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Convergent and parallel evolution in a voltage-gated sodium channel underlies TTX-resistance in the Greater Blue-ringed Octopus: *Hapalochlaena lunulata*

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ABSTRACT

The natural history and pharmacology of tetrodotoxin (TTX) has long intrigued biologists. This toxin has a remarkable distribution that spans two domains of life (Bacteria and Eukarya). Within Eukaryotes, TTX has only been identified in animals but is known to be present in over five-dozen species of phylogenetically distant Metazoans. Despite decades of work, the origin and biosynthetic pathways of TTX remain unresolved. Investigations in puffer fishes and salamanders have provided insights into the acquisition of auto-resistance to TTX through the evolution of voltage-gated sodium ion channels (VGSCs) that have reduced binding affinity for TTX. To date there have been no studies of these proteins in tetrodotoxic Blue-Ringed Octopuses. Here we report data demonstrating that the Greater Blue-ringed Octopus (*Hapalochlaena lunulata*) expresses a VGSC ($HlNa_V1$) gene with mutations that reduce the channel's TTX-binding affinity and likely render the organism TTX resistant. We identified three amino-acid substitutions in the TTX-binding site of $HlNa_V1$ that likely confer TTX-resistance to both the channel and the organism. These substitutions are associated with organismal TTX-resistance in other TTX-bearing taxa and are convergent with substitutions that have evolved in fish, salamanders, and some TTX-resistant invertebrates.

1. Introduction

A wide variety of animal species produce or sequester secondary metabolites that play important roles in their natural history (see Bornancin et al., 2017; Nishida, 2014; Savitzky et al., 2012 for reviews). Toxins represent a common class of these natural products and toxin use is remarkably prevalent across a diverse array of phyla given the potential for negative physiological consequences for species that use them (e.g., Forbey et al., 2009; Riley and Wertz, 2002). One particularly interesting case centers on the neurotoxin tetrodotoxin (TTX) and the evolution of its sodium channel targets.

Tetrodotoxin is a potent neurotoxin with a broad distribution in Metazoans. This toxin has been documented in well over 60 species representing 8 phyla, including Chordata, Echinodermata, Mollusca, Arthropoda, Chaetognatha, Annelida, Platyhelminthes, and Nemertea (see Noguchi and Arakawa, 2008 for review). The ecological role of TTX in many of these species is poorly understood but work in a number of organisms suggests that TTX is primarily used as a defense against predators and parasites at multiple life-history stages (Brodie, 1968; Brodie et al., 1974; Calhoun et al., 2017; Gall et al., 2012; Hanifin et al., 2003, 2008; Itoi et al., 2016, 2014; Johnson et al., 2018; Sakakura et al., 2017; Stokes et al., 2014; Williams et al., 2010, 2011b). In some species, including Blue-ringed Octopuses, TTX may also function as venom to enhance prey capture (e.g., Ritson-Williams et al., 2006; Salvitti et al., 2015; Sheumack et al., 1978; Stokes et al., 2014; Williams and Caldwell, 2009).

The origin and biosynthesis of TTX in Metazoans has intrigued biologists since the discovery that puffer fish (Tetraodontidae) and newts of the genus *Taricha* possessed chemically identical toxins (Brown and Mosher, 1963; Buchwald et al., 1964; Goto et al., 1965; Tsuda et al., 1964; Woodward, 1964). Although TTX has been identified in a wide array of ecologically disparate taxa, decades of research have still not identified the biosynthetic pathways underlying TTX production in either Bacteria or Metazoans. Indeed, the very origin of TTX

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used by any metazoan is still debated (Chau et al., 2011; Hanifin, 2010; Kudo et al., 2017, 2015; 2014; Mebs et al., 2019; Ueyama et al., 2018; Yotsu-Yamashita et al., 2012; Yotsu-Yamashita and Tateki, 2010). Although questions about the source of TTX in Metazoans have complicated examination of the evolution and ecology of TTX in TTX-bearing species, examinations of the physiology and genetic basis of resistance to TTX in these species has provided important insights into the origin of auto resistance to TTX. In TTX-bearing salamanders, the evolutionary history of organismal TTX-resistance has demonstrated that the TTX-bearing phenotype is ancient and likely a synapomorphy associated with the Modern Newt Clade (Hanifin and Gilly, 2015). In puffer fishes, examination of the molecular basis of TTX-resistance suggests that TTX-resistance and the TTX-bearing phenotype have evolved through multiple and repeated evolutionary pathways (Jost et al., 2008).

