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Predictably Convergent Evolution of Sodium Channels in the Arms Race between Predators and Prey

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Abstract
Evolution typically arrives at convergent phenotypic solutions to common challenges of natural selection. However, diverse molecular and physiological mechanisms may generate phenotypes that appear similar at the organismal level. How predictable are the molecular mechanisms of adaptation that underlie adaptive convergence? Interactions between toxic prey and their predators provide an excellent avenue to investigate the question of predictability because both taxa must adapt to the presence of defensive poisons. The evolution of resistance to tetrodotoxin (TTX), which binds to and blocks voltage-gated sodium channels (Na_V1) in nerves and muscle, has been remarkably parallel across deep phylogenetic divides. In both predators and prey, representing three major vertebrate groups, TTX resistance has arisen through structural changes in Na_V1 proteins. Fish, amphibians and reptiles, though they differ in the total number of Na_V1 paralogs in their genomes, have each evolved common amino acid substitutions in the orthologous skeletal muscle $Na_V1.4$. Many of these substitutions involve not only the same positions in the protein, but also the identical amino acid residues. Similarly, predictable convergence is observed across the family of sodium channel genes expressed in different tissues in puffer fish and in garter snakes. Tradeoffs between the fundamental role of Na_V1 proteins in selective permeability of Na^+ and their ability to resist binding by TTX generate a highly constrained adaptive landscape at the level of the protein.

Introduction

The process of adaptation can be characterized as a clash between focused exigency and wandering contingency. On the one hand, natural selection can be exceptionally restrictive, favoring forms that meet a highly specific set of criteria, from the ability to recognize an individual protein on the surface of a pathogen to the expression of a color pattern that exactly mimics a well-protected model. However, the material that natural selection has to work with to build adaptation is limited to the available phenotypic and genetic variation within a population. The mutational forces that generate novel variation introduce a stochasticity that dictates the paths that adaptation can take.

This juxtaposition has led to a core question about evolution – how predictable are the details of phenotypic

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adaptations [DePristo, et al., 2005; Weinreich et al., 2006; Stern and Orgogozo, 2008, 2009; Christin et al., 2010a]? We know that when two populations or species experience a common set of selection pressures, they often converge on the same phenotype [Losos, 1992; Joron et al., 2006; Arendt and Reznick, 2008; Rosenblum et al., 2010; Dobler et al., 2012]. What is less clear is whether such convergent phenotypes are built by changes to the same underlying mechanisms. The pan-adaptationist view leans toward the expectation that selection will always find the best answer to the problem, leading to convergence at the level of physiological and genetic mechanisms. The population genetic view tends to put more stock in the contingent sources of variation, expecting selection to generate common phenotypes but perhaps through different genetic mechanisms.

The picture that is beginning to emerge from the exploration of genetic and genomic patterns of convergence is that adaptation is in fact predictable – sometimes. In many cases of parallel (stemming from a recent common ancestor) and convergent (occurring among distantly related taxa) evolution, taxa are clearly reusing the same genes to solve evolutionary challenges [Arnegard et al., 2010; Christin et al., 2010; Manceau et al., 2010; Rosenblum et al., 2010]. One recent review suggests that the probability of convergent evolution involving the same gene lies somewhere between 0.3 and 0.5, with more closely related taxa having a higher likelihood of gene reuse compared to more distantly related taxa [Conte et al., 2012]. This picture primarily comes from studies of convergence in morphological and color traits. Relatively little is known about convergence and parallelism in the genetic mechanisms involved with neurophysiological traits. Even less is known about the predictability of convergence among gene paralogs that code for proteins expressed in different tissues [Jost et al., 2008; McGlothlin et al., 2014].

Predator-prey interactions provide an exceptional context in which to explore convergence across wide expanses of evolutionary diversity. Although selection pressures on predator and prey may differ because they are combating one another, both species also have to deal with some of the same weaponry, albeit from different sides of the battleground. Predators may adapt to resist a defensive compound used against them, but prey must also evolve mechanisms that allow them to possess and tolerate the same compound. Because predators and their prey typically belong to vastly different phylogenetic lineages, we would expect the probability that evolution involves common genes in both species to be especially low, whereas closely related species of predators exploiting the same prey (with the same defense) might have a high probability of parallel genetic evolution.

Neurophysiological phenotypes may be expected to play an important role in adaptation in the predator-prey interactions between snakes and amphibians that possess the poison tetrodotoxin (TTX). TTX binds to and blocks voltage-gated sodium channels (Na_V) in nerves and muscles, preventing Na^+ movement across the membrane and resulting in arrest of action potentials (AP) [Hille, 2001; Narahashi, 2008]. This simple but deadly effect of TTX makes it a powerful selection pressure that demands response or avoidance by predators. TTX has been found in a wide variety of organisms, including many species of newts and several other amphibian lineages [Hanifin, 2010]. TTX toxicity reaches its pinnacle in the North American newts of the genus *Taricha*, some of which possess enough TTX to kill 56 humans [Stokes et al., 2015]. The origins and synthesis of TTX are not well understood in any taxon. The diverse taxonomic distribution of the molecule, as well as its detection in free-living bacteria, has led to the expectation that bacterial symbionts are responsible for TTX production in many taxa [Hanifin, 2010; Hanifin and Gilly, 2015].

