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Investigation of the Pallid Bat's (*Antrozous pallidus*) Resistance to Scorpion Venoms

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Chapter Two of this thesis utilizes a technique called Constellation Pharmacology which was developed by the lab of Dr. Baldomero Olivera. I would like to thank Dr. Olivera and his lab for helping me set up and run their techniques for this thesis.

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Abstract

The pallid bat (Antrozous pallidus), a gleaning bat found in the western United States and Mexico, hunts a wide variety of ground-dwelling prey, including scorpions. Anecdotal evidence suggests that the pallid bat is resistant to scorpion venom, but no systematic study has been performed. Here we show with behavioral measures and direct injection of venom that the pallid bat is resistant to venom of the Arizona bark scorpion, Centruroides sculpturatus. Our results show that the pallid bat is stung multiple times during a hunt without any noticeable effect on behavior. In addition, direct injection of venom at mouse LD50 concentrations (1.5 mg/kg) has no effect on bat behavior. At the highest concentration tested (10 mg/kg), three out of four bats showed no effects. One of the four bats showed a transient effect suggesting that additional studies are required to identify potential regional variation in venom tolerance. Scorpion venom is a cocktail of toxins, some of which activate voltage-gated sodium ion channels, causing intense pain. Dorsal root ganglia (DRG) contain nociceptive neurons and are principal targets of scorpion venom toxins. To understand if mutations in specific ion channels contribute to venom resistance, a pallid bat DRG transcriptome was generated. As sodium channels are a major target of scorpion venom, we identified amino acid substitutions present in the pallid bat that may lead to venom resistance. Some of these substitutions are similar to corresponding amino acids in sodium channel isoforms responsible for reduced venom binding activity. The substitution found previously in the grasshopper mouse providing venom resistance to the bark scorpion is not present in the pallid bat, indicating a potentially novel

mechanism for venom resistance in the bat that remains to be identified. Taken together, these results indicate that the pallid bat is resistant to venom of the bark scorpion and altered sodium ion channel function may partly underlie such resistance.

Introduction

Animal venoms used for predation, defense and/or intraspecific competition are typically a complex mixture of toxins that can cause intense pain, tissue damage and death. Given the relative abundance of venomous species across phyla, it is not surprising that various predators and prey of venomous animals have developed resistance to one or more of these toxins [1–8]. There are two fundamentally important reasons for studying venom resistance. First, mechanisms of pain modulation can be identified with potential utility in human pain management. These studies will provide insights on how excitability of neurons can be adaptively modified by changes in ion channel sequences. Second, a comparison across species will provide insights into different mechanisms of venom resistance, including evolution of ion channel and receptor modifications and blood serum based mechanisms [1–8]. In this study, we present evidence that the pallid bat (Antrozous pallidus) is resistant to venom of the Arizona bark scorpion (Centruroides sculpturatus), North America's most venomous scorpion. Transcriptome analysis of bat dorsal root ganglia (DRG) was employed to identify potential mechanisms that may contribute to such resistance.

Bats use a variety of foraging strategies. The most common strategy amongst insectivorous bats is 'aerial hawking' wherein echolocation is used to detect, localize and hunt prey in flight. Another strategy, observed in a small group of bat species across families, is known as 'gleaning'. Gleaning bats use a combination of echolocation and passive hearing of prey-

generated noise to hunt prey from various substrates. The pallid bat is a gleaner, depending extensively on prey-generated noise (rustling, walking, etc.) to hunt terrestrial prey, while echolocation is used mostly for obstacle avoidance and general orientation [9]. Pallid bats localize prey-generated noise and land on or near potential prey. This foraging strategy puts the pallid bat in close proximity to scorpions.

Numerous scorpion genera are sympatric with the pallid bat, the most venomous being Centruroides [10]. This includes the Arizona bark scorpion (C. sculpturatus), whose sting induces extreme pain and occasionally death in humans [11]. Observations of night roosts indicate that pallid bats consume various species of scorpions including members of the Centruroides genus [9, 12–15]. Anecdotal evidence suggests that the pallid bats hunt and consume Arizona bark scorpions, but whether they simply avoid stings or are resistant to effects of the venom is unclear. If the latter, the pallid bat would provide an opportunity to determine mechanisms of venom resistance and pain modulation. In addition, studies of the pallid bat would provide comparative insights on mechanisms of venom resistance, given that at least one mechanism of Arizona bark scorpion venom resistance is known in the grasshopper mouse (Onychomys torridus) [16].

The first aim of this study was to use high-speed video to determine if Arizona bark scorpions sting the pallid bat during predation. Given the potential variability in the amount of venom delivered by a bark scorpion in a hunt, the second aim was to inject a known concentration of Arizona bark scorpion venom directly into the pallid bat. For comparative purposes, the same concentration was injected in mice. Upon determination that the pallid bat is indeed resistant to bark scorpion venom, we initiated the third aim: exploring possible molecular mechanisms of

resistance. To this end, we performed a transcriptome analysis of pallid bat dorsal root ganglia (DRG). Although multiple mechanisms of venom resistance have been identified across species [1, 3, 6, 7, 17, 18], we focused here on sequencing voltage sodium channels for two main reasons. First, these ion channels are principal targets of bark scorpion venom and mutations in these channels are known to confer resistance to venom. Second, we wanted to determine if the grasshopper mouse and the pallid bat have converged on similar mechanisms for venom resistance. Many sequence motifs in voltage gated sodium channels are important for venom toxin binding (alpha toxin binding sites:[19–23] beta toxin binding sites: [24–28], review [29]). The rationale for the third aim was to identify substitutions in pallid bat DRG that potentially confer resistance to the painful effects of Arizona bark scorpion venom. Previous studies of grasshopper mouse sodium channels revealed that a switch of a glutamine and a glutamate in IIIS5-S6 of Nav1.8 was sufficient for resistance to bark scorpion venom [16]. An important goal of this study was to determine if the same mechanism of resistance is observed in the pallid bat Nav1.8. We found that the pallid bat is resistant to Arizona bark scorpion venom and describe amino acid substitutions in voltage gated sodium ion channels (Nav 1.7 and 1.8) in the DRG that may confer such resistance. However, the mutation described in the grasshopper mouse is not found in the pallid bat, suggesting a potentially novel mechanism of pain modulation.

Materials and Methods

Animal husbandry

This study was carried out in strict accordance with the animal welfare guidelines of the National Institutes of Health and the Institutional Animal Care and Use Committee (IACUC) at the University of California, Riverside. The protocol was reviewed and approved by the IACUC.

Pallid bats, mist-netted in Arizona, New Mexico and California, were housed on a reversed 12:12 light:dark cycle in an 11 x 14 ft2 room, which allowed them to fly freely. Bats were obtained using scientific collecting permits issued by each of these states. Crickets and/or mealworms and water were supplied ad libitum. Food was withheld 24 hours before encounters with scorpions to ensure motivation to hunt. Scorpions were purchased from Scorpion Sweepers, LLC (Scottsdale, AZ).

Bat-scorpion encounters

Scorpion-bat interactions were filmed in a behavior room (13 x 14 x 8 cu. ft.) in which the scorpion was placed in an open top box (3 x 3x 4 cu.in.) and the bat performed a detect, land and hunt task. No training was required because this is a natural behavior. Additional filming environments included an empty terrarium (1.5 x 0.5 x 1 cu. ft.) with ~1" soil covering the bottom. After the scorpion was in the aquarium for a few minutes, a pallid bat was placed in the same enclosure. The aquarium was chosen to constrain high speed filming to a limited area to record the interaction in more detail. A Canon XA10 video camera and Phantom high-speed camera were positioned to capture the interaction to determine if the pallid bat was stung during the attack. For quantification purposes, a 'sting' is defined as any time the aculeus tip touched the bat.

Venom injection

Freeze dried C. sculpturatus venom was obtained from Spider Pharm (Yarnell, AZ) and kept at -80°C until use. Venom was diluted in saline 1–2 hours before injection. To ensure venom toxicity and to obtain more detailed behavioral response quantification than is currently available in the literature [30, 31], venom was injected into mice (n = 4 at 1.0 mg/kg b.w.) in the range of LD50

previously established for C. sculpturatus [30, 31]. While previous investigators [16] injected venom into the soft tissue of the paw, pallid bat limb extremities have very little soft tissue. To maintain consistent injection sites across mice and bats, the area between the scapulae was chosen for venom injection. As a control, on the day before venom injection, each mouse was injected subcutaneously between the scapulae with 30 µl saline and observed. After saline or venom injection, mice were observed for up to 10 minutes, and signs of pain were quantified. Behaviors associated with pain were quantified as the number of whole body jerky movements (convuslions), vocalizations and time spent grooming. Venom was injected into bats (n = 13) according to the same protocol. A venom dose of 1.0 mg/kg was used in 2/13 bats, 1.5 mg/kg was used in 7/13 bats and 10 mg/kg was used in 4/13 bats. Bats were observed for one hour following venom injection and then placed in a cage in the colony room and observed periodically for an additional 24–48 hours before being released back in the colony room. Humane endpoints were established for both mice and bats. Mice were to be euthanized with sodium pentobarbital (100–125 mg/kg b.w., i.p.) at the end of the 10 minute observation period or if the total time the mouse exhibited abnormal behaviors (convulsions, immobility or prostration) was longer than 1 minute. For bats as well, a 10 minute observation period was used to study effects of venom. Bats that showed abnormal behaviors for more than 10 minutes after injection were to be euthanized with sodium pentobarbital (100–125 mg/kg b.w., i.p.). If euthanasia was not necessary because the effects were minimal or non-existant, buprenorphine (0.05–0.1 mg/kg) was injected after the 10 minute observation window. As described in the Results section below, none of the bats were euthanized because the effects, if present, were transient. There were no deaths prior to the 10 minute observation periods for mice because the injected dose was less than the known LD50. There were no deaths in bats because as we

report below, venom only had transient effects and that too only in 2/13 bats. A fatal injection of sodium pentobarbital was used to euthanize all four mice within the 10 minute observation period.

