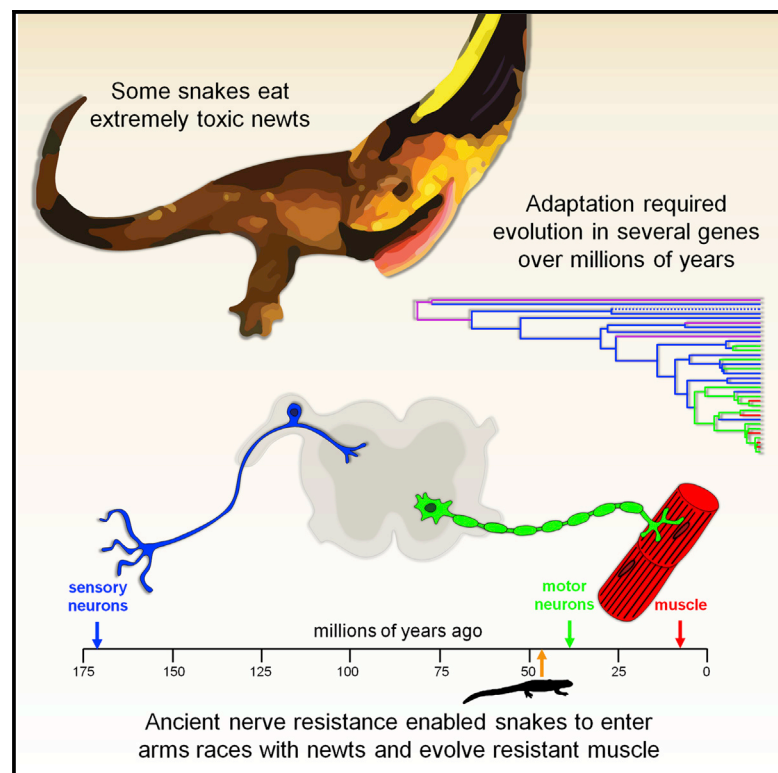


# Current Biology

## Historical Contingency in a Multigene Family Facilitates Adaptive Evolution of Toxin Resistance

### Graphical Abstract



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### In Brief

Some snakes are extremely resistant to toxins found in their prey, an adaptation requiring changes in several genes. Such changes must either arise simultaneously or accrue in a stepwise fashion over millions of years. To explore these alternatives, McGlothlin et al. reconstructed the history of toxin-resistance genes in snakes.

### Highlights

- Toxin resistance in sensory neurons evolved in reptiles before the origin of snakes
- Resistant motor neurons evolved four times in snakes that eat amphibians
- Coevolutionary arms races with toxic prey were facilitated by resistant nerves

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# Historical Contingency in a Multigene Family Facilitates Adaptive Evolution of Toxin Resistance

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## SUMMARY

Novel adaptations must originate and function within an already established genome [1]. As a result, the ability of a species to adapt to new environmental challenges is predicted to be highly contingent on the evolutionary history of its lineage [2–6]. Despite a growing appreciation of the importance of historical contingency in the adaptive evolution of single proteins [7–11], we know surprisingly little about its role in shaping complex adaptations that require evolutionary change in multiple genes. One such adaptation, extreme resistance to tetrodotoxin (TTX), has arisen in several species of snakes through coevolutionary arms races with toxic amphibian prey, which select for TTX-resistant voltage-gated sodium channels (Na<sub>v</sub>) [12–16]. Here, we show that the relatively recent origins of extreme toxin resistance, which involve the skeletal muscle channel Na<sub>v</sub>1.4, were facilitated by ancient evolutionary changes in two other members of the same gene family. A substitution conferring TTX resistance to Na<sub>v</sub>1.7, a channel found in small peripheral neurons, arose in lizards ~170 million years ago (mya) and was present in the common ancestor of all snakes. A second channel found in larger myelinated neurons, Na<sub>v</sub>1.6, subsequently evolved resistance in four different snake lineages beginning ~38 mya. Extreme TTX resistance has evolved at least five times within the past 12 million years via changes in Na<sub>v</sub>1.4, but only within lineages that previously evolved resistant Na<sub>v</sub>1.6 and Na<sub>v</sub>1.7. Our results

show that adaptive protein evolution may be contingent upon enabling substitutions elsewhere in the genome, in this case, in paralogs of the same gene family.

## RESULTS AND DISCUSSION

The role of historical contingency in adaptive evolution has been a longstanding debate in evolutionary biology [1–3]. On the one hand, past evolutionary change can be viewed as a type of negative constraint, limiting the scope of what is achievable by natural selection. On the other hand, historical quirks may open novel and previously inaccessible evolutionary pathways. Because of the pervasiveness of biological interactions, both within a protein and among genes in the genome, single amino acid replacements often change the fitness consequences of other alleles [17–19], suggesting that many or most polygenic adaptations are likely to have been facilitated by substitutions that arose in the distant past. Despite these predictions, little is known about whether adaptive evolutionary changes in natural populations tend to be contingent on previous substitutions in other genes.

Here, we use a comparative approach to assess the role of historical contingency in the evolution of tetrodotoxin (TTX) resistance in snakes. TTX is a neurotoxin used as an antipredator defense in a number of animals, including newts [20] (Caudata: Pleurodelinae). TTX binds to voltage-gated sodium channels (Na<sub>v</sub> proteins), preventing the influx of sodium ions and impairing excitable tissue such as nerves and muscle [21]. Resistance to TTX evolves by amino acid substitutions in the channel's outer pore ("P-loops"), which are normally highly conserved across vertebrates [12, 14, 22]. Snakes possess nine Na<sub>v</sub> channels with tissue-specific expression encoded by the nine genes of the SCNA family [23–25]. Because TTX can potentially bind to several of these channels, physiological resistance to high TTX

levels is a complex adaptation that requires evolutionary changes at several loci [15, 22]. Furthermore, as exemplified by the progressive stages of TTX poisoning in humans [26], tissues vary in their sensitivity to TTX. This observation suggests that in predators that consume TTX, tissues impaired by lower doses of TTX are likely to evolve resistance before those affected by higher doses.

The garter snake *Thamnophis sirtalis* preys upon highly tetrodotoxic *Taricha* newts [27, 28]. Various populations of *T. sirtalis* display physiological resistance to TTX, which can be attributed to amino acid substitutions in at least three Na<sub>v</sub> paralogs [12, 15]: the skeletal muscle channel, Na<sub>v</sub>1.4, and two peripheral nerve channels, Na<sub>v</sub>1.6 and Na<sub>v</sub>1.7. In mammals, Na<sub>v</sub>1.6 is located in the nodes of Ranvier of myelinated axons [29], while Na<sub>v</sub>1.7 is expressed in sensory fibers, sympathetic ganglia, and smooth muscle [30]. Although the precise expression patterns of these two channels are unknown in reptiles, transcriptomic data from lizards [31, 32] and snakes [33] suggest that they are expressed in peripheral nerves (see the [Supplemental Experimental Procedures](#)). The P-loop sequence of Na<sub>v</sub>1.4 varies within and among *T. sirtalis* populations, with different alleles providing different levels of TTX resistance roughly matching the toxicity of local newts, suggesting a relatively recent origin of resistant skeletal muscle [12, 27, 28, 34]. In contrast, substitutions conferring resistance to Na<sub>v</sub>1.6 and Na<sub>v</sub>1.7 are fixed across *T. sirtalis* populations [15], suggesting that resistance in peripheral nerves has a more ancient origin. We hypothesize that the origin of resistant peripheral nerves provided baseline TTX resistance to the ancestors of garter snakes, facilitating later evolution of resistant muscle and the consequent ability to consume highly toxic prey.

