predictions and recent microcosm work that suggest a predictable path is followed during an adaptive walk along a mutational landscape, and that natural selection may be frequently constrained to produce similar genetic outcomes even when operating on independent lineages.

coevolution | molecular evolution | pleiotropy

The degree to which adaptive evolution is predictable at the molecular level remains controversial. On the one hand, convergent phenotypes are expected to arise from unique and unpredictable genetic changes because of the distinct evolutionary history and genetic makeup of independent species and the stochastic nature of mutation and drift. On the other hand, functional constraints (1, 2), patterns of pleiotropy (3–6), and developmental biases (7–10) might filter the available spectrum of mutations to a limited but tolerable cast (3, 5, 6). If adaptive evolution is constrained by the biophysical properties of interacting molecules (1, 2, 11) or limited by developmental and structural constraints (7–10), then adaptive substitutions might be concentrated at a relatively few genetic hotspots and fix in a repeated and predictable fashion (5, 6, 12).

We evaluated the predictability of adaptation at the molecular level by investigating the genetic basis of resistance to tetrodotoxin (TTX) in lineages of predatory snakes that consume toxic amphibians. TTX is a lethal defensive compound found in the skin secretions of a wide variety of amphibians (13, 14). Roughly 500 times deadlier than cyanide, TTX is one of the most potent natural toxins ever discovered (15). TTX operates by binding to the outer pore of voltage-gated sodium channels in nerves and muscles, blocking the movement of sodium ions (Na⁺) across cell membranes and halting action potentials that control nerve impulses (16). Despite the lethality of this neurotoxin, diverse lineages of snakes have evolved the ability to exploit TTX-bearing prey (Fig. 1 and Fig. S1) and are exceptional as the only known vertebrate predators of tetrodotoxic organisms (17). In western North America, populations of Pacific newts (*Taricha*) harbor extreme levels of TTX (18) but are preyed on by multiple

garter snake species (*Thamnophis*): *Thamnophis sirtalis* preys on *Taricha granulosa* (17, 19); *Thamnophis couchii* preys on *Taricha torosa* (20) and *Taricha sierrae* (21); and *Thamnophis atratus* consumes *Taricha granulosa* (22). In Central and South America, the only known predator of highly poisonous *Atelopus* toads (23, 24) is the Neotropical ground snake, *Liophis epinephelus* (25, 26). In southern Japan, the tetrodotoxic newt *Cynops ensicauda* (27) is taken by Pryer's keelback, *Amphiesma pryeri* (28, 29), and in East Asia the TTX-bearing tree frog *Polypedates cf. leucomystax* (30) is preyed on by the tiger keelback, *Rhabdophis tigrinus* (31). Natural selection on these independent lineages of predators is expected to have led to convergent resistance to TTX at the organismal level but not necessarily via the same physiological or genetic mechanisms.

The genetic basis of TTX resistance is known for only one group of predators, the garter snakes (*Thamnophis*). Structural changes in the skeletal muscle sodium channel (Na_v1.4) modify the molecular environment of the channel pore and dramatically alter TTX-binding affinity to the protein (32). Functional allelic variation in Na_v1.4 correlates tightly with whole-animal resistance to TTX and suggests that changes in this single gene are largely responsible for within- and among-population differences in resistance (32–34). Because TTX binds so selectively to the pore of sodium channels, we predict that biophysical constraints associated with channel function have led to a limited set of convergent molecular adaptations to the challenge of TTX-bearing prey worldwide.

We examined sequence variation in the four domains of Na_v1.4 (DI–DIV) that encode the outer pore of the channel (P-loops) and whose residues interact with TTX (35–38) for six species of snakes known to prey on TTX-bearing amphibians, their sister groups, and additional taxa (74 species; $n = 121$ individuals) to provide a robust phylogenetic perspective (*Methods*). We scored organismal TTX resistance for nearly half these species (29 species; $n = 424$) using a performance bioassay (*Methods*). We then compared the observed substitutions in snakes with the spectrum of mutations in mammalian constructs that are known to increase the resistance of sodium channels to TTX to determine whether adaptive evolution in snakes has capitalized on these options randomly or used a restricted subset of these options (*Methods*).