RNA extraction and transcriptome methods.

Dorsal root ganglia (DRG) were extirpated from two pallid bats following a fatal dose of sodium pentobarbital (125 mg/kg b.w.). Cervical and thoracic DRGs were placed immediately in TRIzol and homogenized. Total RNA was purified with PureLink RNA mini kit (Ambion) according to manufacturer's instructions. Agilent Bioanalyzer was used to assure the quality of the RNA and only samples with a RNA Integrity Number (RIN) greater than 9 was accepted for sequencing. RNAseq libraries were made using NEBNext Ultra Directional RNA Library Prep kit for Illumina (New England Biolabs; Ipswich, MA) (prepared by the Institute of Integrative Genome Biology at University of California, Riverside) and libraries were multiplexed and run on the same lane of a NextSeq RNA sequencer (Illumina).

Resultant reads were assembled using the TRINITY [32] software pipeline with custom settings (S1 Fig). The software Benchmarking Universal Single-Copy Orthologues (BUSCO) [33] was run to assess the assembly was complete (76%-80% of all BUSCOs found) suggesting the assembly captured most genes expressed in DRG. Putative open reading frames (ORF) were extracted from the transcriptome assembly via the TransDecoder plugin for Trinity. ORFs were then aligned to two databases using BLAST, one database constructed from genes from Myotis lucifugus, Myotis davidii, Myotis brandtii, and Pteropus alecto (referred to as 4 Bats Database) and the other from the Swiss-Prot database. Duplicate gene hits were eliminated by keeping the hit with the lowest e-value as the pallid bat gene. If two ORFs had the same e-value, then the

ORF with the higher fragments per kilobase of exon per million fragments mapped (FPKM) value was chosen as the representative isoform, referred to as Unique Gene Hit. An overview of the assembly and quality control can be found in S1 Table. Sequences of Nav1.7 and Nav1.8 were then compared to other species using Clustal Omega [34] and Jalview [35].

Results

Bat-scorpion predator-prey encounters

Pallid bats were video-recorded attacking Arizona bark scorpions to determine whether bats are stung or avoid stings. Next we injected Arizona bark scorpion venom directly into pallid bats to determine resistance of pallid bats to the venom.

AZ bark scorpions stung pallid bats, sometimes multiple times, during a hunt. Five high-speed video recordings provided a clear view of the scorpion behavior during pallid bat attacks (see S1 Video for an example). Table 1 provides analysis of pallid bat attacks and scorpion defense, scored as number of stings. Bats 1, 3–5 consumed the scorpion at the end of the encounter, demonstrating that pallid bats eat Arizona bark scorpions. None of the bats reacted to stings during or after the encounter. Bat 2 abandoned the attack, likely because the aculeus became caught in the bat's lip and caused injury likely unrelated to venom injection. Observation of this bat after the encounter showed no behavioral response to envenomation. These videos clearly show that the aculeus contacts the pallid bat multiple times during a hunt. It is presumed that venom was injected in at least some of these instances. However, we observed no mortality, morbidity, or noticeable effect on behavior. It did not appear that the bat was specifically trying to grab the scorpion in any specific manner that prevented aculeus contact.

	Bat 1	Bat 2	Bat 3	Bat 4	Bat 5
Length of Encounter in Sceonds	6.02	1.42	2.5	1	4.13
# Stings	3	1	10*	1	4

*During this trial, the scorpion aculeus was oriented on or near the bat head for most of encounter, resulting in many aculeus-bat contacts that may not have been genuine stings.

Table 1.1 Time Required for Bats to Subdue Scorpions or Abandon Attack and the Number of Observed Stings During Each Encounter

Venom injection

Tables 2 and 3 describe venom injection experiments in mice and pallid bats, respectively. All four mice showed behavioral signs of envenomation (Table 2). These included intense grooming, particularly of the face, and vocalizations, convulsions and disoriented movements. These behaviors were not seen following saline injections. Likely because the concentration tested was less than reported LD50, none of the mice died during the first 10 minutes of post-injection observation. However, altered behaviors were consistent and obvious even at the 1 mg/kg venom dose.

	Dose (mg/kg)	Number of convulsions	Time spent grooming (min:sec)	Number of audible vocalizations	Time to first convulsion (min:sec)	Time to first grooming (min:sec)
M1	1	19 (0)	6:17 (1:13)	0 (0)	1:19 (N/A)	1:38 (0:50)
M2	1	60 (0)	7:43 (0:49)	21 (0)	2:49 (N/A)	2:37 (1:35)
M3	1	46 (0)	6:30 (0:56)	35 (0)	4:14 (N/A)	5:09 (1:40)
M4	1	43 (0)	5:45 (0:34)	36 (0)	5:29 (N/A)	4:15 (2:47)

Responses to saline injection are shown in parenthesis.

Table 1.2 Behavioral Responses of Mice Following Scorpion Venom Injection

Animal	Venom Dose mg/kg	Number of convulsions	Time spent vocalizing (seconds)	Time Spent walking backward
Bat_1	1	0	0	0
Bat_2	1	0	0	0
Bat_3	1.5	0	0	0
Bat_4	1.5	0	0	0
Bat_5	1.5	0	0	0
Bat_6	1.5	0	0	0
Bat_7	1.5	0	0	0
Bat_8	1.5	0	0	0
Bat_9	1.5	0	39	114
Bat_10	10	22	0	0
Bat_11	10	1	0	0
Bat_12	10	0	0	0
Bat_13	10	0	0	0

For 11/13 bats, no observable behavioral modifications were present following injection. Convulsions are defined as whole body jerky movements. Unlike the mouse, venom induced grooming was absent in bats.

Table 1.3 Dose and Indication of Response to Arizona Bark Scorpion Venom When Injected into Pallid Bats

For eight out of nine bats injected with 1 or 1.5 mg/kg dose, venom did not produce noticeable effects on behavior (Table 3). One out of nine injected bats (Bat 3) produced audible vocalizations and lumps on its snout that appeared to be an allergic reaction. Backward walking was also elicited following injection. Vocalizations and backward walking were absent after 10 minutes. At the highest dose tested (10 mg/kg), 3/4 bats showed no noticeable effects. However, one of the bats showed abnormal jerky movements for the first 7 minutes. None of the bats showed any effects after 10 minutes. Taken together, these data indicate that almost all pallid bats tested were resistant to Arizona bark scorpion venom at doses up to 10 mg/kg, with the possibility of reactions in some bats that cannot be fully discounted.

Transcriptome analysis

Assembly of raw Illumina reads were separated into two groups based on biological replicates and labeled DRG1 and DRG2. Assembly is summarized in Supplementary Materials (S1 Table).

The N50 for both samples was ~1500 and individual voltage-gated sodium ion channels were examined to ensure full-length transcripts were present. The software BUSCO was run to assess if the assembly was complete (76%-80% of all BUSCOs found). TransDecoder extracted 94,522 ORFs from DRG1 assembly and 109,948 from DRG2 assembly which yielded approximately 24,500 unique gene hits per tissue sample when processed in our BLAST pipeline.

Sequence analysis of voltage gated sodium ion channels

Transcriptome analysis revealed three voltage-gated sodium channels expressed in pallid bat DRG: Nav1.7, Nav1.8, and Nav1.9. Due to limited information on the effect of scorpion venom on Nav1.9, it was not further analyzed. A recent study of the grasshopper mouse reported substitutions of glutamate and glutamine in domain 2 of Nav1.8, which enhance binding affinity of Arizona bark scorpion toxins leading to channel block [16]. Since Nav1.8 is necessary for action potential propagation, block of Nav1.8 functions effectively as an analgesic, shutting down the pain-signaling pathway [16]. To determine if venom resistance seen in the pallid bat can be attributed to the same mutations, we analyzed domain 2 of Nav1.8. Sequence data indicates that this mechanism does not operate in the pallid bat (Fig 1, S2 Fig shows additional comparative details of pallid bat Nav1.8 sequence in domains known to be important for venom binding in Nav1.7). Indeed, the pallid bat sequence in this region is identical to that of humans and other species susceptible to scorpion venom. Thus, the pallid bat likely has a novel mechanism for Arizona bark scorpion venom resistance.

