Constraint shapes convergence in tetrodotoxin-resistant sodium channels of snakes

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Abstract
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\textsubscript{v}1.4, 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\textsubscript{v}1.4 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.

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\textsuperscript{+}) 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 sierra (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 leucomystax (30) is preyed on by the tiger keelback, Rhodophis tigrinus (31).

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\textsubscript{v}1.4) modify the molecular environment of the channel pore and dramatically alter TTX-binding affinity to the protein (32). Functional allelic variation in Na\textsubscript{v}1.4 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\textsubscript{v}1.4 (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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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. FJ570810–FJ571064, GQ154075–GQ154084, and JQ067537–JQ067861).

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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 Ateleplus 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 (Na1,4) showing the four domains (D1–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 Na1,4 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 (28, 29); and Asian R. couchii (32). The single M1276T replacement in Th. atratus produces a 15- to 30-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).

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). None 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 independently at least six times in snakes. Functionally similar (and sometimes identical) P-loop replacements occur in Na1,4 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 Na1,4 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).
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 DII β-strand. The first mutation, a D1568S replacement, occurs at the same position where a D→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. pyreri* 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. leucomeystax* (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 alphatic 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

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**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).
in the P-loops that decrease TTX-binding affinity also tend to reduce Na⁺ permeability or Na⁺ selectivity (Fig. 3 and Table S1). This tradeoff between TTX resistance and sodium channel performance is a consequence of the neutralization of negative charges that line the outer pore and changes in pore volume or aperture (45, 56). Thus, a number P-loop replacements with charges that line the outer pore and changes in pore volume or aperture (45, 56). Thus, a number P-loop replacements with charges that line the outer pore and changes in pore volume or aperture (45, 56).

**Methods**

**Bioassays.** To provide a phylogenetic perspective on the evolution of elevated TTX resistance in snakes and to aid in our interpretation of Na⁺,1.4 sequence data, we collected TTX-resistance data from a diverse sample of colubrid snakes. We collected data from 16 snake species (n = 116 mut) representing most of the major colubrid lineages (57, 58) and from one lizard outgroup taxon (n = 5). We augmented these data with results from some of our other work (19, 20, 42, 59) to provide the most complete picture of TTX resistance in squamate reptiles to date (29 species; n = 424) (Fig. S1).

We measured TTX resistance using a bioassay of whole-organism performance (17, 60). We first established an individual’s “baseline speed” by racing it down a 4-m racetrack equipped with infrared sensors that clock sprint speed over 0.5-m intervals. We ran each snake twice and averaged the speed of the two time trials to obtain an individual’s baseline crawl speed. Following a day of rest, we gave each snake an i.p. injection of a known, mass-adjusted dose of TTX (Sigma). Thirty minutes after injection we raced snakes on the track to determine postinjection speed. We repeated this process, resting snakes for a day then increasing the dose of TTX (0.5 μg, 1 μg, 2 μg, 5 μg, and 10 μg) and running snakes, up to five total sequential TTX tests per snake.

We scored resistance as the reduction of an individual’s baseline sprint speed following an injection of TTX (postinjection speed/baseline speed). Snakes greatly impaired by TTX slither at only a small proportion of their normal speed, whereas those unaffected by a dose of TTX crawl at 100% of their baseline speed. We then calculated a population (or species) dose response curve from individual responses to the serial TTX injections using a simple linear regression (see ref 61). From this regression model we estimated the 50% dose, defined as the amount of TTX required to reduce speed of the average snake to 50% of its baseline. This measure is analogous to a 50% inhibition concentration (IC₅₀). Because TTX resistance is related to body size (60, 61), we transformed doses into mass-adjusted mouse units, the amount of TTX (in mg) required to kill a 20-g mouse in 10 min (see ref 61). This correction allows us to compare TTX resistance directly between individuals, populations, or species. Further details of the bioassay and information on captive care of snakes can be found elsewhere (17, 61).

**Sequence Data.** To determine whether snake lineages have independently acquired TTX resistance through similar genetic modifications, we examined DNA sequence variation in portions of the four domains (DI–DIV) that code for the P-loops of Na⁺,1.4. We sequenced snakes known to prey on TTX-laden amphibians, their sister groups, and additional taxa to provide a robust phylogenetic perspective (75 species; n = 138) (Fig. S1). We focused on the P-loops because TTX interacts with residues of the outer pore (see refs. 35–38, and changes in P-loop sites appear to be responsible for TTX resistance in animals (34, 39–42, 62).

