An electrophysiological approach to understanding the evolution of tetrodotoxin resistance in *Thamnophis sirtalis*.

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Abstract

A main objective of evolutionary biology is the exploration of the mechanisms of adaptation from a molecular perspective. Many adaptive traits constitute complex phenotypes involving several gene products. Toxin resistance can be considered a complex phenotype when multiple proteins are each targeted by a specific toxin. Tetrodotoxin (TTX) resistance in garter snakes (*Thamnophis sirtalis*) adapted to consuming toxic prey fits this model of a complex phenotype because the potent neurotoxin TTX targets multiple proteins within snake tissues. These TTX target proteins in resistant *T. sirtalis* are paralogous voltage-gated sodium channels (Na_V), which evolved amino acid substitutions that decrease binding affinity of TTX. To better understand evolution of TTX resistance in Na_V channels, I reviewed examples of resistant channels across a wide range of taxonomic groups encompassing vertebrate and invertebrate species. This exploration revealed a high degree of molecular convergence in the protein changes that confer resistance. This convergence likely results from a combination of the binding specificity of TTX and functional constraints in Na_V that are shared among unrelated taxa.

Evolution of physiological TTX resistance in *T. sirtalis* seems to require the acquisition of TTX-resistant substitutions in multiple Na_V paralogs. The resistance-related changes found in one paralog expressed in peripheral nerves ($Na_V1.7$) show ancient evolution. The substitutions in $Na_V1.7$ were acquired before garter snakes evolved to consume TTX-bearing prey. In fact, these substitutions are found in most snakes, suggesting that these changes were acquired to serve a function unrelated to TTX

resistance. I used channel expression in a heterologous system and electrophysiology to explore the hypothesis that evolution of ancient resistance in $Na_V1.7$ is a by-product of ancestral selection for biophysical changes in this paralog. Evolution of resistance in the skeletal muscle paralog ($Na_V1.4$) of *T. sirtalis* shows signs of recent evolution in direct response to TTX exposure. Different snake populations exposed to TTX-bearing prey present few variants of $Na_V1.4$ exhibiting TTX-resistant amino acid substitutions. However, there is a dearth of intermediate channel variants between the ancestral TTX-sensitive $Na_V1.4$ (lacking substitutions) and the derived channel forms found in highly resistant snakes. This implies that selection may disfavor intermediate forms of the channel, possibly due to molecular constraints limiting disadvantageous combinations of channel substitutions. I investigated molecular constraint in the evolution of TTX resistance in $Na_V1.4$ by recreating the intermediate steps in one possible mutational trajectory from a sensitive channel to a TTX-resistant channel variant. I used electrophysiology to assess TTX resistance and different aspects of channel function. This work helped reveal pleiotropic effects of amino acid mutations involved in the evolution of TTX resistance in $Na_V1.4$. The exploration of molecular evolution in two of the paralogs contributing to physiological resistance in *T. sirtalis* can help us understand how molecular factors shaping evolution of proteins influence evolution of a complex trait.

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Introduction

Understanding the genetic basis of phenotypic evolution is a main objective of evolutionary biology. Much of the knowledge about the genetics governing adaptive trait evolution comes from studying traits with simple genetic architecture (Hoekstra and Coyne 2007; Steiner et al. 2007; Stern and Orgogozo 2008); while relatively less is known about the genetics involving adaptation of complex traits (Rockman 2012; Travisano and Shaw 2013). In order to study the evolution of a complex phenotype, it is important to distinguish the different units that contribute to the phenotypic whole. It is also critical to identify the selective pressures driving change in those units and the intrinsic forces that shape the direction of evolution (Breen et al. 2012; DePristo et al. 2005; Stern 2000).

Complex phenotypes involve the products of multiple genes (Pavlicev and Wagner 2012). An opportunity to study polygenic trait evolution arises when phenotypes are the combined product of genes in a multimember gene family that consists of paralogs. Traits involving evolution in paralogs provide a simplified model of genetic adaptation in multiple genes given that paralogous genes are similar in sequence and function, which facilitates their examination (McGlothlin et al. 2014). Tetrodotoxin (TTX) resistance in garter snakes (*Thamnophis sirtalis*) is a good evolutionary example of a complex phenotype involving evolution in paralogous genes. The molecular demands of the neurotoxin TTX require paralogous protein targets to evolve resistance in snakes; and resistance involves multiple amino acid changing substitutions within a protein (Geffeney et al. 2002; McGlothlin et al. 2014). Those substitutions, in turn, are affected by historical constraints dictated by the structure and function of the target proteins (Feldman et al. 2012, Toledo et al. 2016).

Tetrodotoxin resistance evolved as a predatory adaptation allowing resistant snakes to consume otherwise lethal TTX-bearing newts in the genus *Taricha* (Brodie and Brodie 1991; Brodie et al. 2005). Garter snakes are involved in an evolutionary arms race with toxic newts, in which snakes have developed increasing levels of resistance in response to reciprocal selection from exposure to increasing levels of prey toxicity (Hanifin et al. 2008). Tetrodotoxin exhibits a specific mode of intoxication by binding to the pore of voltage-gated sodium channels (Na_V) and blocking Na^+ currents necessary for action potential formation in muscle fibers and neurons (Moczydlowski 2013; Narahashi et al. 2008).

Voltage-gated sodium channels are a family of proteins that include multiple paralogs in all vertebrates and invertebrates; these paralogs are distributed in different types of excitable membranes (Catterall 2000; Kwong and Carr 2015). Not all paralogs are exposed to TTX in the same degree when the toxin enters the body through digestion. The channels expressed in the vertebrate central nervous system are thought to be protected because of TTX's inability to cross the blood-brain barrier (McGlothlin et al. 2014). In addition, some channels are protected because they are inherently insensitive to TTX. For example, the channel that is expressed in the mammalian cardiac muscle is resistant to TTX blockage due to an amino acid replacement that removes an important site of TTX contact (Satin et al. 1992). However, there are Na_V channels expressed in peripheral nerves and in skeletal muscles that can be blocked by TTX in most known vertebrates (McGlothlin et al. 2014).

Several species of animals are exposed to TTX in different ecological contexts, which offers a unique opportunity to explore how different taxa evolved resistance against this toxin. Organisms exposed to TTX comprise both vertebrates and invertebrates; some use TTX as a defensive chemical, some are predators of TTX-bearing prey, and some use TTX as a predatory venom (Du et al. 2009; Feldman et al. 2010; Hanifin and Gilly 2015; Jost et al. 2008). Prey animals that use TTX as a chemical deterrent do not appear to posses a means to sequester the toxin as a way to protect their own sodium channels (Hanifin and Gilly 2015). In fact, it seems clear that tetrodotoxic prey evolved physiological TTX resistance through the same mechanisms as the predators exposed to the toxin: by acquiring amino acid substitutions in their sodium channels that decrease TTX binding (Brodie and Brodie 2015; Toledo et al. 2016).

The evolution of TTX-resistant Na_V channels in unrelated taxa allows the exploration of the causes of molecular convergence. Contrasting the molecular changes in different TTX-resistant Na_V channels can reveal insights about the possible forces influencing mechanistic parallels in convergent phenotypes. Chapter One of this dissertation reviews the literature to reveal broad patterns of convergence across taxa at the level of amino acid changes in Na_V channels. Although the chapter focuses primarily on better-known examples of channel resistance in vertebrate prey and predator species exposed to TTX, the chapter also includes less studied cases of reported resistance in invertebrate species. This review exposes a profound degree of convergence in the evolution of TTX resistance across unrelated phylogenetic groups. This convergent evolution can be appreciated at the level of the protein, the specific functional regions within the proteins and even at the level of similarities in the

distribution of the amino acids that change inside those regions (Toledo et al. 2016). Uncovering a fine degree of convergence suggests that, when it comes to evolving TTX resistance, there may be a limited number of adaptive solutions available forcing distant lineages to evolve strikingly similar molecular traits. A high degree of molecular convergence strongly suggests the presence of molecular constraint limiting protein evolution.

Snakes have nine Na_V paralogs that are expressed in different muscle and neuronal tissues (McGlothlin et al. 2014, 2016). Symptoms of intoxication from exposure to low dosages of TTX are believed to involve blockage of a channel paralog expressed in peripheral nerves ($Na_V1.7$) (McGlothlin et al. 2016). Resistance in $Na_V1.7$ is hypothesized to have played a role in permitting garter snakes to consume low-toxicity newts during the early stages of the arms race (McGlothlin et al. 2016). Snake $Na_V1.7$ presents substitutions that predate the interaction of garter snakes and tetrodotoxic newts; these substitutions are fixed in most snakes. Therefore, it is unlikely that these substitutions evolved in direct response to TTX. Studies in mammalian Na_V channels revealed that TTX-resistant Na_V substitutions have the potential to affect channel function (Lee et al. 2011, Terlau 1991). In this light, it is possible that the ancient substitutions seen in snake $Na_V1.7$ were selected because they could modify function adaptively. In Chapter Two, I use channel expression in a heterologous system and electrophysiology to explore the hypothesis that amino acid replacements seen in garter snake $Na_V1.7$ confer resistance in this channel and also that these changes could affect channel function. Resistance in this paralog may be considered a functional exaptation

conferring snakes a baseline level of resistance, predisposing snakes to start consuming toxic newts and allowing them to enter the arms race.

Eventually, as the arms race progressed, snakes became exposed to larger concentrations of TTX found in their prey, and their skeletal muscle channels ($Na_V1.4$) likely became affected (McGlothlin et al. 2016). When present in large concentrations in the body, TTX causes muscle paralysis that can result in death (Isbister and Kiernan 2005; Noguchi and Arakawa 2008). Most snakes have TTX-sensitive skeletal muscle channels, except garter snakes that are involved in the arms race with highly toxic newts. These snakes have amino acid mutations in $Na_V1.4$ that can decrease TTX-binding and can confer channel resistance (Geffeney et al. 2002; Geffeney et al. 2005). There is some evidence that snake Nav1.4 substitutions expressed in a mammalian channel background could modify channel biophysical properties (Lee et al. 2011). This suggested that TTX-resistant substitutions could carry costs to channel function, which could be a source of molecular constraint in the evolution of TTX resistance in these channels.

One approach to evaluate the degree of molecular constraint affecting evolution of proteins is to recreate evolutionary trajectories experimentally (Dean and Thornton 2007; Tufts et al. 2015). One of the main forces shaping protein evolution is the pleiotropic effect of amino acid mutations (Weinreich et al. 2006). A way to expose this pleiotropy is to measure the consequences of the sequential addition of evolutionarily relevant mutations on multiple aspects of protein function. In Chapter Three, I evaluate molecular constraint in the evolution of TTX resistance in garter snake skeletal muscle channel ($Na_V1.4$) by synthesizing snake channels in which I varied substitutions relevant to resistance. I follow a mutational

pathway going from a sensitive to a resistant snake muscle channel allele via stepwise increments of the number of amino acid replacements. Then, I assessed the function and TTX resistance of all the channels created. I learned that the evolution of TTX resistance in the snake muscle channels could be costly as I identified significant shifts in channel biophysical properties associated with TTX resistance. Also, one of the intermediate channel forms presented a combination of substitutions that could be especially disadvantageous for the function of the channel. These findings support the hypothesis that the mutational pathway toward a well-adapted combination of TTX-resistant amino acid substitutions in garter snake $Na_V1.4$ could be constrained by epistatic interactions of substitutions that negatively affect some channel intermediates.

The results presented in this dissertation ultimately show that revealing the pleiotropic nature of substitutions is important to understand the evolution of TTX resistance. This work shows that resistance in Na_V channels could evolve as a direct result from pressures arising from exposure to TTX, or as a side effect of substitutions that affect function. Substitutions in $Na_V1.7$ are probably exaptive because these changes were already fixed in most snakes before they could offer garter snakes an advantage in terms of resistance to tetrodotoxin. The fact that these substitutions were ancient suggests that a functional shift by these substitutions was likely adaptive in this channel. Therefore, an "accidental" increase in resistance in $Na_V1.7$ that helped snakes survive encounters with toxic newt could be considered pre-adaptive. On the other hand, similar substitutions in $Na_V1.4$ could be considered costly because the changes in function are likely a by-product of substitutions selected to increase resistance. In an ionic protein that serves a conserved role in the

functioning of the central nervous system, unintended functional shifts are likely costly. Understanding the molecular evolution of a highly functionally conserved protein can shed light into the mechanisms that influence the direction of protein evolution.

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Chapter One:

Convergent evolution of tetrodotoxin-resistant sodium channels in predators and prey[1]

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Abstract

Convergent evolution of similar adaptive traits may arise from either common or disparate molecular and physiological mechanisms. The forces that determine the degree of underlying mechanistic similarities across convergent phenotypes are highly debated and poorly understood. Some garter snakes are able to consume newts that possess the channel blocking compound tetrodotoxin (TTX). Despite belonging to unrelated lineages, both the predators and prey have independently evolved remarkably similar physiological mechanisms of resistance to TTX that involve chemical and structural changes in voltage gated sodium channels (Na_V). The evolution of TTX-resistance in this predator-prey pair constitutes a natural experiment that allows us to explore the causes of molecular convergence. Here, we review broad patterns of convergence at the level of amino acid changes in Na_V channels of animals that evolved TTX-resistance and make comparisons to known TTX-resistant channels that did not evolve under the selective pressures imposed by TTX. We conclude that convergence likely stems from the interplay of the target specificity of TTX and functional constraints of Na_V that are shared among taxa. These and other factors can limit channel evolution to favor a few functionally permissible paths of adaptation, which can explain the observed predictability of changes to channel structure. By studying the functional causes of convergence in Na_V channels, we can further our understanding of the role of these important channel proteins at the center of the evolution of the nervous system.

Introduction

Convergent phenotypes arise when unrelated taxa evolve similar adaptive solutions to meet demands imposed by similar environmental pressures. Explorations of the mechanisms of adaptation from a molecular perspective have revealed that phenotypic convergence can arise from either common or disparate underlying developmental and physiological bases (Arendt & Reznick, 2008; Arnegard, Zwickl, Lu, & Zakon, 2010). In some cases, convergent phenotypes involve modifications of the same genes and even the same substitutions within genes. In other examples, different structural and molecular building blocks contribute to phenotypic similarities (Manceau, Domingues, Linnen, Rosenblum, & Hoekstra, 2010). The probability that a common gene will contribute to convergence in different taxa increases with phylogenetic relatedness, but convergence associated with a common gene can still occur in unrelated lineages (Conte, Arnegard, Peichel, & Schluter, 2012). The phenomena influencing the level of underlying mechanistic similarity in convergent phenotypes are not well understood and resolving the role of functional constraint in this process is critical.

Predator-prey systems in which the fitness of each species is governed by the presence of, or resistance to, a toxin are ideal for testing hypotheses about convergent evolution. In these systems, the animals that feed on toxic organisms as well as the animals that use the toxin for defense must evolve resistance to the same toxin. Although the strength of selection will vary for prey and predators, both organisms must converge at some point along the adaptive path to resistance. Because predators and prey typically belong to evolutionarily divergent taxa, convergent phenotypes are less likely to arise through parallel adaptation at the molecular level because of differences in the physiological and functional

requirements of these organisms. As a result, parallel changes associated with such predator-prey systems can provide important insight into the phenomena underlying mechanistic similarity in convergent phenotypes.

The broad taxonomic range of the neurotoxin tetrodotoxin (TTX) provides an exceptional case study of convergent evolution of toxin resistance. Tetrodotoxin has a very specific mode of action and has been extensively studied in the context of structure/function models of its protein target: voltage-gated sodium channels (Na_V) (Moczydlowski, 2013). Studies of the adaptive history and molecular basis of TTX-resistance have been able to focus directly on the specific regions of Na_V channels that regulate TTX-binding (Choudhary, Yotsu-Yamashita, Shang, Yasumoto, & Dudley, 2003; Fozzard & Lipkind, 2010; Santarelli, Eastwood, & Dougherty, 2007).

This toxin has been identified in a remarkable array of species that includes prokaryotes, multiple invertebrates including cephalopods, flatworms, gastropods, as well as many vertebrate taxa including fish and amphibians (Williams, Hanifin, Brodie, & Brodie, 2010; Hanifin, 2010). In animals, TTX serves a defensive role that protects prey species from predation. However, some predators, including snakes, do successfully prey on TTX-bearing taxa. Ultimately, this broad taxonomic distribution of a toxin that targets fundamental physiological processes allows examination of the mechanistic basis of phenotypic convergence across a broad spectrum of species and ecological roles.

Here we will focus on the mechanistic basis of TTX-resistance in TTX-bearing salamanders and garter snakes that prey on those salamanders. The modern newts (family: Salamandridae) are a monophyletic clade of salamanders that include both new world and

old world genera. All genera in this group likely possess TTX, but the best studied of these is the genus *Taricha,* which includes four species known to possess high levels of TTX (Hanifin, Brodie, & Brodie, 2008). This genus is distributed along the western coast of North America and co-occurs with garter snakes in the genus *Thamnophis*. Some species and populations of *Thamnophis* are known to eat TTX-bearing *Taricha* and this predator-prey interaction has become one of the best-studied examples of arms-race coevolution (Brodie, Ridenhour, & Brodie, 2002; Hanifin et al., 2008; Feldman, Brodie, Brodie, & Pfrender, 2012; Thompson, 2005). Both predator and prey lineages have evolved resistance to TTX through adaptive substitutions in their Na_V channels (Geffeney, Fujimoto, Brodie, Brodie, & Ruben, 2005; Feldman et al., 2012; Hanifin & Gilly, 2015).

Tetrodotoxin as an Agent of Selection

The ability to initiate and propagate action potentials (APs) is a fundamental component of vertebrate nerve and muscle function. These signals are generated by the influx of sodium (Na^+) ions through Na_v channels, which are ubiquitous in vertebrates. Tetrodotoxin disrupts the initiation and propagation of APs in excitable tissues. The toxin selectively binds and blocks the outer pore of Na_v channels and prevents the influx of Na^+ ions that carry the current responsible for membrane depolarization during the initial phase of the AP (Hille, 2001; Narahashi, 2008). The detrimental consequences of blocking AP generation include the inability of nerve signals to reach targets and prevention of muscle fibers contraction. A primary symptom of TTX intoxication in animals is paralysis resulting from loss of muscle function (Clark, Williams, Nardt, & Manoguerra, 1999). This muscle paralysis can also interrupt a predator from ingesting a toxic prey animal, allowing the prey to survive the

encounter. Ultimately, the effects of TTX kill the majority of nonresistant predators that might encounter the prey (Williams et al., 2010; Brodie, 1968). The rapid, lethal effects of TTX make it a powerful anti-predator weapon for animals that can either produce or sequester high levels of the toxin (Hanifin & Gilly, 2015; Jost et al., 2008).

Because of the fundamental role of Na_V channels in neuromuscular function, the presence of endogenous TTX in TTX-bearing animals and exposure to TTX in predators of those animals exerts strong convergent selection on both predators and prey. Even exposure to non–lethal levels impairs locomotor performance and likely has significant negative effects on survivorship (Brodie & Brodie, 1999). Studies of coevolution between TTX-bearing newts and snake predators indicate that multiple populations of snakes have evolved elevated TTX-resistance in response to evolution of elevated TTX levels in prey (Hanifin et al., 2008). Newts do not posses a means of sequestering the toxin and the internal organs including heart, muscle and lungs are exposed to high levels of TTX (Wakely, Fuhrman, Fuhrman, Fischer, & Mosher, 1966) generating strong selection favoring TTX-resistance (Hanifin & Gilly, 2015).