Around the world, snakes represent one of the most common groups of predators on amphibians. All snakes swallow their prey whole, which means that snakes ingest a full dose of a prey's toxicity with each predation event. Probably as a result of this feeding behavior, many snakes have evolved resistance to various toxic amphibians [Smith and White, 1955; Brodie, 1968; Brodie et al., 1991; Ujvari et al., 2012; Mohammadi et al., 2013]. In western North America, several species of garter snakes of the genus *Thamnophis* cooccur with the highly toxic *Taricha* and have evolved resistance to the TTX they deploy as a defense [Brodie and Brodie, 1991; Brodie et al., 2005]. Moreover, populations of garter snakes vary dramatically in their resistance to TTX. Snakes from localities where *Taricha* are highly toxic are highly resistant to TTX, but outside the range of *Taricha* or where *Taricha* have low toxicity, garter snakes exhibit ancestral levels of resistance [Brodie et al., 2002; Hanifin et al., 2008]. The physiological resistance of other snakes to TTX has not been measured, but it is fair to assume that, if a snake preys on a tetrodotoxic amphibian, it must have some ability to tolerate the poison. Thus, snake predators represent a series of repeated evolutionary experiments with which to test the predictability of neurophysiological mechanisms during convergent evolution.

The Mechanism of TTX Resistance

Moving from the recognition that an animal is resistant to a poison to understanding the mechanism by which resistance is achieved can be an open-ended treasure hunt. Even identifying the tissues in which a toxin is blocked or disabled can be a daunting challenge. In the case of TTX, however, we can stand on decades of research about the action of the compound and its utility as a workhorse of experimental manipulation in neurophysiology [Terlau et al., 1991; Hille, 2001; Catterall et al., 2007; Fozzard and Lipkind, 2010; Tikhonov and Zhorov, 2011]. Because the physiological effects of TTX are first manifest in reduced coordination and performance, skeletal muscle is a logical first place to look for resistance phenotypes.

To test whether skeletal muscle sensitivity to TTX underlies population differences in resistance, Geffeney et al. [2002] examined individual snakes that had been previously characterized for whole-animal resistance using a locomotor bioassay. The baseline sprint speed of each snake was scored on a racetrack equipped with infrared sensors. After 2–3 days, snakes were injected intraperitoneally with a dose of TTX given 30 min before they raced again for the toxin to induce maximal intoxication. The proportional deficit in sprint speed was taken as a measure of the animal's whole-body response to TTX. A score of 100% represents an individual whose sprint speed is unaffected by a given dose of TTX, whereas a score of 0% is an animal totally immobilized by that dose of toxin.

For each tested snake, the costocutaneous muscle was removed and tested for its ability to propagate AP when exposed to TTX in a bath. Muscle fibers from snakes representing a range of resistance across three orders of magnitude were examined. Control AP rise rates in muscle preparations from all populations were comparable to those observed in rat muscle in a similar preparation. However, when TTX was added to the bath solution in which the snake muscle fibers were tested, AP propagation continued at TTX concentrations that would block channels in rat muscle (up to 1×10^{-5} M). By testing fibers in varying concentrations of TTX, Geffeney et al. [2002] were able to construct dose-response curves for each population as well as for individual snakes. The dissociation constant (K_d), the concentration of TTX at which half the receptors were bound to toxin, was estimated from the curves as a measure of skeletal muscle resistance.

The results of the experiment demonstrated that the binding affinity of TTX to skeletal muscle sodium channels differed among populations and among individual snakes within populations. Resistance measured from isolated muscles and whole-animal tests produced the same rank order of population differences, suggesting the underlying physiological mechanism explained the majority of variation in evolutionary response (fig. 1). The fact that individual differences within populations show a similar correlation between whole-animal and muscle resistance indicates that this mechanism could be available for natural selection to act upon.