Characterizing resistance in TTX-bearing species is straightforward because the mode and action of TTX is well understood (Furukawa et al., 1959; Hille, 2001, 1975; Moore et al., 1967; Narahashi, 1974; Narahashi et al., 1964). Tetrodotoxin selectively binds to and blocks the ion-conducting pore of voltage-gated sodium ion channels (VGSCs). These channels are responsible for the initiation and propagation of action potentials in neurons and many muscle cells. Action potential generation by VGSCs results from the rapid influx of sodium ions through the ion-conducting pore (Catterall, 2012; Hodgkin and Huxley, 1952). Tetrodotoxin binds to the outer portion of this pore and occludes it, thereby preventing sodium ion influx (Narahashi et al., 1964). In all but one of the species that are known to be TTX-resistant, organismal TTX-resistance results from amino acid substitutions in the TTX-binding site of VGSCs that dramatically reduce the binding of TTX to the channel pore (Feldman et al., 2016; Toledo et al., 2016).

The binding site of TTX in the pore is well characterized (Choudhary et al., 2003; Santarelli et al., 2007; Shen et al., 2018, 2019; Terlau et al., 1991). Metazoan VGSC alpha-subunits are formed from four homologous protein domains (Noda and Numa, 1987). The outer pore of the channel is formed by the linker region between two transmembrane helices from each of these domains. The four linkers include amino acids that form the ion selectivity filter, the TTX-binding site, and other structures that modulate channel activity (Capes et al., 2012; Cervenka et al., 2010; Favre et al., 1996; Huang et al., 2000; Otagiri et al., 2008; Sun et al., 1997; Terlau et al., 1991; Todt et al., 1999; Vilin et al., 2001). Two highly-conserved amino acid motifs, DEKA and EEMD (EEID in invertebrates) form two rings of mostly negatively charged amino acids at the opening of the pore (Du et al., 2009; Terlau et al., 1991). Amino acids in the DEKA motif form the selectivity filter and altering amino acids in this motif disrupts sodium ion selectivity and permeability as well as TTX-binding (Favre et al., 1996; Huang et al., 2000; Sun et al., 1997; Terlau et al., 1991). Tetrodotoxin binds to a highly conserved aromatic amino acid positioned one residue downstream of the domain I aspartic acid (D) in the DEKA motif (Santarelli et al., 2007; Shen et al., 2018). Altering amino acids in the EEMD motif also alters ion permeability and TTX binding (Choudhary et al., 2003; Terlau et al., 1991). Tetrodotoxin binds directly to the aspartic acid (D) in the EEMD ring (Choudhary et al., 2003; Shen et al., 2018). Substitutions associated with the adaptive evolution of TTX-resistance show remarkable convergence and parallel evolution across phyla and are typically at positions in or near residues in either the DEKA or EEMD motif. These repeated patterns of substitutions suggest that the evolutionary pathway to TTX-resistance may be highly constrained across species (Toledo et al., 2016).

Blue-ringed Octopuses (genus: *Hapalochlaena*) have long been known to possess TTX (Sheumack et al., 1978) and extensive work on the distribution and ecology of TTX in this genus suggests that it plays an important role in the natural history of the group (Asakawa et al., 2019; Hwang et al., 1989; Sheumack et al., 1978, 1984; Williams et al., 2011a, 2011b; Williams and Caldwell, 2009, 2012; Yotsu-Yamashita et al., 2007). As with other taxa, the origin and evolutionary history of TTX in *Hapalochlaena* is still poorly understood. Whitelaw reviewed what little is known of TTX acquisition in this group but there have been no studies of *Hapalochlaena* VGSCs (Whitelaw et al., 2019). Arthropods and Mollusks appear to have two VGSC orthologs: Na_V1 and Na_V2 (Liebeskind et al., 2011; Moran et al., 2015; Zhou et al., 2004). One of these (Na_V1) includes insect *para* channels (Dong et al., 2014) and is homologous to the entire family of VGSCs found in vertebrates (Liebeskind et al., 2011; Moran et al., 2015; Zakon, 2012). Studies of squid giant axons have shown that Na_V1 is responsible for propagation of action potentials in Cephalopod neurons (Liu and Gilly, 1995; Rosenthal and Gilly, 2003). The other ortholog (Na_V2) is categorized as a VGSC based on sequence and expression patterns but is permeable to Ca^{2+}, insensitive to TTX, and does not appear to propagate action potentials in any group but Cnidarians (Anderson, 1987; Gur Barzilai et al., 2012; Spafford et al., 1996). This physiological background suggests that evolution of TTX-resistance in Na_V1 orthologs of Blue-Ringed Octopuses is likely a critical component of the TTX-bearing phenotype in this genus.