The interaction of TTX with Na_V

The alpha-subunit of Na_v channels, which forms the pore of the voltage-gated ion channel, is encoded by the *SCNA* gene family. In vertebrates, whole genome and tandem gene duplication events have given rise to multiple Na_v paralogs. The number of paralogs varies among lineages; amphibians have six, non-mammalian amniotes have nine, and placental mammals have 10; (Lopreato et al., 2001; Zakon, 2012; Zakon, Jost, & Lu, 2011; Zakon, Jost, Zwickl, Lu, & Hillis, 2009). Na_v channels are composed of four domains (DI-

DIV), each of which includes six trans-membrane segments (S1-S6). The extracellular linkers between S5 and S6 form the outer portion of the channel pore that controls ion selectivity and conductance of the channel (Terlau et al., 1991; Heinemann, Terlau, Stuhmer, Imoto, & Numa, 1992; Chiamvimonvat, Perez-Garcia, Tomaselli, & Marban, 1996; Yamagishi, Li, Hsu, Marbán, & Tomaselli, 2001; Favre, Moczydlowski, & Schild, 1996). These linkers form a constriction in the ionic pathway, called the selectivity filter, that allows entry of Na^{+} while inhibiting the influx of other cations such as calcium (Ca^{++}) and potassium (K^{+}) (Figure 1; Schlief, Schönherr, Imoto, & Heinemann, 1996; Sun, Favre, Schild, & Moczydlowski, 1997). The selectivity filter consists of four amino acids, Asp-Glu-Lys-Ala (DEKA), one from each of the four domains. Three positions downstream from the selectivity filter in each domain is a ring of charged amino acids represented in mammals by the sequence Glu-Glu-Met-Asp (EEMD) (Penzotti, Fozzard, Lipkind, & Dudley, 1998; Terlau et al., 1991; Tikhonov & Zhorov, 2011; 2012). Negative charges in this outer ring create an electrostatic cloud that serves to attract Na^{+} ions into the pore outer vestibule and alterations in the amino acids of the selectivity filter or outer charged ring can severely reduce channel conductance of sodium ions (Mahdavi & Kuyucak, 2015; Terlau et al. 1991).

Tetrodotoxin blocks ion conduction through Na_v channels by binding to amino acids in the outer pore of the channel, thereby occluding the pore. The binding affinity of the channel for TTX can be dramatically altered by substitutions in a limited number of amino acids in the region that includes the selectivity filter, the downstream outer charged ring, and the resides that link these two rings (Noda, Suzuki, Numa, & Stühmer, 1989; Terlau et al., 1991; Choudhary et al., 2003). For example, substitution of a non-aromatic amino acid for an aromatic amino acid at a single position in the domain I outer pore decreases TTX-binding affinity of the channel by several orders of magnitude (Backx, Yue, Lawrence, Marban, & Tomaselli, 1992). Less dramatic shifts in channel binding affinity can be induced by

substitutions outside this region in portions of the pore loops that affect the position of amino acids in the selectivity filter and outer charged ring (Yamagishi, Janecki, Marban, & Tomaselli, 1997). The specific role of many of the observed substitutions associated with TTX-resistance in newts and snakes is unclear. However, some substitutions appear to alter electrostatic interactions between TTX and the pore while others seem to shift the position of electrostatically interacting residues in ways that reduce the binding affinity of TTX in the pore.

Molecular Patterns of Convergence in TTX-Resistant Na_V channels

Convergent molecular evolution associated with TTX-resistance in predators and prey can be viewed at multiple levels: the genes/protein involved, the specific region of the protein where changes occur, the location of amino acid changes within these regions and the identity of the substitutions themselves. In newts, snakes, and puffer fish, TTX-resistance is not associated with a physiological means of sequestering TTX such as a TTX-binding protein or other form of detoxification. In all these taxa, resistance is associated with adaptive evolution in Na_v channels. Furthermore, adaptive TTX-resistance in all these taxa does not appear to be associated with up-regulation or other changes in expression patterns of Na_v channels. Instead, the evolution of TTX-resistance in these taxa is associated with repeated patterns of amino acid substitutions across channel paralogs that also display remarkable patterns of convergence (but see Feldman et al., 2015).

The best-studied channel isoform in newts and snakes is $Na_v1.4$ (*SCN4a*). This channel is present in all tetrapod lineages. Although the channel is expressed at low levels in multiple tissues, it is the primary channel expressed in skeletal muscle of both newts and snakes and is responsible for propagating APs in this tissue in both lineages. $Na_v1.4$ is also present in puffer fish, but in puffers and other fish an independent whole genome duplication event has given rise to two copies of this channel (Novak et al., 2006). Examination of

multiple populations and species of snakes and newts has painted a picture of dramatic convergence in this channel (Figure 2). In this ortholog, TTX-resistant garter snakes and newts exhibit amino acid substitutions in Na_v channels that are restricted to the pore loops of DIII and IV. Many of these changes occur at equivalent positions between newts and snakes, and in some instances the amino acid substitutions are identical.

TTX-resistant $Na_V1.4$ channels in predators

The original identification of TTX-resistance in a predator resulted from the observation that some garter snakes (*Thamnophis sirtalis*) were capable of eating TTX-bearing *Taricha granulosa*. Early work showed that these snakes were not entirely immune to TTX but that exposure to TTX resulted in a loss of righting reflex and a reduction in crawl speed (Brodie, 1968). Subsequent studies showed that skeletal muscle resistance to TTX was correlated with whole-animal resistance among populations of garter snakes (Geffeney, Brodie, & Ruben, 2002). Sequence analysis of the voltage-gated sodium channel from skeletal muscle ($Na_v1.4$) from these populations revealed four sites of amino acid substitution in the pore loops of DIV (Geffeney et al., 2005). To investigate whether these pore loop substitutions were responsible for the differences in resistance, Geffeney et al. (2005) created chimeric channels in which pore regions of mammalian $Na_v1.4$ contained snake sequence. These channel clones were used for electrophysiological recordings in frog oocytes to measure current transmission in the presence of TTX. The measured K_d (TTX concentration necessary to block 50% of Na^+ currents) mirrored the pattern of muscle resistance among populations of *T. sirtalis*. Channels with whole-snake sequence that were tested along with chimeras did not have significantly different K_d values, indicating that the substitutions in the DIV pore loops accounted for the differences in TTX sensitivity of the channels.

Interactions of amino acid replacements with TTX

In garter snakes, a single mutation (I1561V) is shared by all resistant *Thamnophis sirtalis* populations that have been studied (Figure 2). This substitution is associated with the lowest level of physiological resistance, and alone reduces the binding affinity of the channel for TTX by approximately two-fold (Geffeney et al., 2005). The I1561V substitution appears to have arisen at least twice independently in distinct lineages of *T. sirtalis*. Because this amino acid site is not known to interact directly with TTX, its effect on affinity may be the result of outer pore shape alterations that could hinder interactions between TTX and key residues in the outer vestibule (Tikhonov & Zhorov, 2011). A single substitution, D1568N found in snakes from the *T. sirtalis* Willow Creek population increases resistance 30–40-fold in rat $Na_v1.4$ (Choudhary et al., 2003; Penzotti et al., 1998;). This substitution may eliminate or weaken the hydrogen bond between the carboxyl group in Asp and a hydroxyl group in TTX (Choudhary et al., 2003). Neither the individual effects of the remaining changes found in *T. sirtalis* populations, nor the possible additive or epistatic effects of combinations of these changes on the binding affinity of TTX have been explored.

Other snake species that prey on TTX-bearing amphibians were subsequently investigated and revealed additional pore loop amino acid substitutions in DIII and DIV of $Na_v1.4$ (Figure 2; Feldman et al., 2012). Character state reconstruction revealed that resistant mutations had evolved independently at least six times in colubroid snakes. Some of these substitutions occurred in sites already known to interact with TTX from previous functional studies of the channel. For example, the Asp-Asn substitution in DIV (D1568N) that confers high resistance in *T. sirtalis* from Willow Creek, CA was also found in another garter snake species, *T. atratus*. A change to an alternate residue in the same position, D1568S, was observed in *Liophis epinephalus*, a South American snake that consumes tetrodotoxic *Atelopus* toads. Changes at this position are often accompanied by substitutions of the adjacent Gly (1569), suggesting the possibility of combinatorial effects on resistance. Finally,

a change in DIII (D1277E) evolved independently in the $Na_v1.4$ of *T. atratus* and also in the Asian snake *Amphiesma pryeri*, which consumes tetrodotoxic newts in the genus *Cynops* (Figure 2).

TTX-resistant $Na_V1.4$ channels in prey

Recent investigations have revealed that skeletal muscle and its related sodium channel $Na_v1.4$ is also resistant to TTX in the TTX-bearing newts (Hanifin & Gilly, 2015). This group of "modern newts" evolved roughly 50 MYA and includes taxa from Europe, Asia, and the New World. Hanifin and Gilly (2015) showed that fibers from *Taricha* skeletal muscle could fire APs in high concentrations (30 uM) of TTX. A total of four amino acid substitutions in the DIII and DIV pore loops appeared to explain the resistance to TTX in newts. As in the garter snake *T. couchii*, a Met-Thr substitution occurs in the DIII pore loop (M1116T in the newt protein) in all toxic newts. This same substitution is present in puffer fish and has been shown to reduce TTX-binding affinity in $Na_v1.4$ channels by approximately 15-fold (Jost et al., 2008). In addition, toxic newts present the I1424S substitution located in the position equivalent to the Ile-Val (I1561V) in snakes (Figure 2). Substitutions at positions equivalent to D1568 and G1569 in snakes were also identified in TTX-bearing newt species (positions D1431S and G1432D respectively). This pair of substitutions is present in all genera of newts that possess TTX.

TTX-bearing salamanders are not the only lineage of TTX-bearing animals to evolve resistant Na_v channels. Jost et al. (2008) found resistant amino acid substitutions in the pore loops of all eight Na_v paralogs from puffer fish species. These paralogs are expressed in skeletal muscle, heart and nervous system (Novak et al., 2006). Phylogenetic analyses showed that substitutions in puffer fish channels evolved independently in multiple genera, yet some changes were shared among paralogs and across species. Some of the substitutions found in tetrodotoxic puffer fish Na_v channels are similar or identical to those observed in the

skeletal muscle channels of resistant snakes and newts (Figure 2).

Convergence Across other Na_V Paralogs

Different Na_v proteins, encoded by unique but related paralogous genes, are expressed in different tissues across vertebrates. The specific number of the channels varies among tetrapod lineages and the orthology and evolutionary history of these genes is still being explored (Zakon et al., 2011). Ecologically relevant whole-animal resistance to TTX likely requires that TTX-resistant Na_v channels are expressed in most, if not all, tissues. Other channels are less well studied than $Na_v1.4$ but recent work has also shown strong patterns of convergence in these channel paralogs.

McGlothlin et al. (2014) investigated whether Na_v paralogs other than $Na_v1.4$ were resistant in garter snakes using genomic data to examine sequence from two channels expressed primarily in peripheral nervous system. They identified amino acid substitutions in sites associated with TTX-resistance in the pore loops of DIII and DIV of both the $Na_v1.6$ and the $Na_v1.7$ ancestor channels in snakes (Figure 3A). In mammals, these channels are identified as $Na_v1.6$ (*SCN8a*) and $Na_v1.7$ (*SCN9a*). Snakes and other reptiles do not possess a true $Na_v1.7$ (*SCN9a*) ortholog, but instead express the ancestral copy that gave rise to $Na_v1.7$ (*SCN9a*) and Na_x (*SCN10a*) in mammals.

In garter snakes, $Na_v1.6$ exhibited the Ile-Val substitution in DIV (I1709V), that is common in $Na_v1.4$ of resistant *T. sirtalis* and that halves TTX-binding affinity (Figures 2, 3A). The $Na_v1.7$ ancestor presented four substitutions including the DIII Asp-Glu substitution (D1393E) also found in the $Na_v1.4$ of resistant snakes *T. atratus* and *A. pryeri*, the DIV Asp-Asn substitution (D1684N) that confers extreme resistance also found in $Na_v1.4$ of *T. atratus* and *T. sirtalis* from Willow Creek, and the DIV A1681G substitution that occurs at the selectivity filter also found in the skeletal muscle channel $Na_v1.4a$ of resistant puffer fish,

which can reduce TTX-binding affinity by 1.5-fold (Figures 2, 3A; Jost et al., 2008).

Other Na_v paralogs in snakes do not have derived amino acid substitutions in the pore region that are expected to lower TTX binding affinity (McGlothlin et al. 2014). In mammals, three channel types ($Na_v1.1$, $Na_v1.2$, $Na_v1.3$) are expressed in CNS tissue. If expression patterns in reptiles are similar, these channel isoforms would be protected by the blood-brain barrier, which is thought to prevent exposure to ingested toxins like TTX. Consistent with the hypothesis that they are not under selection for TTX-resistance, none of these three paralogs show evidence of derived amino acid substitutions in the pore region of Na_v in garter snakes.

Amphibians do not possess true orthologs of any of the CNS channel isoforms but instead possess the ancestor of $Na_v1.2$ and $Na_v1.3$ (Zakon et al., 2011). In TTX-bearing newts this channel has evolved resistance to TTX and provides an exception to patterns observed in other channels. In the TTX-bearing newt *Cynops pyrrhogaster* TTX-resistance is associated with an Ala substitution in the domain I pore that replaces an aromatic amino acid residue and mirrors substitutions identified in mammalian $Na_v1.5$, $Na_v1.8$, and $Na_v1.9$ channels. This change renders the channel highly resistant to TTX (Figure 4; Hirota, Kaneko, Matsumoto, & Hanyu, 1999).

Convergence of Na_V in invertebrates

Invertebrates possess two channel isoforms: Na_v 1 and Na_v2 (Moran, Barzilai, Liebeskind, & Zakon, 2015). The Na_v2 isoform is present only in invertebrates and is thought to represent an evolutionary intermediate between voltage-gated Ca^{++} channels and true Na_v channels. The Na_v1 isoform is thought to be orthologous to the entire family of Na_v channels present in vertebrates (Moran et al., 2015). TTX-insensitive currents have been reported in multiple invertebrate species including jellyfishes, flatworms, annelids, some insects and

arachnids (Du, Nomura, Liu, Huang, & Dong, 2009). Resistance in these channels mostly results from changes to Na_v1 outer ring EEMD in DIII and IV (Figure 4). Some of these changes have been tested functionally to confirm they reduce TTX binding affinity. Changes to single residues in the EEMD outer ring of the mite *Varroa destructor* channel ($VdNa_v1a$) to mimic the changes seen in other invertebrate channels resulted in TTX-resistant forms (Du et al., 2009). These invertebrate channels share pore loop amino acid substitutions with the resistant channels in snakes, newts, and puffer fish. Notably, the negatively charged Asp (position 1717 in invertebrate channel alignments, analogous to position 1431 in newts and position 1568 in snakes) is replaced by other residues.

Implications of Molecular Convergence in Na_V

Convergent patterns of evolution have long intrigued biologists because convergence suggests fundamental rules that underlie adaptation (Losos, 2011). Convergence at the molecular level suggests that protein evolution also follows predictable rules (Stern & Orgogozo, 2009; Weinreich, Delaney, DePristo, & Hartl, 2006). For Na_V channels, convergent evolution of TTX-resistance across lineages can shed important light into the structure and function of these proteins.

In contrast to other voltage-gated ion channel gene families, the voltage-gated sodium channel gene family expanded during a time that tetrapod vertebrates evolved greater forebrain complexity and novel sensory receptors (Zakon et al., 2011). This expansion appears to have allowed for greater specialization of sodium channel gene expression in tissues and subcellular locations, as well as enhanced processing of sensory input from the skin and body. Yet, because of their key role in signaling of nerve and muscle tissue, Na_V

channels are under strong functional constraint and appear to have limited evolutionary lability. If adaptation has produced sequence level convergence in taxa as widely diverged as newts and snakes, it suggests that there are universal processes linked to Na_V channel structure and/or function that generate that convergence.

Repeated solutions to the TTX-resistance "problem" might result from multiple non-mutually exclusive sources. Convergence could result from the molecular specificity of the mode of action of TTX. Tetrodotoxin interacts with only a small number of amino acids located in the outer pore of the channel. Changes that neutralize the negative charges in the pore that make contact with TTX have the largest effect on resistance (Choudhary et al., 2003; Penzotti et al., 1998). Convergence can also result from functional constraints on the channel itself. The pore of the Na_V channel is responsible for the rapid but regulated influx of Na^+ ions. TTX interacts with charged amino acids that form a region of the channel responsible for the selective influx of Na^+ and blocks the influx of other ions. For example, disruption of the selectivity filter can increase TTX-resistance, but simultaneously causes increased calcium ion (Ca^{2+}) permeability, which would disrupt the signaling properties of the channel (Heinemann et al., 1992). Similarly, substitution of pore residues can both decrease the TTX-binding affinity of the channel and severely disrupt the permeability of the channel to Na^+ and reduce Na^+ conductance (Terlau et al., 1991). As a result, convergence could result because only a small number of amino acid site changes render a channel resistant and an even smaller number of these changes may be permissible due to functional constraints.

Although experimental data suggests that amino acid substitutions in DI can greatly reduce TTX-binding (Terlau et al., 1991; Satin et al., 1992), the molecular pattern of TTX-resistant amino acid changes that evolution has fixed in snakes and newts is restricted to DIII and IV (with the exception of the $Na_V1.2/1.3$ ancestor in newts). This pattern suggests that functional constraints may exist that limit the advantage of charge-changing substitutions in DI of the pore. Although this hypothesis has not been tested directly, there is evidence that pore substitutions in DI and DII, which increase TTX-resistance more than a thousand-fold, can also reduce Na^+ conductance to less than 1% of that seen in wild-type channels (Terlau et al., 1991). In contrast, the DIV substitution Asp to Asn observed in garter snakes (D1568N) significantly increases resistance by removing the negative charge of Asp, but its effect on Na^+ conductance is less dramatic than the effects of similar substitutions in DI and DII (Terlau et al., 1991). Although not tested directly, the same effect could be true for the analogous D1431S substitution found in newts.

In both snakes and newts, negative charge-neutralizing substitutions are accompanied by other pore loop substitutions whose contribution to TTX-resistance is not well-understood. Electrophysiological data show that some of these substitutions alone can have a small to moderate effect in reducing TTX-binding (Du et al., 2009; Geffeney et al., 2005; Jost et al., 2008). This reduction could be due to alterations in the shape of the pore that indirectly hamper TTX-docking in other sites (Tikhonov & Zhorov, 2011). These small effect substitutions also exhibit a convergent pattern between snakes and newts, which suggests they may have an important role in the evolution of TTX-resistance in these channels.

There is a dearth of studies looking at epistatic interactions between these substitutions, but it is possible that changes occur together because they interact non-additively to increase the degree of resistance. For example, the mite *Varroa destructor* possesses a TTX-resistant channel $VdNa_V1$ that exhibits two pore loop substitutions in the outer charged ring in DIII and IV (positions 1425 and 1717 respectively) (Figure 4). These substitutions change the arthropod EEID outer charged ring motif into EETS. Du et al. (2009) introduced mutations in the resistant $VdNa_V1$ (containing EETS) to change this channel to the sensitive type by changing each site individually resulting in EEIS and EETD and a double mutation EEID. Each single change resulted in approximately a 10-fold increase in sensitivity, but the sensitive double mutant exhibited a remarkable 400-fold increase in TTX-sensitivity. This work is a clear demonstration that single amino acid changes in different channel domains can interact non-additively to affect TTX-resistance.

Costs of TTX-resistance

A correlated amino acid substitution could be favored if it helped compensate for negative shifts in biophysical properties caused by large-effect TTX-resistant substitutions. Resistant substitutions, though adaptive, can have detrimental pleiotropic effects on other channel properties. As discussed above, ionic conductance and permeation can be affected by charge-changing or neutralizing amino acid substitution near the selectivity filter. However, proper channel function relies closely on gating properties as well. In addition to controlling ion flow, amino acid substitutions in the pore region of the channel can affect channel gating, including the voltage-dependence of activation and slow inactivation (Hilber et al., 2005; Lee, Jones, Ahern, Sarhan, & Ruben, 2011; Vilin & Ruben, 2001; Xiong et al., 2006)

Lee et al. (2011) used electrophysiological recordings of the mammalian skeletal muscle channel $Na_V1.4$ expressed in frog oocytes to examine the possible functional consequences of pore TTX-resistant snake substitutions. They measured biophysical properties of $Na_V1.4$ containing DIV pore loop substitutions seen in resistant *T. sirtalis* from populations of low and high-resistance. Their study showed that some of these substitutions could significantly shift the voltage-dependence of slow inactivation. To explain these findings the authors postulated that changes in the pore might result in a channel structure that hinders voltage sensor movement and therefore shifts the voltage threshold at which the channel undergoes slow inactivation.