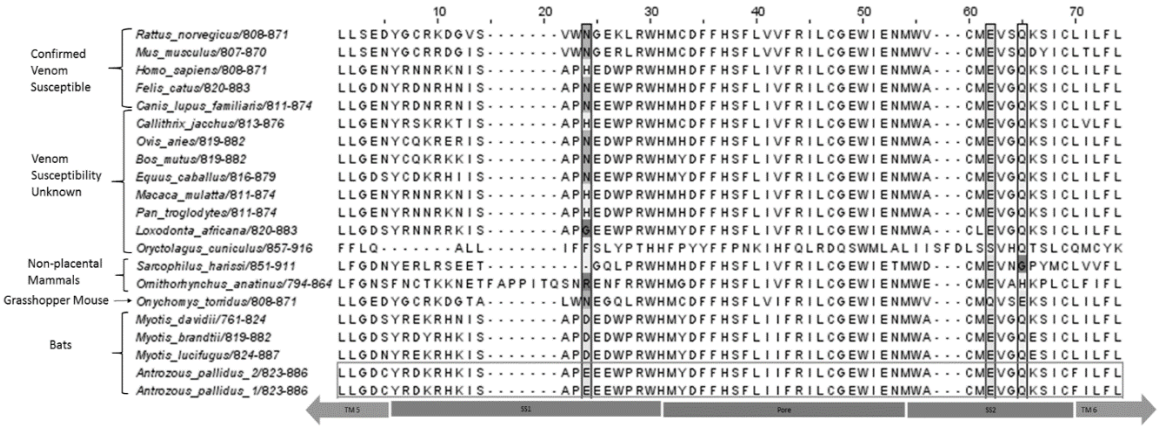

Fig 1.1 Alignment of Extracellular Region IIS5-S6 of Nav1.8

Highlighted columns in SS2 show residues important for granting venom resistance in grasshopper mouse. Most species have a glutamate at position 62 in the alignment shown and a glutamine at position 65, including the pallid bat. However, the grasshopper mouse has these two amino acids switched. This switch has been shown to confer venom resistance in this species [16].

Nav1.7 is the main target of scorpion venom toxins [36–38]. Fig 2A shows known scorpion toxin binding regions in extracellular regions of sodium channels [19–25, 27, 28, 39, 40]. Fig 2B–2E shows alignments of selected extracellular regions of Nav1.7 across various species; colored regions are locations where the pallid bat either has an amino acid substitution known to be important for venom binding in other sodium channel isoforms or has a significant change in amino acid chemistry. Special attention is given to changes in acidic residues, as they are crucial for toxin binding [21, 23, 41]. Substitutions of special note are described in Fig 2.

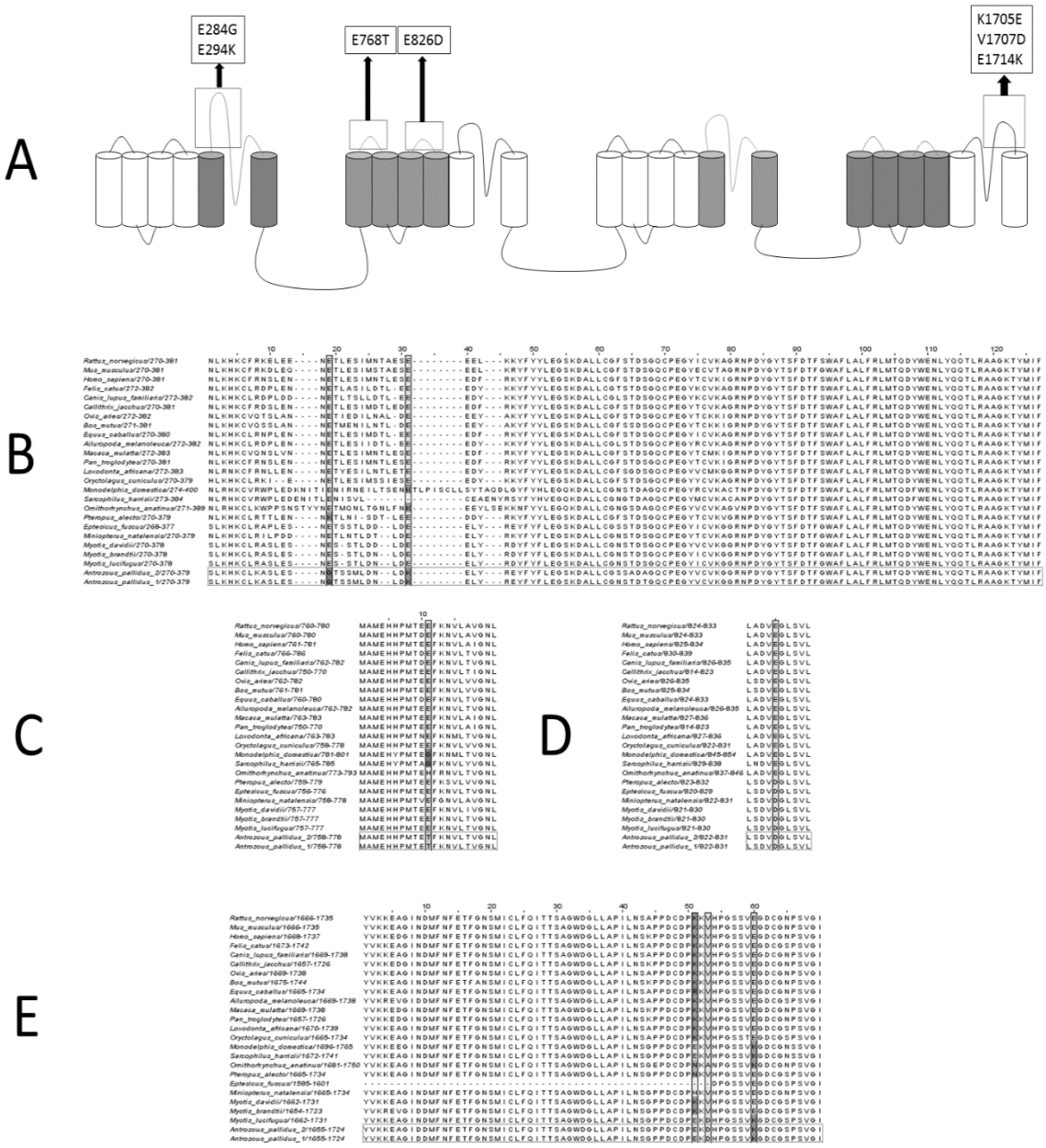

Fig 1.2 Comparison of Selected Extracellular Loops in Nav1.7 Known to be Involved in Scorpion Toxin Binding

While Nav1.7 displays normal activity in the grasshopper mouse, it may be altered in the pallid bat providing venom resistance. (A) Schematic of Nav1.7 showing known scorpion toxin binding

regions and regions of special note in the pallid bat; red are known alpha scorpion toxin binding regions and blue are known beta scorpion toxin binding regions. (B) Extracellular IS5-S6. (C) Extracellular region IIS1-S2. (D) IIS3-S4. (E) Extracellular region IVS5-S6.

Availability of sequences

The sequences for SCN10a (Nav1.8) and SCN9a (Nav1.7) have been deposited in GenBank

Nav1.7 (GenBank Accession MF616470) and Nav1.8 (GenBank Accession MF616471)

Discussion

Observations of bat-scorpion interactions indicate that the pallid bat is stung while hunting the Arizona bark scorpions. In all but one instance, the bat successfully killed and ate the scorpion with the exception most likely attributable to mechanical damage caused by the scorpion. Moreover, direct injection of venom at a dose known to induce strong pain responses in mice caused no evident pain responses in eight out of the nine bats tested. Of the additional four bats tested with 10 mg/kg venom, one bat displayed a transient behavioral reaction that lasted less than 10 minutes. The other bats were unaffected. We interpret these data to mean that the pallid bat is resistant to Arizona bark scorpion venom. Because at least two bats showed a reaction, albeit transiently, the possibility of regional variation in venom tolerance [25] cannot be discounted based on the current study. Grasshopper mice populations that are sympatric with the Arizona bark scorpion exhibit a higher LD50 (~18 mg/kg) compared to populations that are parapatric (~12 mg/kg) or allopatric (~10 mg/kg) [42]. Given variation in sympatry between the pallid bat and C. sculpturatus, future studies with different populations of pallid bats [43]

and additional venom doses are required to evaluate population differences in venom tolerance.

One mechanism underlying venom resistance in the grasshopper mouse is known [16]. An amino acid substitution in the Nav1.8 sodium ion channel (Fig 1) causes the venom to act as an analgesic by inactivating pain sensing neurons of the DRG. Sequence analysis showed that this mechanism is not present in the pallid bat, suggesting that this form of venom resistance occurs through a different, hitherto unknown mechanism. Pallid bat sodium channel sequences show several substitutions in toxin binding regions that may contribute to resistance. A number of these changes involve acidic residues. While studies [21,23,40,41] have shown that changing acidic residues in various isoforms of sodium channels alters toxin binding, little work has focused on voltage-gated sodium channels in DRG (Nav1.7, Nav1.8, and Nav1.9). However, given that acidic residues are important in toxin binding to isoforms of Nav1.7, it is possible that substitutions involving acidic amino acid side chains observed in the pallid bat alter binding affinity of scorpion venom toxins.