We isolated and purified genomic DNA from muscle or liver tissue with the DNeasy Tissue Kit (Qiagen, Inc.). We amplified the four P-loops of Na⁺,1.4 using primers we designed specifically to capture the regions between the SS and S6 transmembrane segments that form the outer pore (6). We cleaned amplified products using the ExelPure PCR Purification Kit (Edge Biosystems) and used purified template in cycle-sequencing reactions with Big Dye chemistry (Applied Biosystems, Inc.). Following an isopropanol/ethanol precipitation, we ran cycle-sequenced products on an ABI 3130 automated sequencer (Applied Biosystems, Inc.). We sequenced all samples in both directions to obtain reading overlaps. For some species this bidirectional sequencing was difficult for Di and DIII because of long introns, and we resequenced these individuals two additional times to confirm exon sequences. We also reamplified and resequenced samples showing adaptive replacements to validate P-loop variation.

We edited and aligned sequences in Sequencer 4.2 (Gene Codes Corp.) and translated coding regions into amino acid sequences using MacClade 4.08 (63). We deposited all sequences in GenBank (accession nos. FJ507810–FJ5071064, GQ154075–GQ154084, and JQ687537–JQ687861).

**Visualizing P-Loop Replacements and TTX in Na⁺,1.4.** We used the 3D model of the outer pore of the sodium channel developed by Lipkind and Fozzard (35) based on structural homology between rat Na⁺,1.4 P-loops and the crystal structure of the pore region of the bacterial potassium channel (KcsA) and refined with data on toxin binding and the biophysics of Na⁺ permeation (35, 36). Lipkind and Fozzard kindly provided the Protein Database coordinates to their model, which we used in UCSF Chimera (64) to visualize the P-loop replacements in TTX-resistant snakes. Finally, we used UCSF Chimera to visualize TTX occluding the outer pore, following the current model of docking orientation (36). Note that Th. atratus A1281P cannot be visualized because this replacement is just C-terminal to the DII model coordinates.

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Fig. 3. Tradeoff between TTX sensitivity and sodium channel performance at the molecular level. Mutations that improve TTX resistance tend to reduce Na⁺ permeability and Na⁺ selectivity. (A) Linear regression (r² = 0.417; F-value = 20.739; P < 0.0001) of the amount of TTX (in nm) required to inhibit peak Na⁺ current by 50% (IC₅₀) against single-channel conductance (pS) in wild-type (triangles) and single-site Na⁺ mutants (circles). The dark circle is a change to the DII selectivity filter that completely abolishes Na⁺ selectivity and would be lethal. Data from literature (refs. 45, 65, and 69; see text). (B) χ² analysis of a larger set of mutants scored as either augmenting or diminishing TTX resistance and Na⁺ permeability or Na⁺ selectivity compared with wild-type sodium channels. In both cases, there is a statistical excess of mutants that increase TTX resistance but decrease Na⁺ permeability (χ² = 71.22, P < 0.0001) or Na⁺ selectivity (χ² = 31.00, P < 0.0001) compared with null expectations (gray boxes; expected counts are given in parentheses). Data from literature (refs. 41, 43–45, 65–70, and 74–81; see text and Table S1).

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We determined whether the pattern of mutations we observed in snakes is clustered or follows a random distribution. If the clustered mutations are distributed 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) (23, 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 115667 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 = variance/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 1.5 distributed (72), we calculated χ-scores for our CD measures to obtain P values against the random expectation (CD = 1). Here χ² = (n−1) where i is our CD and n−1 is the degrees of freedom (72), in this case the number of sites minus 1.

Assessing Tradeoffs in Na1.4. 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 permeation can also interact strongly with TTX through a combination of hydrogen and ionic bonds, steric action, 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 Na1.4, 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 χ² 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% (IC50) in the wild-type and single-mutant channels, and the data on Na+ permeability are measures of single-channel conductance (g). We log-transformed the TTX data and adjusted the Na+ conductance data by segregating the measurements and then 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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