Disturbances of voltage-dependence of slow inactivation generate channel hyperexcitability that can result in a slower rate of current decay or an increase in window-current amplitude (Bendahhou, Cummins, Kula, Fu, & Ptácek, 2002; Bennett & Wood, 2014; Cannon, 2000, 2015). Substitutions at I1561 in garter snakes result in a depolarized shift in the voltage-dependence of slow inactivation of a magnitude comparable to shifts in the same biophysical property associated with human channelopathies (Jurkat-Rott, Holzherr, Fauler, & Lehmann-Horn, 2010; Vicart, Sternberg, Fontaine, & Meola, 2005). Newts also possess substitutions at this position S1424, but their effects have not been quantified. Although there is no direct evidence linking the TTX-resistant $Na_V1.4$ mutations that shift biophysical properties to a detrimental whole-body phenotype, there is some evidence that suggests the evolution of TTX-resistance in garter snakes could involve physiological tradeoffs. Brodie & Brodie (1999) compared crawl speeds of non-resistant and resistant garter snakes and found that resistant snakes crawled more slowly than non-resistant snakes. This pattern was true at

the individual, family and population levels, suggesting that, as with human muscle channelopathies, changes in the snake $Na_V1.4$ channels could result in deleterious whole-body phenotypes.

In wild populations, mutations to $Na_V1.4$ channels that cause deleterious or sub-optimal phenotypes would normally be selected out of the population. However, if these normally "deleterious" mutations confer an adaptive advantage in the ecological context of a predator-prey interaction, they could rise to fixation despite the negative effects. Whole-body effects of Na_V channels with altered biophysical properties could explain why resistant snakes crawl slower. Snakes that crawl more slowly may be at higher risk of predation and less apt to capture prey. However, the fitness benefits of resistance may outweigh the physiological disadvantages in snake populations under high selective pressures to survive encounters with highly toxic newts. The fact that pore loop mutations spread through the population despite possible cost to channel function indicates that functional tradeoff may be unavoidable and that there are very few changes that would decrease TTX-binding while minimally upsetting important channel biophysical properties.

Compensatory changes associated with TTX-resistance

As correlated negative effects may be an inescapable consequence of acquiring amino acid substitutions that lower TTX-binding, these may be more likely to co-occur with other changes that ameliorate or compensate for the negative shifts in biophysical properties. Although such compensatory substitutions are not necessarily restricted to the channel pore, they could arise in the pore loops as changes in this region of the channel have been shown to affect channel properties. The acquisition of compensatory amino acid substitutions after

adaptive ones is a very common phenomenon in the evolution of drug resistance in microbes. Although harder to study in larger organisms, the fine-tuning of adaptations through the evolution of compensatory mechanism is believed to be as important in the evolution of phenotypes in eukaryotes (Pavlicev & Wagner, 2012).

The skeletal muscle channels of resistant newts exhibit the D1431S substitution, which, along with the analogous D1568N in snakes, removes a negative charge that interacts with TTX. Elimination of this charge decreases but does not eliminate ionic conductance (Terlau et al., 1991). In toxic newts, the residue immediately downstream from the S1431 is changed to an Asp from the ancestral Gly. The G1432D change could be compensatory as it replaces the lost negative charge at the upstream positions 1431, which could restore reduced conductance (Figure 1, 2). Interestingly, the same pair of substitutions evolved independently in the unrelated snake *Liophis epinephelus* as well as multiple invertebrate taxa, suggesting this combination of substitutions may effectively confer TTX-resistance while reducing loss of ionic conductance.

In the evolution of TTX resistant Na_V channels, changes that ameliorate negative effects of highly resistant substitutions can either be favored after the acquisition of pleiotropic substitutions or they may constitute functional prerequisites predating the acquisition of highly resistant but functionally costly substitutions. Amino acid substitutions could predispose the acquisition of other (TTX-resistant) substitutions if these initial changes either confer limited resistance that allows the organism to survive exposure to low levels of TTX or reduce the level of functional/structural barriers constraining the acquisition of resistance substitutions.

Coincidental TTX-resistance

Substitutions in the pore region of Na_V proteins can have pleiotropic effects that alter both fundamental biophysical properties and TTX-binding. Some of the channel types that are functionally "TTX-resistant" appear to have evolved that resistance in the absence of, or prior to, exposure to TTX in the environment. Pore loop substitutions could be adaptive if they modify channel biophysical properties to modulate channel function in other adaptive ways. Na_V paralogs and orthologs differ in biophysical properties as a result of functional specialization in different tissues. The $Na_V1.5$ channel, for example, is expressed in the mammalian cardiac muscle. Its specific role in controlling excitability of cardiac myocytes suggests it has been selected for unique biophysical properties that enhance performance within the cardiac tissue. In TTX-sensitive channels, an aromatic residue (Tyr or Phe) at pore loop in DI forms important chemical interactions with TTX. This interaction is negated in $Na_V1.5$ due to the presence of a non-aromatic Cys at this position, therefore rendering the channel TTX resistant (Figure 4; Satin et al., 1992). The presence of a Cys instead of an aromatic residue in mammalian $Na_V1.5$ DI pore loop probably serves a group-specific adaptive role. Because pore loop changes have the potential to make the channel TTX-insensitive by changing the charge or the availability of TTX contact sites, it is possible that TTX-resistance was acquired as the corollary during the acquisition of a pore loop substitution in $Na_V1.5$ that was favored because it changed channel properties in a way that was adaptive within the cardiac muscle. A similar argument might hold for the mammalian nerve channels $Na_V1.8$ and $Na_V1.9$ that are TTX-resistant due the presence of a non-aromatic

residue (Ser) in the same DI pore loop position (Figure 4; Leffler, Herzog, Dib-Hajj, Waxman, & Cummins, 2005).

The presence of TTX resistant channels in taxa that are not exposed to TTX presents an intriguing question. Comparisons with other vertebrate channels suggest that TTX resistant forms of $Na_V1.5$, $Na_V1.8$, and $Na_V1.9$ evolved independently in mammals (and from each other). However, there is no evidence that ecological exposure to TTX played any role in this process and it is generally assumed that TTX-resistance in these channels is a byproduct or secondary artifact associated with selection that favored substitutions associated with changes in other biophysical properties of these channels. Furthermore, these channels show strong patterns of convergence; changes associated with resistance in these channels all occur in one specific amino acid position in the DI pore. Changes in DI pore loop that result in TTX-resistant channels may be the result of selection to modulate function, whereas pore loop changes in DIII and IV may be associated with evolution to withstand TTX. This pattern of protein evolution could reveal important details about the inherent functional constraints in the evolution of the Na_V protein family.

Evolutionary predisposition for TTX-resistance

The "accidental" acquisition of TTX-resistance by means of Na_V amino acid substitutions that adaptively modulate channel biophysical properties could function as a pre-adaptation (or "exaptation") that allows organisms to exploit environments or resources where TTX is present. Mildly resistant organisms could then harbor TTX for defense or could exploit new TTX-containing dietary niches. In fact, it is possible that this type of exaptation was present in both snakes and newts that evolved TTX-resistance. In garter

snakes, two peripheral nerve channels ($Na_V1.6$ and $Na_V1.7$) possess pore loop mutations previously associated with TTX-resistance. These substitutions are shared among TTX-resistant and non-resistant garter snakes, suggesting that peripheral nerve TTX-resistance preceded ecological interactions with toxic *Taricha* (McGlothlin et al., 2014).

As in puffer fish, whole-body TTX-resistance may be the product of possessing several resistant Na_V paralogs. Resistant peripheral nerves may be crucial to organismal resistance in snakes. The peripheral nerve $Na_V1.6$ is found in nodes of Ranvier in mammals, where it is involved in AP propagation (Caldwell, Schaller, Lasher, Peles, & Levinson, 2000). In snakes, which lack a diaphragm, axial muscles innervated by peripheral nerves regulate expansion of the lungs. Possible disruption of the signal to these muscles due to TTX blockage of $Na_V1.6$ could result in death by asphyxiation in these reptiles (McGlothlin et al., 2014). $Na_V1.7$ is expressed in sympathetic peripheral nerves that regulate vasodilation. One detrimental symptom of TTX intoxication is severe hypotension (Zimmer, 2010), possibly as a result of Na_V channel blockage in sympathetic nerves (Dib-Hajj, Yang, Black, & Waxman, 2013). Due to the severe detrimental effect of TTX in these tissues, resistance of $Na_V1.6$ and $Na_V1.7$ could be a requirement for exposed snakes.

Similarly, TTX-resistance precedes the origin of the TTX-toxic clade of newts. Moderate skeletal muscle resistance was identified in basal salamandrid lineages that lack TTX. Sequence analysis of the channel protein showed that this moderate resistance results from substitutions in pore loops of DIV (Figure 3B). In the non-toxic *S. salamandra* the Ile1424 residue is a Thr and in non-toxic *T. shanjing*, which is evolutionarily intermediate to *S. salamandra* and the modern newts, this position is a Ser, but these outgroup genera lack

other substitutions in DIII or DIV. Skeletal muscle resistance in modern newts is an order of magnitude higher than in any snake measured (Hanifin & Gilly, 2015). It is not clear why ancestral salamanders possess pore loop substitutions in $Na_V1.4$, but it is possible they also constitute an exaptation resulting from group-specific adaptive modifications of channel properties.

Tetrodotoxin-resistant Na^+ currents have been identified in several invertebrate Na_V1 channels (Du et al., 2009). It is possible that some of these animals are exposed to TTX or STX (saxitoxin, a marine toxin chemically similar to TTX) since these toxins are prevalent in environments where some of these animals occur (Zimmer & Ferrer, 2007). However, in many cases there is no definitive evidence of exposure to these toxins. In these cases, the evolution of biophysical functions could have led to coincidentally TTX-resistant forms. For example, Na^+ currents in the motor neurons of jellyfishes differ from the currents in the excitable cell of higher metazoan in that they fire fast transient overshooting APs. This results in rapid contraction of the myothelium of the medusa wall, which is a crucial adaptation for fast escape swimming. In the jellyfishes *Polyorchis penicillatus*, *Cyanea capillata* and *Aglantha digitale*, voltage-clamping of these motor neurons showed that these fast currents, though dependent on Na^+, are resistant to TTX (Meech & Mackie, 1993; Spafford, Grigoriev, & Spencer, 1996; Anderson, 1987). It is possible that pore substitutions in areas of the jellyfish channel affecting TTX-binding were favored as part of evolution to modify a swimming behavior and not because jellyfishes were evolving to the presence of TTX.

Conclusion

The deep and specific convergence of TTX-resistant Na_v channels points to a confluence of molecular evolutionary phenomena including protein sequence conservation, functional constraints, epistasis, and pleiotropy. This combination of factors has resulted in highly predictable molecular routes to adaptation across deeply divided lineages. In each of the resistant taxa, TTX-resistance involves amino acid changes in the pore loops of Na_v that reduce the binding affinity of the toxin by either changing the charge of specific residues that interact with TTX or by altering the availability of toxin contact points in the channel pore. These mutations are remarkably repeatable and often occur in predictable combinations suggesting limits to the permissible structural variation at the pore region accompanied by the existence of sites that interact epistatically. This bias may limit the number of possible mutational paths to a few that are frequently seen in TTX-resistant Na_v channels across widely separated lineages.

Adaptive evolution has produced remarkable patterns of convergent evolution in the Na_V channels of predators and prey. Interpreting observed patterns of substitutions suggests that multiple overlapping mechanisms likely explain this convergence at the molecular level. Tetrodotoxin interacts with a very specific region of Na_V channels and, as a result, only a small number of substitutions in these channels can increase TTX resistance of the channel. Cost associated with function may also play a role in driving convergence in these lineages. Substitutions in the outer pore can negatively impact important biophysical properties of the channel and a subset of substitutions observed in newts and snakes seem to maximize increases in TTX resistance while simultaneously minimized this negative effect. However, there is an additional subset of convergent changes whose effects are unclear. These changes

do not occur at positions that interact with TTX but appear to be correlated with substitutions at other sites that do interact with TTX. One possibility is that epistatic interactions between these residues and other substitutions may be driving their convergent patterns. Finally, convergence between newts and snakes suggests the possibility that exaptation and pleiotropy may play an important role in the evolution of TTX resistance.

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Figures

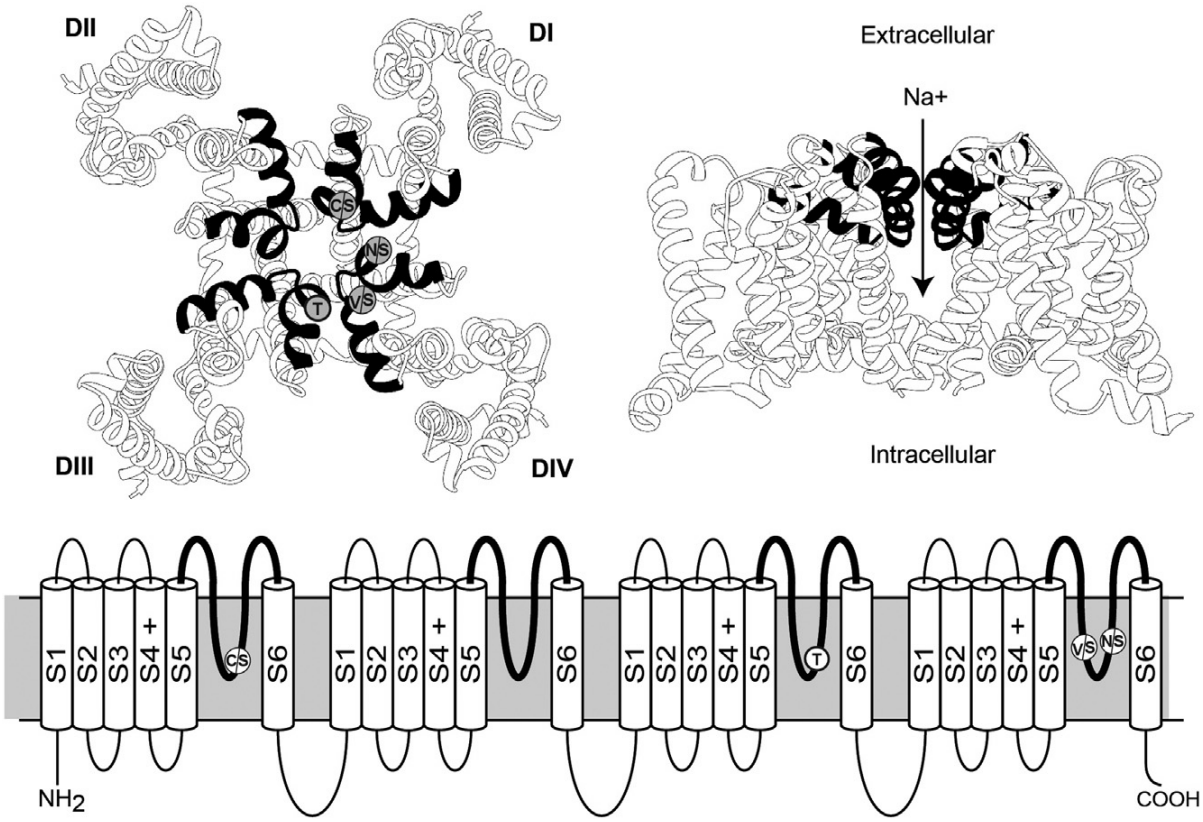

Figure 1. Position of TTX-resistant amino acid sites and substitutions in the pore loops of the voltage-gated sodium channel. The Cys in domain I is present in the mammalian cardiac muscle channel $Na_V1.5$, while a Ser is found in the same position in mammalian neuronal channels $Na_V1.8$ and $Na_V1.9$. In this position, non-aromatic residues (Cys or Ser) decrease TTX-binding by several orders of magnitude compared to channels with an aromatic residue (Tyr or Phe). The Thr substitution in domain III can reduce TTX-binding affinity in $Na_V1.4$ channels by approximately 15-fold. This substitution is found in the garter snake *Thamnophis couchii*, in all TTX-bearing newts, and in some puffer fish channels. In domain IV, the Val

substitution is common to all resistant *Thamnophis sirtalis*. This substitution reduces TTX-binding affinity by approximately two-fold and all toxic newts present a Ser in the same position. Also in domain IV, the Asn substitution is associated with high levels of organismal resistance in garter snakes and can increase channel TTX resistance 30–40-fold; toxic newts exhibit a Ser at the same position. Channel protein model based on the crystal structure of the bacterial sodium channel and Tikhonov and Zhorov (2012). Figure modified from Hanifin and Gilly (2015).

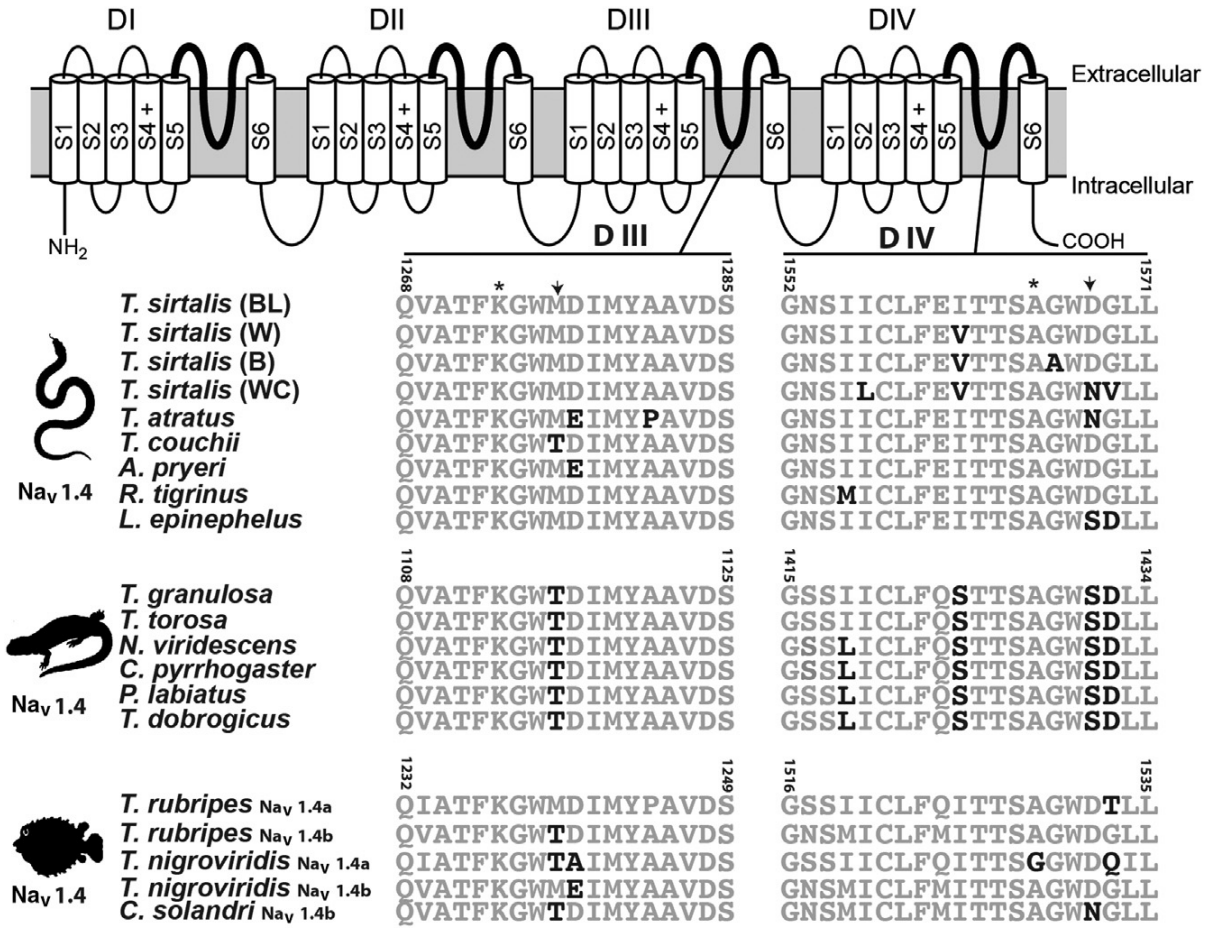

Figure 2. Sequence alignments of skeletal muscle voltage-gated sodium channel pore loops of domains III and IV showing pattern of convergent amino acid substitutions between TTX-resistant predators (snakes) and TTX-bearing prey (newts and puffer fish). The positions of several of these substitutions in the pore loops are shared among the different lineages and in some cases the changes are the same. The predators are represented by snakes known to consume TTX-bearing amphibians including *Thamnophis sirtalis*, *Thamnophis atratus*, *Thamnophis couchii*, *Amphiesma pryeri*, *Rhabdophis tigrinus*, and *Liophis epinephelus*, with the exception of *T. sirtalis* from Bear Lake, ID (BL) that does not eat toxic newts and it

possesses a TTX-sensitive channel. Within *T. sirtalis*, pore loop substitutions have evolved independently in different populations: W, Warrenton, OR; B, Benton County, OR; WC, Willow Creek, CA. The salamanders are represented by *Taricha granulosa*, *Taricha torosa*, *Notophthalmus viridensces*, *Cynops pyrrhogaster*, *Pachytriton labiatus* and *Triturus dobrogicus*. The puffer fish species are *Takifugu rubripes*, *Tetraodon nigroviridis* and *Canthigaster solandri*. Amino acid substitutions are shown in black; the positions of selectivity filter residues for DIII (K, lysine) and DIV (A, alanine) are marked with an asterisk, and the positions of the outer charged ring residues for DIII (M, methionine) and DIV (D, aspartic acid) are marked with an arrowhead. Amino acid positions for snakes, newts, and puffer fish follow those used in (Feldman et al., 2012) , (Hanifin & Gilly, 2015) and (Jost et al., 2008) respectively. Sequences from snakes, newts and puffer fish were obtained from (Geffeney et al., 2005) and (Feldman et al., 2012), (Hanifin & Gilly, 2015), and (Jost et al., 2008) respectively.