This study examined the VGSCs of a single species of TTX-bearing octopus, the Greater Blue-ringed Octopus: *H. lunulata* as well as a sympatric species, the Pacific Brown Octopus (*Abdopus aculeatus*), that is not known to possess TTX. We used a combination of transcriptomics and direct sequencing of expressed VGSCs to gain insight into the physiological and mechanistic basis of TTX-resistance in *H. lunulata*. We found that organismal resistance in this species likely arises from a combination of amino acid substitutions in the TTX-binding site of the primary voltage-gated sodium channel of *H. lunulata* that are convergent (functionally homologous but divergent) or parallel (identical) with described TTX-resistant voltage-gated sodium channels from other metazoans that either possess or are resistant to TTX. We identify both Our results add to a body of literature documenting a striking degree of convergent and parallel evolution in Metazoan VGSCs associated with TTX-resistance.

2. Materials and Methods

2.1. Samples

We investigated the structure of VGSCs present in two species of octopuses from the eastern portion of the Indo-Pacific (i.e. Greater Indonesia). The Greater Blue-ringed Octopus (*Hapalochlaena lunulata*) and the Pacific Brown Octopus (*Abdopus aculeatus*). These two species are sympatric but only *H. lunulata* is known to possess TTX. Live animals (two for each species) were obtained through the commercial pet trade and shipped to Utah State University. Animals were held briefly and euthanized in compliance with IACUC protocols for work with invertebrates at Utah State University.

2.2. Sample preparation and sequencing

Immediately following death, stellate ganglia and optic lobes were surgically removed and total RNA extracted. We used either an RNeasy Mini or Micro kit (Qiagen) to extract total RNA from each individual ganglion or optic lobe. RNA was resuspended in RNase-free water. A portion of this total RNA was used for RNAseq analysis and a portion was saved at −80 °C for later rtPCR analysis. Total RNA used for transcriptomes was treated with DNase (University of Rochester Genomics Research Center (URGRC)) and RNA integrity checked with an Agilent 2100 Bioanalyzer. We used four samples for transcriptome sequencing: HL0714-230SG (RIN > 8), HL0714-230OL (RIN > 4), AA714-233OL (RIN N/A), and AA714-232SG (RN N/A). All libraries and sequencing were completed at the URGRC. Libraries from each of the four samples were generated using poly-A selection with the Illumina TruSeq Stranded mRNA library preparation kit LT. We pooled libraries prior to sequencing. These samples were then sequenced using the Illumina HiSeq 2500 Rapid Run flow cell with Rapid SBS reagents. All samples were sequenced using 2 × 100 bp paired-end sequencing

cycles on a single lane producing 154, 263, 318 read pairs. Base-calling was done by Illumina Real Time Analysis (RTA) and RTA output was demultiplexed and converted to FastQ format. Final transcriptome data are based only on a single animal for each species because of challenges associated with RNA quality.

2.3. Transcriptome processing, assembly, and annotation

Post-sequencing processing and assembly were completed in the Rosenthal lab at the University of Puerto Rico's Recinto de Ciencias Medicas with additional analysis (MAB) at the Marine Biological Laboratory (MBL). Sequencing data were pooled for each species. Data went through two stages of read trimming: first quality-based trimming using Trimmomatic v0.33 (Bolger et al., 2014) was performed. A sliding window of 4 bp and trimming threshold of phred score equal to 2 were chosen to maximize sensitivity. This process was followed by K-mer spectral analysis to remove low abundance k-mers using "filter-abund.py-V" from the Khmer 2.0 package (Crusoe et al., 2015). FastQC v0.11.3 was used to check data quality before and after trimming (Heiman et al., 2014). After filtering, the remaining 150, 155, 243 high-quality read pairs were used for *de novo* transcriptome assembly using Trinity v6.0.2 (Grabherr et al., 2011) producing 166,402 and 139,657 transcripts for *H. lunulata* and *A. aculeatus,* respectively. Seq-Clean was used to trim poly-A tails and remove low complexity sequences. For functional annotation, assembled transcripts were blasted against the Swiss-Prot database and best hits with *E*-values less than 1 x $e10^{-3}$ were selected. Assembly statistics are provided in the Supplemental Materials.