TTX Binding and Na_V

The specific action of TTX within the nervous system is well understood; it is a fundamental tool for unveiling the shape and structure of membrane-bound Na_V channels [Terlau et al., 1991; Hille, 2001; Catterall et al., 2007; Fozzard and Lipkind, 2010; Tikhonov and Zhorov, 2011]. The α subunit of the Na_V protein is comprised of four homologous domains (DI–DIV) that fold together to form a transmembrane pore. Each domain includes a voltage-sensing domain and a 'P-loop' that connects the inner and outer helices. This P-loop forms the outer edge of the pore, which acts as the selectivity filter that is responsible for Na^+ conductance. TTX is known to specifically bind to this outer pore vestibule, thereby blocking the flow of Na^+ across the membrane. The entire region of the Na_V protein that is thought to interact with TTX includes just over 80 amino acid residues [Terlau et al., 1991; Payandeh et al., 2011; Tikhonov and Zhorov, 2012]. Site-directed mutagenesis experiments have demonstrated that changes in the P-loop sequence can alter the electrostatic charges that influence binding, or may affect the shape of the outer pore in ways that interfere with docking, thereby reducing the binding affinity of TTX [Terlau et al., 1991; Penzotti et al., 1998; Tikhonov and Zhorov, 2011, 2012].

The α subunits of vertebrate sodium channels (Na_V1) are encoded by a family of genes (SCNA) that diversified through ancient duplication events, resulting in 6–10 different Na_V1 in the major vertebrate lineages [Lopreato et al., 2001; Zakon et al., 2009, 2011; Zakon, 2012]. Each unique channel is expressed in a different group of nerve or muscle tissues. The four domains that form the pore are highly conserved both among taxa and among the different channel proteins expressed within a species. The four P-loops of the $Na_V1.4$ channel expressed in skeletal muscle, for example, differ by only a few amino acids across all vertebrates.

To determine if structural changes to channels underlie variation in TTX resistance among populations of gar-