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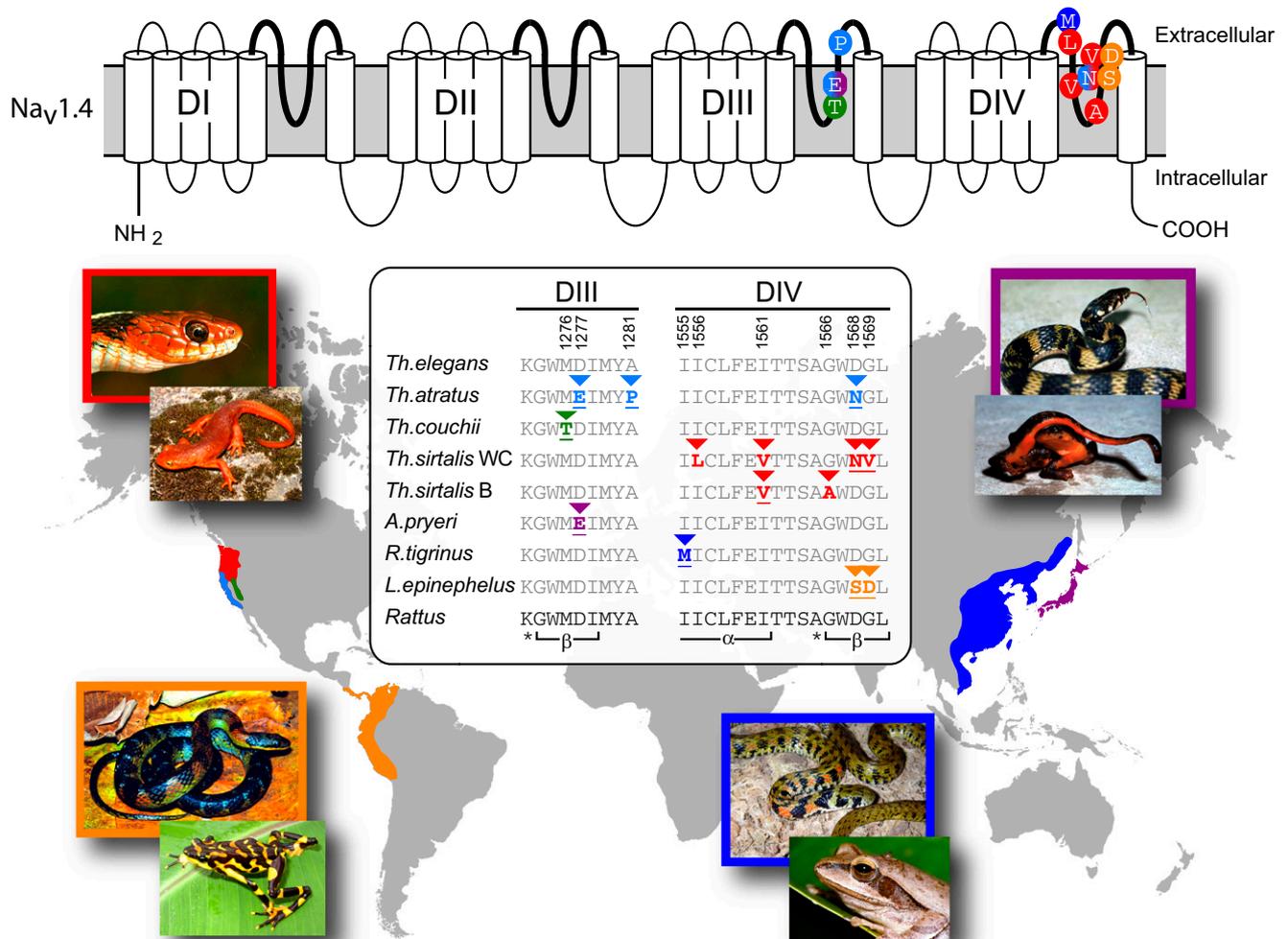