2.4. Identification and sequence confirmation of VGSC genes and $HlNa_V1$

Voltage-gated sodium channel alpha-subunit transcripts were identified in the annotated transcriptome manually and by comparison of BLAST results. All transcripts identified as VGSCs were aligned using Clustal Ω and were placed in a simplified gene tree using the UPGMA tool in simple phylogeny (Clustal Ω). This preliminary analysis confirmed the presence of two different VGSC genes in both species. A general examination of these octopus VGSC orthologs showed that they are broadly similar in terms of structure and, likely, function to other invertebrate VGSCs. Each included the typical structure composed of four homologous domains with clearly identifiable pore regions as well as the other functional regions associated with metazoan VGSCs (e.g., voltage sensors, inactivation gate, and a sodium selectivity filter). An initial examination using the DEKA-DEEA polymorphism that differentiates invertebrate Na_V1 and Na_V2 channels followed by alignments using Codoncode v6.02 allowed us to identify the presence of complete ORFs as well as any sequence variation associated with different transcripts and orthologs. The final sequence for $HlNa_V1$ was then confirmed using 3′ and 5′ rapid amplification of cDNA ends (RACE) and reverse transcriptase PCR (rtPCR). We generated both 5′ and 3′ cDNA from reserved HL714-231 stellate ganglia RNA using the SMARTer RACE 5'/3′ kit (Clontech). Overlapping gene-specific primers were designed using the built-in primer design function in Codoncode. These primers amplified portions of both the 5′ and 3' untranslated regions (UTRs) of the gene as well as the complete coding sequence of the channel. We used PCR (Takara ExHotstart TAQ) with a touchdown protocol to generate 20 overlapping amplicons that covered the entire $HlNa_V1$ gene. Overlapping regions and additional PCR amplicons gave us at least 6X coverage of the pore regions of the channel and a minimum of 2x coverage in all other regions of the gene. Primer sequences for $HlNa_V1$ as well as PCR protocols are provided in Supplemental Materials.

For the three orthologs ($HlNa_V2$, $AaNa_V1$, and $AaNa_V2$) that were not independently confirmed by PCR, read alignment scores using Bowtie 2 (Langmead and Salzberg, 2012) and per-residue read depth coverage were calculated for the consensus amino acid sequences to quantify the read support for the assembled transcripts (see Supplemental Materials).

2.5. Gene identification and orthology

We established the evolutionary relationships and gene orthology of the four VGSCs identified above using both Maximum Likelihood and Bayesian analyses. We converted cDNA sequences from our transcriptome data and rtPCR data to protein sequences using either CodonCode or ExPASy (https://www.expasy.org/). We obtained additional invertebrate and vertebrate VGSC protein sequences from either Genbank or the Joint Genome Institute protein database (JGI; https://jgi.doe.gov/) to build a gene tree that integrated our samples with previously identified VGSC orthologs from both invertebrates (both Na_V1 and Na_V2) and vertebrates. We included nine VGSC $Na_V1.x$ orthologs (Na_V 1.1–1.9) from each of two vertebrates (*Homo sapiens* and *Rattus norvegicus*) and known invertebrate Na_V1 orthologs from eight species. Invertebrate Na_V1 orthologs included *para* channels from insects as well as cephalopod Na_V1 sequences. We included Na_V2 sequences from five species of invertebrates. A table of species and Genbank accession numbers are provided in the Supplemental Materials. *Hapalochlaena lunulata* and *A. aculeatus* VGSC sequences are archived at Genbank (accession numbers: MN384671-MN384676). Protein sequences were aligned using MUSCLE (CIPRES; (Miller et al., 2010); and then trimmed using the gappyout algorithm in trimAI (Phylemon2; Sánchez et al., 2011). We used Modeltest (CIPRES) to determine the best evolutionary model for our data. Trimmed alignments were then analyzed using both RAxML (Stamatakis, 2014); Maximum-likelihood) and MrBayes (Huelsenbeck and Ronquist, 2001; Bayesian). We used a general 'variable time' (VT) matrix (Müller and Vingron, 2000) model for both Bayesian and Maximum-likelihood (ML) analyses with *Drosophila melanogaster* voltage-gated calcium channel (DmCa1) set as the outgroup sequence. Bootstraps were set to 1000 for ML analyses. Four Markov chains of 5,000,000 generations were run at the default temperature setting (0.2) with every 1000th tree save to a file for Bayesian analysis. Burn in was set at the default value (0.25) and a majority rule consensus was calculated to estimate posterior probabilities in MrBayes. Tree topologies for both analyses were congruent when DmCa1 was used as the outgroup sequence.

3. Results and discussion

We identified two distinct VGSC orthologs, Na_V1 and Na_V2, expressed in both the stellate ganglia and optic lobe of *Hapalochlaena lunulata* and *Abdopus aculeatus* (Fig. 1). Predicted ortholog sequences were well supported by the reads as demonstrated by high alignment scores and mean per-residue read depth coverage exceeding 110 reads for all four orthologs (Supplementary Materials). Three key amino acid substitutions in the TTX-binding site of the *H. lunulata* Na_V1 ortholog $HlNa_V1$ (I1406T, D1699H, and G1700S) are likely associated with reduced TTX-binding affinity of $HlNa_V1$ (Fig. 2). These substitutions occur in a region (and at amino acid positions) of the channel associated with TTX-binding and are absent in the pore of *Abdopus aculeatus* Na_V1 ($AaNa_V1$) as well as Na_V1 channels from other TTX-sensitive invertebrates (Fig. 2). Furthermore, natural studies of TTX-resistant species and experimental manipulations of Na_V1 provide extensive evidence that amino acid replacements at these positions contribute to adaptive TTX-resistance in multiple taxa (see below and Fig. 2).