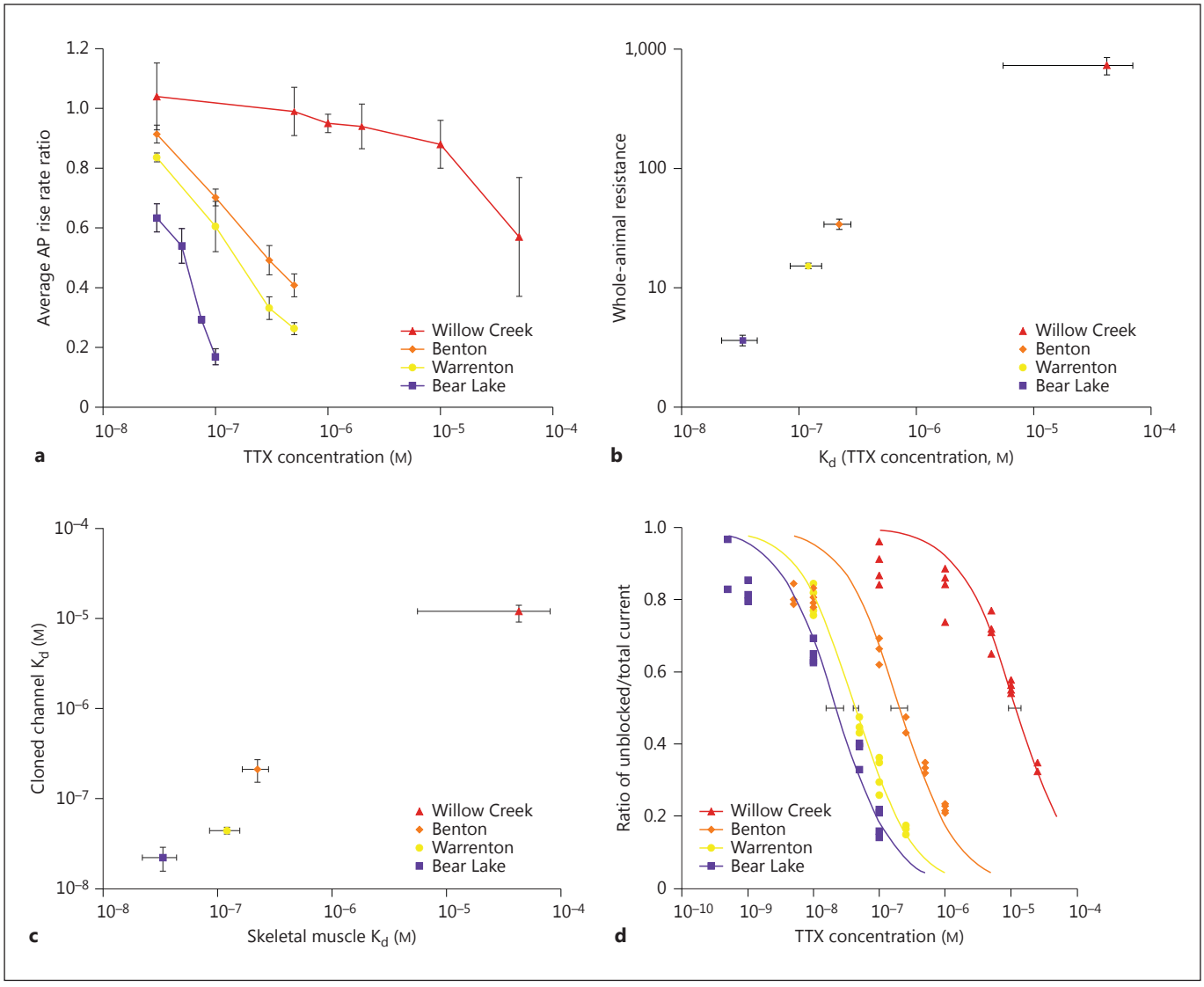

Fig. 1. The relationships between population differences in whole-animal, muscle fiber, $Na_V1.4$ and chimeric resistance of *T. sirtalis*. **a** Population differences in skeletal muscle fiber response to TTX concentration for four populations. **b** Among-population correlation of K_d of muscle fiber with whole-animal resistance measured by racetrack locomotor bioassay (in mass-adjusted mouse units required to reduce a snake to 50% baseline crawl speed). **c** Among-population correlation of K_d of complete snake $Na_V1.4$ protein expressed in *Xenopus* oocytes with skeletal muscle fiber K_d. **d** Concentration response curves of chimeric channels (human $Na_V1.4$ with only DIV amino acid replacements observed in each population substituted). Colors denote the same populations in each panel (colors refer to the online version only). Bars indicate standard errors for each estimate; horizontal bars in **d** indicate standard errors around the interpolated 50% K_d. Drawn from data in Geffeney et al. [2002, 2005].

ter snakes, Geffeney et al. [2005] examined cDNA of $Na_V1.4$ expressed in skeletal muscle. Although most of the outer pore of snake channels exhibited almost complete homology with rat and human channels, a limited number of unique amino acid substitutions were observed in the DIV P-loop. The number of substitutions correlated roughly with the average level of resistance of snakes from each locality, with the most resistant snakes exhibiting four unique residues. The least resistant population had only a single novel amino acid, a switch from Ile to Val in the pore helix (I1561V). A nonresistant population of snakes allopatric to toxic newts had an outer pore structure identical to that of other vertebrates susceptible to TTX.

To move beyond this correlative association between sequence variation and organismal resistance, Geffeney et al. [2005] expressed whole-snake $Na_V1.4$ in *Xenopus* oocytes and investigated how TTX concentrations blocked their current (fig. 1). Chimeric channels in which human channels were engineered to have only the P-loop substitutions found in snakes were also tested. The K_d of snake channels expressed in oocytes correlated closely with that measured from whole-muscle preparations. Moreover, the K_d of chimeric channels did not differ from those of whole-snake channels from the same population, demonstrating that the specific amino acid substitutions identified in the DIV P-loop of garter snakes explained the majority of variation in TTX resistance among populations (fig. 1). By mutating some of the individual amino acids, Geffeney et al. [2005] further showed that the most common substitution found in all populations, I1561V, altered the binding affinity of TTX approximately twofold. The populations of *Thamnophis sirtalis* tested represent at least two independent evolutionary lineages, indicating that amino acid substitutions conferring resistance in $Na_V1.4$ evolved at least twice within this single species.

TTX-Resistant Predators

The most convincing answers to the question of predictability in evolutionary convergence come from comparisons across a variety of phylogenetic scales. The only vertebrate predators that are known to successfully ingest TTX-bearing prey are snakes, but, in addition to *T. sirtalis* discussed above, at least two other North American species of the garter snake, *Thamnophis atratus* and *Thamnophis couchii*, prey on *Taricha* and are, in some localities, resistant to TTX. Around the world, other amphibian specialist snakes prey on frogs or salamanders that are known to secrete TTX, including *Amphiesma pryeri* that preys on the newt *Cynops ensicauda* in southern Japan, *Liophis epinephelus* that feeds on toads of the genus *Atelopus* in Central and South America, and *Rhabdophis tigrinus* that eats the treefrog *Polypedates* sp. in East Asia. If convergence occurs through common mechanisms, we would expect mutations to the SCN4A gene that encodes $Na_V1.4$ and possibly even some of the same positional amino acid changes observed in *T. sirtalis* populations.

Feldman et al. [2012] examined the pore sequences of SCN4A from over 70 species distributed around the globe and throughout the phylogeny of snakes. Using genomic DNA and focusing on the exons that encode the four pore domains, they asked whether the six taxa above that are known to prey on TTX-laden prey show changes in SCN4A different from other snakes that are not known to ingest the toxin. The results supported the overall conservatism of SCN4A. Across all the species of TTX-sensitive snakes, only eight substitutions were found in SCN4A, and none of these were in the outer pore. TTX sensitivity of $Na_V1.4$ is clearly the ancestral condition for snakes (fig. 2).