Fig. 1. Parallel arms races between lethal, TTX-bearing amphibians and resistant snakes are expected to have evolved independently across the globe through convergent amino acid substitutions. In parts of North America, *Th. sirtalis* (red), *Th. atratus* (light blue), and *Th. couchii* (green) prey on *Taricha* newts (19–22); in parts of Central and South America *L. epinephelus* (orange) consumes *Atelopus* toads (25, 26); Japanese *A. pryeri* (purple) eats the newt *C. ensicauda* (28, 29); and Asian *R. tigrinus* (dark blue) preys on the tree frog *P. leucomystax* (31). (Upper) 2D structure of the α -subunit of skeletal muscle sodium channel ($\text{Na}_v1.4$) showing the four domains (DI–DIV), their six transmembrane segments, and the linkers that connect segments. Four polypeptide chains link the final two transmembrane segments of each domain to form the ion-conducting outer pore (P-loops) of the sodium channel (bold). Although a number of residues in each P-loop are known to be important in TTX binding to the pore, all the adaptive variation in snakes is clustered in DIII and DIV (circles on P-loops: T, threonine; E, glutamic acid; P, proline; M, methionine; L, leucine; V, valine; A, alanine; N, asparagine; S, serine; D, aspartic acid). (Lower) Detailed alignment of the DIII and DIV P-loop sequences in TTX-resistant snakes highlighting the adaptive replacements (triangles above residues). Identical or functionally equivalent replacements occur at most of the same residues (underlined) in $\text{Na}_v1.4$ of tetrodotoxic pufferfish. Note that separate alleles have proliferated in western populations of *Th. sirtalis* (B, Benton County; WC, Willow Creek), but we assume they share I1561V through common descent. Rat sequence (NM013178) and nonresistant garter snake (*Th. elegans*) sequence are given for comparison; positions follow $\text{Na}_v1.4$ coding sequence from *Th. sirtalis* AY851746. Structures of the pore labeled below rat sequence (*, selectivity filter; α , α -helix; β , β -strand). Colors are coded by species as above.

Results and Discussion

Genetic Basis of TTX Resistance in Snakes. Amino acid sequences of the pore-forming structures (pore α -helix, selectivity filter, β -strand) are highly conserved across colubroid snakes and are nearly identical to mammalian sequences. In the P-loops of TTX-sensitive reptiles we found only eight replacements, all at sites not implicated in TTX binding. However, in the six species that consume TTX-bearing prey, we found 13 derived substitutions, all at positions that influence TTX affinity (Fig. 1 and Movie S1). Nine of these substitutions replace amino acids in the β -strand, whose side chains face the pore and interact directly with TTX (Fig. 2 and Movie S1) (35–38). Mapping these changes and the TTX-resistance data onto the colubroid phylogeny (Fig. S1) clearly demonstrates that high sensitivity to TTX is the ancestral condition and that TTX resistance has evolved inde-

pendently at least six times in snakes. Functionally similar (and sometimes identical) P-loop replacements occur in $\text{Na}_v1.4$ of TTX-bearing pufferfish (39–41) (the only other TTX-resistant vertebrate whose sodium channels have been characterized) in all but two of the sites modified in TTX-resistant snakes (Fig. 1).

Garter snakes (*Thamnophis*) that prey on Pacific newts (*Taricha*) exhibit multiple replacements in DIII and DIV; one in *Th. couchii* (42), three in *Th. atratus* (34), and up to four in *Th. sirtalis* (32). The single M1276T replacement in *Th. couchii* (positions follow $\text{Na}_v1.4$ CDS from *Th. sirtalis* AY851746) produces a 15-fold decrease in TTX binding to the channel (40); the I1561V substitution in *Th. sirtalis* halves TTX-binding affinity (32); and the shared D1568N replacement in *Th. atratus* and *Th. sirtalis* produces a 30- to 40-fold decrease in TTX binding to the channel (43, 44).