In Nav1.7, we see various substitutions that are either pallid bat specific, bat specific, or bat and non-placental mammal specific. For example, Fig 2B shows the known venom binding region in the extracellular region of domain 1 between TM 5 and 6 (IS5-S6) [27], where the pallid bat has an E284G (pallid bat numbering) substitution with respect to venom-susceptible species. The only other species examined that does not have a glutamate in this location is the black flying fox (Pteropus alecto), which has a lysine. Also in IS5-S6 the pallid bat has an E294K substitution. All other species examined have a glutamate at this position with the exception of the three non-placental mammals: Gray short-tailed opossum (Monodelphis domestica), Tasmanian devil

(Sarcophilus harrisii), and Duckbilled platypus (Ornithorhynchus anatinus). Both the opossum and platypus have a glutamate to lysine substitution, while the Tasmanian devil has a deletion in this region. The change in charge between the pallid bat and two non-placental mammals may indicate convergent evolution of venom resistance in these three species. However, it is unclear if the opossum and platypus are scorpion venom resistant. Scorpion venom resistance of non-placental mammals is in general unclear, but all have overlapping ranges with venomous species. Snake venom resistance is reported in other species of opossum [1] and arthropods are a known prey item of gray short-tailed opossum. The Tasmanian devil is a known generalist predator whose diet includes arthropods and venomous snakes [44]; however its venom resistance status is unknown. The platypus employs venom for intraspecific mate competition [45]. Given the high potency of this venom in humans, platypuses most likely possess some level of resistance to their own venom. In non-placental mammals, sodium channel sequence similarities to the pallid bat in scorpion toxin binding regions suggests they have a mechanism of venom resistance similar to that of the pallid bat.

In IIS1-S2, we see that the pallid bat again shares more sequence similarity with non-placental mammals. The pallid bat E769T substitution contrasts with marsupial glycine and platypus histidine substitutions. The investigators in [40], showed that changing glutamate to either a glutamine or cysteine greatly reduces the binding affinity of the beta scorpion toxin CssIV to Nav1.2 and we may be seeing a similar toxin binding altering substitution in the pallid bat Nav1.7

One intriguing result of the comparative analysis is that all bats with known sequences have aspartate instead of glutamate in a specific locus in IIS3-S4 (Fig 2D). The functional implications

of this substitution are presently unclear. At least one other bat species, Hemprich's Long-eared bat (Otonycteris hemprichii), is resistant to scorpion venom [5]. This species is also a gleaning bat found in the Negev desert, where it is observed to hunt the highly venomous Deathstalker scorpion (Leirus quinquestriatus) [5]. A few studies have documented scorpion parts in the diet of other bats [46–49], but identities of these scorpions are not known. Future comparative analyses of sodium ion channel sequences from bats that hunt scorpions versus aerial hawking bats will inform studies of evolution of venom resistance and gleaning behavior in bats.

While IVS5-S6 is not a known venom toxin-binding region, altered amino acid side chain charge highlighted in Fig 2E could alter toxin binding allosterically. For example, in venom susceptible animals, two consecutive lysine residues (K1705 and K1706) occur adjacent to valine at V1707. The K1705E and V1707D substitutions in the pallid bat result in a local charge alteration from to +2 in venom susceptible animals to -1. This is the same type of substitution seen in naked mole rats Nav1.7, which reduces nociceptor firing in response to acidic conditions [50]. Taken together, these differences in chemical properties become compelling targets for functional analysis. Although the focus here has been on sodium ion channels, other mechanisms of venom resistance could include neutralization of toxic proteases/phospholipases by inhibitors in pallid bat blood, as has been seen in other species [51]. Future studies will mix bat serum with venom for injection into mice to determine if this mechanism is involved in venom resistance.

Conclusions

This study presents the first evidence that pallid bats are resistant to Arizona bark scorpion venom at concentrations that causes significant pain and death in mice. Sequencing of the voltage gated sodium ion channels present promising sites to begin investigating precise

mechanisms that confer venom resistance. Some of these changes are confined to the pallid bat, while others are observed across the various bat species examined. Future investigations will focus on regional differences in venom tolerance, functional consequences of sodium channel sequence alterations for pain tolerance, and amino acid substitutions seen in bats that may have been subject to positive selection. Together these data indicate that the pallid bat has evolved novel mechanisms of pain modulation involving altered ion channel function.

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Chapter 2

Abstract

The pallid bat (Antrozous pallidus) is a gleaning bat found in western North America and is a well known hunter of scorpions. Previous work has shown the pallid bat is highly resistant to the venom of the Arizona barks scorpion, Centruroides sculpturatus. Here we build upon that work and show how the pallid bat may overcome the venom via cellular and genetic adaptations. Pallid bats were injected with doses of C. sculpturatus venom up to 20 mg/kg and showed only minor symptoms and made full recoveries. The serum neutralizing effects of pallid bat blood serum was tested via incubation of pallid bat blood serum with the venom before injection into mice. The serum incubated venom retained equivalent potency as the non-serum-incubated venom suggesting the pallid bat does not inactivate the venom via serum-based mechanisms. The sensory neurons in pallid bat dorsal root and trigeminal ganglia were tested via the Constellation Pharmacology method. Pallid bat sensory neurons experienced more calcium signal when challenged with C. sculpturatus venom than mouse sensory neurons, suggesting pallid bat venom resistance is not dependent on preventing increases in somatic calcium concentrations. The sequences of two voltage gated sodium channels were compared to other species and possible sites influencing venom action showed signs of positive selection in scorpion hunting bats.

Introduction

Venoms are a diverse array of toxins which can induce pain, injury, or death in its victims and are often used in predation, defense, feeding, and even intraspecific mate competition. Venoms have evolved in many phyla from mollusks to mammals. The would be

targets of these venomous species have thus been put under a strong selective pressure to adapt to this evolutionary weapon. There are many documented cases of venom resistance such as honey badgers resistance to their venomous snake prey, ground squirrels developing resistance to their rattlesnake predators, snakes' resistance to their own and other snake species' venom and much more across the animal kingdom.

Of particular interest are scorpion toxins due to their worldwide range and medical importance. The Arizona bark scorpion (*Centruroides sculpturatus*) is the most toxic scorpion in the United States and is found mainly in the deserts of southwestern United States and northern Mexico. The venom of *C. sculpturatus* is composed of many molecules, the most biologically active of which target voltage gated sodium channels (VGSCs). At least two species have been shown to be resistant to this lethal arthropod: the grasshopper mouse (*Onychomys torridus*) and the pallid bat (*Antrozous pallidus*). *O.* torridus is able to withstand venom doses up to (~16 mg/kg) and *A.* pallidus is able to withstand at least 10 mg/kg although an upper limit was not established. A key mutation was identified in *O. torridus* which prevents the primary VGSC toxin in *C. sculpturatus* from inducing pain. The mutation is in SCN10A, normally unaffected by venom but crucial for action potential propagation in nociceptors. In the grasshopper mouse a mutation in the extracellular region allows scorpion toxin binding. The toxin then blocks SCN10A and thereby prevents any signal from nociceptors to reach the CNS resulting in an analgesic effect. No mechanism has been found in the pallid bat however mutations in key toxin binding regions have been identified which may alter toxin function.

In this study we set out to test the venom resistance of the pallid bat to *C.* sculpturatus venom. Building on previous work we injected pallid bats with 20 mg/kg venom doses and

hypothesized that pallid bats would show more envenomation symptoms than at lower doses. Bats showed much more severe signs of envenomation however none died as a result of venom injection indicating a high level of resistance in the pallid bat. Further we cultured pallid bat trigeminal ganglia and used calcium imaging to measure their responsiveness to venom. Trigeminal ganglia contains sensory neurons which are the primary targets for scorpion venom. We hypothesized pallid bats would have fewer cells responding to venom than mouse. This turned out to be incorrect as pallid bat neurons were more responsive to scorpion venom than mouse. Finally we used the phylogenetics software suite PAML to look for signs of positive selection in the pallid bat. It was hypothesized that signs of positive selection would appear in known venom binding regions of voltage gated sodium channels. Multiple sites were found and are prime candidates for future mutagenic analysis.

Materials and Methods

Animal husbandry

This study was carried out in strict accordance with the animal welfare guidelines of the National Institutes of Health and the Institutional Animal Care and Use Committee (IACUC) at the University of California, Riverside. The protocol was reviewed and approved by the IACUC. Pallid bats, mist-netted in Arizona, New Mexico and California, were housed on a reversed 12:12 light:dark cycle in an 11 x 14 ft^2 room, which allowed them to fly freely. Bats were obtained using scientific collecting permits issued by each of these states. Crickets and/or mealworms and water were supplied *ad libitum*.