On the other hand, the six species of TTX-resistant snakes (including *T. sirtalis*) had 14 derived substitutions, all in the outer pore vestibule that interfaces with TTX. Every one of these amino acid replacements was found in DIII or DIV, and nine involved positions, and in some cases the same amino acids, as those seen in other resistant snakes. Character state reconstruction indicated that resistant amino acid replacements in $Na_V1.4$ have evolved independently no less than six times within the radiation of colubrid snakes.

The functional consequences of many of these replacements are known from site-directed mutagenesis and expression, or from inference based on models of the interactions between TTX and the outer pore [Feldman et al., 2012]. The DIV replacements discovered in *L. epinephelus* alter the charge at the ancestral D1568 that forms a hydrogen bond with TTX. Changes to this residue also are found in *T. sirtalis* and *T. atratus* and, along with substitutions at the neighboring G1569, are known to cause major reductions in TTX-binding affinity. *T. atratus* also shares a change in D1277E with Asian *A. pryeri*. Like the I1561V found in all resistant *T. sirtalis* populations, these substitutions involve biochemically similar amino acids, but are still capable of generating significant reductions in TTX binding affinity [Geffeney et al., 2002].

TTX-Resistant Prey

Taricha belong to the family Salamandridae that includes two major clades referred to as 'primitive' and 'modern' newts [Zhang et al., 2008]. All of the modern newt genera (including *Taricha*) tested have been found to bear TTX in the skin, whereas none of their sister clade, the primitive newts *(Echinotriton*, *Tylototriton* and *Pleurodeles)*, do [Hanifin, 2010]. *Taricha granulosa* are further known to be resistant to injected TTX [Brodie, 1968; Brodie and Brodie, 1991], but the mechanism was unclear until Hanifin and Gilly [2015] demonstrated that *T. granulosa* have skeletal muscle fibers that are able to