Two substitutions located in the EEM(I)D motif of $HlNa_V1$ (I1406T in domain III and D1699H in domain IV), likely render $HlNa_V1$ TTX-insensitive (Fig. 2). Amino acid substitutions at these two positions can disrupt TTX-binding and are associated with TTX-resistance in other metazoans including TTX-resistant newts, snakes, and puffer fishes (Fig. 2; see Toledo et al., 2016 for review). An identical isoleucine to threonine replacement and a homologous aspartic acid to serine amino acid replacement in the EEM(I)D ring of a TTX-resistant Na_V1 channel

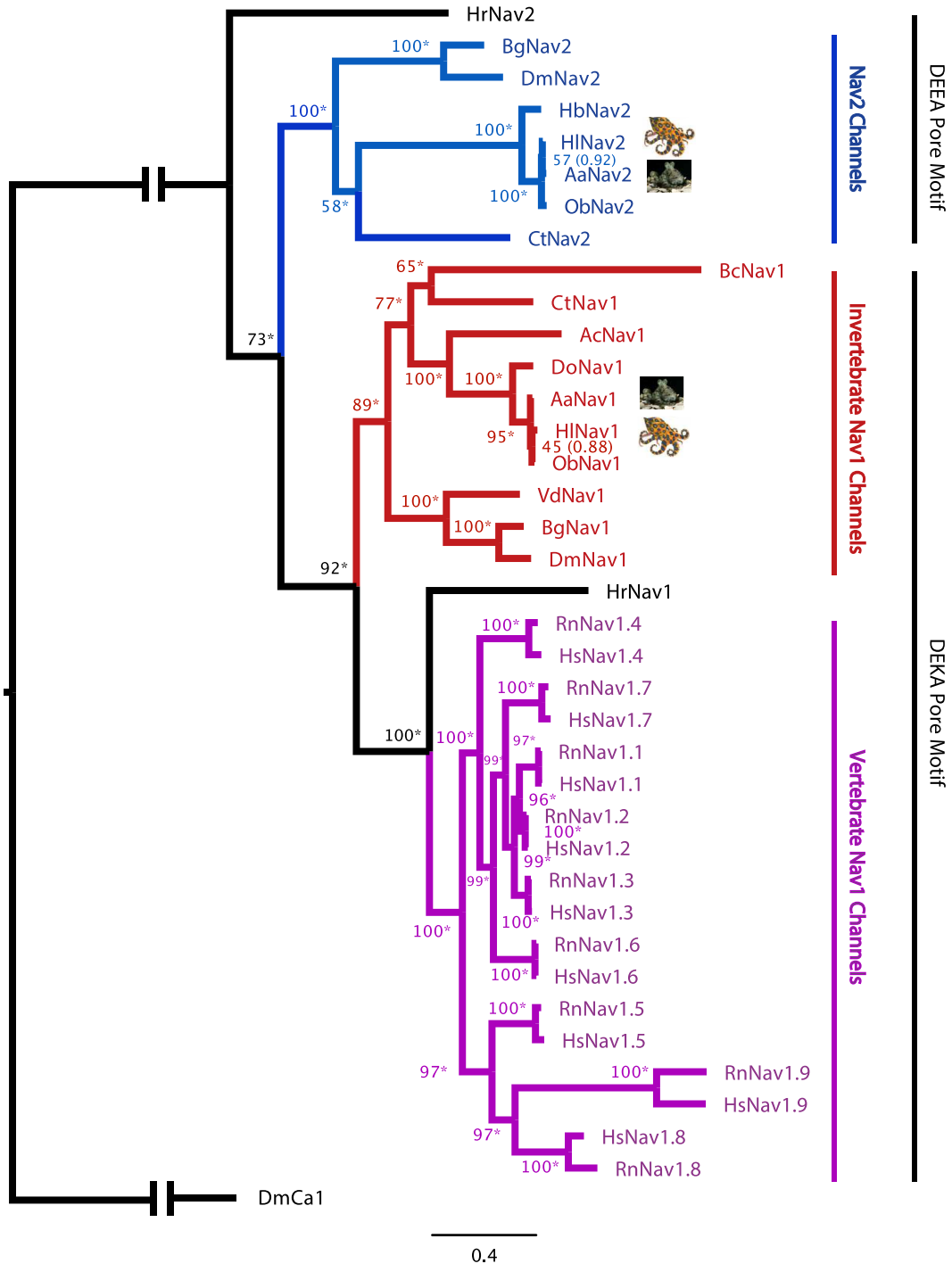

Fig. 1. Phylogeny of metazoan VGSCs demonstrating orthology of VGSCs identified in *Hapalochlaena lunulata* and *Abdopus aculeatus*. Orthologs of the invertebrate VGSC Na_V1 (in red) are present in both *H. lunulata* ($HlNa_V1$) and *A. aculeatus* ($AaNa_V1$). Orthologs of invertebrate Na_V2 (in blue) are present in *H. lunulata* and *A. aculeatus* ($HlNa_V2$ and $AaNa_V2$ respectively). Vertebrate VGSCs (Humans and Rat; in purple) show homology and a shared DEKA selectivity pore motif with invertebrate Na_V1 while Na_V2 orthologs possess a DEEA selectivity pore motif. Chordate VGSCs (Tunicate; $HrNa_V1$ and $HrNa_V2$) are shown in black and included to clarify the evolutionary history of Na_V1 and Na_V2. Tree topology is based on a maximum-likelihood analysis using a general VT model. Percent bootstrap support out of 1000 are shown for each node. A Bayesian analysis returned a similar topology (see Materials and Methods sec. 2.5). Nodes marked with * had 100% posterior probability support, lower support is indicated in (). All sequences are predicted amino acids from cDNA. All sequences are predicted amino acids from cDNA. Accession numbers and species names are provided in Table S4 (Supplemental Materials).

($VdNa_V1$) from mites each decrease TTX-binding affinity by 10-fold (Du et al., 2009). In vertebrates, the EEM(I)D motif is characterized by a methionine in domain III. Substitutions of this methionine with threonine have been identified in at least 17 TTX-resistant vertebrate VGSCs and are associated with organismal TTX-resistance in multiple species of unrelated snakes, salamanders, and puffer fishes (Fig. 2; reviewed in Toledo et al., 2016) Replacements of the aspartic acid in domain IV demonstrate convergent patterns in vertebrates and substitutions at this position are associated with high levels of TTX-resistance in salamanders ($TgNa_V1.4$; D1431S; Hanifin and Gilly, 2015), and TTX-resistant garter snakes ($TsNa_V1.4$; D1574N; Geffeney et al., 2005).