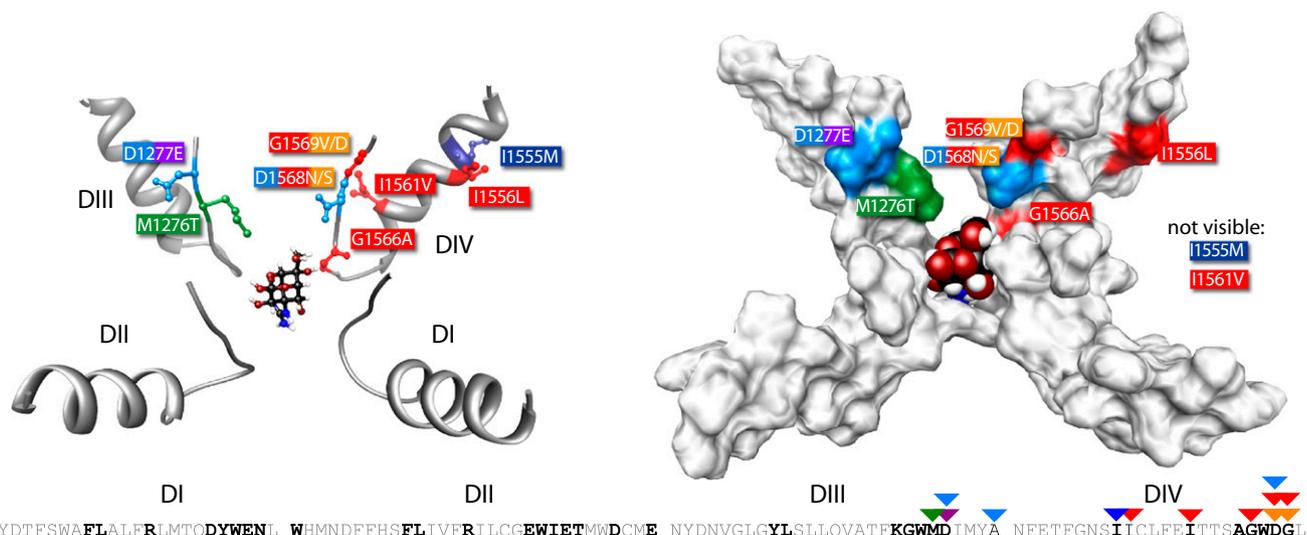


Fig. 2. Structural model of the Na⁺1.4 outer pore (following ref. 35) with TTX docking in the pore (also see [Movie S1](#)). Adaptive replacements in DIII and DIV P-loops of snakes (colors follow Fig. 1) change the electrostatic environment and geometry of the outer pore, weakening TTX binding to the pore (see text for details). (Upper Left) Ball-and-stick model. (Upper Right) Space-filling model. (Lower) Amino acid sequence of the four P-loops (Rat NM013178) highlighting residues known to affect TTX binding to the pore (bold; see text) and positions where adaptive variation in snakes is clustered (triangles above residues).

The Neotropical ground snake, *L. epinephelus*, the only known predator of the highly toxic harlequin toads (*Atelopus*) (25, 26), displays two flanking mutations in the DIV β -strand. The first mutation, a D1568S replacement, occurs at the same position where a D \rightarrow N replacement is seen in highly resistant *Th. atratus* and *Th. sirtalis*. This site plays a critical role in TTX binding because a hydrogen bond forms between TTX and D1568 (37, 44). Changes that neutralize the charged D1568, such as S, dramatically reduce TTX affinity to the outer pore (43, 45). The second P-loop change, G1569D, should alter the docking orientation of TTX into the outer pore (37, 44) and may weaken the affinity of TTX to the neighboring D1568 residue further (41). In *L. epinephelus*, the G1569D substitution involves dramatically different amino acids and likely changes the conformation of the external mouth of the outer pore and possibly pore volume. *L. epinephelus* also is one of the few predators of deadly dart-poison frogs (25), some of which possess batrachotoxin (BTX) and pumiliotoxin (PTX-B) (46). Both toxins interfere with Na⁺ channel inactivation, causing the channels to remain persistently open (16, 47, 48). However, these toxins do not bind to the outer pore but instead to the inner pore (BTX) (48–50) or to other transmembrane helices (PTX-B) (47, 51). Thus, although overall Na⁺ channel structure in *L. epinephelus* likely is influenced by multiple prey toxins, we are confident that the P-loop replacements we discuss have been shaped by selection from TTX, because the outer pore is the target of TTX, and these functional mutations are at sites critical to TTX binding but uninvolved in BTX or PTX-B binding (47–51).