To test this hypothesis, we reconstructed the evolutionary history of TTX resistance in snakes by sequencing portions of the genes *SCN4A*, *SCN8A*, and *SCN9A* (encoding the proteins Na<sub>v</sub>1.4, Na<sub>v</sub>1.6, and Na<sub>v</sub>1.7, respectively) from 78 snake species. We sequenced regions known to underlie TTX resistance in *Thamnophis* [12, 13, 15]: the P-loops in domain III and IV (DIII and DIV) of Na<sub>v</sub>1.4, DIV of Na<sub>v</sub>1.6, and DIII and DIV of Na<sub>v</sub>1.7 (see the [Supplemental Experimental Procedures](#)). We obtained sequences from snake species known to consume TTX-bearing prey, their sister taxa, and a number of other lineages representing the breadth of snake diversity, to date the origins of resistance-conferring substitutions. We included sequences from three published snake genomes, *Boa constrictor* [35], *Python molurus* [36], and *Ophiophagus hannah* [37], and from one unpublished snake genome, *Ramphotyphlops bituberculatus*. As outgroups, we added sequences from the genomes of two lizards (*Anolis carolinensis* [38] and *Ophisaurus gracilis* [39]), a turtle (*Chrysemys picta* [40]), and a bird (*Gallus gallus* [41]). Many amino acid substitutions causing TTX resistance have been characterized experimentally [14, 22], allowing us to infer resistance from DNA sequences. Substitutions putatively conferring TTX resistance were identified from predicted translations and mapped to a time-calibrated phylogeny [42, 43] to reconstruct the origins of TTX resistance.

### Stepwise Evolution of TTX Resistance in the Na<sub>v</sub> Family

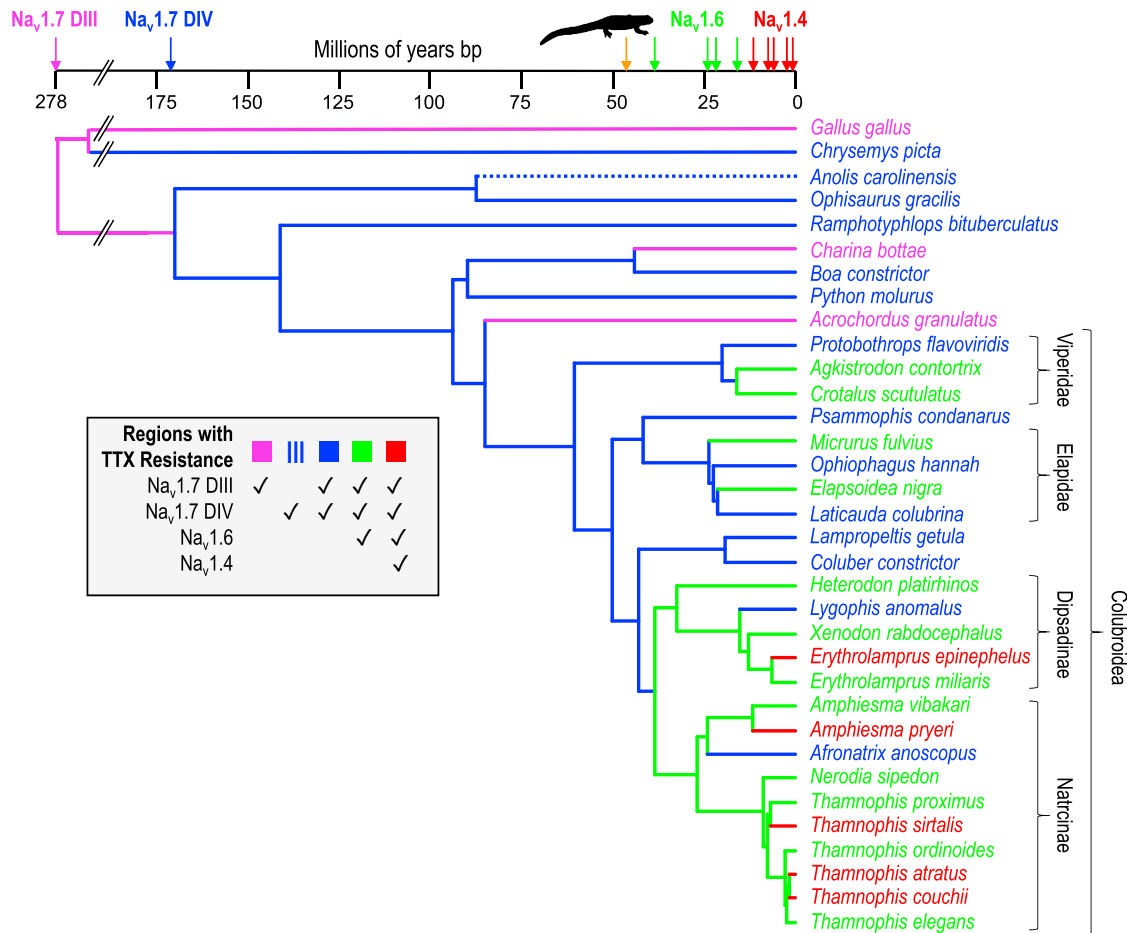
Our results show that TTX resistance of both peripheral nerve channels (Na<sub>v</sub>1.6 and Na<sub>v</sub>1.7) predated TTX resistance of muscle channels (Na<sub>v</sub>1.4; [Figure 1](#)). Resistant Na<sub>v</sub>1.7 had the most

ancient origin. One substitution in DIV known to provide very high (30-fold) TTX resistance [12, 44, 45], D1684N, was present in the common ancestor of all snakes ([Figures 1, 2, S1, and S2](#); positions refer to *T. sirtalis* sequence [15]). This substitution also occurs in the lizard *O. gracilis*, suggesting that D1684N originated in ancestral squamates at least 170 million years ago (mya), in the Middle Jurassic [42]. *A. carolinensis* has a D1684A substitution in this position instead, which provides even stronger resistance (150-fold [45]; [Figure S1](#)). In *Epicrates* sp., we found D1684H, which presumably also interferes with TTX binding, although this has not been directly tested ([Figure S1](#)).

Two additional DIV substitutions (A1681G and G1685Y) that likely contribute to TTX resistance of Na<sub>v</sub>1.7 arose twice in snakes: once in *Leptotyphlops* (63 mya) and independently in the common ancestor of advanced snakes (Colubroidea, 61 mya), a group that includes garter snakes ([Figures S1 and S2](#)). The former, which occurs at the selectivity filter of the channel, is known to provide mild (1.5-fold) TTX resistance and is found naturally in several channels in TTX-bearing pufferfish [22]. We also found this substitution in the turtle *C. picta* ([Figure S1](#)). The substitution G1685Y has not been tested experimentally, but this position is thought to be associated with TTX binding, and substitutions here are often found in naturally TTX-resistant channels, either alone or together with substitutions at position 1684 [12, 14, 22]. This substitution should interfere with TTX binding, as it replaces the very small side chain of glycine with the large side chain of tyrosine. Position 1685 was quite variable across species, and many of the detected substitutions likely also interfere with TTX binding ([Figure S1](#)). An additional DIV substitution known to provide 2-fold resistance to TTX [12], I1677V, arose recently in the genus *Carphophis*.

