**Interaction between voltage-gated sodium channels and the neurotoxin, tetrodotoxin**

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**Introduction**

Voltage-gated sodium channels (Na\textsubscript{V}) are a class of transmembrane proteins expressed in nerve and muscle tissue that regulate the flow of sodium ions across the membrane of a cell. Activation (opening) of the channel allows sodium ions to move down their electrochemical gradient into the cell. This inward flow of sodium ions initiates the rising phase of the action potential (AP) and allows AP propagation. The activity of Na\textsubscript{V} is thus a crucial component of membrane excitability, and its loss leads to dysfunction in nerve and muscle tissues, including paralysis. Molecular analysis revealed that the Na\textsubscript{V} is a large, membrane-bound protein complex composed of an α-subunit and one or more smaller accessory β-subunits. To date, nine mammalian Na\textsubscript{V} isoforms, and a related tenth isoform, have been identified. Different members of Na\textsubscript{V} isoforms are expressed in different types of excitable tissues.

Tetrodotoxin (TTX) is a potent toxin that specifically binds to voltage gated sodium channels. TTX binding physically blocks the flow of sodium ions through the channel, thereby preventing action potential (AP) generation and propagation. TTX has different binding affinities for different sodium channel isoforms. These differences are imparted by amino acid substitutions. Such substitutions confer TTX resistance to a variety of species. Tetrodotoxin resistance, however, may come at a cost to performance caused by changes in the biophysical properties and/or ion selectivity of the TTX resistant sodium channels. We here review the properties of sodium channels and their interaction with TTX, and look at some special examples of TTX resistant channels wherein the benefit of toxin resistance may be offset by other behavioral costs.

**Voltage-Gated Sodium Channel Structure**

Na\textsubscript{V} is comprised of a highly processed α-subunit (260 kDa) and one or more smaller accessory β-subunits (33–37 kDa). The β-subunits are important in modifying voltage dependency and kinetics of Na\textsubscript{V} gating. In addition, they play important roles in cell adhesion, signal transduction and channel expression at the plasma membrane. The α-subunit is formed from four homologous domains (D1–D4) and each domain contains six α-helical transmembrane segments (S1–S6). Transmembrane segments are connected through small intracellular and extracellular loops, and larger intracellular loops connect homologous domains (Fig. 1). The α-subunit is important in channel function, including voltage sensitivity and ion selectivity. The S4 transmembrane segments of the α-subunit have been shown to be the voltage sensors in Na\textsubscript{V}, as in other voltage-gated ion channels. They include positively charged...
Interaction between NaV and TTX

arginine and lysine residues at every third amino acid position. Depolarization of the membrane triggers the positively charged S4’s to translocate toward the extracellular side of the cell membrane and initiates channel activation.16 This movement of the charges within the protein and primarily confined to the intramembrane region of S4’s, gives rise to gating currents and is suggested to mediate channel gating.17 Neutralization of the positive charges in S4 transmembrane segments of all four domains results in altered channel gating.18,19 The voltage sensor is also associated with the voltage dependence of channel inactivation.20,21 There are different types of NaV inactivation, including fast inactivation (FI), slow inactivation (SI) and ultra slow inactivation (USI).22 Inactivation plays a crucial role in membrane excitability by contributing to the regulation of resting sodium channel availability. FI arises from a group of hydrophobic residues, isoleucine, phenylalanine and methionine (IFM), which constitute the inactivation gate of the intracellular DIII–DIV linker. The outward movement of the voltage sensor opens the channel and also exposes an intracellular binding site for the IFM motif of the inactivation gate. The subsequent movement and binding of the IFM motif inactivates the channel by blocking the pore and preventing the channel from reopening until the channel is restored to its resting state.23 SI and USI are proposed to involve a conformational change of the channel via rearrangement of the pore22 and they are reported to be biophysically, pharmacologically, and molecularly different from FI. The structural underpinnings of SI and USI, however, are poorly understood.22,24