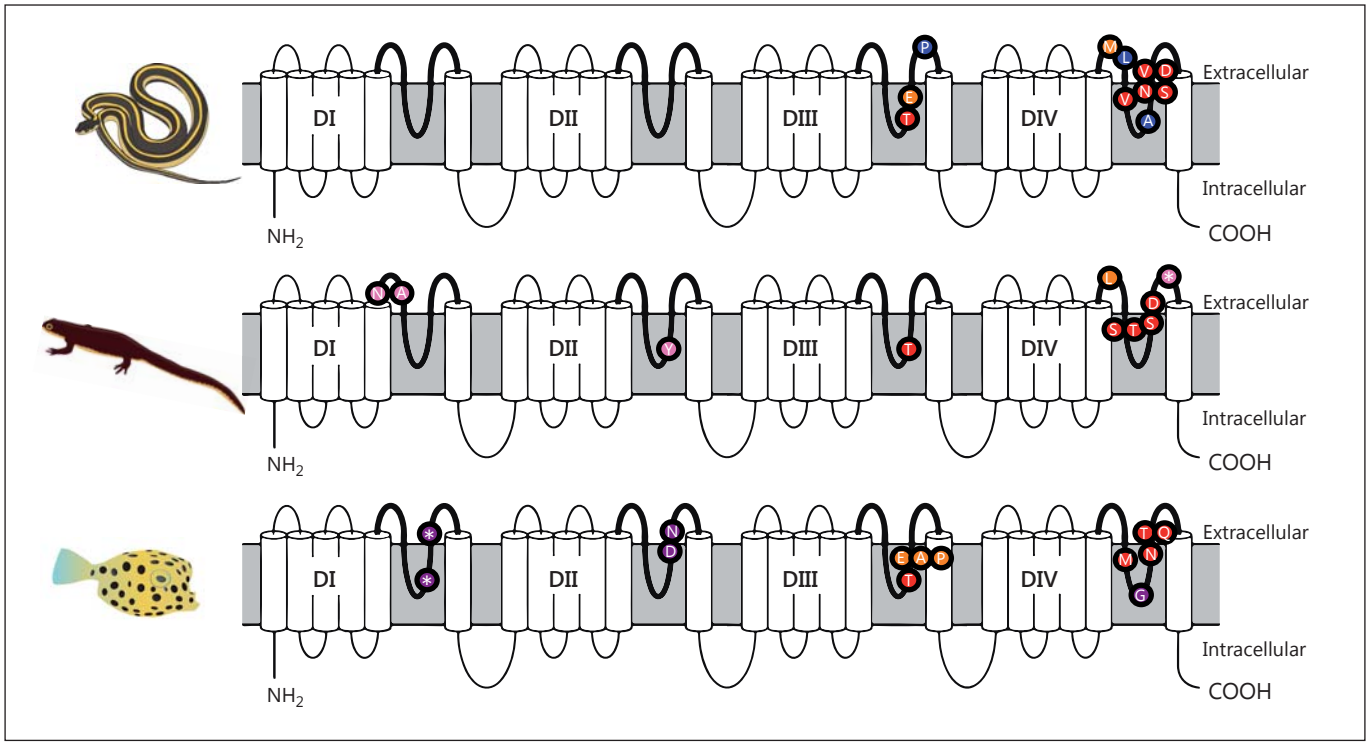

Fig. 2. Convergent amino acid substitutions in $Na_V1.4$ in predators and prey. A schematic representation of the voltage-gated sodium channel protein expressed in muscle is shown for snakes, newts and puffer fish (including both $Na_V1.4a$ and 1.4b) with amino acid replacements in the outer pore noted. Substitutions include the accumulated derived changes seen in any of the species tested within each taxonomic group: snakes [Feldman et al., 2012], newts [Hanifin and Gilly, 2015] and puffer fish [Jost et al., 2008]. Not all of the substitutions illustrated are known to reduce TTX binding (e.g. DI and DII substitutions unique to newts). Red circles indicate convergent position substitutions shared by all three groups, orange for those shared by two groups, blue unique to snakes, pink unique to newts and purple unique to puffer fish. The derived amino acid replacement is denoted by the single letter abbreviation in each case. Where two or more substitutions are present at a convergent site in a single group, side-by-side circles are shown; where more than one substitution is observed at taxonomically unique sites, an asterisk is shown. Drawn from data of Jost et al. [2008], Feldman et al. [2012] and Hanifin and Gilly [2015].

transmit AP even when exposed to high TTX concentrations. Primitive newts have slightly resistant muscle fibers compared to other salamanders, but not nearly as extreme as those of their sister clade. As with garter snakes, this finding implicates $Na_V1.4$ as a likely mechanism of TTX resistance in prey.

In salamanders, the evolutionary transition to withstand TTX and harbor large quantities of the poison for defense appears to have occurred at the split between primitive and modern newts. Amino acid replacements in the outer pore of $Na_V1.4$ enabled this transition [Hanifin and Gilly, 2015]. Both clades of newts have a single amino acid replacement, I1424S, in DIV that confers slight reductions in binding affinity of TTX, consistent with the low-level muscle resistance measured in primitive newts. This residue is analogous to the Ile-Val substitution common in the DIV of garter snakes (I1561V; alignment numbers differ among orthologs in different taxa). Within the modern newts, three additional substitutions confer much greater resistance. In DIII, newts share the same M1116T that is observed in *T. couchii* and known from mutagenesis studies to reduce TTX binding 15-fold [Terlau et al., 1991; Feldman et al., 2012]. In DIV, resistant newts have two charge-neutralizing replacements, D1431S and G1432D, in the identical P-loop position as the analogous substitutions (D1568N/S and G1569V/D) discovered in *T. atratus*, *T. sirtalis* and *L. epinephelus*, which are known to reduce TTX binding 300-fold (fig. 2) [Geffeney et al., 2005; Feldman et al., 2012; Hanifin and Gilly, 2015].

Newts are not the only TTX-bearing prey. In fact, TTX was first identified from, and named for, tetraodontid

puffer fish [Hanifin, 2010]. A comparison of four genera of puffer fish [Jost et al., 2008] revealed that they too have altered $Na_V1.4a$ and $Na_V1.4b$ structure consistent with resistance to TTX (teleosts have two paralogs expressed in skeletal muscle due to an ancient whole-genome duplication). Character state reconstruction suggests these two proteins have evolved replacements in the P-loops six to nine times within the radiation of the tetraodontids. Of these, three amino acid replacements are analogous to those found in both newts and snakes – the Met-Thr found in DIII and the Asp-Asn and Ile-Val (seen as Ile-Met in puffer fish) in DIV. Unlike the pattern observed in newts and snakes, puffer fish have several other modifications to the skeletal muscle channels in DI and DII, each of which has been shown to reduce binding affinity (fig. 2).

Parallel Evolution across a Gene Family

Because unique sodium channels are expressed in different tissues, an organism cannot be wholly resistant to TTX simply by evolving changes in one channel type [Zakon, 2002]. Each of the susceptible tissue fields somehow must evolve resistance. In teleosts, eight Na_V1 proteins are known to have arisen during ancient duplications. Jost et al. [2008] examined each of the paralogous genes that encode these proteins in tetraodontid puffer fish. Despite the high level of conservatism in the pore region of these proteins across vertebrates, puffer fish exhibit derived substitutions in all eight of the known SCNA genes (fig. 3). With only one exception ($Na_V1.5La$ in *Tetraodon*), every genus showed at least one derived substitution that is known to alter TTX binding affinity in each paralog. Several key substitutions seen in other taxa have evolved independently in multiple proteins, including the familiar Met-Thr in DIII discussed above, which arose in five of the eight paralogs. Other patterns of repeated parallel evolution appear to be unique within puffer fish, including one change each in the DI and DII regions and an Ala-Gly change in DIV that arose in four different Na_V1 proteins. This latter replacement is especially surprising because it occurs in the highly conserved DEKA motif (DI-Asp, DII-Glu, DIII-Lys and DIV-Ala) that is thought to be fundamental to Na^+ selectively [Jost et al., 2008].