In DIII of Na<sub>v</sub>1.7, a potentially TTX-resistant substitution (D1393E) was observed in all sampled taxa except for *A. carolinensis* and *C. picta* ([Figures 1, 2, S1, and S2](#)). Here, the ancestral D refers to typical mammalian sequence (data not shown). This substitution occurs at the TTX-binding site [21], and although it has not been tested experimentally, it is found in other TTX-resistant channels [13, 14]. The lizard *A. carolinensis* has the mammalian D (aspartate) in this position, while the turtle *C. picta* has the very dissimilar proline (P). The latter has not been tested for TTX resistance but is found in Na<sub>v</sub>1.4a of the tetrodotoxic pufferfish *Arothron nigropunctatus* [22]. The adjacent position also displays two different substitutions, M1392A (*R. bituberculatus*) and M1392T (*B. constrictor* and *P. molurus*). The former has not been tested, but the latter is known to provide 15-fold resistance to TTX [22]. Although further sampling across reptiles and experimental verification of the effects of D1393E on TTX binding are necessary for confirmation, our results suggest that the common ancestor of all reptiles may have possessed Na<sub>v</sub>1.7 with at least mild TTX resistance.

Because Na<sub>v</sub>1.7 in *T. sirtalis* and other advanced snakes contained a large number of substitutions that had never been experimentally tested in combination, we verified the resistance of this channel by expressing it in *Xenopus* oocytes and recording sodium currents in the presence and absence of TTX (see the [Supplemental Experimental Procedures](#)). Compared to rat Na<sub>v</sub>1.7 ( $K_d \pm 95\% \text{ CI} = 1.34 \times 10^{-8} \text{ M} \pm 3.9 \times 10^{-9} \text{ TTX}$ ), snake Na<sub>v</sub>1.7 displays 900-fold greater resistance to TTX ( $K_d \pm 95\% \text{ CI} = 1.21 \times 10^{-5} \text{ M} \pm 5.3 \times 10^{-6} \text{ TTX}$ ; [Figure 3](#)), which is



**Figure 1. Extreme Resistance to TTX Evolved in a Stepwise Fashion in Snakes**

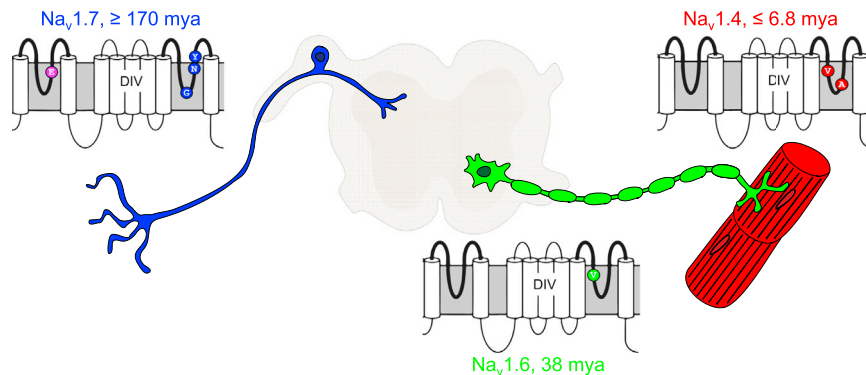
A substitution in the domain III (DIII) TTX-binding region of the voltage-gated sodium  $\text{Na}_v1.7$  was present in the common ancestor of all living reptiles (purple arrow on the timeline). A substitution providing extreme levels of resistance to the same channel arose in DIV at least 170 mya (blue arrow). Other substitutions conferring TTX resistance in  $\text{Na}_v1.7$  DIV arose independently in turtles (*Chrysemys*) and anoles (*Anolis*), with the DIII substitution being lost in *Anolis*. Following the origin of TTX-bearing newts 44–48 mya (orange arrow), an identical I1709V substitution arose in  $\text{Na}_v1.6$  in four different lineages (green arrows). Finally,  $\text{Na}_v1.4$  showed five independent origins of TTX resistance in lineages with resistant  $\text{Na}_v1.7$  and  $\text{Na}_v1.6$  (red arrows). Branches are color-coded from fewest to most TTX-resistant regions as shown in the key above. The phylogeny [43] is pruned to a limited number of species, and the time axis (based on [42]) is truncated at the left for clarity. See also Figures S1 and S2.

comparable to the level of TTX resistance displayed by the most resistant known  $\text{Na}_v1.4$  allele in *T. sirtalis* [12]. This combination of  $\text{Na}_v1.7$  P-loops arose ~61 mya (Figure S2), indicating that advanced snakes inherited extremely TTX-resistant  $\text{Na}_v1.7$  from their common ancestor.

A second nerve channel,  $\text{Na}_v1.6$ , evolved TTX resistance independently at least four times within snakes: once in the common ancestor of two large subfamilies (Natricinae, which includes garter snakes, and Dipsadinae, 38 mya), once in the New World Viperidae (16 mya), and twice within the family Elapidae ( $\leq 24$  mya; Figures 1, S1, and S2) [42]. In each case, TTX resistance arose by an identical substitution in DIV (I1709V) (Figures S1 and S2). Although this substitution has not been tested in  $\text{Na}_v1.6$ , it is known to confer a 2-fold increase in TTX resistance when expressed in  $\text{Na}_v1.4$  [12], which has a nearly identical TTX-binding region [15]. Each of the origins of I1709V occurred in lineages that had possessed TTX-resistant  $\text{Na}_v1.7$  for over 100 million years. A second substitution likely to confer TTX resis-

tance, G1717M, is found within the species *Erythrolamprus (= Liophis) epinephelus* ( $\leq 6.4$  mya). This substitution has not been experimentally tested, but it is found naturally in the channel  $\text{Na}_v1.1\text{Lb}$  in *A. nigropunctatus* [22]. TTX resistance was lost from domain IV on at least two occasions: in the natricine clade containing *Afronatrix*, *Rhabdophis*, and *Xenocrophis* (23 mya) and in the dipsadine *Lygophis anomalus* ( $\leq 15$  mya).

TTX-resistant skeletal muscle channels ( $\text{Na}_v1.4$ ) arose only in lineages that historically expressed resistance in both  $\text{Na}_v1.7$  and  $\text{Na}_v1.6$ , suggesting that the presence of two resistant channels in peripheral nerves facilitated the evolution of resistant muscle (Figures 1 and 2). Indeed, the origin of resistance in  $\text{Na}_v1.4$  was significantly contingent on the presence of resistance in  $\text{Na}_v1.6$  ( $\chi^2_1 = 5.28$ ,  $p = 0.02$ , Pagel's Discrete [46]). TTX resistance in  $\text{Na}_v1.4$  evolved independently in five snake species that consume toxic amphibians via substitutions in DIII and/or DIV [14] (Figure S1). One species, *E. epinephelus*, is found in the subfamily Dipsadinae, and four are found in the related



**Figure 2. History and Physiological Context of TTX-Resistance Substitutions in Garter Snakes**

Evolutionary changes in DIII and DIV of Na<sub>v</sub>1.7 provided the common ancestor of all snakes with TTX resistance of small sensory neurons (blue). TTX resistance of larger myelinated axons subsequently evolved four times via a substitution in DIV of Na<sub>v</sub>1.6 (green). These changes provided certain snake lineages with baseline resistance to low TTX levels, facilitating predator-prey arms races and recent evolution of resistant skeletal muscle via substitutions in Na<sub>v</sub>1.4 (red). The substitutions illustrated above derive from *T. sirtalis* in Benton County, Oregon [15]. Although nerve channels do not vary across the species range, Na<sub>v</sub>1.4 is polymorphic and varies according to the toxicity of local newts [12, 15, 28, 34]. See also Figures S1 and S2.