The mode of action for each of these substitutions is likely shared

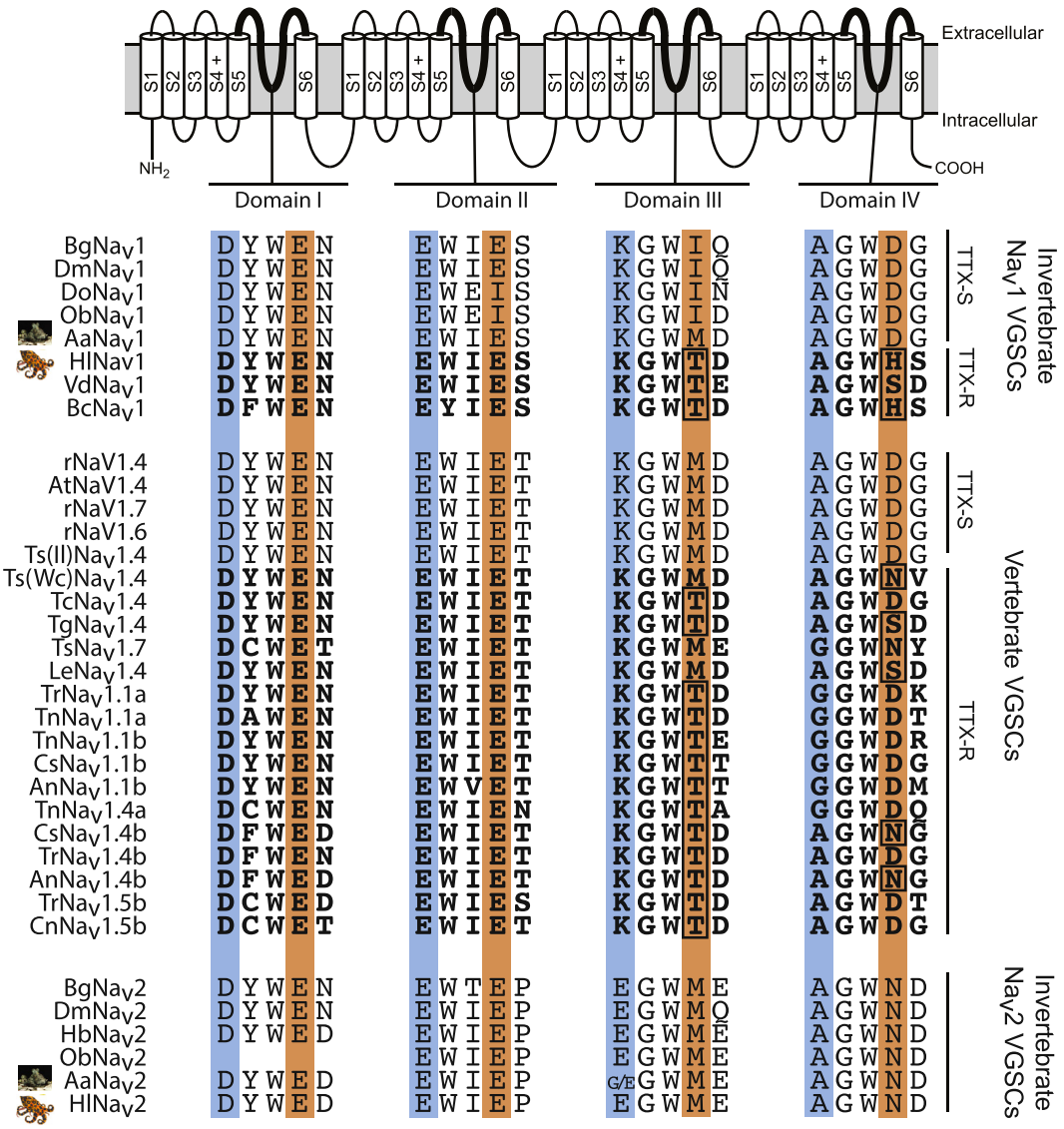

Fig. 2. Sequence Alignment and Comparison of Key Amino Acids Associated with TTX-binding and the Pore of $HlNa_V1$, $AaNa_V1$, as well as Select Metazoan VGSCs. The $Na_V1/1.x$ gene family is above and divided into TTX-sensitive (TTX-S) and TTX-resistant (TTX-R) groups (top). The DEKA and EE(M/I)D pore motifs are shaded (blue and orange respectively). Substitutions associated with TTX-resistance are boxed. Animals that are known to be TTX-resistant are in **bold.** Also included are $HlNa_V2$ and $AaNa_V2$ (below) with other invertebrate Na_V2 sequences (bottom). A diagram of a typical VGSC is included to identify the functional regions on the channel and is based on the vertebrate $Na_V1.x$ VGSC group. Accession numbers and species names are provided in Table S4 (Supplemental Materials). Because a clear domain I pore sequence for $ObNa_V2$ could not be identified it is not shown in this figure.

across TTX-resistant VGSCs. In $HlNa_V1$ and $VdNa_V1$, a hydrophobic isoleucine is replaced with a hydrophilic threonine in the domain III EEM(I)D motif (I1406T and I1699T respectively; Fig. 2). In TTX-resistant vertebrate VGSCs, replacement of a hydrophobic methionine in the EEM(I)D motif with threonine is likely functionally homologous (e.g. $TcNa_V1.4$, $TgNa_V1.4$, and $TnNa_V1.4a$; Fig. 2). In domain IV, a reduction in TTX-binding affinity likely results from disruption of a hydrogen bond or salt bridge between the aspartic acid in the EEM(I)D ring and TTX (Shen et al., 2018). Tetrodotoxin binds to the aspartic acid in the EEM(I)D motif and loss of an acidic side chain at this position disrupts TTX binding (Choudhary et al., 2003; Shen et al., 2018; Terlau