Predators are also expected to evolve resistance in multiple tissues, though the selective landscape is somewhat different. Prey must deal with toxin that is present in multiple tissues and is probably circulating throughout the body on a regular basis, thus demanding resistance in all sodium channel paralogs, as observed in puffer fish. Predators, on the other hand, ingest TTX and are exposed in a more episodic fashion, so selection may not target all channel forms equally. In mammals, and probably reptiles, three of the sodium channel forms ($Na_V1.1$, $Na_V1.2$ and $Na_V1.3$) are expressed in the CNS and protected from ingested toxins like TTX by the blood-brain barrier [Goldin, 2001, 2002; Catterall et al., 2005; Zimmer, 2010]. Three other channel forms in mammals are ancestrally insensitive to TTX – the cardiac muscle channel, $Na_V1.5$, and two channels of the peripheral nervous system, $Na_V1.8$ and $Na_V1.9$ [Goldin, 2001].

Garter snakes encountering TTX, therefore, are expected to evolve parallel resistance in only three sodium channels – $Na_V1.4$, which is known to be resistant, $Na_V1.6$ and $Na_V1.7$. Analysis of the coding sequence of these genes from *T. sirtalis* revealed familiar amino acid replacements in both of these newly explored channels (fig. 3) [McGlothlin et al., 2014]. $Na_V1.6$, which is expressed in peripheral nerves, had the same Ile-Val substitution in DIV that is common in the $Na_V1.4$ of resistant populations of the same species. $Na_V1.7$, also expressed in the peripheral nervous system, was found to have four replacements. These include the DIII Asp-Glu substitution observed in the $Na_V1.4$ of *T. atratus* and Asian *A. pryeri* and the DIV Asp-Asn substitution associated with extreme resistance of $Na_V1.4$ in *T. sirtalis*. The two other substitutions are found in the TTX-resistant puffer fish, including the DIV Ala-Gly observed in the DEKA motif. Sequencing of the *T. sirtalis* $Na_V1.1$–1.3 expressed in CNS confirmed that these channels retained their ancestral TTX-sensitive forms as expected for channel types protected by the blood-brain barrier.

Conclusion

The evolution of TTX-resistant Na_V1 in vertebrates follows a remarkably predictable path across the ecological guilds of predator and prey, deep phylogenetic divides and among paralogous genes within species. Not only has evolution reused the same genes to adapt to this neurotoxin, in many cases it has also struck on the same position and even the same amino acid substitution in the proteins. Of the roughly 80 amino acid positions that TTX is thought to interact with, only a tiny fraction shows any evidence of evolving replacements that alter binding affinity, and those changes represent a highly biased subset of the available substitutions [Feldman et al., 2012].