The α-subunit forms the ion permeation pathway, a critical structural determinant of normal channel function. The extracellular end of the pore is formed by the highly conserved P-regions of the membrane-spanning S5 and S6 linkers of the four domains while the wider intracellular end of the pore is lined S6 segments.25-27 The amino acid residues of outer (EEDD: E403, E758, D1241, D1532) and inner (DEKA: D400, E755, K1237, A1529) rings in the P-regions serve as the ion selectivity filter.27 The traditional view of the NaV ion conducting pore is a single pore similar to the potassium channel found in the bacteria Streptomyces lividans. Sato et al.,28 proposed the 3D structure of NaV to consist of a bell shaped outer surface, square-shaped bottom and a hemi-spherical top with four small pores on the extracellular side of the cell. These four small pores are connected to a central body that diverges to four outlet pores in the intracellular side and twisting and untwisting of the central cavity corresponds to the closed and open states of the channel (“twist-sprinkler” model).29 This multi-pore structure of the channel allows the massive influx of sodium channels during rapidly changing membrane potential and consequently induces AP. Due to its crucial role the pore region of the channel is highly conserved. Studies using site-directed mutagenesis in the pore forming P-loops have shown that mutations in the pore region produce a marked decrease in sodium ion conductance and TTX-sensitivity,10,27,30-33 and affect voltage-dependent gating in activation34,35 and slow inactivation.36,37

Voltage-Gated Sodium Channel Isoforms

To date, nine mammalian NaV isoforms have been identified and functionally expressed (NaV1.1, NaV1.2, NaV1.3, NaV1.4, NaV1.5, NaV1.6, NaV1.7, NaV1.8 and NaV1.9), and a tenth isoform (NaX) has been recognized as a related protein that does not encode a voltage-gated sodium channel.1 These isoforms are all greater than 50% identical in amino acid sequence in the transmembrane and extracellular domains and the functional properties are relatively similar.1 All of the nine sodium channel isoforms are therefore suggested to be members of a single family. As expected, some

Figure 1. Transmembrane diagram of α-subunit of NaV. The α-subunit is formed from four homologous domains with six α-helical transmembrane segments in each domain. The polypeptide chains connect subunits and domains. S4 segments are voltage sensors and IFM motif in intracellular loop between DIII and DIV act as inactivation gate. P-loops of all four domains and S6 segments forms extracellular and intracellular ends of the pore and TTX binds to the selectivity filter in P-loops (outer EEDD; inner DEKA).
TTX binds to the neurotoxin receptor site located at the outer pore of $\text{Na}_v$. In addition, the inhibition of toxin binding by pH titration,\textsuperscript{51} carboxyl-modifying reagents,\textsuperscript{52} and some monovalent cations, divalent metal ions and protons\textsuperscript{53} supported the idea of the guanidinium group and the hydroxyls of TTX interact with the pore region of $\text{Na}_v$. The guanidinium group of TTX is proposed to form an ion-pair with negatively charged functional groups located in P-loops of DI (Asp-384, Glu-387) and DII (Glu-942), while the hydroxyl groups C9 and C10, and C11 of TTX form hydrogen bonds with Glu-945 in DII and Asp-1532 in DIV, respectively.\textsuperscript{49,54,55} The binding to the pore sterically and/or electrostatically occludes sodium ion permeation into the cell, leading to inhibition of AP generation and propagation. TTX binding consequently causes conduction block in muscle and nerves, and leads to diaphragm paralysis and death from respiratory failure.

The binding affinity of the channel for TTX is affected by changes in electrostatic interactions between TTX and the amino acid side chain lining of the pore. Neutralizing the negatively charged residues in the P-loops such as glutamate acid at 387, 942 and 945 and aspartic acid at 384 and 1532 cause marked decrease in TTX-sensitivity.\textsuperscript{27,30,55} Altering the shape of the selectivity filter region of the pore affects the TTX-sensitivity of the channel.\textsuperscript{32,33,46} Substitution of an aromatic ring residue to a non-aromatic ring residue at 401 in DI of TTX-resistant channel isoforms produces substantial reduction in TTX-sensitivity while the TTX-resistant channel isoforms gains TTX-sensitivity by substituting an aromatic ring residue to the same site.\textsuperscript{32,33,46} A strong non-bonded interaction between the aromatic ring and the non-polar surface of TTX is proposed to be hindered by the substitution.\textsuperscript{49} The substitution is also proposed to distort the positions of critical channel residues in the selectivity filter such as Glu-758 and Asp-1532 and reduces binding of TTX indirectly.\textsuperscript{31} TTX-sensitivity of the channel is also affected by changes in membrane potential; toxin-binding increases following repeated depolarizing pulses by increasing availability of the TTX binding site.\textsuperscript{56,57}