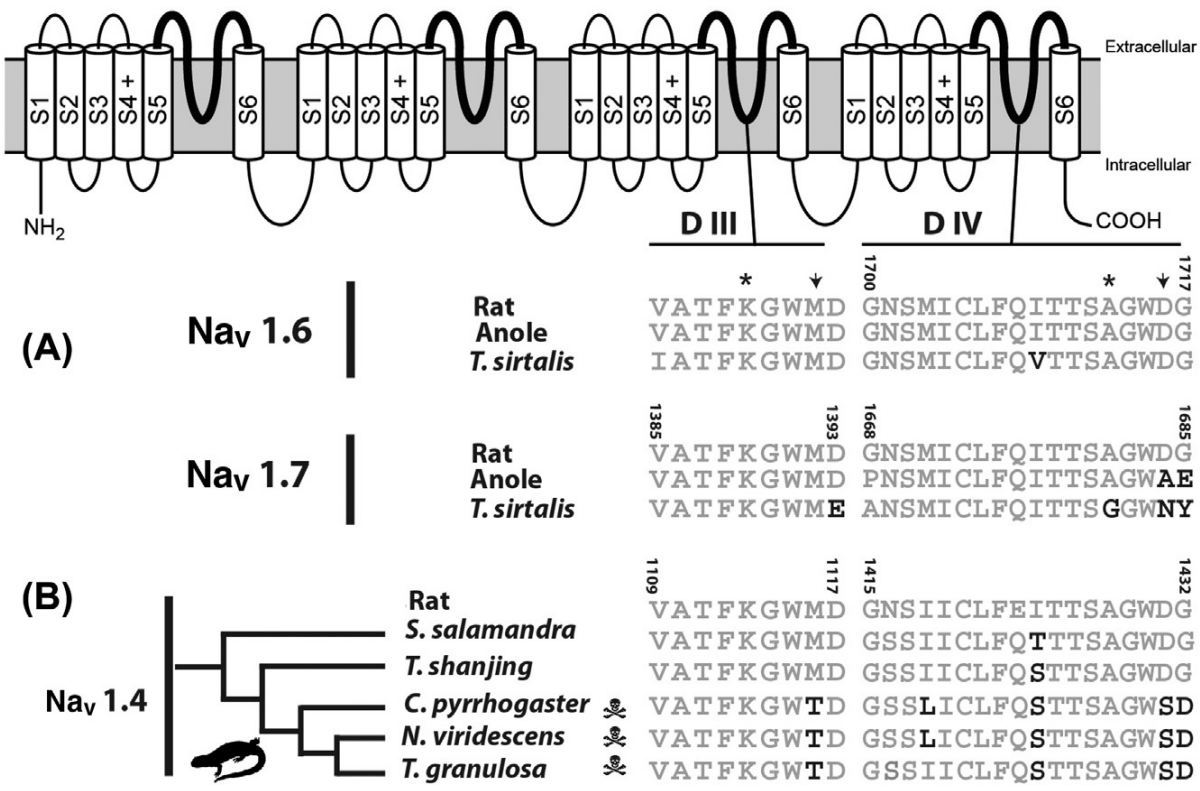

Figure 3.Sequence alignments of voltage-gated sodium channel pore loops of domains III and IV showing amino acid substitutions (black) in sites known to influence TTX-binding. **A.** Garter snake peripheral nerve channels $Na_V1.6$ and $Na_V1.7$ present substitutions previously associated with increased TTX-resistance. These substitutions are lacking in the sensitive mammalian channels, but the anole $Na_V1.7$ channel also presents substitutions suggesting channel changes in $Na_V1.7$ could have appeared ancestrally in reptiles. Snake sequences and amino acid positions taken from (McGlothlin et al., 2014). Mammalian sequences obtained from GenBank: $Na_V1.6$ (rat AF049240.1, anole NC_014777.1) $Na_V1.7$ (rat AF000368.1), anole H9G4U9 obtained from UniProt. **B.** Phylogenetic distribution of

amino acid substitutions in pore loops of salamander and newt skeletal muscle channel $Na_V1.4$ in domains III and IV. Substitutions M1116T, D1431S, and G1432D are present in toxic TTX-bearing species (skull and crossbones). These three substitutions are associated with extreme increases in the TTX resistance in modern newts while substitutions Thr and Ser at I1424 are associated with moderate increases in resistance in non-toxic species suggesting moderate resistance may be the ancestral state of this group possibly predating evolution of toxicity. Salamander and newt species are *Salamandra salamandra, Tylototriton shanjing, Cynops pyrrhogaster, Notophthalmus viridensces and Taricha granulosa.* The TTX-sensitive rat sequence is presented for comparison. Salamander sequences, phylogenetic tree, and amino acid positions correspond to (Hanifin & Gilly, 2015). The positions of selectivity filter residues for DIII (K, lysine) and DIV (A, alanine) are marked with an asterisk; and the positions of the outer charged ring residues for DIII (M, methionine) and DIV (D, aspartic acid) are marked with an arrowhead.

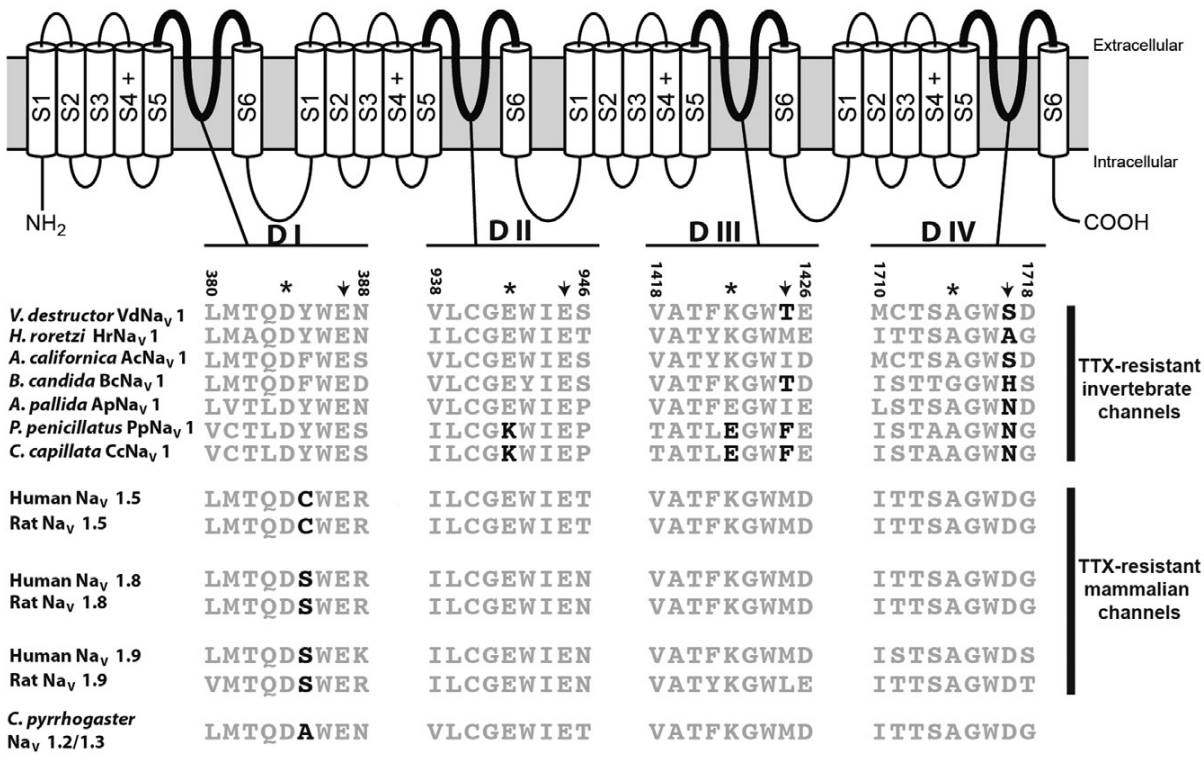

Figure 4. Sequence alignments of voltage-gated sodium channel pore loops of domains I-IV showing amino acid substitutions (black) in sites known to influence TTX-binding in invertebrate and mammalian channels. With the exception of flatworms (Salvitti, Wood, Taylor, McNabb, & Cary, 2015), there is no clear evidence that these species are exposed to TTX suggesting the acquisition of these substitutions may respond to selective pressures to change channel biophysical properties. Substitutions in invertebrate channels constitute deviations from the selectivity filter DEKA motif and the outer charged ring EEMD motif. Invertebrate sequences include the mite *Varroa destructor* (AY259834), the tunicate *Halocynthia roretzi* (D17311), sea hare *Aplysia californica* (U66915), flatworm *Bdelloura*

candida (U93074), sea anemone *Aiptasia pallida* (AF041851), hydrozoan jellyfish *Polyorchis penicillatus* (AF047380), and scyphozoan jellyfish *Cyanea capillata* (L15445) with amino acid positions corresponding to rat $Na_V1.2$ (X03639) as reviewed in Du et al. (2009). TTX-resistant mammalian cardiac muscle channel $Na_V1.5$ (human BC051374.1, rat AF353637.1), and neuronal channels $Na_V1.8$ (human AC116038, rat AJ623271.1) and $Na_V1.9$ (human AY686224.1, rat AF059030.2) show DI residues Cys and Ser in the same positions as the aromatic residue Tyr present in other paralogs. Non-aromatic residues (Cys or Ser) in this position decrease TTX-binding by several orders of magnitude. Interestingly, the amphibian neuronal channel thought to be the ancestor of $Na_v1.2$ and $Na_v1.3$ (*Cynops pyrrhogaster*, AF123593.1) presents a non-aromatic residue Ala in DI suggesting this channel is TTX-resistant. The DEKA motif residues for all domains are marked with asterisks; and the EEMD motif residues are marked with arrowheads.

Chapter Two:

Amino acid substitutions in peripheral nerve sodium channel $Na_V1.7$ play a role in the evolution of tetrodotoxin resistance in garter snakes[2]

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Abstract

Genetic history can either limit or foster future evolution. In instances of exaptation, ancestral states can potentiate evolution as pre-existing traits are co-opted to allow species to adapt to novel environmental challenges. Although the evolutionary importance of exaptations is well recognized, there is a dearth of studies that examine the role of exaptation during the process of adaptation. Here we investigate a case of possible exaptation during the evolution of tetrodotoxin (TTX) resistance in garter snakes (*Thamnophis sirtalis*) adapted to preying on tetrodotoxic newts. Tetrodotoxin binds to and blocks voltage-gated sodium channels (Na_V). Garter snakes possess amino acid replacements in their peripheral nerve channels ($tsNa_V1.7$) that are thought to reduce binding affinity of the toxin. The substitutions in $tsNa_V1.7$ evolutionarily predate snake contact with TTX-bearing newts, suggesting these changes were acquired in response to selective pressures unrelated to predation of toxic newts. Changes in this area of the channel protein are known to influence channel function. Because these substitutions are fixed in snakes, we hypothesize instead that $tsNa_V1.7$ substitutions evolved because they modified channel biophysical properties to modulate excitability of tissues expressing $tsNa_V1.7$. To explore this, we synthesized a $tsNa_V1.7$ channel for expression in *Xenopus* oocytes and measured TTX resistance and channel biophysical properties. We confirmed that $tsNa_V1.7$ is highly TTX-resistant compared to the known TTX-sensitive rat $Na_V1.7$ ($rNa_V1.7$). We also found differences in channel biophysical properties between $tsNa_V1.7$ and $rNa_V1.7$, which could indicate that channel substitutions affecting resistance in $tsNa_V1.7$ accumulated because they modified channel function in the ancestor of modern snakes. The channel $Na_V1.7$ is expressed in nervous tissue

of the snake vomeronasal organ, which is important for mate and prey detection. One possible explanation for the functional evolution in snake $Na_v1.7$ is that substitutions in $tsNa_V1.7$ served an adaptive evolutionary role modifying firing thresholds in the neurons found in the vomeronasal organ. In garter snakes, TTX resistance afforded by resistant $tsNa_V1.7$ could be an exaptation if physiological resistance developed as a by-product of such adaptations of channel function.

Introduction

Organisms adapt to their environment through systematic changes in their genomes powered by the forces of natural selection, which acts on available heritable variation and on random changes that arise on well-established genetic backgrounds. The fact that every novel trait evolves within the confines of already-existing genomes has been recognized as a major source of constraint as every new change has the potential to form negative interactions with pre-adapted genetic complexes (Bloom 2010; Shah et al. 2015; Stern and Orgogozo 2009; Weinreich et al. 2006). In some cases, however, genetic history can serve to foster new evolutionary outcomes because ancient modifications allow organisms to adapt to novel environmental challenges (Blount et al. 2008; Kryazhimskiy et al. 2014). Traits that were previously molded by natural selection for a particular function and are later co-opted into new functions are examples of how past changes can potentiate future adaptations. Such traits are sometimes referred to as exaptations or pre-adaptations (Gould 1982). Exaptations can be a driving force of evolution since it has been postulated that historical contingency is able to reach adaptive innovation faster than gradual and cumulative selection (Blount et al. 2008).

Evolutionary instances of exaptive traits abound, including classical anatomical examples such as feathers in reptiles, which may have originally evolved for functions unrelated to flight such as thermoregulation, but were later co-opted for flight (Lewin 1982). However, many examples involve protein evolution, especially during the evolution of metabolism since many enzymes and hormones appear to have evolved via functional co-option (Barve and Wagner 2013; Szappanos et al. 2016; Vianello et al. 2012). Although

instances of exaptations can be found from the macroscopic to the molecular scale there are few studied examples of molecular exaptations with a clear molecular change and a developed functional test of those changes. Here, we combine gene synthesis and electrophysiology to explore the hypothesis that evolution of toxin resistance in garter snakes may constitute an exaptation for function in a snake ionic channel protein.

Some garter snake *(Thamnophis sirtalis*) populations have entered an evolutionary arms race with sympatric tetrodotoxin-bearing newts (*Taricha granulosa*) in which reciprocal selection provided the pressure leading to the exaggeration of defensive (toxicity in newts) and predatory (toxin resistance in snakes) traits (Brodie and Brodie 1990; Hanifin et al. 2008). Although the source of tetrodotoxin (TTX) production in *Taricha* remains elusive, it is recognized that populations of newts have increased in toxicity by increasing the concentration of TTX in their tissues (Hanifin el al. 2008). Non-snake predators of *Taricha* including birds and mammals have the advantage of using appendages such as limbs or beaks to separate the organs that contain the most TTX as a behavioral strategy to avoid intoxication (Stokes et al. 2011). Snakes, on the other hand, are limited by having to swallow their prey whole and therefore are exposed to a full dose of the toxin (Williams et al. 2010). As a result, garter snakes that prey on toxic *Taricha* were pushed to adapt physiologically to resist increasing levels of newt toxicity over thousands of years (Toledo et al. 2016; Brodie and Brodie 2015; Wood and Baker 2001).

Tetrodotoxin is a potent neurotoxin that binds to voltage-gated sodium channels (Na_V) (Hille 2001; Narahashi 2008). Voltage-gated sodium channels regulate the cellular entry of Na^+ necessary for the formation of action potentials (AP) in excitable membranes

(Wood and Baker 2001; Yu and Catterall 2003; Hille 2001). Tetrodotoxin blocks Na_V channels by binding to the channel outer pore preventing Na^+ inflow. The effect of this blockade of Na^+ currents precludes formation of AP in native tissues. Voltage-gated sodium channels belong to a protein family that expanded through multiple events of duplication in vertebrates (Yu and Catterall 2003). In snakes, Na_V channels are represented by nine paralogs that are expressed in different neuronal and muscle tissue types (McGlothlin et al. 2014).

In mild cases of intoxication, TTX is believed to affect mostly the Na_V channels expressed in peripheral nerves (McGlothlin et al. 2014). However, in severe cases of intoxication, TTX reaches and blocks sodium channels of skeletal muscles, resulting in paralysis that could be lethal (Isbister and Kiernan 2005; Noguchi and Arakawa 2008). Evolved resistance is afforded by the acquisition of amino acid substitutions that decrease the binding affinity of TTX (Brodie and Brodie 2015; Feldman et al. 2012; Hanifin et al. 2008; Jost et al. 2008; Toledo et al. 2016). These substitutions occur at the channel pore loops, which are the structures that form the outer-pore of the channel and therefore come into contact with the TTX molecule (Chong and Ruben 2008; Payandeh et al. 2012).

The best studied examples of Na_V pore loop substitutions conferring resistance in garter snakes involve populations found in the most advanced stages of the arms race in which snakes have adapted to survive large concentrations of TTX (consuming highly toxic newts). These snakes show substitutions in their skeletal muscle channels ($Na_V1.4$) that protect them from the paralytic effects of high-doses of TTX (Feldman et al. 2010; Geffeney et al. 2002; Geffeney et al. 2005). However, recent data point to the possibility that other sodium channel paralogs in garter snakes played a role, especially during the earlier stages of

the arms race when garter snakes were exposed to lower TTX concentrations (i.e., before the newts evolved extreme levels of toxicity) (McGlothlin et al. 2014). Of special interest is the finding that a garter snake peripheral nerve channel (tsNa$_V$1.7) possesses several pore loop substitutions, one of which (D1684N) has been associated with high resistance when studied in mammalian Na$_V$ orthologs (Figure1) (Choudhary et al. 2003).