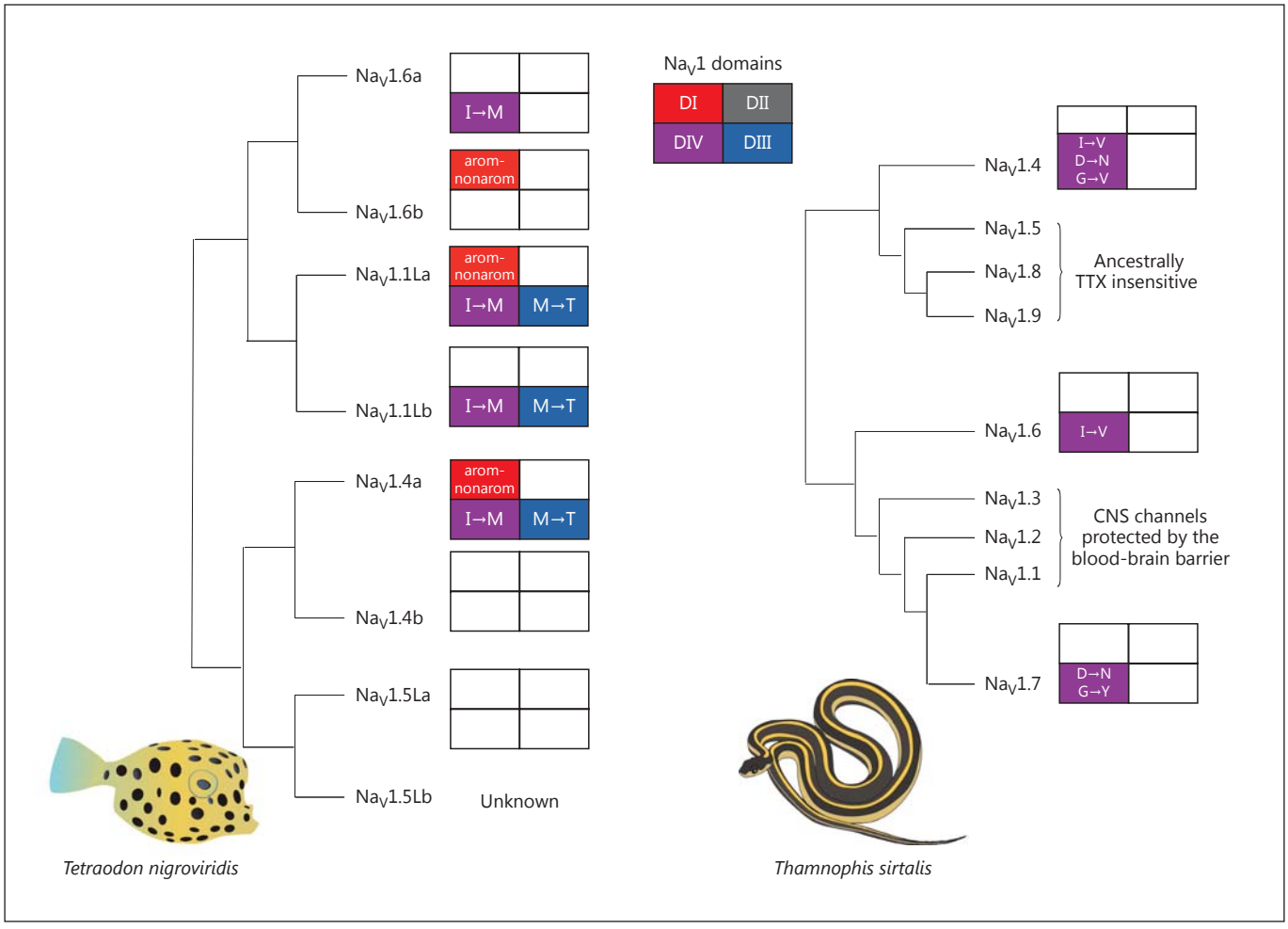

Fig. 3. Parallel evolution of TTX resistance in Na$_V$1 paralogs within species. The gene trees of the family of paralogous Na$_V$1 (SCNA genes) are shown for one species of puffer fish *(Tetraodon nigroviridis)* and one species of garter snake *(T. sirtalis)*. Boxes represent the P-loop of each of the four domains (DI–DIV) of the Na$_V$1 protein. Amino acid substitutions in these regions that are convergent across paralogs within each species are shown (unique replacements and those found in paralogous genes in other taxa not shown). In DI of puffer fish, 'arom-nonarom' denotes several replacements that alter a residue from an aromatic to a nonaromatic amino acid. Drawn from data in Jost et al. [2008] and McGlothlin et al. [2014].

This pattern of parallelism suggests dramatic constraints in the balance between the progressive evolution of new ability (resistance) and the maintenance of critical existing functions (selective Na^+ permeability) that may be somewhat unique to fundamental proteins such as membrane ion channels. Because they perform such a critical and conserved role in AP propagation, there is little room for reduced functionality. The high sequence homology of the pore regions of Na$_V$1 proteins suggests they experience strong purifying selection, at least on the selectivity filter of the channels where TTX binds. Site-directed mutagenesis studies show that amino acid substitutions that reduce TTX-binding affinity also reduce Na^+ permeability and selectivity [Feldman et al., 2012]. This fundamental trade-off suggests there are a limited number of switches that evolution can flip to respond to the selective challenge of TTX, resulting in a highly predictable set of molecular solutions to evolutionary problems.

Although the available data point to a narrow and predictable adaptive path that involves structural changes to SCNA genes, there is no reason to imagine that this is the only conceivable mechanism that could confer TTX resistance. Expression patterns of ancestrally resistant sodium channels, like the cardiac Na$_V$1.5, could be altered

to generate TTX-resistant tissues [Moody and Huey, 2002], and expression of both TTX-sensitive and TTX-insensitive channels in heart muscle has been observed across vertebrates [Zimmer, 2010; Vornanen et al., 2011]. Posttranscriptional RNA editing, common in tissue-specific expression of invertebrate sodium channels, is known to generate TTX-resistant forms [Liu et al., 2004; Dong, 2007]. Finally, TTX-binding proteins have been identified in the hemolymph of some crabs and blood of puffer fish that might offer an alternative mechanism of resistance [Llewellyn, 1997; Matsui et al., 2000; Nagashima et al., 2002]. The diverse and independently derived taxa that face TTX as predators or prey provide untapped opportunities to further test the hypothesis of constraint and predictability in adaptive evolution.

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