Japanese *A. pyrreri* consume the tetrodotoxic newt *C. ensicauda* (28, 29) and have a single D1277E substitution in the DIII β -strand. This replacement involves biochemically similar amino acids, and mutations at this position generally lead to only minor changes in TTX binding (44, 45). Nevertheless, substitutions involving similar amino acids at positions thought to have little involvement with TTX can still produce significant reductions in TTX-binding affinity (e.g., I1561V) (32).

The Asian keelback *R. tigrinus* preys on the TTX-bearing tree frog *P. leucomystax* (31) and displays an I1555M change in the DIV α -helix. This substitution occurs at a TTX-sensing residue (37), and the loss of a rigid aliphatic side chain and gain of a larger functional group is expected to alter the orientation of

the α -helix and thus the geometry of the outer pore, thereby indirectly weakening TTX-binding affinity to the pore (38).

Constraints on Convergent Evolution. Phenotypic convergence, or the evolution of similarity in divergent lineages, is pervasive in biological systems and is thought to demonstrate the primacy of natural selection because the repeated occurrence of similarity by chance seems unlikely. However, the basis of convergence, particularly at the molecular level, can be strongly biased through biophysical or biochemical constraints on proteins (1, 2, 11) and by commonalities in the genetic architecture underlying the phenotype (i.e., genetic channeling; see ref. 52). These constraints may restrict available routes through the adaptive landscape and bias trait evolution (53, 54). Thus, both natural selection and evolutionary constraints likely are involved in convergent evolution (8, 9, 12, 55).

A number of residues distributed throughout the four P-loops contribute to TTX-binding affinity ($n = 35$; *Methods*), and mutations at any of these sites are candidates for adaptation of TTX resistance. For example, TTX-bearing pufferfish possess adaptive changes in the P-loops of all four domains (39–41). However, the replacements fixed during the evolution of resistance in snakes are limited to only two domains (DIII and DIV) and a fraction (26%) of the possible sites. In fact, seven of the 13 substitutions are confined to the same three positions, and two of these substitutions involve the same amino acid (we assume coincident I1561V in *Th. sirtalis* represents a single event). We observe a departure from the null expectation of a Poisson distribution of mutations (random events) whether we liberally consider all P-loop sites available for substitution ($\chi^2 = 338.45$, $P < 0.0001$) or conservatively consider only those sites experimentally verified to reduce TTX binding to the pore significantly ($\chi^2 = 54.31$, $P = 0.015$).

Why has the evolution of TTX resistance centered on such a predictable subset of relevant mutations? Many of the amino acids that form the outer pore to create the optimal environment for the selective permeation of Na⁺ ions also interact strongly with TTX (36), so changes that reduce the affinity of TTX to the outer pore are likely to have pleiotropic effects on the molecular sieving of the sodium channel. Data from ex vivo expression of sodium channels altered through site-directed mutagenesis of specific residues (*Methods*) indicate that amino acid substitutions