subfamily Natricinae: *T. sirtalis*, *T. atratus*, *T. couchii*, and *Amphispma pryeri*. In a sixth natricine, *Rhabdophis tigrinus*, Na<sub>v</sub>1.4 was previously interpreted as TTX-resistant via the substitution I1555M [14]; however, because this methionine is present in nearly every TTX-sensitive Na<sub>v</sub> channel, this characterization is most likely erroneous, and we do not consider this species to possess resistant Na<sub>v</sub>1.4 here. All origins of TTX-resistant Na<sub>v</sub>1.4 are independent, and none are shared with extant sister species. Based on the dated phylogeny we present, all origins have occurred relatively recently, with *E. epinephelus* evolving resistance  $\leq 6.4$  mya, *T. sirtalis*  $\leq 6.8$  mya, *T. atratus* and *T. couchii*  $\leq 1.5$  mya, and *A. pryeri*  $\leq 11.7$  mya. However, most of these origins likely occurred much more recently. In particular, in at least two species, *T. sirtalis* and *T. atratus*, Na<sub>v</sub>1.4 is highly polymorphic within and among populations and covaries with prey toxicity, which both indicates ongoing coevolutionary arms races and suggests a very recent origin [12, 27, 28, 34].

### Historically Contingent Origins of Extreme TTX Resistance

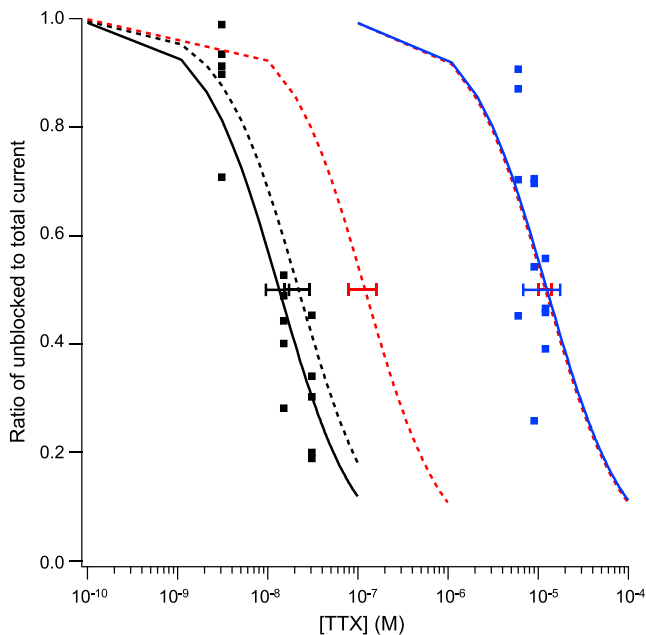
The ancient emergence of resistance in Na<sub>v</sub>1.7 indicates that it did not evolve as a direct response to selection from TTX-bearing newts, which did not appear until  $\sim 44$  mya [20, 47, 48], or from other amphibians, which likely also did not possess TTX in the Jurassic [20]. Because TTX-resistant substitutions are located in the Na<sub>v</sub> outer pore, they typically influence other biophysical properties [14], suggesting that the evolution of TTX-resistant Na<sub>v</sub>1.7 may have occurred as a side effect of selection for other functions. For example, the D1684N substitution observed in snakes is known to decrease channel conductance [45] and may also affect ion selectivity [49]. In contrast, the substitution A1681G is known to increase channel conductance [22]. In mammals, Na<sub>v</sub>1.7 is involved in setting the threshold for action potentials in a number of neuron types including nociceptors and olfactory receptors [30, 50], suggesting that such changes in channel function may have been selected for via effects on neuron excitability. In snakes, Na<sub>v</sub>1.7 appears to be the primary sodium channel expressed in the snake vomeronasal organ [33] (see the Supplemental Experimental Procedures), which implicates the channel's role in chemosensation as a potential driver of the observed evolutionary changes.

In contrast, resistant Na<sub>v</sub>1.6 may have arisen as a direct response to selection by toxic prey. All origins of TTX-resistant Na<sub>v</sub>1.6 postdate the origin of modern newts (Figure 1) and either postdate or roughly coincide with the origin of the more highly toxic North American newts ( $\sim 36$  mya [20, 48]). Further, the groups with resistant Na<sub>v</sub>1.6 contain most of the snake species that commonly feed on amphibians; in particular, many dipsadine and natricine snakes are amphibian specialists [51]. All of these snake groups also overlap geographically with newts, suggesting that Na<sub>v</sub>1.6 resistance may have evolved via past interactions between snakes and newts.

The historical sequence of evolution of TTX-resistance across the Na<sub>v</sub> family in snakes suggests that modern predator-prey arms races were possible only after the sequential accumulation of toxin resistance in more sensitive tissues (Figure 2). The localization of Na<sub>v</sub>1.7 on small-diameter neurons suggests that its function would be impaired by relatively small concentrations of TTX [52]. Accordingly, mild cases of TTX poisoning in humans involve solely sensory symptoms [26], likely mediated by Na<sub>v</sub>1.7. Reduced affinity of Na<sub>v</sub>1.7 to TTX likely would have rendered early snakes less sensitive to the numbing sensation caused by small doses of TTX, which if present, would have resulted in the avoidance of tetrodotoxic prey. The slightly higher concentrations necessary to block Na<sub>v</sub>1.6 in larger neurons [52] could be delivered by ingesting tetrodotoxic prey. The consequent motor impairment [26] caused by blockade of action potentials in peripheral motor neurons would then provide a source of selection for Na<sub>v</sub>1.6 resistance in lineages that frequently consumed such prey. In this genetic background, the appropriate ecological interactions between predators and prey should occasionally trigger escalating arms races such as those seen in *Thamnophis* and *Taricha*, where TTX resistance of Na<sub>v</sub>1.4 in garter snake skeletal muscle coevolves with the magnitude of newt TTX [14, 27, 28].

### Conclusions

Neurotoxins are an effective defense mechanism against many predators because the evolution of physiological resistance requires changes in multiple sensitive proteins. Such adaptations could conceivably arise in a predator species in one of two ways: either all of the proteins in question evolve resistance effectively simultaneously, or they acquire resistance



**Figure 3. Garter Snake  $\text{Na}_v1.7$  Shows Strong Resistance to TTX**

Garter snake  $\text{Na}_v1.7$  is blocked at TTX concentrations 900-fold higher than rat  $\text{Na}_v1.7$ , a level of resistance comparable to the most resistant garter snake  $\text{Na}_v1.4$ . From left to right, the traces are rat  $\text{Na}_v1.7$  (black, solid), garter snake or garter snake-human chimeric  $\text{Na}_v1.4$  from three different populations (dashed lines; Bear Lake chimera [black, non-resistant], Benton garter snake [red, moderately resistant], Willow Creek chimera [red, strongly resistant]; data from [12]), and garter snake  $\text{Na}_v1.7$  (blue). Each symbol corresponds to the ratio of unblocked to total current for an oocyte expressing the indicated channel and exposed to TTX. The TTX concentration that blocked 50% of the channels ( $K_d$ ) for each channel type was calculated from pooled channel data (see the Supplemental Experimental Procedures).  $K_d$  values ( $\pm 95\%$  confidence limits) are shown for each channel type with a bar. The lines represent the equation fitted to the data with the estimated  $K_d$  for each channel type.

sequentially over longer periods of evolutionary time. We have shown that the arms races that drive exaggerated evolution of TTX toxicity and resistance arise only after a stepwise pattern of accumulated changes in paralogous proteins expressed in diverse tissues. Our results emphasize both the predictable and capricious aspects of adaptive evolution. The convergent origins of extreme TTX resistance, which occurred multiple times in snakes through predator-prey coevolution, were facilitated by earlier changes in the lineage's distant evolutionary past.