et al., 1991). In $HlNa_V1$, D1699H replaces an acidic aspartic acid with a basic histidine (Fig. 2). Although the specific replacements associated with TTX-resistance in other species are polymorphic, they all result in a replacement of an acid side chain with either a neutral or basic amino acid (Fig. 2). In *Varroa* mites ($VdNa_V1$; Du et al., 2009), a flatworm ($BcNa_V1$; Du et al., 2009), toad-eating snakes ($LeNa_V1.4$; Feldman et al., 2012), and TTX-bearing salamanders ($TgNa_V1.4$; Hanifin and Gilly, 2015) the replacement is a polar, neutral serine (Fig. 2). In some pufferfish VGSCs ($CsNa_V1.4b$, $AnNa_V1.4b$; Jost et al., 2008), super-resistant garter snake muscle ($Ts(Wc)Na_V1.4$; Geffeney et al., 2005) and nerve ($TsNa_V1.7$; McGlothlin et al., 2016) channels, the replacement is an asparagine, which is polar and neutral (Fig. 2). In all of these cases a hydrogen bond/salt bridge is likely disrupted as a result of the loss of the aspartic acid.

Amino acid substitutions in the EEM(I)D motif of $HlNa_V1$ (i.e. I1406T and D1699H) do not have additive effects on TTX binding but instead are multiplicative when they occur together (Du et al., 2009). Single and double mutant analysis of I1674T and D1967S in $VdNa_V1$ demonstrate that the changes individually reduced TTX-binding affinity by 10-fold but channels including both have a 1000-fold reduction in TTX-binding (Du et al., 2009). Combinations of these two substitutions in the EEM(I)D motif are present in multiple species that possess TTX-resistant VGSCs and are associated with a "super" TTX-resistant phenotype in other vertebrate VGSCs including TTX-resistant newts ($TgNa_V1.4$; Hanifin and Gilly, 2015) and TTX-bearing puffer fishes

(CnNa$_V$1.4b and AnNa$_V$1.4b Jost et al., 2008); Fig. 2).