Assessing Bias in Na_v1.4 Mutations. We determined whether the pattern of mutations we observed in snakes is clustered or follows a random distribution. If the observed mutations are not distributed randomly among P-loop sites but instead are clustered, then we have evidence that the genetic response of snakes has been narrowed. We tallied the number of times a site was hit by a mutation for two sets of potentially available sites: (i) all sites of the four P-loops ($n = 96$) (following ref. 35) or (ii) only sites experimentally verified to reduce TTX sensitivity twofold versus wild type and/or shown to influence TTX binding significantly in protein models ($n = 33$) (32, 37–41, 43, 45, 62, 65–71) but also including sites with parallel substitutions between snakes and pufferfish still unstudied ($n = 2$) (39–41). We then used a simple binomial test to compare the distribution of our data against the null expectation of a Poisson distribution. Note that we counted the I1566V replacement seen in both Benton and Willow Creek *Th. sirtalis* only once, because we assume this replacement appears in these populations because of common descent (i.e., does not represent independent replicated events). We calculated the mean and variance of our samples and then the coefficient of dispersion ($CD = \text{variance}/\text{mean}$). In a Poisson distribution the mean and variance are roughly equal ($CD = 1$), but in a clumped distribution the variance should be much greater than the mean ($CD > 1$) (72). Because the CD is approximately χ^2 distributed (72), we calculated χ^2 scores for our CD measures to obtain P values against the random expectation ($CD = 1$). Here $\chi^2 = l(n - 1)$ where l is our CD and $n - 1$ is the degrees of freedom (72), in this case the number of sites minus 1.

Assessing Tradeoffs in Na_v1.4. Sodium channels are highly specialized proteins, and the amino acids that form the outer pore and selectivity filter interact in complex ways to create the optimal environment for the selective permeation of Na⁺ ions (16). However, the same P-loop residues that permit selectivity and permeability of Na⁺ also interact strongly with TTX through a combination of hydrogen and ionic bonds, steric attraction, and cation- π interaction (36, 37, 43–45, 66, 69, 71, 73). Therefore, changes that reduce the affinity of TTX to the outer pore also might negatively impact the molecular sieving of the sodium channel. If antagonistic pleiotropy exists within Na_v1.4, then we should find convergence at the molecular level, because the pool of replacements that can reduce TTX binding and preserve channel function probably is limited. We quantified this potential tradeoff between TTX resistance and sodium channel function to understand whether constraints have influenced molecular evolution in TTX-resistant snakes.

We evaluated the potential tradeoff between TTX resistance and two essential functions of the sodium channel that are determined by the molecular architecture of the outer pore: (i) Na⁺ permeability and (ii) Na⁺ selectivity. We searched the literature for electrophysiological studies that measured the effects of individual replacements (via site-directed mutagenesis and *ex vivo* expression) on TTX block, Na⁺ permeability, or Na⁺ selectivity. Most studies measured or reported these variables in different ways

(e.g., different cations were tested for Na⁺ selectivity), and few examined the effects of the same replacement on each of these three variables. Therefore, we simply scored a replacement as producing a positive or negative effect on TTX resistance (predictor variable) and either Na⁺ permeability or Na⁺ selectivity (response variables) as follows: we scored a replacement as positive (1) if it produced an effect as well or better than the wild type; we scored a replacement as negative (0) if it produced a statistically worse effect than the wild type. Using this scheme, we tallied 46 mutants for both TTX resistance and Na⁺ permeability (41, 43–45, 66, 67, 69, 70, 74, 75) and 29 mutants for TTX resistance and Na⁺ selectivity (43, 65–68, 74–81) (summarized in Table S1). We then determined whether either Na⁺ permeability or Na⁺ selectivity is statistically independent of TTX resistance using a simple χ^2 test under the null assumption that a mutation could affect traits along both axes with equal probability (expected ratio 1:1:1) (Fig. 3).

We also performed a regression analysis on TTX resistance against Na⁺ permeability using data from three site-directed mutagenesis studies that collected and reported comparable data (45, 65, 69). Here, the data on TTX sensitivity are the concentrations of TTX (in nM) required to reduce peak Na⁺ current by 50% (IC_{50}) in the wild-type and single-mutant channels, and the data on Na⁺ permeability are measures of single-channel conductance (pS). We log-transformed the TTX data and adjusted the Na⁺ conductance data of each study by setting wild-type measures to 1 and scaling the conductance values of each mutant to its respective wild type. We then performed a linear regression of these data ($n = 31$) using SAS 9.2 (SAS Institute Inc.) (Fig. 3).