#### ACCESSION NUMBERS

The accession numbers for new sequences are GenBank: KX063539–KX063606 and KX079340–KX079444. The accession numbers for new annotations from *Ophiophagus* are GenBank: BK009415–BK009419. The accession numbers for previously published sequences are given in Table S1. New annotations of genes from *Ophisaurus* and *Boa*, along with a time-calibrated phylogeny, have been deposited in Dryad: <http://dx.doi.org/10.5061/dryad.tm65d>.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2016.04.056>.

#### AUTHOR CONTRIBUTIONS

J.W.M. provided project leadership, designed the project, collected and analyzed sequence data, and performed bioinformatic analyses. M.E.K. and C.R.F. collected and analyzed sequence data. T.A.C. performed bioinformatic analyses. S.L.G., C.T.H., and G.T. tested TTX resistance of  $\text{Na}_v1.7$ . F.J.V. and M.K.R. sequenced the *Ramphotyphlops* genome. E.D.B., Jr., M.E.P., and E.D.B. III provided project leadership and designed the project. All authors prepared the manuscript.

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**Current Biology, Volume 26**

**Supplemental Information**

**Historical Contingency in a Multigene Family**

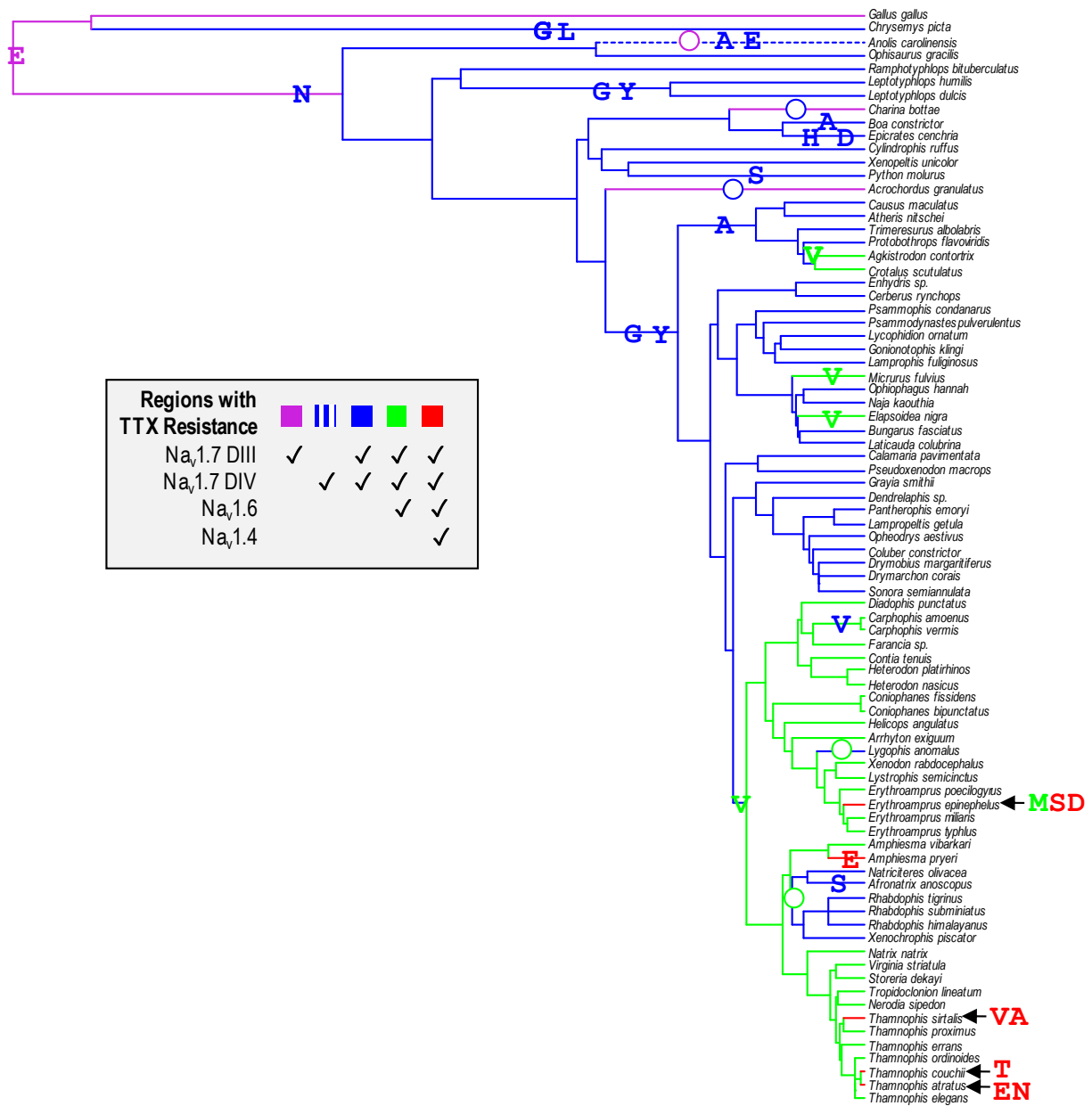
**Facilitates Adaptive Evolution of Toxin Resistance**