The pore loop replacements in tsNa$_V$1.7 are ancient. Substitutions in Na$_V$1.7 appeared around 170 mya (million years ago) in the common ancestor of snakes. The specific combination of substitutions seen in *T. sirtalis* arose around 61 mya while TTX-bearing newts did not appear in the fossil record until 44 mya (Roelants et al. 2007; Zhang et al. 2008). In addition, other amphibians are not thought to have possessed TTX during the time when the substitutions in Na$_V$1.7 of snakes were acquired (Hanifin 2010). The channel Na$_V$1.7 is expressed in small diameter peripheral nerves where it plays a role in the sensory symptoms associated with early signs of TTX intoxication in humans (Isbister and Kiernan 2005; McGlothlin et al. 2016). The fact that tsNa$_V$1.7 has pore loop substitutions that might confer resistance suggests that this channel could have been important in predisposing garter snakes to tolerate low levels of TTX when snakes first encountered toxic newts.

Substitutions similar to the ones present in tsNa$_V$1.7 have been found to affect biophysical properties of the channel such as the voltage-dependence of activation and inactivation (Lee et al. 2011; Wu et al. 2013). These properties describe states of channel activity in response to membrane depolarization. Substitutions that affect channel activation or inactivation could affect sensitivity of nerves expressing Na$_V$1.7 channels. Given the effect pore loop substitutions can have on channel biophysics, we hypothesized that 1) the original

role of substitutions found in tsNa$_V$1.7 was to modify channel biophysical properties and 2) these substitutions may constitute an exaptation by conferring ancestral TTX resistance to the channel. To investigate this, we compared the level of TTX sensitivity of tsNa$_V$1.7 to that of a known TTX-sensitive ortholog, rat Na$_V$1.7 (rNa$_V$1.7). In addition, we compared these two channels functionally by measuring the voltage-dependence of activation, fast inactivation and slow inactivation. These three channel properties describe channel excitably in relation to membrane voltage. Differences between these two channels in biophysical properties may be an indication that pore changes found in tsNa$_V$1.7 were selected because of their affects on channel function.

Methods

Channel synthesis and expression

Complimentary genomic sequences encoding the channel proteins for both tsNa$_V$1.7 and rNa$_V$1.7 were used to make messenger RNAs that were injected into *Xenopus laevis* oocytes (6-12 ng) for channel expression. The sequence encoding rNa$_V$1.7 was provided by Baldomero Olivera from the University of Utah (Fiedler et al. 2008). The tsNa$_V$1.7 sequence used was based on genomic sequences from tissue DNA extraction from a *T. sirtalis* specimen collected in Benton County, Oregon (McGlothlin et al. 2016). Channel tsNa$_V$1.7 was synthesized into a plasmid by the company DNA2.0 with optimized *Xenopus laevis* codon usage. The synthesized Na$_V$1.7 plasmid contained a SP6 promoter site as well as 5′ and 3′ UTRs from the *Xenopus* globin gene and a poly-A tail to aid in translation and expression. The plasmid flanking regions were derived from the pSP64T plasmid (Addgene).

We prepared mRNA from sequence verified PCR product provided by DNA2.0 because the plasmid containing ts$Na_V1.7$ could not be grown in standard plasmid preparations in our lab. RNA was made with the mMessage Machine Kits ULTRA system (Life Technologies) using standard reactions for T7 (r$Na_V1.7$) or SP6 (ts$Na_V1.7$) promoters. Oocytes were obtained from Ecocyte.

Current measurement and TTX assay

Sodium 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). The experiments were performed in an external solution containing 120 mM MES Na, 10 mM HEPES Na, and 1.8 mM CaCl2 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. To record and measure current we used the pClamp software (Molecular Devices), sampling at 100 kHz and filtering at 20 kHz. The holding potential for all experiments was -100 mV. Leak subtraction was performed with the use of a p/4 protocol.

To estimate TTX binding affinity of channel variants, we evoked peak currents at 0.05 Hz with 20-ms pulses to 0 mV following a 500-ms pre-pulse to -150 mV. The size of the current was measured offline with the software IgorPro (WaveMetrics). The ratios of peak currents in the presence and absence of TTX 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 changes of solution) over a range of TTX concentrations. We estimated TTX concentration that blocked 50% of the expressed channels

(K_d) by fitting the data to an equation derived from a single-site Langmuir adsorption isotherm, Current ratio= 1/ [1+ ((TTX)/ K_d)] where (TTX) is the concentration of toxin and K_d is the concentration of TTX at which half of the channels are blocked by the toxin. The K_d and its 95% confidence limits were estimated from the curve in IgorPro (WaveMetrics).

Channel Biophysical Properties

The voltage-dependence of activation was measured from the peak inward current during a 20 ms test pulse to voltages ranging from -100 to 80 mV in 10 mV steps following a 500-ms pre-pulse to -150 mV. The voltage-dependence of fast inactivation was measured from the peak inward current during a 20-ms pulse to 0 mV after a 500-ms, conditioning pre-pulse ranging from -150 to -10 mV in 10 mV increments. The voltage-dependence of steady-state slow inactivation was measured from the peak inward current during a 20 ms test pulse to 0 mV. The test pulse followed three conditioning pulses, the first a 30-s pulse to -130 mV to remove fast and slow inactivation, the second a 60-s pulse to voltages ranging from -130 to -10 mV (in 10 mV increments) and the third a 20-ms pulse to -150 mV to recover channels from fast inactivation. Peak current amplitudes were measured during test pulses offline with Igor Pro (Wavemetrics). Conductance-voltage relationships for the voltage-dependence of activation were derived using the following equation: GNa = Ipeak/(VM – ENa) where GNa represents sodium conductance, Ipeak is the peak-test-pulse current, VM is the test-pulse voltage, and ENa is the measured sodium equilibrium potential. Activation, fast inactivation and slow inactivation curves were fitted by a Boltzmann distribution with the following equation: Normalized conductance or current amplitude = $1/(1+ \exp (-z*e0(VM - V_{1/2})/kT))$ where z is the apparent valence, e0 is the elementary charge, $V_{1/2}$ is the midpoint voltage, k is

the Boltzmann constant, and T is the temperature in degrees Kelvin. $V_{1/2}$ and its 95% confidence limits were estimated from the curve using Igor Pro (Wavemetrics).

Results

TTX resistance

The channel tsNa$_V$1.7 exhibits a 900-fold increase in resistance (K_d =1.21 x 10e-5 M) relative to rNa$_V$1.7 (K_d =1.34 x 10e-8 M) (Figure 2). The level of resistance of tsNa$_V$1.7 is comparable to that exhibited by the most resistant *T. sirtalis* skeletal muscle channel form that has been observed (Figure 1, tsNav1.4 (Willow Creek))(Feldman et al. 2010; Geffeney et al. 2005; Hague et al. 2017).

Channel function

We found a depolarizing (right) shift in midpoint voltage of activation when comparing TTX-resistant tsNa$_V$1.7 ($V_{1/2}$ =-14.52 mV) to the TTX-sensitive rNav1.7 ($V_{1/2}$ =-21.21mV) (Figure 3). The fast inactivation curve, on the other hand, showed a hyperpolarizing shift (left) in midpoint voltage when comparing TTX-resistant tsNa$_V$1.7 ($V_{1/2}$ =-67.39 mV) to the TTX-sensitive rNa$_V$1.7 ($V_{1/2}$ = -63.03mV) (Figure 3). These data together represent a reduction in the level of overall excitability of the snake channel compared to the rat channel. The slow inactivation curves revealed a depolarizing shift in midpoint value when the snake channel ($V_{1/2}$ = -78.09 mV) is compared to the rat channel ($V_{1/2}$ =-93.08 mV) (Figure 4).

Discussion

The snake channel tsNa$_V$1.7 presents pore loop substitutions of an ancient origin in sites that could confer TTX resistance to garter snakes. It had been hypothesized that resistance in this channel may have played a role in the initial stages of the arms race between garter snakes and toxic newts. Our results confirm that tsNa$_V$1.7 is a highly TTX-resistant channel. In our trials, the concentration of TTX had to be increased 900-fold (relative to rat) in order to block elicited Na^+ currents in tsNa$_V$1.7. The fact that pore loop substitutions were present in tsNa$_V$1.7 before garter snakes encountered TTX-bearing newts suggests that these channel changes evolved to serve a function unrelated to toxin resistance. To explore this hypothesis, we compared the biophysical properties of tsNa$_V$1.7 to a TTX-insensitive channel ortholog that does not present pore loop substitutions to test for functional differences that may have been the original target of selection driving evolution in the ancestor of snakes. Our data revealed biophysical differences between tsNa$_V$1.7 and rNa$_V$1.7. These differences may be an indication that pore loop substitutions in tsNa$_V$1.7 were acquired in response to selection for functional evolution in the snake channel.

The notion that the acquisition of TTX-resistant substitutions in tsNa$_V$1.7 is the result of selection for channel function weighs on the fact that Na$_V$ pore loop substitutions can have pleiotropic effects. The pleiotropic nature of Na$_V$ pore substitutions has been demonstrated by several independent studies showing that replacements that increase TTX resistance can influence channel ionic conductance, voltage-dependence of slow inactivation and ionic selectivity (Feldman et al. 2012; Lee et al. 2011; Terlau et al. 1991). This pleiotropy stems from the fact that pore loops are involved in channel gating which is the result of the coordinated movement of several channel units in response to fluctuations in membrane

voltage (Catterall 2000, Hille 2001). Conformational rearrangements within the pore have long distance interactions with other channel structures that can influence gating, therefore substitutions that change the charge and shape of the pore loops could modify gating properties affecting channel function (Hilber et al. 2005; Lee et al. 2011; Vilin and Ruben 2001; Xiong et al. 2006).

We found differences in the biophysical properties measured between rNa_V1.7 and tsNa_V1.7 (Figures 3 and 4). There were shifts in the voltage-dependence of channel activation and inactivation; these properties describe channel activity. Substitutions that shift these measurements could affect the excitability of the tissues where Na_V channels are expressed. Past electrophysiology studies have found that some of the substitutions in tsNa_V1.7 are associated with changes in channel function when those changes are expressed in other Na_V paralogs. For example, the substitution D1684N produces a decrease in channel ionic conductance and it also shifts ionic selectivity (Choudhary et al. 2003). Also, the substitution A1681G has been found to increase channel conductance (Jost et al. 2008).

From studies in mammalian channels, we know that Na_V1.7 plays an important role in setting up firing thresholds for action potentials in small peripheral neurons such as pain receptors (nociceptors) and in olfactory receptors (Ahn et al. 2011; Dib-Hajj et al. 2012). Channel substitutions that change the voltage-dependence of activation, fast inactivation and slow inactivation can change channel excitability resulting in modulation of the firing threshold for pain signaling leading to chronic pain disorders in some cases (Eberhardt et al. 2014; Faber et al. 2012; Han et al. 2012; Huang et al. 2016). It has been shown that Na_V1.7 is

relevant in olfaction and chemical signaling in vertebrates (Ahn et al. 2011; Gingras et al. 2014; Rupasinghe et al. 2012).

Transcriptomic sequence from the vomeronasal organ of snakes showed that $Na_V1.7$ is the principal Na_V channel found in this tissue (McGlothlin et al. 2016). The vomeronasal organ or Jacobson's organ is a vital organ for chemosensation in terrestrial reptiles (Brennan and Keverne 2003). Chemosensation is one of the better developed ophidian senses; the snake tongue is used to gather moisture-borne particles, which stimulate the Jacobson's organ to collect information such as scent of prey and pheromones (Bertmar 1981). Snakes are reliant on this method of chemical recognition for survival and reproduction (Halper and Kubie 1983). It is possible that environmental demands to perfect prey or mate detection imposed selection on the sodium channel controlling action potential firing thresholds in the sensory neurons expressed in the Jacobson's organ ($Na_V1.7$) in the ancestor of snakes. Because some changes in ts$Na_V1.7$ date to the origin of snakes (McGlothlin et al. 2016), functional advantages conferred by replacements in this channel could impact all snakes.

Possessing TTX-insensitive $Na_V1.7$ channels could have prevented garter snakes from experiencing some of the sensory symptoms associated with exposure to the concentrations of TTX (pre-arms race) found in the ancestral *Taricha* newts. This advantage may have imposed predation-based selection on the newts resulting in an increase of defensive toxicity, which could have contributed to starting the arms race. Once newts reached sufficient levels of toxicity to exert selection on snake resistance, the high physiological resistance observed in modern resistant snake populations would have been

favored. This high resistance involved the subsequent acquisition of resistant substitutions in other Na_V paralogs such as the skeletal muscle channel $Na_V1.4$.

We postulate that these early changes in ts$Na_V1.7$ facilitated the evolution of extreme TTX resistance in other paralogs. However, it is possible that TTX-resistant substitutions in $Na_V1.7$ were acquired because they served an adaptive functional role. Amino acid changes in the pore sequence of the Na_V channels are believed to be deleterious unless they serve an adaptive role, given the level of sequence conservation exhibited in this area of the protein. The fact that pore substitutions seen in ts$Na_V1.7$ were ancestrally fixed and remain present in most snakes implies that they were acquired because they were adaptive in the ancestor of garter snakes. All of this may indicate that resistance in ts$Na_V1.7$ could be considered an exaptation on physiological resistance on peripheral nerves. Results from our work show that complex adaptations may be contingent on changes that originated in the distant evolutionary past. These findings support the idea that historic innovations can be a driver for future adaptations. In the case of garter snakes, ancient molecular changes may have opened adaptive pathways permitting the initiation of an evolutionary arms race with toxic prey.

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Figures

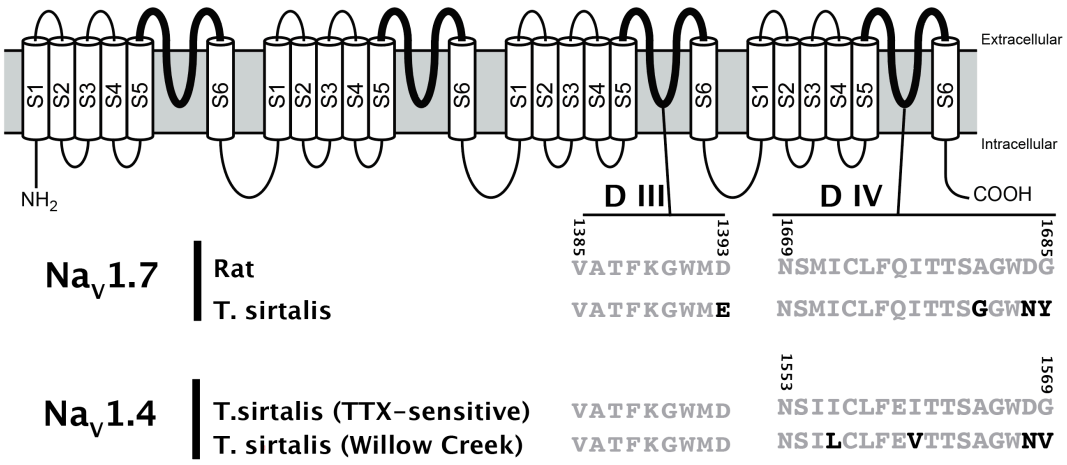

Figure 1. Schematic of a voltage-gated sodium channel protein showing four domains each consisting of six trans-membrane segments (S1-S6). Pore loops (bold lines) are alpha helices connecting segments S5 and S6. The channel tsNa$_V$1.7 presents substitutions in pore loops of domains III and IV. The highly TTX-resistant channel tsNa$_V$1.4 (Willow Creek) presents pore loop substitutions in domain IV compared to the sodium channel of TTX-sensitive snakes. The substitution D1684N is shared between tsNa$_V$1.4 (Willow Creek) and tsNa$_V$1.7. The sensitive *T.sistalis* sequences was obtained from a snake specimen collected at Bear Lake, Idaho and the TTX-resistant sequence was obtained from a specimen collected at Willow Creek, California. Snake sequences and amino acid positions taken from (McGlothlin et al. 2014); rat sequences were obtained from GenBank: (AF000368.1).

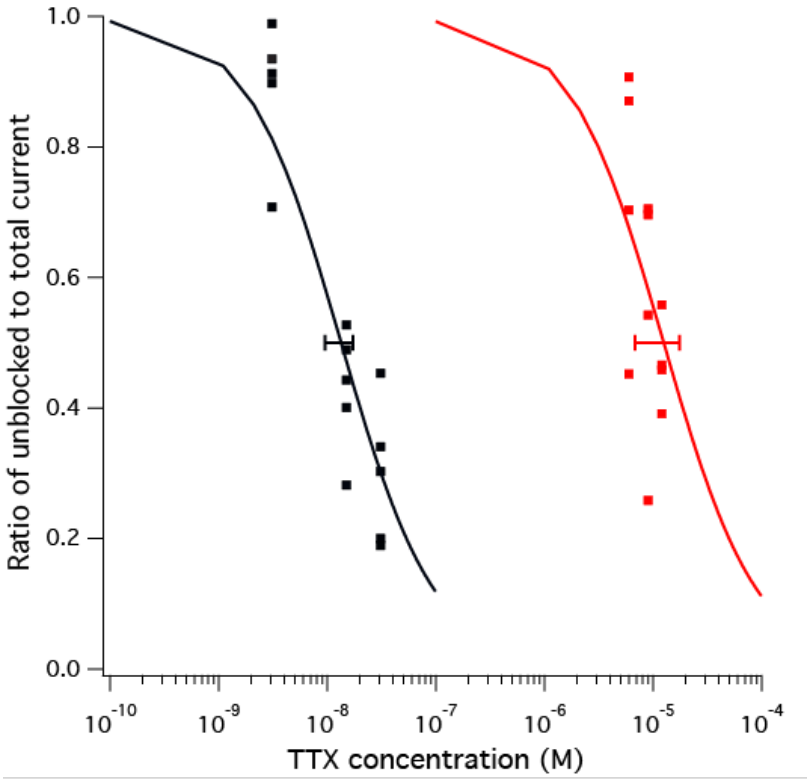

Figure 2. Ratio of unblocked to total current for oocytes expressing the indicated channel and exposed to a range of TTX concentrations. Sodium current of tsNa$_V$1.7 (red) is blocked at 900-fold higher TTX concentrations than rat Na$_V$1.7 (black). The TTX concentration that blocked 50% of the channels (K_d for each channel type was calculated from pooled channel data. K_d values (±95% confidence limits) are shown for each channel type with a bar. Rat Na$_V$1.7 (K_d ± 95% CI = 1.34 x 10e-8 M ± 3.9 x 10e-9 TTX, n=5), tsNav1.7 (K_d ±95% CI = 1.21 x 10e-5 M ± 5.3 x 10e-6 TTX, n=4). The lines represent the equation fitted to the data with the estimated K_d for each channel type.