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natricines *Thamnophis sirtalis* (red), *Thamnophis atratus* (light blue), and *Thamnophis couchii* (green) prey on *Taricha* newts (1–4); the Old World natricines *Rhabdophis tigrinus* (bright blue) preys on the tree frog *Polypedates leucomystax* (5) and *Amphiesma pryeri* (purple) preys on the newt *Cynops ensicauda* (6, 7); the neotropical dipisadine *Liophis epinephelus* (orange) consumes several *Atelopus* toads (8, 9). Phylogeny of colubroid snakes and relatives is based on relationships presented in refs. 10–18. Snakes that prey on TTX-laden frogs or salamanders show derived variation in the P-loops domains DIII and DIV (colored circles); P-loops in other domains (and other taxa) lack adaptive variation (black circles). In a few cases we were unable to obtain P-loop sequences (white circles). Numbers of individuals sequenced (GE) and assayed for TTX resistance (PE) alongside measures of TTX resistance [50% mass-adjusted mouse units (MAMU)]. Direct measures of whole-animal resistance for this study were augmented with some of our previous data (19–21). We inferred elevated levels of TTX resistance (†) for *A. pryeri*, *R. tigrinus*, and *L. epinephelus* based on measures of TTX recorded in their respective prey: 60–7,000 mouse units (MU) of TTX for *C. ensicauda* (22–25); 30–920 MU for *Polypedates* spp. (26); 10–100 MU for *Atelopus* spp. (25, 27, 28).

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Table S1. Sodium channel mutational constructs (from the literature) functionally expressed ex vivo and measured for TTX resistance and/or Na⁺ conductance or Na⁺ selectivity

TTX ≥ WT	Conductance ≥ WT	Selectivity ≥ WT	Sodium channel replacement	Citations and notes
1	0		Y401C	1
1	1		W736C	2 (see figure 1D; conductance the same
1	0		D1532A	3 (see figure 3)
1	0		F385N	3 (see figure 2)
1	0		D400A	4 [TTX]; 5 [conductance]
1	0		E755A	4 [TTX]; 5 [conductance]
1	1		K1237A	4 [TTX]; 5 [conductance]
0	0		I757C	6 [TTX]; 7 [conductance]
0	0		M1240C	6 [TTX]; 7 [conductance]
0	0		D1241C	6 [TTX]; 7 [conductance]
1	0		W402C	6 [TTX]; 7 [conductance]
1	0		E403C	6 [TTX]; 7 [conductance]
1	0		E758C	6 [TTX]; 7 [conductance]
1	0		K1237C	6 [TTX]; 7 [conductance]
1	0		W1239C	6 [TTX]; 7 [conductance]
1	0		A1529C	6 [TTX]; 7 [conductance]
1	0		D1532C	6 [TTX]; 7 [conductance]
1	1		G1530C	6 [TTX]; 7 [conductance]
1	1		W1531C	6 [TTX]; 7 [conductance]
0	0		R379Q	8
0	0		D1426Q	8
0	0		D1426K	8
1	0		Q383E	8
1	0		Q383K	8
1	0		D384E	8
1	0		D384N	8
1	0		W386Y	8
1	0		E387Q	8
1	0		E387S	8
1	0		E387Y	8
1	0		N388R	8
1	0		Q391K	8
1	0		W943Y	8
1	0		E945K	8
1	0		K1422E	8
1	0		M1425Q	8
1	0		M1425K	8
1	0		D1426N	8
1	0		A1714E	8
1	0		D1717Q	8
1	0		D1717K	3 (see figure 3), 8
1	0		D1717N*	3 (see figure 3), 8; *here the D1568N replacement of <i>Th. atratus</i> and <i>Th. sirtalis</i>
1	0		E942Q	8, 9
1	0		E945Q	8, 9
1	0		R395C	10
1	0		R750C	10
1		0	Y401C	1 [TTX]; 11–13 [selectivity]
1		0	Y401A	12
1		0	Y401D	12
1		0	Y401S	13
1		1	W736C	2 (see figure 5C); selectivity mostly the same, but more sensitive to NH ₄ ⁺
1		1	E942Q	9; selectivity same but only Li ⁺ tested
1		1	E945Q	9; selectivity same but only Li ⁺ tested
1		1	D949N	9; selectivity same but only Li ⁺ tested
1		0	D400A	4 [TTX]; 5, 14 [selectivity]
1		0	E755A	4 [TTX]; 5, 14 [selectivity]
1		0	K1237A	4 [TTX]; 5, 14 [selectivity]
0		0	W756C	6 [TTX]; 7 (see figure 6B) [selectivity]
1		0	W1239C	6 [TTX]; 7 (see figure 6B), 15 [selectivity]