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Species	Na, 1,7 DIII	Na, 1,7 DIV			Na, 1,6 DIV		Na, 1,4 DIII	Na, 1,4 DIV		
	1392 1393	1677	1681	1684 1685	1709	1717	1276 1277	1561	1566 1568 1569	
<i>Gallus gallus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGWDG	LL	NFETFGNSM	ICLFQITTSAGWDG	LL	ATFKGWM	IM
<i>Chrysemys picta</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGWD	LL	NFETFGNSM	ICLFQITTSAGWD	LL	ATFKGWM	IM
<i>Anolis carolinensis</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Ophisaurus gracilis</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Ramphotyphlops bituberculatus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Leptotyphlops humilis</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Leptotyphlops dulcis</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Charina bottae</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Boa constrictor</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Epicrates cenchria</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Cylindrophis rufus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Xenopeltis unicolor</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Python molurus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Acrochordus granulatus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Causus maculatus</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Atheris nitschei</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Trimeresurus albolabris</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Protobothrops flavoviridis</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Agkistrodon contortrix</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Crotalus scutulatus</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Erythris sp.</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Cerberus rynchops</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Psemmophis condanarus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Psemmodynestes pulverulentus</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Lycophidion ornatum</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Gonionophis klingi</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Lamprophis fuliginosus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Micurus fulvius</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Ophiophagus hannah</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Naja kaouthia</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Elapsoides nigris</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Bungarus fasciatus</i>			-----NSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Laticauda colubrina</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Calamaria pavimentata</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Pseudoxenodon macrops</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Grayia smithii</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Dendrelaphis sp.</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Pantherophis emoryi</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Lampropeltis getula</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Ophedys aestivus</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Coluber constrictor</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Dryoblabius margaritiferus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Drymarchon corais</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Sonora semiannulata</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Diadophis punctatus</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Carphophis amoenus</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Carphophis vermis</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Farancia sp.</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Contia tenuis</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Heterodon platirhinos</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Heterodon nasicus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Coniophanes fissidens</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Coniophanes bipunctatus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Helicops angulatus</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Arrhyton exiguum</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Lygophis anomalus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Xenodon rabdocephalus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Lystrophis semicinctus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Erythrolamprus poecilogyrus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Erythrolamprus epinephelus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Erythrolamprus miliaris</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Erythrolamprus typhlus</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Amphiesma vibakari</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Amphiesma pteryi</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Natriciteres olivacea</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Afronatrix anoscopus</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Rhabdophis tigrinus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Rhabdophis subminiatus</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Rhabdophis himalayanus</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Xenochrophis piscator</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Natrix natrix</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Virginia striatula</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Storeria dekayi</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Tropidoclonion lineatum</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Nerodia sipedon</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Thamnophis sirtalis</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Thamnophis proximus</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Thamnophis errans</i>			NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL		
<i>Thamnophis ordinoides</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Thamnophis couchii</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Thamnophis atratus</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM
<i>Thamnophis elegans</i>	ATFKGWM	IM	NFETFGNSM	ICLFQITTSAGW	LL	NFETFGNSM	ICLFQITTSAGW	LL	ATFKGWM	IM

**Figure S1, related to Figures 1 and 2 (previous page).** Amino acid sequences of P-loop regions in three Na<sub>v</sub> paralogs. Na<sub>v</sub>1.4 sequences are shown only for species for which Na<sub>v</sub>1.6 or Na<sub>v</sub>1.7 sequences were also available; 30 additional snake species have also been sequenced previously, none of which possessed TTX-resistant Na<sub>v</sub>1.4 alleles [S1]. *T. atratus* and *T. sirtalis* are both polymorphic for Na<sub>v</sub>1.4 [S2, S3], but only a single resistant allele is shown for each species (*T. atratus* from Santa Cruz Co., California, *T. sirtalis* from Benton Co., Oregon). Unknown amino acids are noted by dashes, and putative TTX-resistance substitutions are shown in color. Positions are numbered based on sequence from *T. sirtalis* [S4].



**Figure S2, related to Figures 1 and 2.** Ancestral state reconstructions for all TTX-resistance substitutions. Changes in Na<sub>v</sub>1.7 DIII are in purple, Na<sub>v</sub>1.7 DIV in blue, and Na<sub>v</sub>1.6 in green, and Na<sub>v</sub>1.4 in red. Branches are correspondingly color-coded by the resistant channels they possess. Gains in resistance are indicated by a single-letter amino acid code, and losses are indicated by open circles. Ancestral states were reconstructed in PAML.

**Table S1, related to Accession Numbers.** Accession numbers of previously published sequences and sources for new annotations. Accession numbers for new sequences and annotations are given in the text.

<b>Gene</b>	<b>Species</b>	<b>Source</b>
SCN4A	<i>Anolis carolinensis</i>	GenBank XM_008113208.1
	<i>Boa constrictor</i>	New annotation of scaffold 1125 [S5]
	<i>Chrysemys picta</i>	GenBank XM_005283115.1
	<i>Gallus gallus</i>	GenBank NM_001318445.1
	<i>Ophiophagus hannah</i>	New annotation of GenBank AZIM01001805
	<i>Ophisaurus gracilis</i>	New annotation of scaffolds 5447, 5313, 3412, 6394, 3096, 4518, 2968 [S6]
	<i>Python molurus</i>	GenBank XM_007424834.1
	<i>Thamnophis sirtalis</i>	GenBank BK008863 [S4]
	All others	GenBank FJ570810–FJ571064, GQ154075–GQ154084, and JQ687537–JQ687861 [S1]
SCN8A	<i>Anolis carolinensis</i>	GenBank XM_008103948.1
	<i>Boa constrictor</i>	New annotation of scaffold 944 [S5]
	<i>Chrysemys picta</i>	GenBank XM_008173851.1
	<i>Gallus gallus</i>	GenBank XM_424477.4
	<i>Ophiophagus hannah</i>	New annotation of GenBank AZIM01001981
	<i>Ophisaurus gracilis</i>	New annotation of scaffold 281 [S6]
	<i>Python molurus</i>	GenBank XM_015889936.1
	<i>Thamnophis sirtalis</i>	GenBank BK008864 [S4]
SCN9A	<i>Anolis carolinensis</i>	GenBank XM_008115140.1
	<i>Boa constrictor</i>	New annotation of scaffold 2823 [S5]
	<i>Chrysemys picta</i>	GenBank XM_005290393.2
	<i>Gallus gallus</i>	GenBank NM_001293282.1
	<i>Ophiophagus hannah</i>	New annotation of GenBank AZIM01003776.1
	<i>Ophisaurus gracilis</i>	New annotation of scaffold 755 [S6]
	<i>Python molurus</i>	GenBank XM_007436013, XM_015890542
	<i>Thamnophis sirtalis</i>	GenBank BK008865 [S4]

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

**Sequencing:** To reconstruct the history of TTX resistance in snakes, we used a combination of previously available and newly generated sequences. For new sequences, targeted regions were amplified via the polymerase chain reaction and sequenced via Sanger sequencing. Most sequences for the gene *SCN4A* (coding for the protein Na<sub>v</sub>1.4) were derived from a previous study, and their generation is described elsewhere [S1]. Sequences for *T. sirtalis* were obtained from a bacterial artificial chromosome library in a previous study [S4]. Full genomic sequences of *SCN4A*, *SCN8A* (coding for Na<sub>v</sub>1.6) and *SCN9A* (coding for Na<sub>v</sub>1.7) were obtained from the genome sequences of four snakes, *Boa constrictor* [S5], *Python molurus* [S7], and *Ophiophagus hannah* [S8], and *Ramphotyphlops bituberculatus*, two lizards, *Anolis carolinensis* [S9] and *Ophisaurus gracilis* [S6], a turtle, *Chrysemys picta* [S10], and a bird, *Gallus gallus* [S11]. For each sequence, the correct paralog was identified using BLAST [S12], BLAT [S13], and/or synteny, which is conserved across tetrapods [S14, S15].

Sequences from the four snake genomes as well as *T. sirtalis* were used to design primers to obtain sequence from previously uncharacterized snake species (see table below). For very closely related species (e.g. within the same genus or subfamily), we used primers designed using a single species. For more distantly related species, we aligned sequences from two or more snake species (using MAFFT [S16] or MAUVE [S17]) and designed primers (using primer3 [S18]) based on regions of high conservation. Alignment and primer design applications were implemented within Geneious [S19] (Biomatters). Desired regions were amplified using the polymerase chain reaction (PCR) and standard conditions, and PCR products were cleaned with a combination of exonuclease I and shrimp alkaline phosphatase (Exo-SAP, USB). Sanger sequencing of cleaned product was performed at Yale University's DNA Analysis Facility. Sequencing reads were trimmed and assembled in Geneious and their identity was confirmed using BLAST or BLAT.