### Voltage-Gated Sodium Channels in Pain Pathways

Voltage-gate sodium channels play a crucial role in signal transduction sensory neurons, whose somata lie within the dorsal root ganglia (DRG). Specifically, the TTX-resistant channels $\text{Na}_v1.8$ and $\text{Na}_v1.9$ are located in pain-sensing peripheral neurons (nociceptors) and are important factors in physiological and pathophysiological pain sensation, and $\text{Na}_v$ blockers have been clinically used as analgesics for both normal neuropathic pain for many years. The chronic-constriction-injury model used in neuropathic pain studies shows that peripheral nerve injury upregulates expression levels of TTX-sensitive $\text{Na}_v1.3$ in damaged peripheral neurons within the DRG.\textsuperscript{58-60} Normally, $\text{Na}_v1.3$ is preferentially localized in central neurons of adults and its expression is greater during embryonic development than in adults (Table 1). One of the key properties of $\text{Na}_v1.3$ is a marked increase in the rate of recovery from fast inactivation compared to TTX-resistant channels, $\text{Na}_v1.8$ and 1.9, normally present in nociceptors.\textsuperscript{61} The abnormal expression of $\text{Na}_v1.3$, and its more rapid recovery from inactivation, suggests that it may play an important part in sustaining high frequency APs in chronic pain.\textsuperscript{62} In fact, reducing expression of $\text{Na}_v1.3$ in nociceptors using anti-sense oligodeoxynucleotides, resulted in decreased hypersensitivity of DRG

**Table 1 Mammalian voltage-gated Na\textsuperscript{+} channel isoforms and their TTX sensitivity**

| Channel | Gene | TTX sensitivity | Distribution |
|---------|------|----------------|--------------|
| $\text{Na}_v1.1$ | SCN1A | EC\textsubscript{50} = 6 nM \textsuperscript{38} | CNS |
| $\text{Na}_v1.2$ | SCN2A | EC\textsubscript{50} = 18 nM \textsuperscript{27,30} | CNS |
| $\text{Na}_v1.3$ | SCN3A | EC\textsubscript{50} = 4 nM \textsuperscript{39} | CNS |
| $\text{Na}_v1.4$ | SCN4A | EC\textsubscript{50} = 25 nM \textsuperscript{41} | skeletal muscle |
| $\text{Na}_v1.5$ | SCN5A | EC\textsubscript{50} = 5.7 µM \textsuperscript{43} | heart |
| $\text{Na}_v1.6$ | SCN8A | EC\textsubscript{50} = 6 nM \textsuperscript{42} | CNS |
| $\text{Na}_v1.7$ | SCN9A | EC\textsubscript{50} = 24.5 nM \textsuperscript{40} | PNS(DRG) |
| $\text{Na}_v1.8$ | SCN10A | EC\textsubscript{50} = 60 µM \textsuperscript{45} | PNS(DRG) |
| $\text{Na}_v1.9$ | SCN11A | EC\textsubscript{50} = 40 µM \textsuperscript{33} | PNS(DRG) |

$^a$EC\textsubscript{50}, median effective concentration; CNS, central nerve system; PNS, peripheral nerve system; DRG, dorsal root ganglion.