Venom Injections

Freeze dried *C. sculpturatus* venom was obtained from Spider Pharm (Yarnell, AZ) and kept at -80°C until use. Venom was diluted in saline 1–2 hours before injection. While previous investigators [16] injected venom into the soft tissue of the paw, pallid bat limb extremities have very little soft tissue. To maintain consistent injection sites across mice and bats, the area between the scapulae was chosen for venom injection.

For serum-neutralization experiments serum was collected from mice and bats using the same protocol. Bat or mouse serum was collected from freshly euthanized animals with sodium pentobarbital (100–125 mg/kg b.w., i.p.). Blood was extracted immediately after an incision into the right atrium. Blood was collected into 1 mL tubes and allowed to sit at room temperature for 1 hour. The tube was then spun at 100 rpm for 30 minutes. Supernatant (serum) was collected and frozen until used. On day of serum-neutralization experiment either bat or mouse serum was mixed with venom stock solution and incubated together for 10 minutes before injection into mouse at a dose of 1 mg venom / kg mouse.

After saline or venom injection, mice were observed for up to 10 minutes, and signs of pain were quantified as described previously (Hopp et al., 2017). Behaviors associated with pain were quantified as the number of whole body twitches (bats and mice), time spent grooming (mice) and number of lip smacks (a unique transient reaction seen in a few bats at the highest dose tested). Venom was injected into bats according to the same protocol. A venom dose of 20 mg/kg was used in 8 bats. Bats were observed for one hour following venom injection and then placed in a cage in the colony room and observed periodically for an additional 24–48 hours before being released back in the colony flight room. Humane endpoints were established for both mice and bats. Mice were to euthanized with sodium pentobarbital (100–125 mg/kg b.w.,

i.p.) at the end of the 10 minute observation period or if the total time the mouse exhibited abnormal behaviors (convulsions, immobility or prostration) was longer than 1 minute. For bats as well, a 10 minute observation period was used to study effects of venom. Bats that showed abnormal behaviors for more than 10 minutes after injection were to euthanized with sodium pentobarbital (100–125 mg/kg b.w., i.p.). Euthanasia was not necessary with bats because the effects were minimal or non-existent. However buprenorphine (0.05–0.1 mg/kg) was injected after the 10 minute observation window to alleviate pain. As described in the Results section below, none of the bats were euthanized because the effects, if present, were transient (<1 min). There were no deaths prior to the 10 minute observation periods for mice because the injected dose was less than the known LD_{50}. A fatal injection of sodium pentobarbital was used to euthanize all mice tested within the 10 minute observation period.

Constellation Pharmacology

Challenge Chemical	Working Concentration
High Potassium	33 mM and 100 mM*
ACh	1 mM
ATP	20 uM
Menthol	200 uM
AITC	100 uM
Capsaicin	300 nM
Low Venom	2 ug/mL
High Venom	16 ug/mL

Table 2.1: Chemicals and Concentrations Used During Constellation Pharmacology Experiments

*100 mM was only used at the end of the experiment as a 'last call' step. Some cells only responded to 33 mM with a very weak, or no, signal. 100 mM allowed for the identification of all cells which respond to a potassium dose. Since it was done at the end each experiment it did not influence other Challenge Chemical responses.

Culturing Protocol

Both mouse and bat trigeminal ganglia were dissected and cultured identically except for the euthanasia. Bats were first anesthesized with isofluorane to facilitate a lethal dose of sodium pentobarbital of 780 mg/kg i.p.. Mice received the same dose of sodium pentobarbitol but did not require isofluorane anesthesia.

An incision was made dorsally from the foramen to just before the eyes. Skull was cut away and an incision was made rostrally to sever the olfactory bulb from the brain. The brain was slowly lifted with forceps and connective tissue snipped with scissors, being careful not to damage the ganglia. Once the brain was removed the trigeminal nerve were clearly visible along the base of the skull. The trigeminal nerve was cut at the most caudal and rostral points and carefully lifted up by the nerve fibers being careful not to touch the ganglia itself. The dissected trigeminal ganglia + trigeminal nerve was then immediately placed into 1 mL of a collagenase/dispase solution (4mg/mL in HBSS). Solution was allowed to incubate for 15 minutes at 37° C agitating the solution every 5 minutes.

Dorsal root ganglia were removed via removal of the spine from a recently deceased animal via cutting at the cervical and lumbar vertebrae. The spine was then cut laterally to open the spine. The spinal cord was gently deflected and the DRG were removed from their sockets. Culture preparation for DRG was identical to TG.

While the ganglia were incubating the well plates were prepared. Previously prepared silicon rings with ~6 mm interior diameters and ~8 mm outside diameters were fit into the wells of a poly-D-lysine coated 24 well plate. 60 uL of laminin solution (10ug/mL) was applied to the interior of each well and incubated at least one hour.

After the 15 minute incubation period for the ganglia + collagenase/dispase solution the tube with the solution was spun at 1400 rcf for 3 minutes on soft mode. Supernatant was removed and replaced with 1 mL warmed DMEM + supplement (approximately 37° C). Tube was spun again at 1400 rcf on soft mode for 3 minutes. Supernatant was removed and fresh DMEM + supplements added. The entire solution was titrated with fire polished pipettes 5-10 times until

the solution took on a cloudy appearance. The cell solution was sent through a 70 um cell filter, washed 2x with 500 uL DMEM + supplements. Elutant was spun at 1400 rcf for 10 minutes on soft mode. Excess supernatant was removed to a final volume of approximately 80-100 uL.

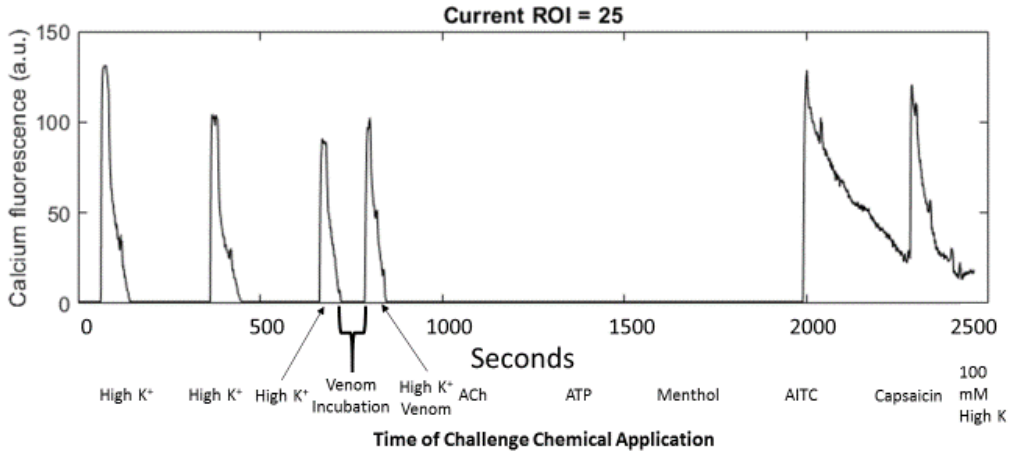
Laminin solution was removed from the 24 well plate and replaced with 60 uL DMEM + supplements. The DMEM + supplements was then removed and replaced with 40-60 uL of cell suspension in DMEM + supplements. Well plate was incubated at 37° C with 5% CO2 for 1 hour. After the hour 1 mL DMEM + supplements + GDNF was added to each well. The solution was left overnight for cell attachment before imaging the next day.

Cultured sensory ganglia cells were incubated with 2 uM Calbryte 520-AM in DMEM + supplements media for 1 hour at 37° C and 15 minutes at room temperature before imaging. After incubation, dye solution was washed with two applications of 1mL DMEM + supplements solution at room temperature. A Keyence BZ-X710 automated microscope was used with the 24 well plate setting and GFP excitation filter. Framerate was set to 1 fps and 920/1080 pixel resolution.

Challenge Chemical Solutions were made by dissolving stock solutions into Observation Solution (Table 1) Each experiment began with washing well 3x with observation solution. After 55 seconds of recording, the first high potassium incubation was given. Fifty-five seconds was chosen to allow for a baseline fluorescence measurement to be obtained before high potassium application. This was used later during analysis to calculate the change in fluorescence activity. Most chemicals followed the same pattern of extracting the observation solution from the well and immediately replacing with 500 uL challenge chemical. This was accomplished with a 10 mL syringe attached to a plastic tube approximately 15 cm long with a snug fit between syringe and

one end of tube. The other end of the tube was placed into the well and held in place with modeling clay. End of tube was placed approximately 1-2 mm above the bottom of the well to ensure thorough emptying. After well was emptied a 500 uL of the next challenge chemical or observation solution was added with a 1 mL pipette. The challenge chemical was incubated in the well for 15 seconds before removal of the challenge chemical solution and replacement with 500 uL observation solution. 40 seconds after this wash the observation solution was replaced with fresh observation solution to ensure all traces of the chemical have been removed. The only exception to this pattern is with the venom incubation challenge. The procedure was the same except the venom was allowed to incubate with the cells for 60 seconds instead of 15 seconds. Immediately after the venom incubation a high potassium + venom solution was applied. After 15 seconds of incubation with the high potassium + venom solution, the well was washed 3x with fresh observation solution with each wash separated by 20 seconds.