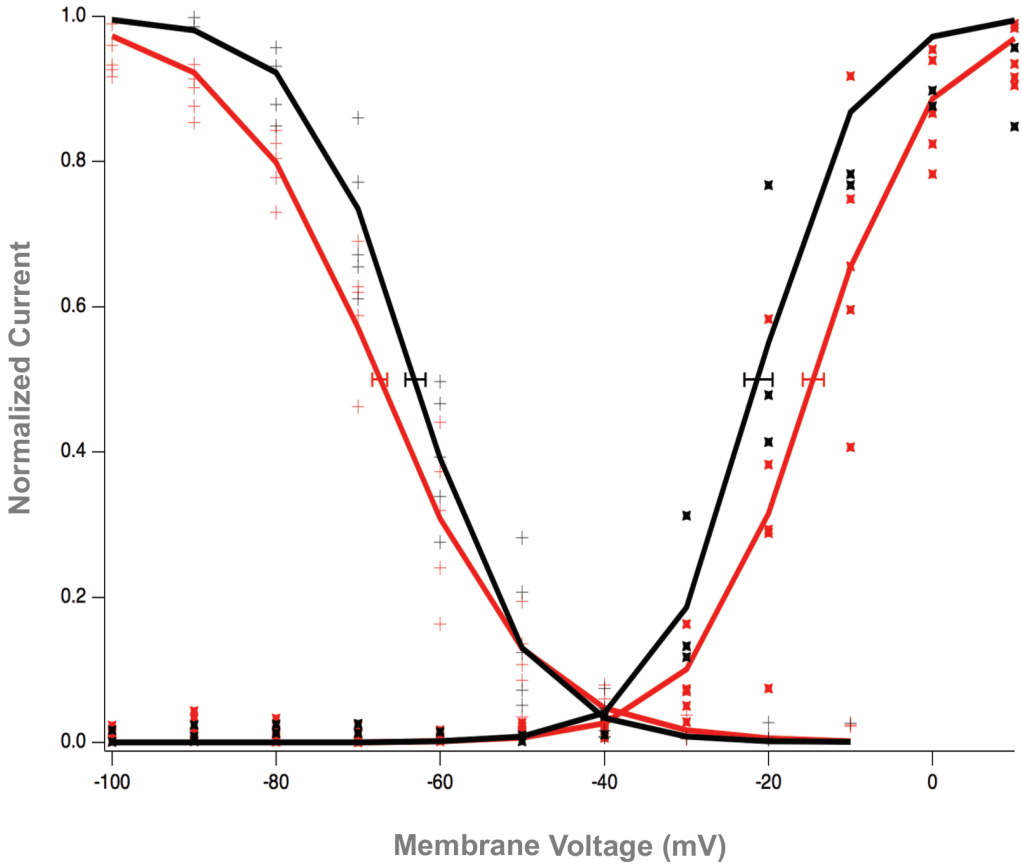

Figure 3. Activation and fast inactivation curves for tsNa$_V$1.7 (red) and rat Na$_V$1.7 (black). The currents for the activation data were converted to conductance (GNa), $V_{1/2}$ values (±95% confidence limits) are shown for each channel type with a bar. Rat Na$_V$1.7 activation ($V_{1/2}$ ± 95% CI = -21.21 ± 1.7 mV, n=6), tsNa$_V$1.7 activation ($V_{1/2}$ ± 95% CI = -14.52 ± 1.34 mV, n=5). Rat Na$_V$1.7 inactivation ($V_{1/2}$ ± 95% CI =-63.03 ± 1.2 mV, n= 5), tsNa$_V$1.7 inactivation ($V_{1/2}$ ± 95% CI = -67.39 ± 0.97 mV, n=5). The data are normalized to maximum peak current (for inactivation) or conductance (for activation) and fit according to the Boltzmann functions (see text for details).

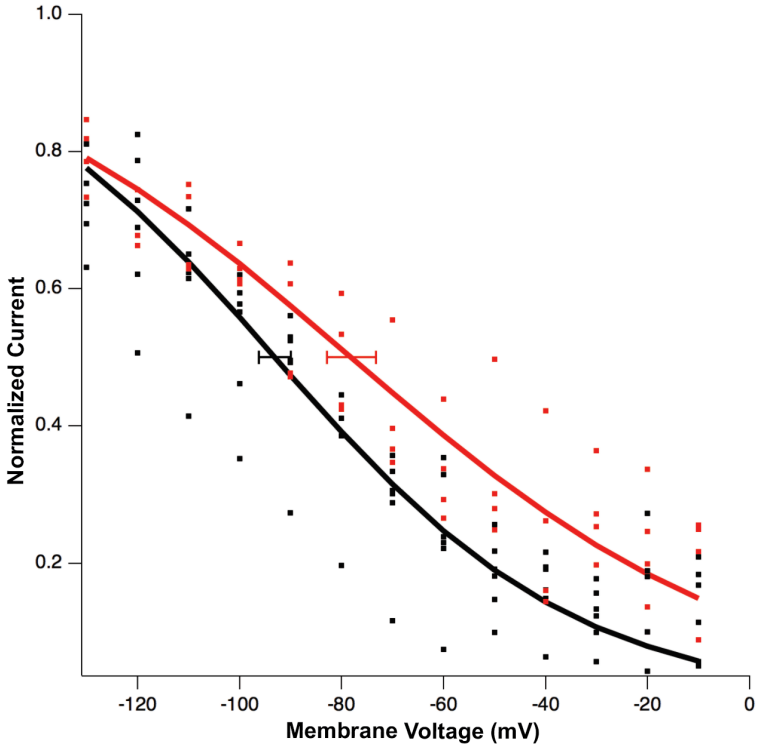

Figure 4. Steady-state slow inactivation of sodium channels. $V_{1/2}$ values (±95% confidence limits) are shown for each channel type with a bar. Rat $Na_V1.7$ ($V_{1/2}$ ± 95% CI =-93.08 ± 3.11 mV, n=6), ts$Na_V1.7$ ($V_{1/2}$ ± 95% CI = -78.09 ± 4.75 mV, n=4). The data are normalized to maximum peak current and fit according to a Boltzmann function (see text for details).

Chapter Three:

Functional constraints shape the evolution of tetrodotoxin-resistant voltage-gated sodium channels in garter snakes (*Thamnophis sirtalis*)

Abstract

A central goal of evolutionary genetics is to understand the roles of mutational pleiotropy and epistasis in shaping trajectories of protein evolution. Here we explore how these mutational forces affect the evolution of tetrodotoxin (TTX) resistance in the skeletal muscle voltage-gated sodium channel ($Na_V1.4$) of the common garter snake (*Thamnophis sirtalis*) by experimentally recreating one possible mutational trajectory from a TTX-sensitive channel to a TTX-resistant channel form. To do this, we synthesized snake $Na_V1.4$ channels via Gibson assembly and we investigated the stepwise accumulation of a number of resistance-related amino acid replacements. We tested one possible evolutionary trajectory containing five $Na_V1.4$ variants; two of the intermediate channel forms possessed hypothetical combinations of substitutions never observed in nature. We then expressed the synthesized channel proteins in *Xenopus* oocytes to measure TTX resistance and biophysical properties of the channels. We found that amino acid substitutions associated with TTX resistance caused significant shifts in channel biophysics. One hypothetical intermediate channel variant exhibited a functional profile that would be extremely disadvantageous for muscle physiology. These results suggest that the evolution of TTX resistance in *T. sirtalis* could be constrained by the functional costs of amino acid substitutions that confer resistance in this channel protein, and that precise combinations of substitutions are needed to get around these costs.

Introduction

Protein evolution may involve the serial acquisition of individual amino acid mutations. Each mutational step potentially represents a fitness improvement resulting in better-adapted proteins, but not all changes will have net benefits. Protein evolution is limited by genetic processes that reduce the number of accessible mutational pathways leading to optimal protein forms (Weinreich et al. 2005). Some of the main genetic phenomena that constrain evolution of proteins are pleiotropy and epistasis (Stern & Orgogozo 2009). Pleiotropy manifests when a single mutation affects multiple traits, and epistasis occurs when mutations have non-additive effects on phenotypes or fitness resulting from interactions with other sites. Pleiotropic effects of mutations can have genome-wide consequences, however, pleiotropy can arise from within proteins as mutations that change amino acid sites affect multiple aspects of a single protein's function and structure (Bloom et al. 2006; DePristo et al. 2005; Weinreich et al. 2006). Similarly, epistasis can influence protein evolution because the magnitude of pleiotropic effects of amino acid mutations taking place within proteins could be contingent on the genetic background in which mutations occur (Bridgham et al. 2009; DePristo et al. 2007; Tokuriki et al. 2008; Tokuriki and Tawfik 2009; Weinreich et al. 2006).

Experimental evaluation of intermediate evolutionary states allows us to explore the forces that influence the distribution of evolutionarily relevant amino acid changes in proteins (Dean and Thornton 2007; Thomson et al. 2005). Studying the function of protein intermediates can provide information on possible fitness consequences of varying amino acid sequences, which can reveal pleiotropy or negative epistatic interactions that constrain

the outcome of evolution (Tufts et al. 2015; Weinreich et al. 2006). Evolution of new functions in proteins usually requires the acquisition of multiple amino acid changing mutations, and there are multiple mutational pathways leading from the ancestral form of the protein to the derived form. By recreating these mutational pathways in the laboratory, it is possible to measure different aspects of protein function that might be affected by each mutational step. It is also possible to evaluate the likelihood of molecular routes based on estimates of how genotypes affect fitness along a given hypothetical trajectory (Poelwijk et al. 2007; Weinreich et al. 2005; Weinreich et al. 2006). Here we explore molecular constraint shaping the evolutionary trajectory of an ionic channel protein involved in the evolution of toxin resistance in garter snakes.

Some populations of the common garter snake (*Thamnophis sirtalis*) found in the Pacific Northwest of North America evolved to withstand consuming newts (*Taricha granulosa*) that contain high levels of the powerful neurotoxin tetrodotoxin (TTX) (Brodie and Brodie 1990; Hanifin et al. 2008). Tetrodotoxin is a small guanidinium molecule that binds to the pore of voltage-gated sodium channels (Na_V) and blocks Na^+ currents necessary for the generation of action potentials in muscle fibers and neurons (Hille 2001; Moczydlowski 2013; Narahashi 2008). During TTX intoxication, positive charges in the TTX molecule are attracted to negative charges in key residues in the channel pore resulting in the binding of TTX to the channel outer pore effectively clogging the channel by physically impeding the entry of Na^+. This elimination of inward Na^+ currents inhibits the signaling pathways necessary for muscle fiber contraction and therefore can lead to paralysis (Hille 2001; Narahashi 2008). Snakes evolved resistance to TTX through the acquisition of

amino acid replacements in their skeletal muscle sodium channels (tsNa_V1.4) (Brodie and Brodie 2015; Feldman et al. 2012; Hanifin et al. 2008; Toledo et al. 2016). These replacements are located in the channel pore loops, which are the helices that form the channel pore (Figure 1). These amino acid substitutions can eliminate TTX-attracting negative charges or decrease the accessibility of TTX to these negatively charged residues (Terlau et al. 1991; Tikhonov and Zhorov 2005, 2012).

The skeletal muscle sodium channel is a member of the Na_V protein family characterized by the presence of an α-subunit consisting of four homologous domains (D1-DIV) that are arranged in the membrane to form a passage that allows the entry of Na^+ (Figure 1)(Catterall 2000). The channel's high selectivity for Na^+ is kept by restrictions imposed by structural and electrical relationships maintained by amino acid residues located in the pore loops (Chiamvimonvat et al. 1996; Favre et al. 1996; Heinemann et al. 1992). The protein sequence at the pore loops is highly conserved because pore loops influence important functional roles (Toledo et al. 2016). The fact that substitutions that confer TTX resistance occur in this highly conserved area of the channel protein increases the likelihood that these substitutions may cause pleiotropic effects. In fact, some studies have revealed that pore loop replacements that decrease TTX binding in mammalian Na_V can also affect channel ionic selectivity (Choudhary et al. 2003) and conductance (Mahdavi and Kuyucak 2015; Terlau 1991).

The levels of toxicity exhibited by *Taricha* newts and the level of physiological resistance of sympatric snakes varies throughout the geographic range where the two species overlap (Hanifin et al. 2008, Feldman et al. 2010). Newt populations have increased toxicity

by increasing the TTX concentrations in their tissues (Hanifin et al. 2008). Snake resistance is believed to be primarily the result of the accumulation of TTX-resistant substitutions in Na_V channels, especially those in the skeletal muscle channel $tsNa_V1.4$. Resistance in garter snakes has been measured as a physiological or whole-body phenotype by assessing deficits in snake locomotor performance after injection of TTX (Brodie and Brodie 1990).

At the protein level, differences have been found in the number and identity of amino acid substitutions at the pore loops in $tsNa_V1.4$ among populations exhibiting different levels of whole-body resistance (Feldman et al. 2010, Hague et al. 2017). Garter snakes from populations in California that exhibit the highest known levels of whole-animal resistance have four pore loop substitutions in their skeletal muscle channel ($tsNa_V1.4^{LVNV}$). These four substitutions produced a large increase in TTX resistance when these changes were recreated and tested on a mammalian channel ortholog expressed in a heterologous system (Geffeney et al. 2005). The same substitutions modified biophysical properties in a mammalian channel background (Lee et al. 2011), suggesting TTX-resistant substitutions in garter snake sodium channels may carry pleiotropic costs to channel function.

In this study we explored whether molecular constraint related to channel function played a role during the evolution of the channel variant $tsNa_V1.4^{LVNV}$ found in highly resistant *T. sirtalis* from California. To do this we recreated a mutational pathway between a sensitive *T. sirtalis* skeletal muscle sodium channel ($tsNa_V1.4^{+}$, lacking pore loop substitutions) and $tsNa_V1.4^{LVNV}$ (Figure 2), and we measured TTX resistance and different aspects of channel function. Phylogenetic comparisons revealed the initial most likely mutational step in the trajectory (I1561V) (Figure 2; Hague et al. 2017). We included this

change first, resulting in the creation of channel variant tsNa$_V$1.4^V. Six possible mutational trajectories could change tsNa$_V$1.4^V to tsNa$_V$1.4^{LVNV}; due to technical limitations we could only test a one of those trajectories. We created a total of five *T. sirtalis* skeletal muscle channels: tsNa$_V$1.4$^+$, tsNa$_V$1.4^V, tsNa$_V$1.4^{VN}, tsNa$_V$1.4^{VNV} and tsNa$_V$1.4^{LVNV} (Figure 2). Three of these channels (tsNa$_V$1.4$^+$, tsNa$_V$1.4^V, and tsNa$_V$1.4^{LVNV}) occur in natural snake populations, while channel forms tsNa$_V$1.4^{VN} and tsNa$_V$1.4^{VNV} constituted hypothetical intermediates never observed in nature.

We used expression in *Xenopus* oocytes and electrophysiological techniques to measure the level of TTX resistance and three aspects of channel function in each of the channel forms created. Measuring TTX resistance allowed us to explore whether the stepwise accumulation of substitutions represents improvements towards higher TTX resistance, as had been suggested by results of past studies (Geffeney et al. 2002; Geffeney et al. 2005). Measurement of channel biophysical properties can reveal whether substitutions are pleotropic affecting resistance and channel function, which could ultimately constrain amino acid evolution in this protein. We examined voltage-dependence of activation, fast inactivation, and slow inactivation to describe the availability/excitability of the channel in dependence of membrane voltage (Hille 2001). Channel activation measurements describe the range of membrane voltages at which channels open to allow Na^+ inflow. Similarly, channel inactivation describes the voltage range at which channels become inactivated or unavailable to Na^+ entry. Pore loop substitutions that affect these biophysical properties can produce changes in channel excitability by changing the level of membrane depolarization necessary to elicit activation or inactivation. If the mutational pathway to resistance is

constrained, negative pleiotropic or epistatic interactions between novel site changes may lead to suboptimal intermediate channel forms. Discovery of a suboptimal intermediate would suggest that an alternative pathway was likely favored by evolution. We evaluated the existence of epistatic interactions among mutated sites by observing how the effect of single amino acid mutations on TTX resistance and channel biophysical properties varied with respect to the presence of other mutations already present in the channel pore. We detected pleiotropy when channel substitutions affected more than one channel measurement.

Methods

Channel construction and expression

We generated synthetic Na_V proteins through Gibson synthesis (Gibson et al. 2009). The complete genomic template for *T. sirtalis* $Na_V1.4$ was obtained from a tissue specimen collected from a population in central North America (Illinois, USA), outside the range of *Taricha* newts (methods in Hague et al. 2017). This *T. sirtalis* population is considered to be ancestral to western *T. sirtalis* and contains the ancestral pore loop sequence of $tsNa_V1.4$ (Hague et al. 2017). This sequence was used as the template to construct the TTX-sensitive channel $tsNa_V1.4^+$; all other snake channels were constructed using this sequence and substituting the experimental pore loop substitutions.

The coding sequence of the template channel $tsNa_V1.4^+$ was codon optimized for expression in *Xenopus laevis* and inserted into a plasmid for cloning. We first used Sanger sequencing to generate the full protein-coding sequence of $tsNa_V14^+$. The synthetic $tsNa_V14^+$ cDNA sequence (1875 aa, 5625 bp) was codon optimized by IDT for expression

in *Xenopus* oocytes. Two silent EcoRV cut sites were included at positions 4482 and 5211 to allow for mutagenesis. We used a commercial supplier (IDT) to generate four synthetic oligonucleotides (≈1400 bp each) that corresponded to the codon-optimized cDNA. The blocks included 20 bp overlapping regions with each other and the target vector to enable Gibson assembly. We assembled gene fragments with a linearized (SmaI, NEB) vector (pGEMHE, courtesy of J. Rosenthal from University of Puerto Rico) that included a T7 promoter for in vitro mRNA synthesis, 3' and 5' *Xenopus* globin UTRs, and a poly-A tail using standard Gibson assembly protocols (NEB). The product of this reaction was transformed into competent JM109 cells (Promega, USA) and selectively screened using standard protocols. Positive clones were sequenced using Sanger sequencing (Sequetech; USA) to ensure correct assembly and orientation of the snake sequences. We chose one correct clone, which was re-transformed and sequence verified for further expression and mutagenesis.

Channel variants were then constructed using Gibson assembly. Sequence-verified plasmids with the complete channel coding sequence were digested with EcoRV (NEB) and purified in agarose gel (0.8%) to isolate the approximately 8.5 kb fragment containing the channel coding sequence. The fragment was further purified and concentrated using standard Phenol:Choloroform protocols and Na^+ acetate precipitation. The resulting linearized plasmid was identical to the native tsNav1.4^+ construct with approximately 700 bp removed from the DIV region of the protein. We constructed the four mutant DIV alleles (1.4^{V}, 1.4^{VN}, 1.4^{VNV}, and 1.4^{LVNV}) with the same approach as the template channel ($tsNa_V1.4^+$). Channel expression in *Xenopus* oocytes was achieved by the injection of messenger RNAs containing

coding sequence for each specific channel variant. Before injection, channel constructs were linearized with Nhe1 digestion (NEB). We used a T7 ultra mMessage mMachine kit (Life Technologies) to synthesize capped and tailed mRNAs and then we injected 5-30 ng of channel mRNA into stage 5 *Xenopus* oocytes (EcoCyte Bioscience) for channel expression.

Current assay and TTX sensitivity

Biophysical properties of channels were recorded from Na^+ current protocols run on channels expressed in *Xenopus* oocytes. Sodium currents were measured at room temperature (22-25 °C) 2-7 days after RNA injections using the cut-open oocyte Vaseline gap voltage-clamp technique (Stefani and Bezanilla 1998) with a CA-1B High Performance Oocyte Clamp (Dagan Instruments). The experiments were performed in an external solution containing 120 mM MES Na, 10 mM HEPES Na, and 1.8 mM CaCl2 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. To record and measure current we used the pClamp software (Molecular Devices), sampling at 100 kHz and filtering at 20 kHz. The holding potential for all experiments was -100 mV. Leak subtraction was performed with the use of a p/4 protocol.

To estimate TTX binding affinity of channel variants we evoked peak currents at 0.05 Hz with 20-ms pulses to 0 mV following a 500-ms pre-pulse to -150 mV. The size of the currents was measured offline with the software IgorPro (WaveMetrics). We calculated the ratios of peak currents in the presence and absence of TTX with peak currents recorded before and after perfusing the selected TTX concentration into the external bath solution for 2.5 min (approximately 36 changes of solution) over a range of TTX concentrations. We estimated TTX concentration that blocked 50% of the expressed channels by fitting the data

to the following equation derived from a single-site Langmuir adsorption isotherm: current ratio= 1/ [1+ ((TTX)/ K_d)] where (TTX) is the concentration of toxin and K_d is the concentration of TTX at which half of the channels are blocked by the toxin. The K_d and its 95% confidence limits were estimated from the curve in IgorPro (WaveMetrics).

Channel biophysical properties

The voltage-dependence of activation was measured from the peak inward current during a 20 ms test pulse to voltages ranging from -100 to 80 mV in 10 mV steps following a 500-ms prepulse to -150 mV. The voltage-dependence of fast inactivation was measured from the peak inward current during a 20-ms pulse to 0 mV after a 500-ms, conditioning prepulse ranging from -150 to -10 mV in 10 mV increments. The voltage-dependence of steady-state slow inactivation was measured from the peak inward current during a 20 ms test pulse to 0 mV. The test pulse followed three conditioning pulses, the first a 30-s pulse to -130 mV to remove fast and slow inactivation, the second a 60-s pulse to voltages ranging from -130 to -10 mV (in 10 mV increments) and the third a 20-ms pulse to -150 mV to recover channels from fast inactivation.