isoforms are more closely related to one another than to others. This variation in relation appears to also correlate with chromosomal location. $\text{Na}_v1.1$, $\text{Na}_v1.2$, $\text{Na}_v1.3$ and $\text{Na}_v1.7$ are highly TTX-sensitive and their genes are localized on human chromosome 2q23-24.\textsuperscript{27,30,38-40} The genes encoding $\text{Na}_v$ localized on human chromosome 2 are mainly expressed in the central and peripheral neurons. $\text{Na}_v1.4$ is localized in chromosome 17, and that encoding $\text{Na}_v1.6$ is located in chromosome 12. $\text{Na}_v1.4$ and $\text{Na}_v1.6$ are both TTX-sensitive channels and predominantly expressed in skeletal muscle and in the central nervous system, respectively.\textsuperscript{51,42} $\text{Na}_v1.5$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$ are TTX-resistant and their genes are located on human chromosome 3p21-24.\textsuperscript{33,43-45} The gene encoding $\text{Na}_v1.5$ is primarily expressed in the heart, and $\text{Na}_v1.8$ and $\text{Na}_v1.9$ are mainly found in dorsal root ganglion (DRG) neurons. TTX-resistant channels from chromosome 3 have a unique amino acid substitution at the pore forming P-loop of DI; a non-aromatic amino acid is found at site 401 in TTX-resistant channels, whereas TTX-sensitive channels have an aromatic ring amino acid at the site. This substitution reduces TTX-sensitivity.\textsuperscript{32,33,45,46} The $\text{Na}_v$ isoforms expressed in a particular excitatory tissue thus determines its TTX sensitivity (Table 1).

**Tetrodotoxin**

TTX is known as an extremely potent inhibitor of sodium currents in nerve and muscle. TTX is a low-molecular weight neurotoxin with a highly unusual chemical structure.\textsuperscript{4} It consists of a positively charged guanidinium group and a pyrimidine ring with six hydroxyl groups at the C-4, C-6, C-8, C-9, C-10 and C-11 position. (Fig. 2) TTX is predominantly isolated from the ovary and liver of puffer fish, and it has been detected in a remarkably wide range of organisms including fish, amphibians, arthropods, nematodes, echi- nomerms, mollusks, dinoflagellate and bacteria. The occurrence and distribution of TTX among a wide variety of organisms gave rise to speculation that TTX and its derivatives originated from symbiotic microorganisms. Indeed, a number of bacteria have been shown to produce TTX including the genera *Aeromonas* and *Alteromonas*, *Escherichia coli*, *oto bacterium phosphoreum*, *Plesiomonas shigelloides*, *Pseudomonas* sp, and some *Vibrio* sp. (reviewed in refs. 2–4).

Site-directed mutagenesis experiments,\textsuperscript{27,30} photo affinity labeling\textsuperscript{47,48} and molecular modeling studies\textsuperscript{49,50} suggested that...
Adaptive Evolution of an Elevated Resistance to TTX in NaV

There are several advantages for organisms to carry TTX. The toxin offers an excellent defense mechanism against potential predators. Puffer fish, for example, have no known predators other than humans.63 Conversely, TTX-resistance enables organisms to selectively feed on tetrodotoxic organisms. Some populations of the garter snake Thamnophis sirtalis feed on tetrodotoxic newts of the genus Taricha.64 TTX has been shown to function as a male-attracting pheromone at the time of spawning. Study of the puffer fish Fugu niphobles has shown that ovulated oocytes release TTX from the vitelline membrane and attracts males, but not females, to the spawning grounds, thereby increasing the chances of fertilization.65 Studying the adaptive evolution of TTX-resistance in various organisms has provided an avenue to determine what changes are possible in a highly conserved portion of the NaV that continue to permit proper channel function. Cloning and sequencing of the cDNA of TTX-resistant channels has allowed us to observe what adaptive molecular changes have occurred in these channels. Studies have revealed that elevated resistance to TTX is due to point and multiple site mutations in the pore region of NaV.4-10