A



B

Figure 2.1: A. Example Fluorescent Trace for Calcium Imaging. Challenge Chemicals added at the times indicated. “High K+” denotes a 33 mM solution while the “100 mM High K+” denotes the ‘last call’ solution used to highlight any cells whose response to 33 mM was undetectable . This particular neuron responded to High K, AITC, and Capsaicin. B. Example photo of a cell culture responding to a high potassium dose (100 mM). White regions signify cells experiencing increases in calcium concentrations.

After imaging, an AVI file was acquired. This AVI file was converted to a TIF stack with the program ImageJ. A Z-stack image of the TIF stack image was made also using ImageJ which displayed a single frame with the maximum intensity for each pixel represented. The TIF stack and Z-stack image was then used as input for the MATLAB program FluoroSNNAP. First ROIs were identified by using the Z-stack image in the FluoroSNNAP segmentation GUI. The threshold method was used with settings of Min Intensity set to 5, Min ROI Size set to 1, and Max ROI Size set to 500. This creates a segmentation file which can then be loaded into the full TIF stack. Analysis was run with FluoroSNNAP with only basic processing modules activated. Template based identification was used with similarity set to 0.75. Other settings were left at their default values. After FluoroSNNAP was run, the raw traces and calcium event detection were exported to selected experiment file.

Files were then analyzed by in house software for a combination automated and manual quality control to ensure responses were valid. See supplementary file “Constellation Pharmacology Tutorial” for a tutorial on how data was processed with Matlab code and Excel sheets. Briefly, the third high potassium dose was used as a standard amplitude. A challenge chemical, including venom, needed to elicit an amplitude at least equal to half the amplitude of the third high potassium dose to be considered a response. The signals matching this criterion were then examined visually by a human and either confirmed to be a response or counted as a no response. A 100 mM High K dose was given at the end of the experiment to activate all neurons in the field of view. A neuron was counted as surviving the experiment if it had a response to either the 100 mM High K or Capsaicin since Capsaicin is the last Challenge

Chemical. If a neuron died after Capsaicin so that no 100 mM High K signal was present it was still counted as surviving.

In addition to identifying neurons which responded to venom with at least a 50% response as seen in the third high potassium dose we also sorted neurons into groups that responded at least to 80%, 110%, 140%, 170%, and 200% of the amplitude seen for the third high potassium dose. Due to variations in total number of cells responding to venom in mice and bats these responses were normalized to the number of cells responding with venom amplitudes at least 50% of the third high potassium dose to facilitate inter-species comparison.

PAML

Positive selection analysis was performed with the codeml program in the PAML suite of software (Yang). Control file settings can be found in Supplemental Figure (codeml.ctl). 24 species' SCN10A and 38 species SCN9A were separately aligned and a consensus tree was made based on available literature. For SCN9A we tested 5 iterations of the tree with a different foreground selected for each run. The foreground lineages were all bats in the alignment, all gleaning bats, then each gleaning bat separately in case of divergent venom resistance strategies, *O. hemprichii, A. pallidus,* and *M. lyra*. For SCN10A the foreground branches were the same except the *M. lyra* only run was replaced with *O. torridus* and the gleaning bat group only included *O. hemprichii* and *A. pallidus*. This was due to lack of availability of *M. lyra* SCN10A and a known mutation in *O. torridus*' SCN10A which grants venom resistance.

Results

Behavioral Analysis

None of the bats tested died and made full recovery, when injected with a 20 mg venom/kg dose of *C. sculpturatus* venom. However, the bats showed a transient increase in the pain categories of involuntary twitching (Fig 1A) and lip smacking behavior (Fig 1B) compared to previously published data for 10 mg/kg (Hopp et all 2017). These data indicate that LD50 is higher than 20 mg/kg for Arizona bark scorpion venom in the pallid bat. Higher doses were not tested because behavioral effects of venom, however transient, indicate an effect at 20 mg/kg.

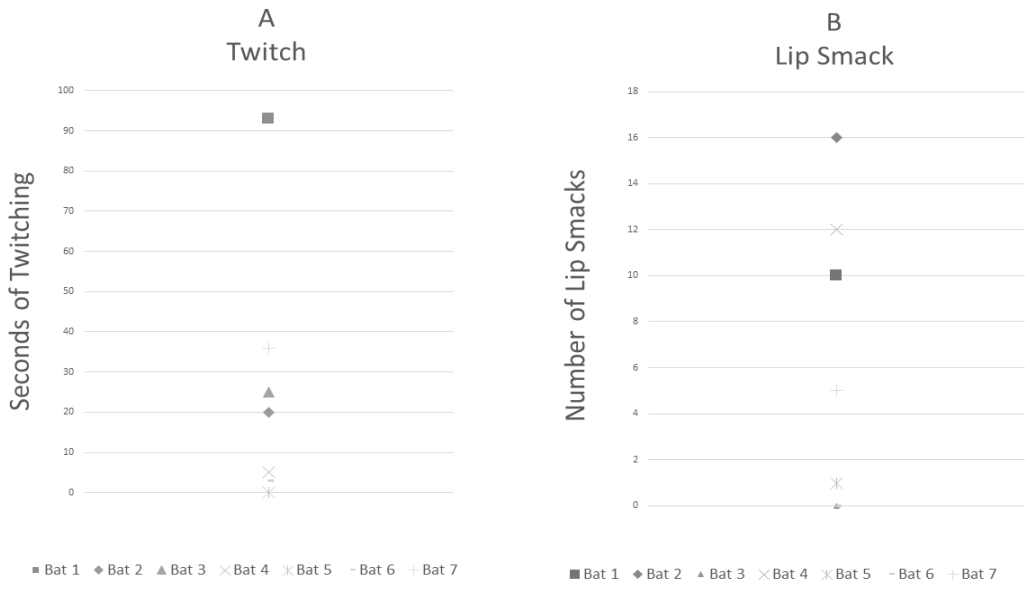


Figure 2.2: Scatter Plot of Pallid Bat Responses to Venom. 20 mg/kg venom injection. A) number of twitches in first 10 minutes after venom injecion. B) number of lip smacking behavior seen in first 10 minutes. All 7 individuals survived and made full recoveries within 24 hours.

Serum Based Venom Neutralization

Bat serum showed no ability to reduce the amount of venom induced grooming in mice (Fig 2).

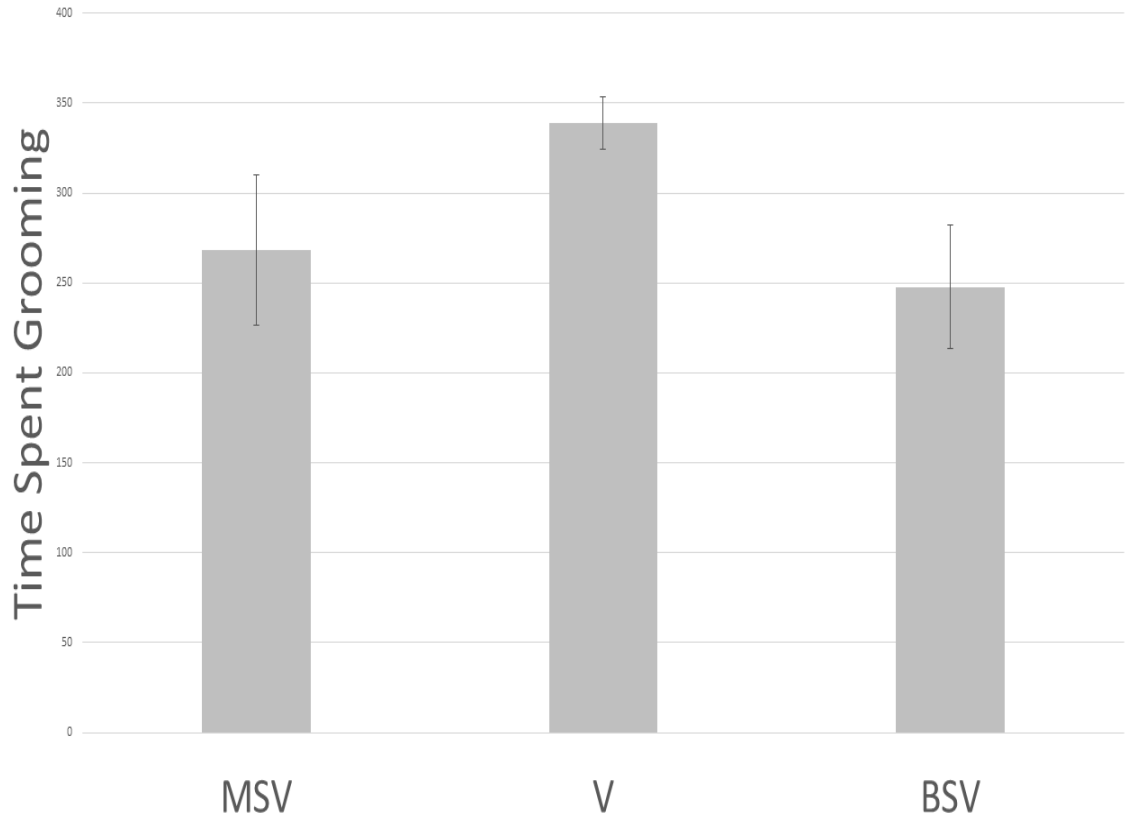


Figure 2.3 Histogram of Mouse Responses to Venom: Grooming of mice in response to venom or venom-serum treatment: Number of seconds mice spent grooming for up to 10 minutes after injection with either C. scultpuratus venom (V), venom which had been incubated with mouse blood serum (MSV) or bat blood serum (BSV) for 10 minutes immediately beforehand. N = 5 for all groups. No significant difference detected between groups using one way ANOVA ($p=0.59116$). Error bars are 1 standard error.