In total, we obtained new sequences of P-loops from 73 snake species (Figure S1). Because of their positions within very long exons, domain IV P-loops were easier to successfully amplify. We obtained domain IV P-loop sequences from *SCN8A* in 67 species and from *SCN9A* in 65 species. By contrast, the domain III P-loop is split between two exons. The second of these exons, which contains the most crucial region for TTX resistance, is very short and often surrounded by long introns, causing amplification by PCR to be difficult in distantly related species. Thus, we obtained sequence of this *SCN9A* DIII from only 37 new snakes.

**Patterns of *SCNA* expression in reptiles:** Although *SCN4A* is known to be expressed in snake skeletal muscle [S2], expression patterns of *SCN8A* and *SCN9A* in reptiles have previously been inferred from work in mammals [S4]. To verify the expression patterns of these two genes, we performed BLAST searches using exon 26 of *A. carolinensis* *SCN8A* and *SCN9A* against a set of transcriptomes from various tissues in *A. carolinensis* [S20, S21] and using exon 26 of *T. sirtalis* *SCN8A* and *SCN9A* against a transcriptome of corn snake (*Pantherophis guttatus*: Colubrinae) vomeronasal organ (VNO), the major chemosensory organ in snakes [S22]. In *Anolis*, we found 100% matches of *SCN8A* in whole embryo (38-somite stage), adult brain, adult dewlap, adult ovary, original tail, tail stump, and regenerating tail. We found 99.8-100% matches for *SCN9A* in all of these tissues except for the three tail stages, as well as in whole embryo (28-somite stage). These results confirm that as in mammals, *SCN8A* and *SCN9A* are expressed in the periphery and the channels they encode (Na<sub>v</sub>1.6 and Na<sub>v</sub>1.7, respectively) are thus vulnerable to ingested TTX. In the snake VNO, we found matches for *SCN9A* but not *SCN8A*. Further blast searches using complete coding sequences of six paralogs from *T. sirtalis* [S4] retrieved a nearly complete (missing only 54 bp) coding sequence of *SCN9A* and fragments covering about half the coding sequence of another paralog, *SCN2A*. This result suggests that in reptiles as in mammals [S23], Na<sub>v</sub>1.7 is the primary sodium channel in olfactory sensory neurons.

**Inference of TTX resistance:** Resistance to TTX was inferred based on changes to amino acid residues known to influence TTX binding [S1, S2, S24-S26]. In many cases, amino acid substitutions we identified have been experimentally tested for TTX resistance via site-directed mutagenesis, expression in oocytes, and single-molecule patch clamping. Most of these studies been conducted in Na<sub>v</sub>1.4 (but see [S27, S28]). However, because of the high degree of sequence conservation in the TTX-binding site across paralogs and across taxa, it is common practice to use studies of Na<sub>v</sub>1.4 to infer resistance in other channels (e.g. [S26, S29, S30]). Further, similar results have been obtained when the same substitution has been tested in more than one paralog (e.g. [S28, S30]). Inference of TTX resistance for untested substitutions is indirect and thus should be treated with appropriate caution.

Because *T. sirtalis* *SCN9A* exhibited many substitutions in its P-loop regions, we directly tested for the effects of the combination of these substitutions on TTX resistance of Na<sub>v</sub>1.7. We measured resistance in channels expressed in *Xenopus* oocytes. Complimentary DNAs encoding each of two Na<sub>v</sub> channels, *T. sirtalis* Na<sub>v</sub>1.7 [S4] and rat Na<sub>v</sub>1.7 ([S31] provided by T. Olivera) were used to transcribe RNAs for injection into *Xenopus* oocytes. Rat

Na<sub>v</sub>1.7 cDNA was produced using standard methods and plasmid preparations. Complimentary DNA encoding *T. sirtalis* Na<sub>v</sub>1.7 was synthesized by DNA2.0 with optimized *Xenopus laevis* codon usage. We added an SP6 promoter site as well as 5' and 3' UTRs from the *Xenopus* globin gene and a poly-A tail to enhance translation and expression. All flanking sequences were derived from pSP64T plasmid (Addgene; courtesy of D. Melton) [S32]. Because the gene construct could not be grown in standard plasmid preparations we constructed mRNA from sequence verified PCR product provide by DNA2.0. RNA was produced using the mMessage Machine Kits ULTRA system (Life Technologies) using standard reactions for SP6 (*T. sirtalis* Na<sub>v</sub>1.7) or T7 (rat Na<sub>v</sub>1.7) promoters. RNA (6-12 ng) was injected into prepared *Xenopus* oocytes (Ecocyte).

Ionic currents were measured at room temperature (22-25 °C) 2-7 days after RNA injection using the cut-open oocyte Vaseline gap voltage-clamp technique with a CA-1B High Performance Oocyte Clamp (Dagan Instruments). Recordings were made in an external solution containing 120 mM MES Na, 10 mM HEPES Na, and 1.8 mM CaCl<sub>2</sub> at pH = 7.2 and an internal solution containing 110 mM MES K, 10 mM MES Na, 10 mM HEPES Na, and 1 mM EGTA at pH = 7.2.

Current records were acquired using pClamp software (Molecular Devices), sampling at 100 kHz and filtering at 20 kHz. Peak currents were evoked at 0.05 Hz with 20-ms pulses to 0 mV following a 500-ms prepulse to -150 mV. The holding potential for all experiments was -100 mV. Leak subtraction was performed before the test pulse (p) with the use of a p/4 protocol. Peak current amplitudes were measured offline with IgorPro (WaveMetrics). The ratios of peak currents in the presence and absence of TTX over a range of TTX concentrations were calculated with peak currents recorded before and after perfusing the selected TTX concentration into the external bath solution for 2.5 min (approximately 36 solution changes). To estimate the TTX concentration that blocked 50% of the expressed channels, the data were fitted to an equation derived from a single-site Langmuir adsorption isotherm,

$$\text{current ratio} = \frac{1}{1 + \frac{[\text{TTX}]}{K_d}}$$

in which [TTX] is the concentration of toxin and  $K_d$  is the concentration of TTX at which half of the channels are bound to the toxin.  $K_d$  and its 95% confidence limits were estimated from the curve using IgorPro (WaveMetrics).

**Inference of evolutionary history:** Sequences from each Na<sub>v</sub> paralog were aligned in Geneious using MUSCLE [S33], and regions outside the P-loops were trimmed from the alignments for analysis. We used an amino-acid model in PAML [S34] to analyze these alignments and reconstruct sequences at ancestral nodes. All PAML analyses used a phylogeny consisting of a pruned species tree of squamates [S35] with an appended outgroup clade containing *G. gallus* and *C. picta*.

Evolutionary transitions were dated by applying estimated divergence dates to our pruned species tree. We used the procedure chronos in the R package ape [S36] to estimate the age of all nodes in the tree using previously estimated divergence dates [S37] as model constraints. Divergence dates outside squamates were applied to our tree from TimeTree [S38]. Dates of evolutionary events given below and in the main text represent dates of the most recent common ancestor (i.e., nodes) of a given clade, and thus two sources of uncertainty must be acknowledged. First, the date of each node is estimated with a certain degree of error, and second, a synapomorphy could have evolved at any time between a clade's most recent common ancestor and its next most recent common ancestor.