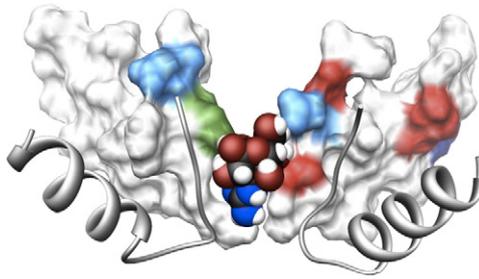
Table S1. Cont.

TTX \geq WT	Conductance \geq WT	Selectivity \geq WT	Sodium channel replacement	Citations and notes
0		1	M1240C	6 [TTX]; 7 (see figure 6B), 15 [selectivity]; selectivity mostly the same
1		0	K1237C	6 [TTX]; 7 (see figure 6B), 15, 46 [selectivity]
1		0	A1529C	6 [TTX]; 7 (see figure 6B), 15, 46 [selectivity]
1		0	W1531C	6 [TTX]; 7 (see figure 6B), 15, 46 [selectivity]
1		0	D1532C	6 [TTX]; 7 (see figure 6B), 15, 46 [selectivity]
0		1	I757C	6 [TTX]; 7 (see figure 6B), 16 [selectivity]; selectivity mostly the same
1		0	W402C	6 [TTX]; 7 (see figure 6B), 16 [selectivity]
1		0	G1530C	6 [TTX]; 7 (see figure 6B), 16 [selectivity]
1		0	E403C	6 [TTX]; 11 (see figure 4D), 16 [selectivity]
0		1	D1241C	6 [TTX]; 16 (see figure 4D), 16 [selectivity]; selectivity mostly the same
1		0	E758C	6 [TTX]; 16 (see figure 4D), 16 (see figure 3)[selectivity]; similar but significantly worse for Li ⁺
1		0	R395C	10
1		0	R750C	10
1		1	F745C	10; selectivity mostly the same
1		0	I1532V*	17; *here the I1561V replacement of <i>Th. sirtalis</i>
1		0	IIDG->LVNV*	17; *here the four DIV replacements in Willow Creek <i>Th. sirtalis</i> (1.4 ^{LVNL} allele of ref. 18)

Replacement notation follows that of the original study. For the χ^2 analyses we tallied a replacement as positive (1) if it produced an effect as well or better than the wild type on TTX resistance (predictor variable) and either Na⁺ permeability or Na⁺ selectivity (response variables) and as negative (0) if it produced a statistically worse effect than the wild type.

*Only two studies (3, 17) have examined some of the naturally occurring mutations in snakes for Na⁺ conductance or Na⁺ selectivity, but these studies show that TTX-resistant mutations (or alleles) compromise sodium channel function.

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Movie S1. 3D model of the Na_v1.4 outer pore showing TTX docked in the pore and adaptive replacements in TTX-resistant snakes. The movie begins with a top-down view of the four pore loops (DI–DIV) and TTX occluding the outer pore; then the outer pore tilts forward to show angled side views of the outer pore. Note that both TTX and Na⁺ enter the pore from the extracellular side of the protein (top). The structural model of the outer pore follows ref. 1.

[Movie S1](#)

1. Lipkind GM, Fozzard HA (2000) KcsA crystal structure as framework for a molecular model of the Na⁺ channel pore. *Biochemistry* 39:8161–8170.