Constellation Pharmacology

Overall Cell Numbers:

Total animals in each venom treatment group is shown in Figure 4. TG cells were cultured from each animal used in the study (Figure 4). However the survival rate of cells to the end of the experiment excluded some ganglia in certain individuals from being included in the final analysis (ganglia needed to yield at least 100 neurons which survived to the end of experiment).

	# Animals TG	# Neurons for TG	Average # Neurons TG
Bat	3	5397	1799
Mouse	6	4433	739

Table 2.2 Number of Neurons Examined in Each Species and Ganglion: Table showing number of animals and neurons for each category. Average neurons per animal and variance is also provided.

Subclass Results:

Figure 4 shows the averaged results for the percent of each animals' neurons which responded to venom.

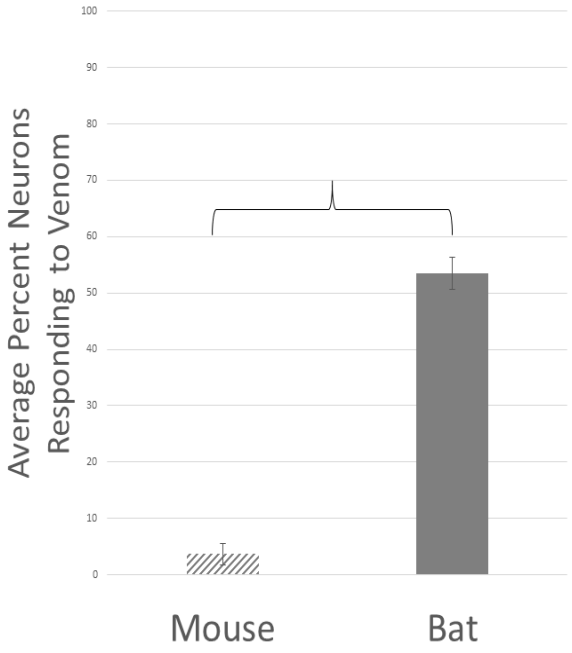


Figure 2.4: Average Percent of Neurons Responding to Venom in Pallid bat and Mouse: Percent of total neurons responding to venom for each group in TG. Significant difference found only in TG ($p = 9.23e-07$). Error bars are 1 standard deviation.

Subclass Venom Results:

Increased responses were seen in bat TG compared to mouse TG with respect to high venom treatment in every subclass except menthol (Fig 2.5).

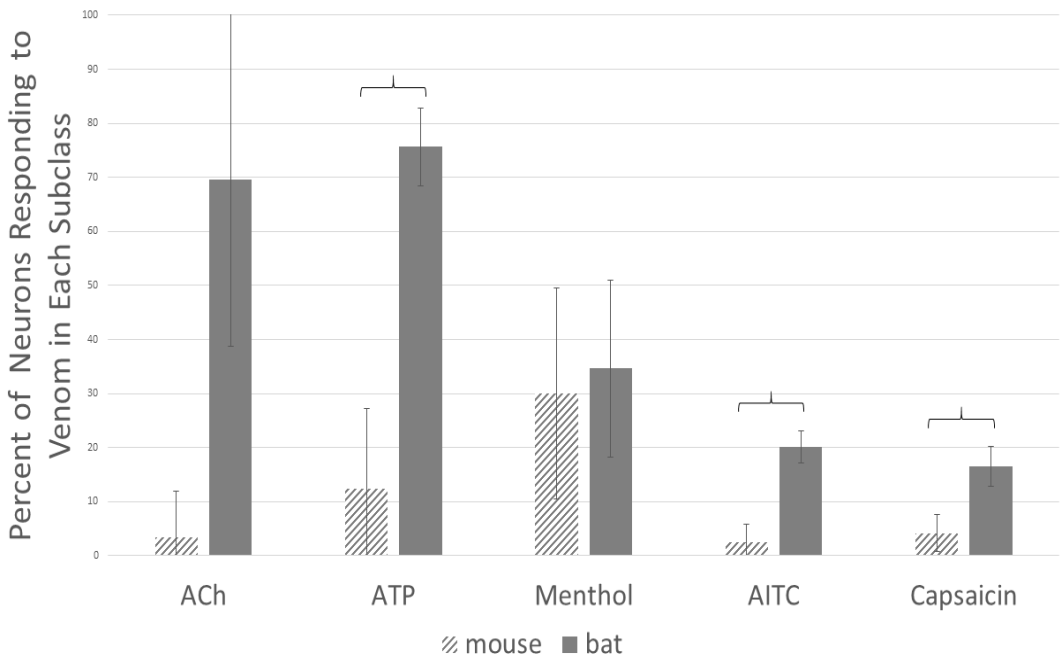


Figure 2.5 Response to Venom by Neuron Subclass in Mouse and Bat: Each subclass normalized to total number of cells in subclass. Percent of each subclass responding to venom in TG. Significant differences between mouse and bat indicated by brackets (p value < 0.05).

Amplitude Response Analysis:

In order to examine differences in the extent of calcium influx between species we normalized each neuron's response to venom to that of the third high potassium dose. The responses were then grouped by this normalized value into > 0.5, > 0.8, > 1.1, > 1.4, > 1.7, and > 2.0. Since bats had many more neurons responsive to venom compared to mice we then normalized each venom amplitude group to that individual's 0.5 group (Fig 6).

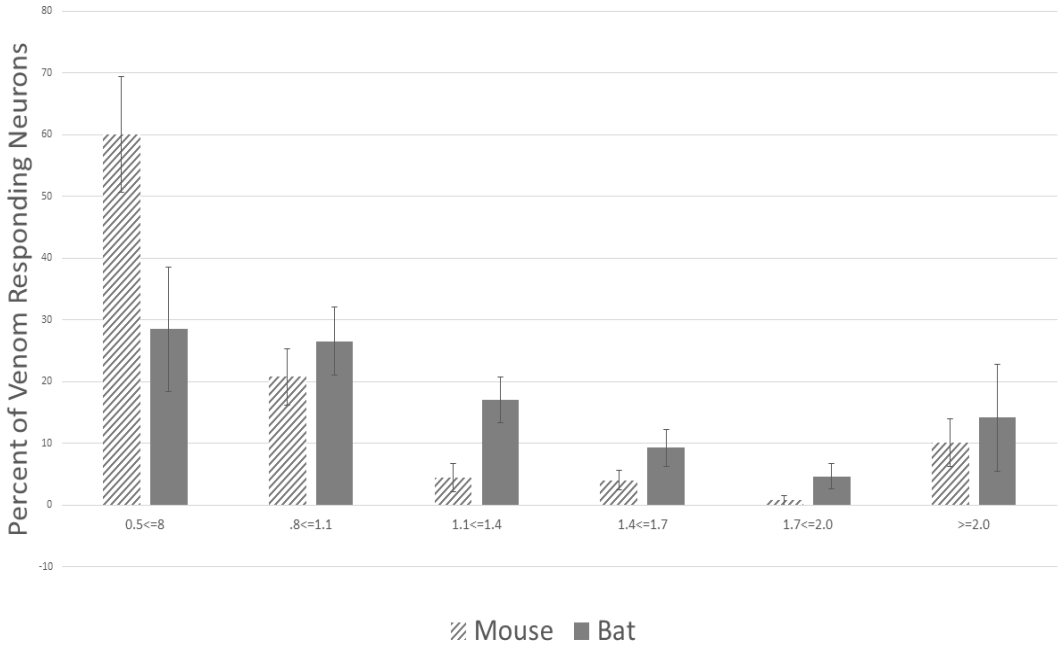


Figure 2.6 Normalized Amplitude Responses to Venom in Mouse and Bat: Normalized to 0.5 of third high potassium dose. Amplitude responses for TG in mice and bats. Bats and mice were statistically different when tested with a chi square test p=3.23432E-16

Positive Selection Analysis with PAML

Positive selection analysis was performed using the codeml program in the PAML software suite. The genes encoding the two voltage gated sodium channels most studied with respect to venom activity on sensory ganglia were chosen for analysis, SCN9A and SCN10A which encodes for Nav1.7 and Nav1.8 respectively.