TTX Resistance in Puffer Fish

It is well known that puffer fish generally carry a high concentration of TTX without any adverse effects. Puffers contain TTX mainly on skin and some internal organs, especially liver and gonads. The toxicity of puffer fishes varies among different species and depends on various factors such as seasonal, individual and local variations.6 It is proposed that TTX-resistance channels first arose in the NaV of an early tetrodontid ancestor before diversification of the Tetraodontidae.9 Yotsu-Yamashita and coworkers reported the genetic basis of TTX-resistant Fugu paradiisalis arises from the substitution of the non-aromatic amino acid Asn for an aromatic amino acid (Tyr or Phe) at 401 in the DI P-loop of NaV1.4a.6 This site of mutation was also found in the NaV1.4a of Takifugu rubripes, Tetradon nigroviridis and Arubron nigropunctatus and in four different genes of puffer fish sodium channels (NaV1.1La, NaV1.5La, NaV1.5Lb and NaV1.6b).4,7 This substitution of aromatic amino acid to non-aromatic amino acid at DI causes up to a 3,000-fold increase in TTX-resistance. Differential TTX-resistance might be determined by the side chain length in the non-aromatic amino acid residue. In addition to the substitution, replacement of Thr-759-Ser (or Asn) was reported in NaV1.4a of Arubron nigropunctatus and Tetradon nigroviridis and NaV1.6b of Cantigaster solandri.9 Mutation at Thr-759 might induce a regional allosteric effect on Glu-758, a residue forming the outer ring, which could produce additional TTX-resistance from 2-fold up to 2,000 fold.55 Mutation at Glu-758-Asp was reported in the NaV1.6b of Tetradon nigroviridis.7 Various other amino acid substitutions in the pore region that confer TTX-resistance have also been reported in different species of puffer fish, including Met-1240-Thr in DIII, and Ala-1529-Gly, Ile-1561-Met, Asp-1568-Asn and Gly-1569-Thr in DIV.9 These findings are good examples of parallel evolution of TTX-resistance in puffer fish.

STX Resistance in Soft-Shell Clams

Saxitoxins (STX) are a family of water-soluble neurotoxins, which also block sodium channels, and a similar mechanism of action has been proposed as for TTX.66 STX selectively binds to the same amino acid residues in the outer pore loops of NaV and occludes the channel pore. STX has a molecular skeleton structurally similar to TTX but with an additional positive guanidinium group and this structural difference of STX causes different binding affinities to NaV.31,50 STX and its analogs are collectively called paralytic shellfish toxins (PST) and are considered to cause lethal paralytic shellfish poisoning (PSP). STX-producing dinoflagellates cause harmful algal blooms (red tides) and bivalve mollusks, which are the primary vectors of PSP in humans, exposed to such algal blooms accumulate PSP in their tissue.11 Some populations of soft-shell clams, Mya arenaria, distributed in the Atlantic North America, from the Gulf of St Lawrence to Chesapeake Bay, were found to carry STX, and amino acid sequence analysis reveals that STX-resistance derives from an amino acid substitution at Glu-758-Asp in the DI of NaV.11 This substitution confers a 1,500-fold increase in STX-resistance and 3,000-fold increase in TTX-resistance.11 This genetic adaptation to the harmful algal blooms permits increased survivorship of STX-resistant soft-shell clams, with reduced fitness of STX-sensitive individuals. Interestingly, identical DII Glu-758-Asp substitution was also observed in NaV1.4b of Tetradon nigroviridis.7 These findings suggest sodium channels have the potential to develop neurotoxin resistance by single amino acid substitutions, and that substitutions in NaV from completely different organisms result in the same molecular evolution under similar selective pressures.