Peak current amplitudes were measured during test pulses offline with Igor Pro (Wavemetrics). Conductance-voltage relationships for the voltage-dependence of activation were derived using the following equation: $G_{Na} = I_{peak}/(V_M - E_{Na})$ where G_{Na} represents sodium conductance, I_{peak} is the peak-test-pulse current, V_M is the test-pulse voltage, and E_{Na} is the measured sodium equilibrium potential. Activation, fast inactivation and slow inactivation curves were fitted by a Boltzmann distribution with the following equation: Normalized conductance or current amplitude = $1/(1+ \exp(-ze_0(V_M - V_{1/2})/kT))$ where z is

the apparent valence, e_0 is the elementary charge, $V_{1/2}$ is the midpoint voltage, k is the Boltzmann constant, and T is the temperature in degrees Kelvin. $V_{1/2}$ and its 95% confidence limits were estimated from the curve using Igor Pro (Wavemetrics). Activation curves were created by plotting conditioning voltage and conductance (G_{Na}) while fast inactivation and slow inactivation curves were created by plotting conditioning voltage and normalized current. Construction of TTX binding curves and channel biophysical property curves was done from current data pooled from 7-13 oocytes expressing a particular channel variant. We considered significant differences when 95% confidence intervals of K_d or $V_{1/2}$ values did not overlap.

Results

TTX resistance

We found striking differences in sensitivity to TTX in the channels tested (Figure 3, Table 1). The sensitive snake channel $tsNa_V1.4^+$ is slightly less sensitive to TTX binding than the known mammalian TTX-sensitive channel rat $Na_V1.4$, though no amino acid differences occur in the TTX binding site of the pore loops between these channels. Incorporating the change I1561V to the channel $tsNa_V1.4^+$ causes a small but not significant increase in TTX resistance. Notably, all the channels that exhibited high TTX resistance (i.e., K_d was in the micro molar concentration range) presented the D1568N substitution. Adding the substitution G1569V to the $tsNa_V1.4^{VN}$ background did not alter TTX resistance. However, adding the change I1556L to the other three changes in $tsNa_V1.4^{VNV}$ doubled resistance.

Biophysical Properties

Both rat $Na_V1.4$ and $tsNa_V1.4^V$ have similar voltage-dependence of activation curves as $tsNa_V1.4^+$ and open at more negative potentials compared to the resistant channels containing the D1568N change ($tsNa_V1.4^{VN}$, $tsNa_V1.4^{VNV}$ and $tsNa_V1.4^{LVNV}$) (Figure 3 and 4, Table 2). Both $tsNa_V1.4^V$ and rat $Na_V1.4$ presented a similar depolarizing shift for measurements of voltage-dependence of steady-state slow inactivation compared to $tsNa_V1.4^+$ (Figure 5 and Table 2). The creation of a highly resistant channel $tsNa_V1.4^{VN}$ by adding the substitution D1568N on the $tsNa_V1.4^V$ background caused a hyperpolarizing shift in slow inactivation. The neighboring substitution G1569V caused a significant depolarizing shift in the voltage-dependence of slow inactivation compared to $tsNa_V1.4^{VN}$. Adding the change I1556L to the other three changes to create $tsNa_V1.4^{LVNV}$ did not significantly shift slow inactivation. All of the channels tested had similar voltage-dependence of fast inactivation curves (Figure 6, Table 2). The values for voltage of half-maximal fast inactivation ($V_{1/2}$) fell within a narrow 10 mV range for all channels.

Discussion

Our results show that pore loop substitutions in $tsNa_V1.4$ have significant effects on sensitivity to TTX and on channel biophysical properties when evaluated in the phylogenetically appropriate genetic background. Tetrodotoxin sensitivity data suggest that the stepwise accumulation of the substitutions tested leads to a great increase in channel resistance in the evolutionary trajectory we recreated. However, not all of the steps that we tested represented improvements in resistance. Some substitutions appeared to have minimal effects on resistance while others had larger contributions. The effect of substitutions on TTX

resistance and channel biophysical properties seemed to be contingent on the presence of other substitutions, suggesting substitutions at different sites interact epistatically. Substitutions in tsNa$_V$1.4 DIV pore loop are pleiotropic affecting TTX binding and also channel biophysical properties, especially channel activation and slow inactivation.

Consequences of pore substitutions on TTX resistance

TTX binding curves revealed that the evolutionary trajectory we explored leads to dramatically higher resistance. Although we did not exhaustively test the single and combinatorial effects of all four substitutions, we could infer from our results that the contribution of substitutions to resistance is highly variable. For example, the replacement I1561V increased resistance of the channel slightly but not significantly, although earlier work that tested the effect of this substitution on resistance in a mammalian-snake Na$_V$1.4 chimera found the small increase in resistance caused by I1561V to be significant (Geffeney et al. 2005). The substitution D1568N causes a large shift in the level of resistance of the channel (Figure 3). This substitution may reduce TTX affinity by impeding the formation of a hydrogen bond between the aspartic acid residue and TTX (Choudhary et al. 2003; Penzotti et al. 1998). The same substitution has been found to increase channel resistance by 30–40-fold in rat Na$_V$1.4 (Penzotti et al. 1998).

The change G1569V did not seem to affect resistance when added to the tsNa$_V$1.4^{VN} genetic background. However, this replacement affected the voltage-dependence of slow inactivation (Figure 5). This finding reveals that some pore loop changes could offer an adaptive advantage that is strictly related to channel function. The replacement of isoleucine for a leucine at position 1556 significantly doubles the level of resistance when added to the

$tsNa_V1.4^{VNV}$ background (Figure 3). Since the residues exchanged by this replacement are not extremely different chemically, it was surprising to find a doubling in resistance caused by this substitution. However, protein models of the sodium channel have shown that seemingly minimal changes have the potential to hinder the ability of TTX to make contact with pore binding sites and therefore can contribute to increase TTX resistance (Tikhonov and Zhorov 2012). The channel variant $tsNa_V1.4^{LVNV}$ exhibited the highest resistance out of all the channels we tested. This channel form is the only one known that can withstand the highest levels of newt toxicity (Hague et al. 2017; Hanifin et al. 2008).

Functional consequences of channel substitutions

The consequences of pore substitutions on channel function were most dramatic in two of the biophysical properties measured: the voltage-dependence of activation and the voltage-dependence of slow inactivation. We found that all channels with the substitution D1568N presented a shift in activation towards more depolarized potentials (Figure 4). This shift means that a higher degree of membrane depolarization is necessary for channel activation, making channels with this shift less excitable relative to the sensitive snake channel $tsNa_V1.4^+$ and to $rNa_V1.4$.

The slow inactivation data showed that most snake channels with pore loop substitutions shifted towards more depolarized voltages with respect to $tsNa_V1.4^+$. The exception was the hypothetical intermediate channel $tsNa_V1.4^{VN}$ that shifted towards hyperpolarized potentials (Figure 5). This hyperpolarizing shift in slow inactivation indicates that relatively little depolarization of the membrane is sufficient to slow inactivate $tsNa_V1.4^{VN}$ eliminating overall channel availability and impairing Na^+ currents in

hypothetical muscle fibers expressing this channel variant.

Shifts in activation and slow inactivation together can affect the window current, which is the area below the overlapping activation and inactivation curves that represents an estimated range of voltages available for the channel to open (Figure 7). The hyperpolarizing shift in slow inactivation seen in the hypothetical intermediate $tsNa_V1.4^{VN}$ caused a considerable contraction on the window current for this channel, not seen in the other channels tested (Figure 7). In fact, more than half of the Na^+ current is unavailable in $tsNa_V1.4^{VN}$ at membrane values close to resting membrane potential (measured for garter snakes in Geffeney et al. 2002) due to slow inactivation (Figure 7). These data suggest that there is a severe reduction in channel availability for $tsNa_V1.4^{VN}$ that would impair muscle excitability in snakes expressing this channel.

Although the consequences of these shifts in channel biophysical properties for snake muscle physiology remain to be investigated, studies of the human skeletal muscle channel provide links between similar biophysical shifts and human muscle disease phenotypes. Multiple studies have found that $Na_V1.4$ substitutions that produce hyperpolarizing shifts in the voltage-dependence of slow inactivation (such as the ones seen in the hypothetical intermediate $tsNa_V1.4^{VN}$) are linked to human muscle diseases including hypokalemic periodic paralysis and paramyotonia congenita (Bendahhou et al.1999; Jurkat-Rott et al. 2000; Richmond et al. 1997; Richmond et al. 1997; Struyk et al.2000; Takahashi et al. 1999).

Evolutionary trajectory to extreme resistance

If some combinations of amino acid mutations produce channel intermediates that are dysfunctional in the mutational pathway from $tsNa_V1.4^{+}$ to $tsNa_V1.4^{LVNV}$, we expect that some mutational routes to high resistance would be inaccessible due to constraint by impaired biophysical functions. This is because the introduction of a single or a combination of substitutions has the potential to alter important site contacts that influence function due to unexpected epistatic interactions between novel sites that can negatively affect channel activity. One of the hypothetical intermediate channels we tested ($tsNa_V1.4^{VN}$) showed a striking reduction in excitability (Figure 7), which would result in a deleterious phenotype. The discovery of this suboptimal and probably deleterious intermediate suggests that some combinations of substitutions in the pore loop of $tsNa_V1.4$ may be selected against because they of their effects on channel function.

The fact that intermediate forms between $tsNa_V1.4^{V}$ and $tsNa_V1.4^{LVNV}$ cannot be found in nature suggests that a highly limited number of DIV pore loop substitutions in $tsNa_V1.4$ are capable of producing a functionally stable TTX-resistant channel. Most other combinations of pore substitutions may be functionally deleterious, which explains why the sequence at the pore loops is highly conserved in Na_V channels and there are few instances of sequence variation observed in natural populations. We only explored one possible evolutionary trajectory between $tsNa_V1.4^{V}$ and $tsNa_V1.4^{LVNV}$. This trajectory did not seem to be the most likely direction undertaken by evolution since one intermediate may have possessed low fitness. The most likely trajectory is one where each successive amino acid mutation along the trajectory results in improvements to resistance without great pleiotropic costs to channel biophysical properties. Given our results from the functional assessment of

the channels in the trajectory we tested, the next logical evolutionary route to assess should be one where $Na_V1.4^{VV}$ is an intermediate to $Na_V1.4^{VNV}$ since this intermediate might mask the deleterious effects of the D1568N substitution.

Conclusion

Our work supports the hypothesis that the evolutionary trajectory towards a TTX-resistant $Na_V1.4$ in garter snakes is constrained by the functional consequences of changing pore loop residues. When adaptive change involves mutations with pleiotropic effects, evolutionary solutions may strike a balance between the level of adaptive novelty and the level of negative consequence to other stable traits. In the case of resistant snakes, selection to increase TTX resistance may favor combinations of pore loop replacements that confer the necessary channel resistance while also maintaining permissive values of channel performance. High sequence conservation at Na_V pore loops and the high specificity of TTX attraction to selected pore loop residues suggests there is a limited number of sites in this regions that can change to effectively reduce TTX binding affinity while simultaneously keeping biophysical properties under permissible values imposed by muscle performance requirements.

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Tables

Channel variant	K_d ± 95% confidence levels (nM)	n
Rat $Na_V1.4$	35±6.3	10
$TsNa_V1.4^+$	50±5.2	13
$TsNa_V1.4^V$	65±11	11
$TsNa_V1.4^{VN}$	4800±1300	11
$TsNa_V1.4^{VNV}$	5300±1400	10
$TsNa_V1.4^{LVNV}$	13000±1800	11

Table 1. The concentration of TTX required to block 50% of the elicited currents (K_d) was pooled from several cell recordings.

	Channel activation			Channel slow inactivation			Channel fast inactivation		
Channel variant	$V_{1/2}$ (mV)	z	n	$V_{1/2}$ (mV)	z	n	$V_{1/2}$ (mV)	z	n
Rat $Na_V1.4$	-39.6±1.5	6.10±2.17	8	-69.7±1.9	-2.14±0.29	7	-51.5±0.7	-3.06±0.21	9
$TsNa_V1.4^{+}$	-36.2±1.0	4.49±0.71	7	-83.6±1.4	-1.77±0.16	7	-56.6±0.7	-4.14±0.41	9
$TsNa_V1.4^{V}$	-34.7±1.9	3.78±0.92	8	-67.0±3.2	-1.18±0.17	7	-49.2±0.8	-4.29±0.54	8
$TsNa_V1.4^{VN}$	-19.3±0.8	4.30±0.52	8	-97.8±1.5	-2.04±0.24	8	-53.7±0.9	-3.49±0.36	9
$TsNa_V1.4^{VNV}$	-18.8±1.3	4.37±0.83	7	-68.6±5.1	-1.04±0.22	7	-58.5±1.4	-2.27±0.25	12
$TsNa_V1.4^{LVNV}$	-16.4±0.5	4.03±0.29	7	-64.1±1.7	-1.79±0.19	7	-54.7±0.7	-4.27±0.43	10

Table 2. Channel biophysical parameters. From left to right, voltage of half-maximal activation ($V_{1/2}$) for each channel, voltage of half-maximal steady-state slow inactivation ($V_{1/2}$) for each channel, voltage of half-maximal fast inactivation ($V_{1/2}$) for each channel; z, apparent valence, n, number of cells. Values represent means ± 95% confidence intervals.

Figures

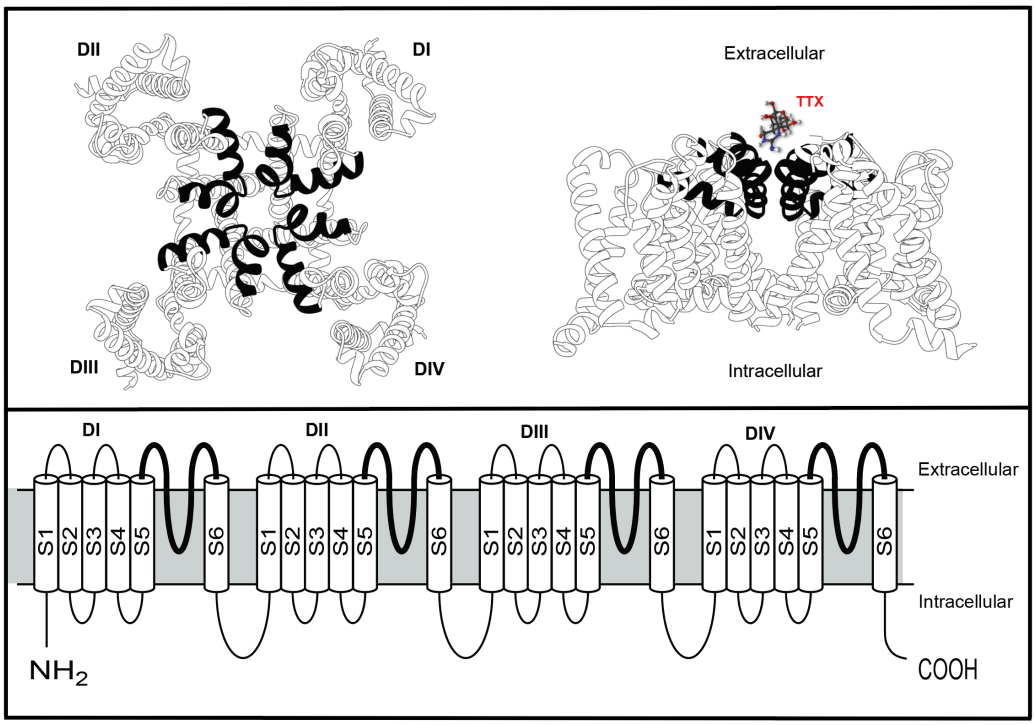

Figure 1. Upper panel: Channel protein model based on the crystal structure of the bacterial sodium channel with pore loop helices highlighted in black. Left: Channel view from above. Right: Lateral view of channel with TTX docking at the pore. Figure modified from Hanifin and Gilly 2015. Lower panel: Illustration of the unfolded α-subunit of the voltage-gated sodium channel inserted in the cell membrane. The α-subunit consists of four homologous domains (DI-DIV) each composed of six trans-membrane segments (S1-S6). The pore of the channel is formed by the pore loops, which are the helices connecting trans-membrane segments S5 and S6.

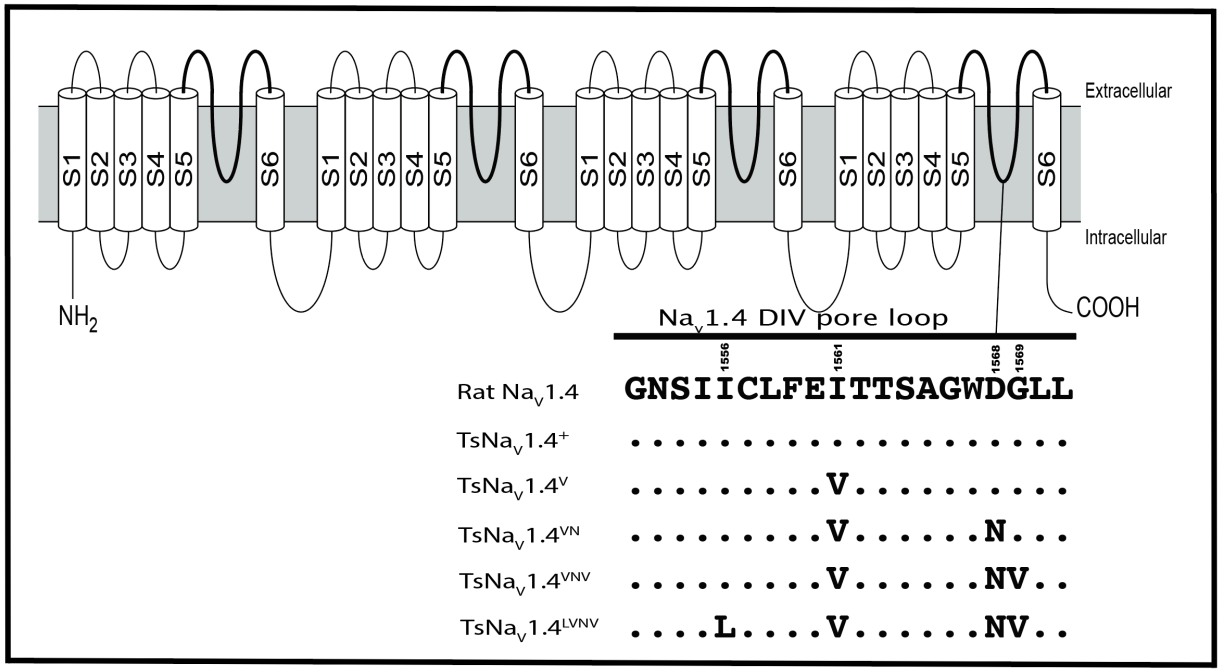

Figure 2. Schematic of a voltage-gated sodium channel protein showing the location of pore loop substitutions in domain IV that were mutated for our experiment. A section of a known sensitive channel (rat $Na_V1.4$) DIV pore loop amino acid sequence is shown as a reference. Pore loop sequences are conserved in most vertebrates; therefore there are no differences between pore loop sequences of snakes that are not exposed to TTX and the rat ortholog. The TTX-sensitive *T. sirtalis* sequence ($tsNa_V1.4^+$) was obtained from garter snakes in populations outside of the range of *Taricha* newts. We recreated one mutational pathway from $tsNa_V1.4^+$ to $tsNa_V1.4^{LVNV}$ (a DIV pore loop variant expressed in *T. sirtalis* populations from California that consume highly toxic *Taricha*) through the stepwise addition of amino acid replacements at the DIV pore loop as depicted in the figure. The first step (replacement of isoleucine for a valine at position 1561) is the only step supported by phylogenetic reconstruction (Hague et al. 2017). There are no intermediate channel variants in nature exhibiting variations of DIV pore loop substitutions between $tsNa_V1.4^V$ and $tsNa_V1.4^{LVNV}$.