The functional effects of the G1700S replacement in domain IV are not well characterized but substitutions at this position are present in multiple TTX-resistant taxa that possess replacements at the upstream aspartic acid (e.g. mite VdNa$_V$1, newt TgNa$_V$1.4, and snake Ts(Wc) Na$_V$1.4; Fig. 2). In HlNa$_V$1, glycine is replaced with a larger, polar serine. In TTX-resistant mites (VdNa$_V$1) and TTX-resistant newts (TgNa$_V$1.4), glycine is replaced with a larger, acidic aspartic acid. In TTX-resistant snakes (TsNa$_V$1.4), the glycine is replaced with a larger, non-polar valine. This repeated pairing of substitutions in domain IV associated with the evolution of extreme TTX-resistance (e.g. D1574N and G1575V in Ts(Wc)NaV1.4; Fig. 2) suggests that D1699H-G1700S combination is important in HlNa$_V$1 but the specific functional contribution of the changes (e.g. TTX resistance or other functional properties) are, as yet, unclear.

We saw no substitutions in the pore DEKA motif associated with either Na$_V$1 ortholog from octopuses (HlNa$_V$1 and AaNa$_V$1; Fig. 2). These results are not surprising given how highly conserved these residues are in metazoan Na$_V$1 VGSCs. Sequence comparisons did document expected differences between the selectivity filter motif (DEKA vs. DEEA) of our two classes of orthologs (Na$_V$1 and Na$_V$2 respectively), with one exception (Fig. 2). HlNa$_V$2 possesses a glutamic acid residue in domain III and the *A. aculeatus* Na$_V$2 VGSC (AaNa$_V$2) appears to be polymorphic with both glycine and glutamic acid at this position. Although this sequence is based solely on transcriptome data and has not been confirmed through direct rtPCR, the residue in question is covered by 310 reads (Fig. S1). Of these, 29% contain adenosines (leading to glutamic acid) and 71% contain guanines (leading to glycine). Given the abundance of A-to-G mRNA editing activity in cephalopod nervous tissue (Liscovitch-Brauer et al., 2017), this may be an RNA editing site (see Supplemental Materials) that generates a glycine/glutamic acid polymorphism in the expressed channel. The importance of this polymorphism is unclear. Unlike Cnidarians (Anderson, 1987; Gur Barzilai et al., 2012; Spafford et al., 1996), Na$_V$2 does not appear to be responsible for action potential propagation in Cephalopod neurons (Liu and Gilly, 1995; Rosenthal and Gilly, 2003). In insects Na$_V$2 may play a role in regulating cell excitability in a small number of neuronal circuits (Liu and Gilly, 1995; Rosenthal and Gilly, 2003; Zhang et al., 2013), but this role has not been demonstrated in Cephalopods.

Alternative splicing is common in invertebrate VGSC channels and plays an important role in modulating the properties of excitable tissues in these taxa (Olson et al., 2008; Zhang et al., 2011). We saw some evidence of splice variants in the VGSC orthologs (both Na$_V$1 and Na$_V$2) present in our transcriptome data (data not shown). However, rtPCR data and alignments of transcriptome data provided no evidence of alternative splicing in the pore regions that form the TTX-binding site in any of our identified VGSCs. These results suggest that differential expression of splice variants of octopus VGSCs does not play a role in organismal resistance in *H. lunulata* or the evolution of the TTX-bearing phenotype in this species.

4. Conclusions

We identified three amino acid replacements in the VGSC ortholog, Na$_V$1, from the Greater Blue-ringed Octopus (*Hapalochlaena lunulata*) that likely render this channel highly resistant to TTX. Na$_V$1 is both structurally and functionally homologous to the VGSCs responsible for action potential propagation in the neurons of most Metazoans (the Na$_V$1.x gene family in vertebrates and Na$_V$1 in other invertebrates) suggesting that TTX-resistance in this channel likely confers organismal resistance to TTX in *H. lunulata*. Our results and patterns of convergence and parallelism associated with the evolution of TTX-resistance within the Na$_V$1 gene family suggest two additional dynamics. Firstly, that a core mix of channel functionality and constraints are applicable across Na$_V$1/1.x orthologs despite the deep evolutionary time that separates invertebrate Na$_V$1 VGSCs from vertebrate VGSCs. Secondly, that underlying functional similarities and constraints across metazoan VGSCs may facilitate the evolution of auto-resistance to TTX and, in turn, the evolution of the TTX-bearing phenotype in Metazoans.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2019.09.013.

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