Protein Time Machine:

Creating an Ancestral Voltage Gated Sodium Channel of *Thamnophis sirtalis*

College of Science
UtahStateUniversity

Jennie Cordingley, Nicole Cook, Kaleb Hamann, Shauntel Johnson *Utah State University* | Drs S. Geffeney & C. Hanifin *Utah State University*

Introduction

Predator-prey interactions between garter snake predators (*Thamnophis sirtalis*) and tetrodotoxin (TTX) bearing prey (*Taricha* newts) have generated extreme TTX-toxicity in newts and extreme TTX-resistance in snakes. Tetrodotoxin resistance in snakes results from the expression of TTX-resistant voltage-gated sodium channels (VGSCs). Although extensive work has identified the specific amino-acid changes that make these proteins TTX-resistant, the evolutionary history or "adaptive walk" associated with TTX-resistance in snakes is poorly understood. Fully assessing this history requires replicating the last 50,000 years of molecular evolution in snake VGSCs using modern electrophysiology and modelling.

Here we report a "protein time machine" solution to the question of the evolution of TTX resistance in *T. sirtalis*. We estimated the putative sequence of the VGSC expressed in the muscle tissue of the ancestor of modern *T. sirtalis* (TsAnc). We modelled the interactions of TsAnc with TTX and generated a synthetic expressible form of the gene. This approach will allow us to directly measure the functional effects of changes as they likely occurred in snakes over the last 50,000 years.

Photo Credit: Richard Greene Om Nom Newt

Methods

We used a phylogenetic analysis and ancestral sequence reconstruction (ASR) to estimate the amino acid sequence of TsAnc (Geffeney et. al. in prep). We estimated binding energy of TTX and hydrogen bond formation with TsAnc using Swiss Model, Autodock Vina, UCSF Chimera, and Pymol.

Construction of the TsAnc expressible clone followed the methods outlined in Geffeney and Hanifin (2020). We used the predicted amino acid sequence of TsAnc to generate codon-optimized cDNA and four synthetic oligonucleotide blocks (1500 bp each) These were assembled with a linearized PGEM HE *Xenopus* oocyte expression vector using standard isothermal Gibson assembly cloning (NEB HIFI). We used standard protocols to transform reaction products and screen for positive colonies using commercial Sanger sequencing.

Results

Estimates of TTX binding energy and hydrogen bond formation with TTX for TsAnc and TTX-sensitive snake VGSCs were comparable suggesting that TsAnc is TTX-sensitive and represents an appropriate genetic background to examine the evolutionary history of snake TTX-resistance.

Gene synthesis and assembly generated five positive clones. Sequential restriction digest analysis and insert sequencing (minimum 2X) ultimately identified a single plasmid that included the correct sequence for TsAnc as well as all required regulatory sequences for in vitro mRNA synthesis.

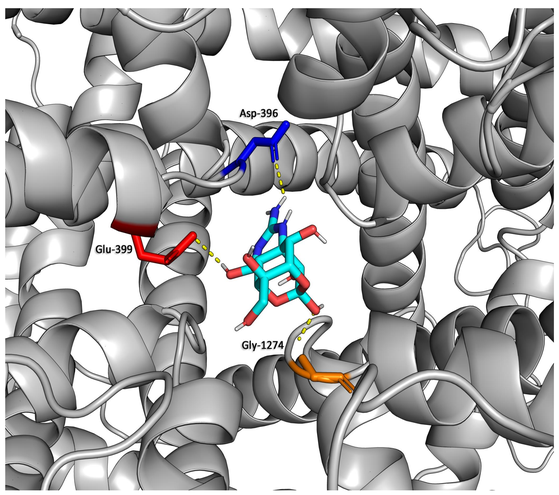

Figure 2: Model structure of TsAnc with TTX in the pore. TTX-binding energy estimates (7.4 kCal/mol) and hydrogen bond formation with TTX (3 bonds) are comparable with known TTX-sensitive VGSCs. Yellow dashed lines represent hydrogen bonds.

Highlighted node is the location of the ancestral sequence within the phylogenetic tree of *T. sirlatis*

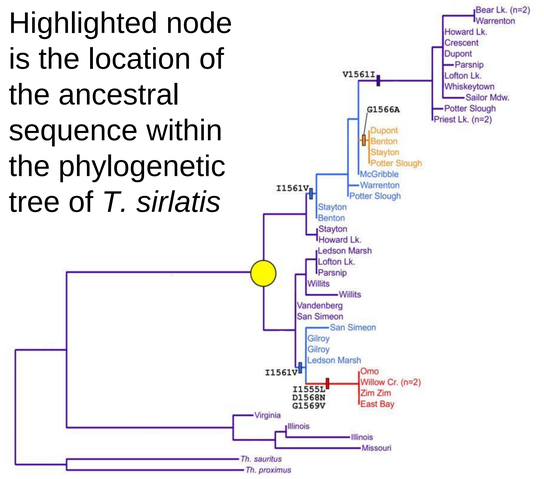

Figure 1: Phylogenetic tree of T.sirtalis modified from Hauge et al. Tree shows the progression of mutations within the T. sirtalis sequence leading to different levels of resistance to tetrodotoxin. Amino acid sequences listed at each node.

Conclusions

Work reported here demonstrates that the skeletal muscle VGSC present in the ancestor of modern western *Thamnophis sirtalis* was likely TTX sensitive. Models of the channel suggest that our predicted gene sequence likely encodes a functional VGSC protein that can be expressed. Gibson assembly cloning produced an expression vector that includes a modified form of this gene that is optimized for use in *Xenopus* oocyte expression. These results, in turn, will allow us to examine the "adaptive walk" that generated extreme TTX-resistance in some populations of *T. sirtalis* over the last 50,000 years.

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