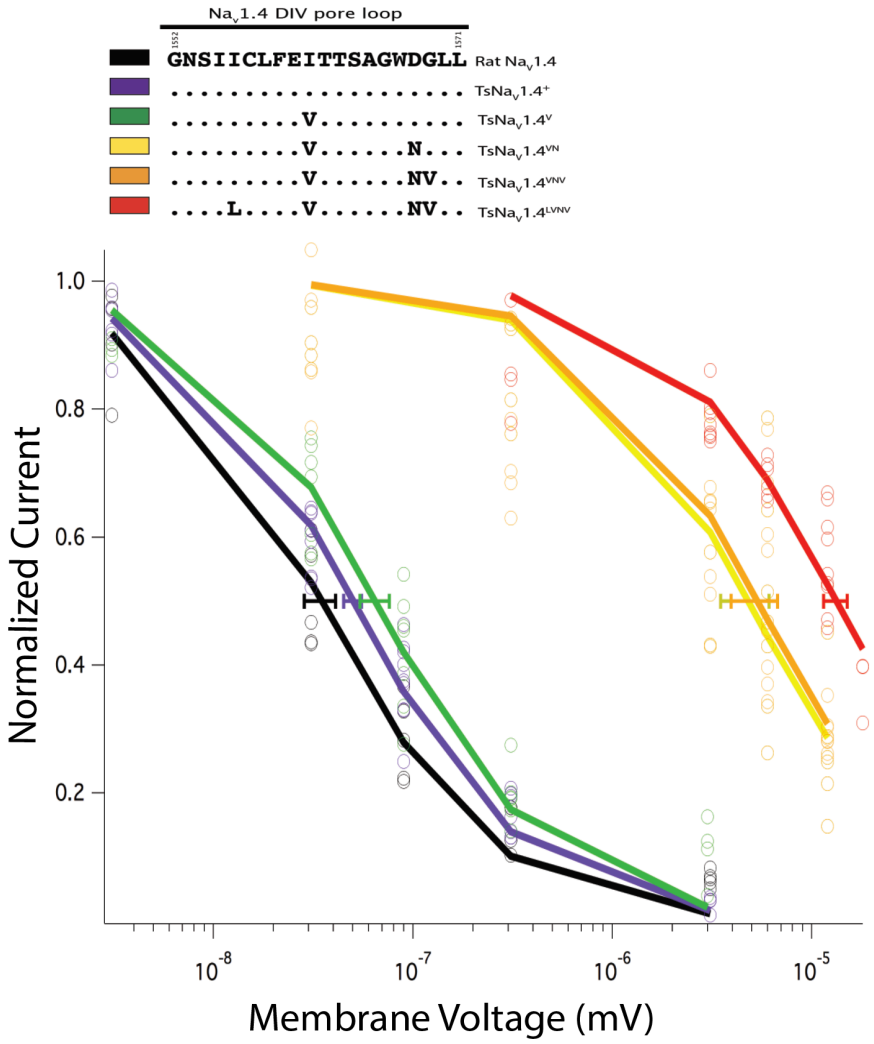

Figure 3. The effect of TTX on $Na_V1.4$ channels. TTX binding curves for each channel describe the reduction caused by TTX concentration on sodium-induced current size. The three snake channels containing the substitution D1568N (yellow, orange, red) exhibited a large increase in channel resistance. The TTX concentration that blocked 50% of the channels (K_d) ±95% confidence limits are shown for each channel type with a bar. Rat $Na_V1.4$ (black), $tsNa_V1.4^+$ (purple), $tsNa_V1.4^V$ (green), $tsNa_V1.4^{VN}$ (yellow), $tsNa_V1.4^{VNV}$ (orange), $tsNa_V1.4^{LVNV}$ (red).

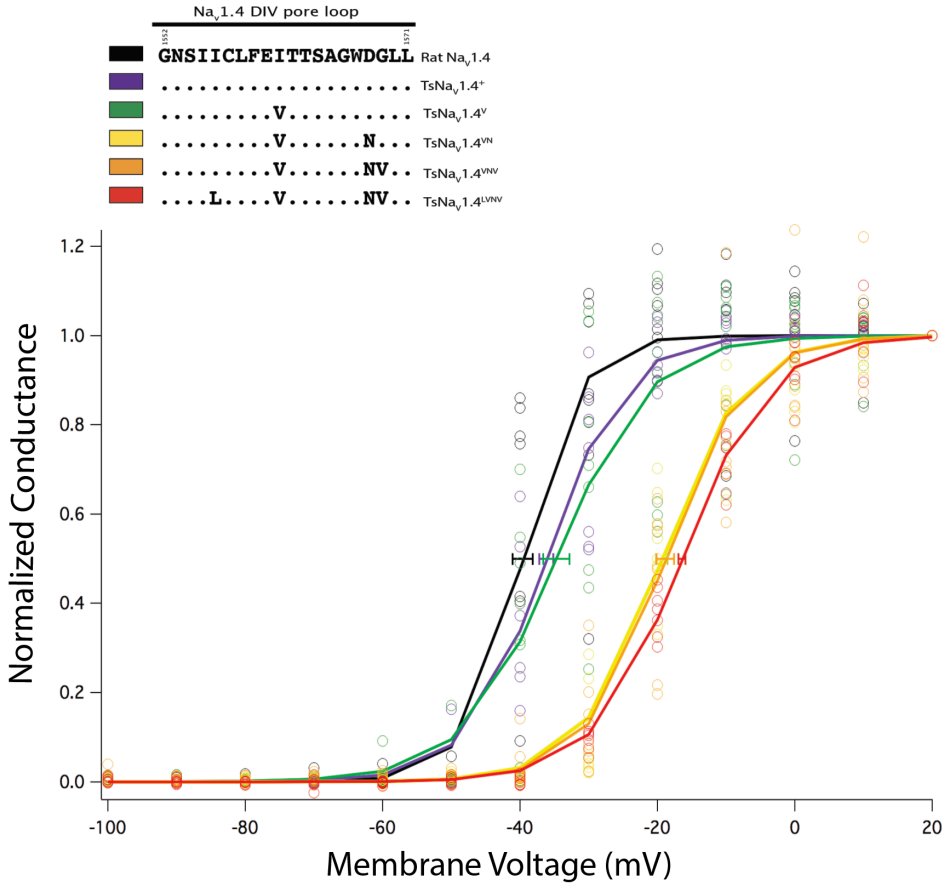

Figure 4. Voltage-dependence of activation of channels. Curves describe normalized sodium conductance versus membrane potential for each channel. Voltage at half normalized conductance ($V_{1/2}$ ± 95% confidence intervals) is denoted with a bar for each curve. The three snake channels containing the substitution D1568N (yellow, orange, red) exhibited a depolarizing shift in activation with respect to the other channels. The current data were normalized to maximum conductance and fit according to the Boltzmann function described in Methods.

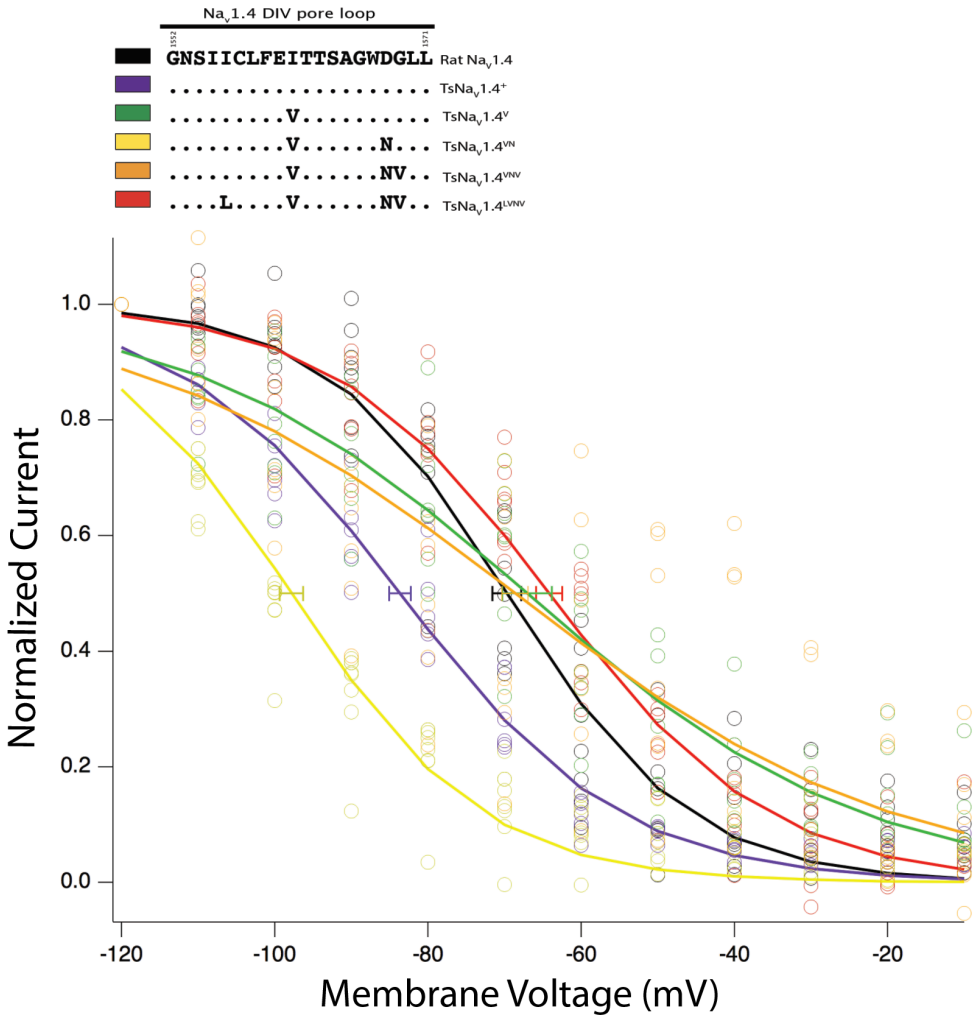

Figure 5. Voltage-dependence of steady-state slow inactivation of $Na_V1.4$ channels. Curves show normalized mean current versus membrane potential. Voltage at which 50% of the channels slow inactivate ($V_{1/2}$ ± 95% confidence intervals) is denoted with a bar for each curve. All snake channels with DIV pore loop substitutions exhibited a depolarizing shift in slow inactivation with respect to the sensitive snake channel $tsNa_V1.4^+$, except for the hypothetical intermediate channel $tsNa_V1.4^{VN}$ (yellow), which shifted to hyperpolarizing potentials. The current data were normalized to maximum peak currents and fit according to the Boltzmann function described in Methods.

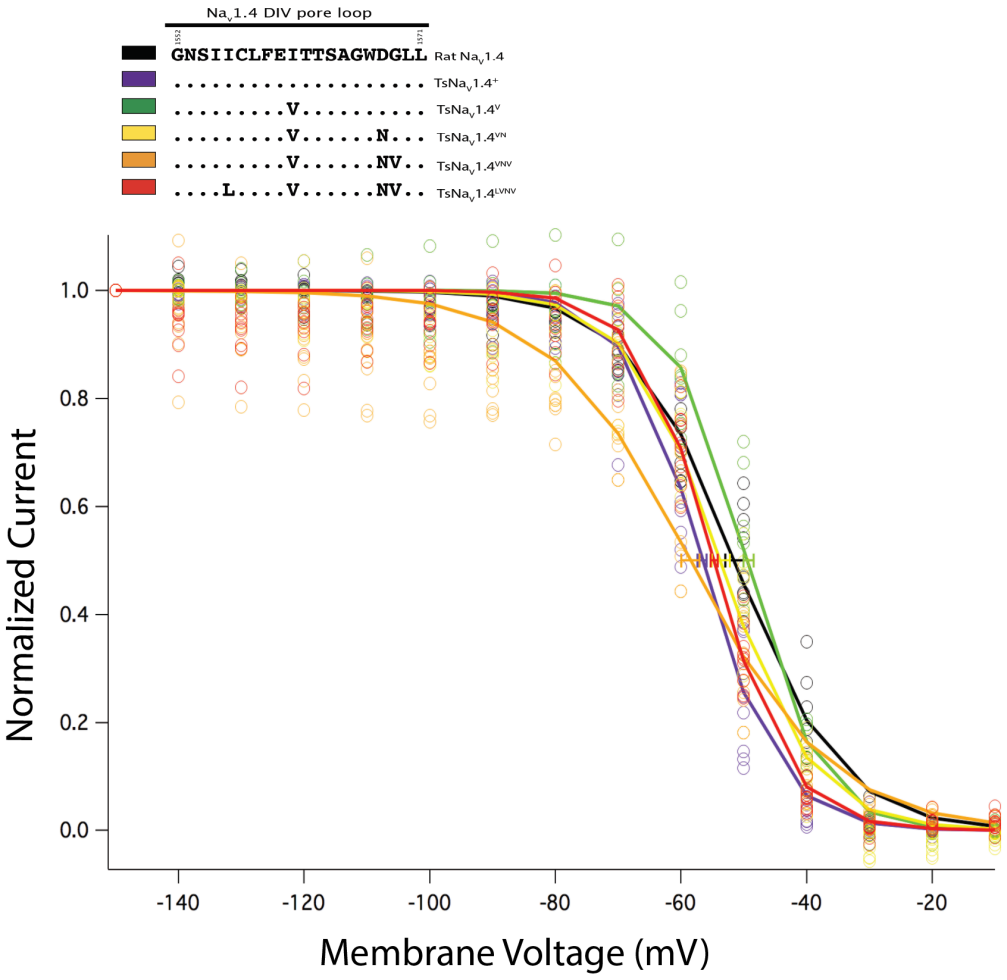

Figure 6. Voltage-dependence of fast inactivation of $Na_V1.4$ channels. Curves show normalized mean current versus membrane potential. Voltage at which 50% of the channels fast inactivate ($V_{1/2}$ ± 95% confidence intervals) is denoted with a bar for each curve. Differences in fast inactivation among the channels were minimal; $V_{1/2}$ varied within a narrow a range of 10 mV for all channels tested. The current data were normalized to maximum current and fit according to the Boltzmann function described in Methods.

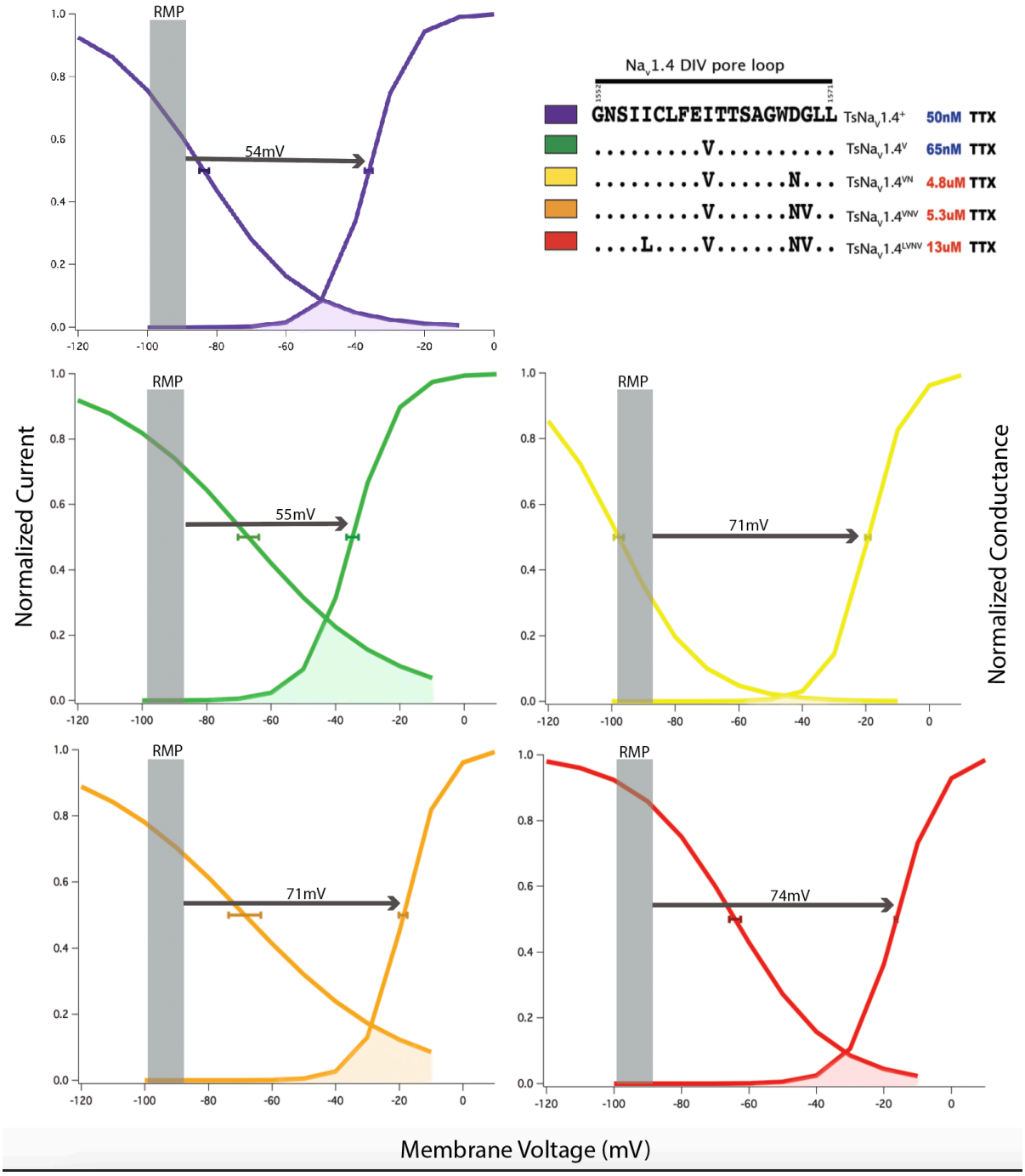
RMP
54mV
Na$_v$1.4 DIV pore loop
GNSIICLFEITTSAGWDGLL TsNa$_v$1.4$^+$ 50nM TTX
.....V..... TsNa$_v$1.4^V 65nM TTX
.....V.....N... TsNa$_v$1.4^{VN} 4.8uM TTX
.....V.....NV.. TsNa$_v$1.4^{VNV} 5.3uM TTX
....L....V.....NV.. TsNa$_v$1.4^{LVNV} 13uM TTX
RMP
55mV
RMP
71mV
Normalized Current
Normalized Conductance
RMP
71mV
RMP
74mV
Membrane Voltage (mV)

Figure 7. Window currents for $Na_V1.4$ channels are represented as the shaded area below the overlapping activation and slow inactivation curves. The vertical grey bar marks the resting membrane voltage recorded for snake skeletal muscle (Geffeney et al. 2002); the length of the arrow represents how much membrane depolarization the channel requires to reach half-maximal channel activation from a state of resting membrane potential (RPM). The highly resistant channels $tsNa_V1.4^{VN}$, $tsNa_V1.4^{VNV}$ and $tsNa_V1.4^{LVNV}$ exhibited shifts in voltage-dependence of activation to more positive voltages resulting in channels that require more depolarization in order to activate. In the case of resistant channels $tsNa_V1.4^{VNV}$ and $tsNa_V1.4^{LVNV}$, the shifts in activation were accompanied by shifts in steady-state slow inactivation in the same direction therefore the overall window current shifted to more depolarized potentials, but the window area did not get reduced. However, the hypothetical intermediate $tsNa_V1.4^{VN}$ showed a hyperpolarizing shift in slow inactivation causing an extreme reduction of the window current. As a result, the range of voltages available for this channel to fire is extremely contracted which would result in severe reduction of channel excitability.