We tested for historical contingency of the origin of Na<sub>v</sub>1.4 resistance on previous evolution of resistance in Na<sub>v</sub>1.6 using a constrained contingency test in Pagel's Discrete [S39] implemented in BayesTraits 2.0. For this test, we used our 82-species dated phylogeny and the data presented in Figure S1 to assign resistance. To be conservative, Na<sub>v</sub>1.4 resistance was considered to be unknown if we were missing sequence from either DIII or DIV. The null model contained four rate parameters representing the rate of origin and loss of resistance in each channel, while the alternative model fit separate rate parameters for the origin of resistance in Na<sub>v</sub>1.4 in the presence and absence of resistance in Na<sub>v</sub>1.6. In the latter model, the origination rate for Na<sub>v</sub>1.4 resistance was estimated as 0.076 origins per million years in lineages possessing resistant Na<sub>v</sub>1.6 and 0 origins per million years in lineages with non-resistant Na<sub>v</sub>1.6. These models were compared using a likelihood-ratio test with one degree of freedom.

Primers used in this study. Forward and reverse primers within blocks were used interchangeably to create pairs.

<b>Region</b>	<b>Name</b>	<b>Forward Primer Sequence</b>	<b>Name</b>	<b>Reverse Primer Sequence</b>
<i>SCN8A</i> DIV	SCN8AIVFA1	GTGCAGTCAGGTGGCGGTGA	SCN8AIVRA1	TCCAACGGAAGGATTCCCACA
	SCN8AIVFA2	ACCCATCCTCAACCGTCCTCCA	SCN8AIVRA2	ACAGGTGGTGGATCACTGCTTTG
	SCN8AIVFB1	CCGCCTGGCCCGTATTGGTC	SCN8AIVRB1	ACTGGGTAGCGTCGGGGTCA
	SCN8AIVFC1	TGTTTTGGCAGAAATCATAGAGA	SCN8AIVRC1	TGGGCTTAGGAACACGAAG
	SCN8AIVFC2	TTGTCAACATTGGCTCCT	SCN8AIVRC2	TCTTCACTCAGTGGATCAGCA
	SCN8AIVFD1	GCCCGTATTGGTCGAATCCT	SCN8AIVRD1	TGAAGCCACTCCCAGGATG
	SCN8AIVFD2	GCCCGTATTGGTAGAATCCT	SCN8AIVRD2	ACCATTGGCAAATCCATGGC
	SCN8AIVFD3	TTCAACATTGGCTCCTGCT	SCN8AIVRD3	ATCCCCTCCTTGCTAAACGG
<i>SCN9A</i> DIII	SCN9AIIIFA1	GAAGCTTTTCATTTTCATTCCAAA	SCN9AIIIRA1	TGAACCAAATATAATGAAGCCAAC
	SCN9AIIIFB1	ATGCCCTGATAGGAGCTATA	SCN9AIIIRB1	GTTGAATCAACAGCAGCATTCA
	SCN9AIIIFB2	ATGCCCTGGTAGGAGCCATA	SCN9AIIIRB2	GTTGAATCAACAGCAGCGTACA
	SCN9AIIIFB3	ATGCCTTGGTAGGAGCCATA	SCN9AIIIRB3	GTTGAATCAACAGCAGCATACA
	SCN9AIIIFC1	TCATGGGTGTAAATCTGTTGCT	SCN9AIIIRC1	CTGCCAAGAGAAGTGAGGGA
	SCN9AIIIFC2	TCTGGCTAATTTTCAGCATCATGG		
	SCN9AIIIFD1	CAGGGTCATTTGTAGACTAGCACA	SCN9AIIIRD1	ATCTTGTCTCAGTATTCTTGGCT
	SCN9AIIIFD2	TGCAGAACATTTACTTGGCCA		
	SCN9AIIIFE1	CCAGGTGGTGGTGAATGCC	SCN9AIIIRE1	ATCTTGACCTCCTAAGTAAAGAAGT
	SCN9AIIIFF1	TCTCTCCAGGTGGTGGTAAA	SCN9AIIIRF1	ACACATGAAGATTTGCCATCCT
	SCN9AIIIFF2	TCTCTCCAGGTGGTGGTGAA	SCN9AIIIRF2	ACATACGAAGATTTGCCATCAT
	SCN9AIIIFF3	GTTCTCCAGGTGGTGGTGAA	SCN9AIIIRF3	ACACATGGAGATTTGTATCCT
	SCN9AIIIFF4	TTTCTCCAGGTGGTGGTGAA	SCN9AIIIRF4	ACACATGAAGATTTGCCATCAT
			SCN9AIIIRF5	ACACATGTAGATTTGTATCCT
			SCN9AIIIRF6	AAACACTAAATTTTGCATCCT
	<i>SCN9A</i> DIV	SCN9AIVFA1	AGGGGATAGAGCCAATTTGCGA	SCN9AIVRA1
SCN9AIVFA2		ACAAGGAGCCAGACTGTGACCC	SCN9AIVRA2	TGGCATAAGCTTTCAGTGTGTGGT
SCN9AIVFB1		TCTCTCCTTTTCTGGTCA	SCN9AIVRB1	TGGTTCATAGGAGACTTTTGAGG
SCN9AIVFB2		TTTTTGCCTTGATGATGTCTCT	SCN9AIVRB2	TTGCACACTGGTTTCTCAG
SCN9AIVFC1		CCGACTTGCCAGGATTGGTC	SCN9AIVRC1	ATGGGCAGGTCCATTGCAAT
SCN9AIVFC2		CCGACTTGCCCGGATAGGTC	SCN9AIVRC2	ATGGGCAGATCCATTGCGAT
SCN9AIVFC3		CCGACTTGCCAGGATAGGTC	SCN9AIVRC3	ATGGGCAGGTCCATTGTAAC
SCN9AIVFC4		CCGTCTGGCCAGGATAGGTC		
SCN9AIVFD1		GGATCCGCACTCTGCTCTTT	SCN9AIVRD1	AACACGCTTTGTAAAGGCAAA
SCN9AIVFD2		GAATCCGCACCCTGCTTTT		
SCN9AIVFE1		ACGCTATATTTGGAATGTCCCA	SCN9AIVRE1	AGCTGACAAAGAAGAAAATCCCA
			SCN9AIVRE2	TTCTTCTTTTCTGACTTTTCTTGTC
SCN9AIVFF1		GTTCCGAGTGGTCCGACTTG	SCN9AIVRF1	GTCACAGTCTGGCTCCTTGT
SCN9AIVFF2		GGTCGAGTCCTGCGTCTAAT		

SCN9AIVFF3	CACCCTGCTTTTTGCCTTGAT		
SCN9AIVFG1	GATGTCTCTCCCTGCCTTGT	SCN9AIVRG1	GTCACCACTCACAATGGGCA
SCN9AIVFG2	GGTCTCCTCCTTTTCCTGGT	SCN9AIVRG2	TGGTATTAGCAGAGGGGGCT
SCN9AIVFH1	GGTCTCCTCCTTTTCCTGGTC	SCN9AIVRH1	TCTTCATCTGTGCAAGCTGGT
SCN9AIVFI1	CTCCTCCTTTTCCTCGTCATGT	SCN9AIVRI1	CTAAAACACGCTTTGTAAAGGC
SCN9AIVFI2	CTCCTCCTTTTCCTGGTCATGT	SCN9AIVRI2	CCAAAACACGCTTTGTAAAGGC
SCN9AIVFI3	CTCCTCCTTTCCTGGTCATGT	SCN9AIVRI3	CCAAAACATGCTTTGTAAAGGC
		SCN9AIVRI4	CCAAGACACGCTTTGTAAAGGC

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