TTX-Resistance in Garter Snake

Geffeney and coworkers studied four different garter snakes of the species Thamnophis sirtalis acquired from different geographical locations in the western United States: Bear Lake, Idaho; Warrenton...
and Benton County, Oregon; and Willow Creek, California.\textsuperscript{10,67} Three of these populations have co-evolved with the TTX-toxic newts \textit{Taricha granulosa}, and phylogenetic information indicates that their TTX-resistance has evolved independently.\textsuperscript{64} TTX-resistance in these populations was observed by measuring the concentration of TTX required to block AP propagation in the skeletal muscle.\textsuperscript{67} Garter snakes of Bear Lake, Idaho are the least TTX-resistance (1.0 x 10^{-7} M TTX), Warrenton and Benton, Idaho are intermediate in TTX-resistance (5.0 x 10^{-7} M TTX and 17.5 x 10^{-7} M TTX, respectively) and the most TTX-resistant Willow Creek snakes required 1.0 x 10^{-5} M TTX to block AP propagation. Similar to these findings, Brodie and coworkers reported that garter snake populations from California (Willow Creek, San Mateo, East Bay and Omo) showed greatest organismic TTX-resistance, individuals from Oregon showed intermediate TTX-resistance (Warrenton, Benton and Tenmile) and Idaho had least TTX-resistant garter snake populations (Bear Lake, Latah and Selway).\textsuperscript{64} These findings showed geographic differentiation in resistance to TTX within species. TTX-resistance in garter snakes was also related to the expression of TTX-resistant \textit{Thamnophis sirtalis} Na\textsubscript{v}1.4 (tsNa\textsubscript{v}1.4) in the skeletal muscle.\textsuperscript{10} Mutations of genes that encode tsNa\textsubscript{v}1.4 of garter snakes underlie TTX-resistance. The functional expression of tsNa\textsubscript{v}1.4 revealed that the resistance to TTX was a consequence of substitutions of several amino acids in the DIV pore region.\textsuperscript{10} Warrenton, Benton and Willow Creek populations shared amino acid substitution in which isoleucine is replaced by valine at 1561 in DIV.\textsuperscript{10} The substitution occurs within the pore helix and may consequently alter the pore structure, thereby interrupting TTX binding affinity. This common substitution in geographically isolated populations within the same snake species might represent parallel evolution. Sodium channels in the Benton County populations have an additional substitution at Gly-1566-Ala, and tsNa\textsubscript{v}1.4 of Willow Creek garter snake possesses two more substitutions, Asp-1568-Asn and Gly-1569-Val. These additional mutations are proportional to organismic, action potentials and channel TTX-resistance.\textsuperscript{10,67} How the substitutions affect TTX binding is unknown. Similar patterns were observed in puffer fish Na\textsubscript{v}1.4, including \textit{Takifugu rubripes}, \textit{Tetraodon nigroviridis} and Canthigaster solandri. Additional mutations at the homologous Gly-1569 were observed to contribute significant TTX-resistance.\textsuperscript{6-10} These findings suggest multiple amino acid substitutions impart greater neurotoxin resistance in sodium channels, and that similar substitutions occur in widely divergent organisms.

The geographic, expression and mutational differences between TTX-resistant garter snake populations could result from differences in availability of the nontoxic prey and/or TTX content on the skin of the toxic prey. Although an increased number of amino acid substitutions that cause greater TTX-resistance in the garter snake populations are beneficial for their fitness for preying on toxic newts, the substitutions may also cause negative impacts on survival, since more resistant snakes have a slower maximum run speed.\textsuperscript{68} Run speed is determined, at least in part, by the influx of sodium ions through Na\textsubscript{v}1.4 that generates and propagates APs in skeletal muscle. It has been reported that TTX-resistant Na\textsubscript{v} ions tend to have lower conductance, slower kinetics and a more positive current-voltage relationship than TTX-sensitive isoforms.\textsuperscript{61} These data suggest the amino acid substitutions in TTX-resistant snakes may have biophysical sequelae resulting in slower maximum run speeds. A more complete assessment of how amino acid substitutions differentially affects the selectivity, permeability and gating of the channel is required. Interestingly, substitutions found in the most TTX-resistant Willow Creek populations of garter snake, Ile-1561-Val and Asp-1568-Asn, were also observed in the DIV of T-type Ca\textsuperscript{2+} 3.3 channel, and permitted TTX to directly interact with the channel, blocking ion permeation.\textsuperscript{59}

Future Studies

How is sodium channel function affected by the amino substitutions that impart TTX resistance? This question is being addressed by our present investigations into sodium channel function in chimeric channels carrying the amino acid substitutions that confer TTX resistance in \textit{Thamnophis} garter snakes. Since the pore region is associated with channel gating and selectivity, and since the amino acid substitutions are predicted to alter channel structure sufficiently to impair TTX binding, it seems reasonable to speculate that selectivity, permeability and gating may be similarly affected. Our present experiments are designed to test this idea. The answers to this question will help us understand whether there are biophysical, and perhaps organismal, tradeoffs for the advantage of resistance to TTX.

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