Sequences form 38 species in Boreoeutheria were aligned and consensus trees formed based on available literature for SCN9A. Four amino acid sites were found to be under positive selection in SCN9A when the three gleaners used in the analysis, *A. pallidus*, *Megaderma lyra*,

and *Otonycteris hemprichii* were selected as the foreground (Figure 11). Of these four sites significant positive selection was found in one of the sites when *A. pallidus* was selected as the sole foreground and two were significant when *M. lyra* was the sole foreground. Interestingly despite being included in the gleaner foreground group, *O. hemprichii* did not show any sites under positive selection by itself.

24 species orthologs of SCN10A were aligned and a consensus tree was formed to conform to the established literature. None of these sites reach the 0.950 probability cutoff we had established for SCN9A. This could be due to less positive selection in SCN10A or greater sequence diversity may dilute any positive selection signal that may exist. For this reason we included all sites with probability of at least 0.900 for SCN10A.

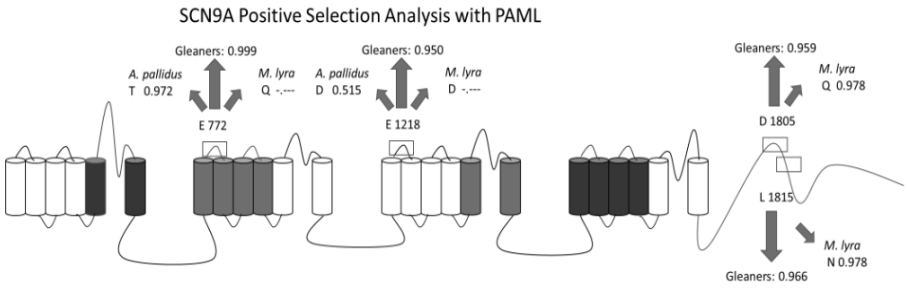


Figure 2.7 Diagram of Nav1.7 and with Residues Under Positive Selection Highlighted: Residues found to be under positive selection are highlighted and probability of positive selection for the given foreground lineage is given. If blank, then when set as the foreground that lineage was not found to be under positive selection.

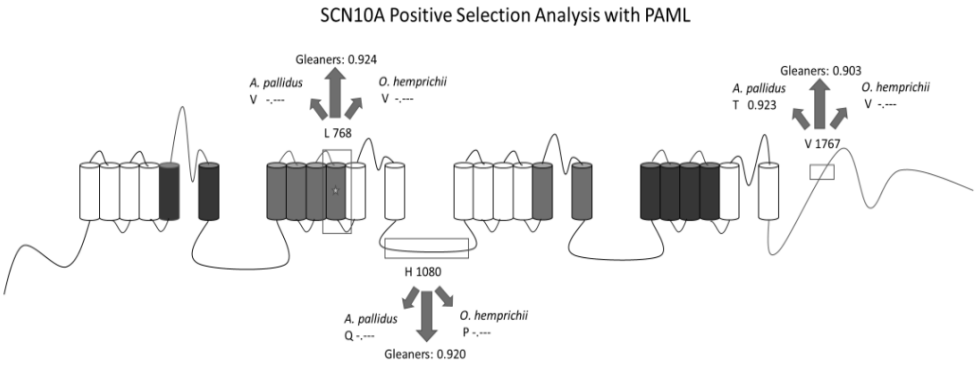


Figure 2.8 Diagram of Nav1.8 and with Residues Under Positive Selection Highlighted: Residues found to be under positive selection are highlighted and probability of positive selection for the given foreground lineage is given. If blank, then when set as the foreground that lineage was not found to be under positive selection.

Serum-Neutralization

Blood-serum based venom neutralization is found in some species of snakes, opossums, and mongooses (Kilmon Sr; Heatwole and Poran; Ovadia and Kochva; Voss and Jansa). Our current survey of the literature found no documentation of scorpion venom resistance that included a serum-neutralization component. This may be due to the neurotoxic nature of scorpion venom while snake venom can include both neurotoxic and hemotoxic toxins. The hemotoxic component of snake venom including metalloproteinases and phospholipase A2 myotoxins can be neutralized by factors found in the blood of resistant species (For Review: (Voss and Jansa)). Since scorpion venom's primary components target voltage gated ion channels it may be evolutionarily challenging to adapt serum borne molecules to neutralize them.

While pallid bats lack blood serum which neutralizes venom it is possible other components of the innate and acquired immune response enhance their resistance. Mast cell enzymes enhance the survivability of mice to venom injections from scorpions, snakes, honeybee, and Gila monster (Akahoshi et al.; Metz et al.; Schneider et al.). Further, previous sub-lethal exposure to a venom can enhance survivability of later normally lethal doses (Marichal et al.) suggesting the immune system helps combat venom. While bat blood serum is not able to neutralize venom it is possible other aspects of the bat's immune system influence venom reactions. We studied wild caught bats which means it is possible the bats in this study had encountered scorpion venom before and acquired enhanced resistance through generation of immune antibodies. Since scorpions routinely kill mammals of similar size, it is reasonable to assume that even if exposure can enhance pallid bat venom resistance they must have some innate resistance to scorpion venom. Otherwise the bat would die in its first encounter and be unable to enjoy the benefits of acquired resistance. Further study is needed in chiropteran immunology with respect to venom resistance.

20 mg/kg venom injection

Pallid bats show a remarkable resistance to Arizona bark scorpion venom. Previous work has shown that pallid bats are almost completely unfazed by venom concentrations up to 10 mg/kg (Hopp et al.). This work expands to 20 mg/kg and while the bats showed more signs of twitching and lip smacking all 7 bats in this study made full recoveries within 24 hours. The LD50 values for scorpion venom across species vary widely with *C. sculpturatus* 1-1.5 mg/kg, *Androctonus australis* 0.32 mg/kg, *Hadrurus arizonensis* 168 mg/kg i.p., *Centruroides limpidus tecomanas* 0.69 mg/kg (Stahnke; Watt and Simard). The LD50 of *C. sculpturatus* venom in grasshopper mice

range from 3.91 mg/kg (northern grasshopper mouse) to 18.35 (southern grasshopper mouse sympatric with *C. sculpturatus*) (Rowe and Rowe) suggesting that even among venom resistant species, the pallid bat is exceptional. The LD50 in the pallid bats tested in this study is >20 mg/kg, the highest known value so far for *C. sculpturatus* venom.

Whether pallid bats show geographic differences in venom resistance is unclear. Although bats used in this study were caught in California, Arizona and New Mexico, there appeared to be no clear individual differences in reactions. This may be due to the volant nature of pallid bats combined with their wide geographic distribution. Pallid bats display female philopatry and male mediated genetic dispersal (Weyandt and Van Den Bussche; Lack, Wilkinson and Van Den Bussche). In this way a selective pressure on one population of pallid bats lead to adaptations which are then spread across the bat's range. In the context of venom toxicity *C. sculpturatus* is not the most lethal North American scorpion. If they were the only selective force acting on pallid bats the resistance of pallid bats may not be as high compared to other predators of *C. sculpturatus*. However there are various Mexican and Central American scorpions with nastier stings than *C. sculpturatus* whose range overlaps with pallid bats in northern and central Mexico. The bats in this sympatric region may have had a strong selective pressure placed on them resulting in extreme adaptations. These adaptations may have then been dispersed to their northern neighbors via male mediated dispersal resulting in southwestern US pallid bats acquiring their 'super resistance' even if faced with less toxic prey. This concept of 'hot spots' having effects on adjacent populations is predicted the geographic mosaic theory of coevolution (Review (Thompson)) . Since bats can disperse their genetic material over much greater distances than ground based animals the effects of these "hot spots" might cover a larger

geographic area. Future studies should examine bats caught near the northern extremes of the range, which are farther removed from the most toxic scorpions sympatric with pallid bats.

Constellation Pharmacology

Our results deviate in overall subclass distribution for sensory ganglia (Teichert, Raghuraman, et al.; Teichert, Schmidt and Olivera; Teichert, Memon, et al.). This may be due to different culturing protocols or the effects of whole venom on cell survivability as only cells which complete the experiment were counted for analysis. This is especially interesting given that 4 of 7 bat individuals failed to meet the criteria of 100 surviving neurons despite starting with similar number of cells to other cultures. This low survival rate may be related to the elevated activity seen in bat cells in response to venom compared to mouse. For purpose of this discussion, however, we will focus on the cells which survived through the experiment.

The most profound difference is seen in the total number of cells which respond to venom in mice versus bats (Figure 4). Within each subclass, bat TG had more cells responding to venom than mouse except for the menthol and acetylcholine subclasses. This soundly disproves our hypothesis that bat sensory neurons would show less activity than mouse in response to venom. However, since calcium imaging is only a measure of intracellular calcium we cannot say if fluorescent neurons are sending more action potentials or even significantly depolarizing, only that the calcium inside the cells is increasing. If calcium is entering the neurons but not sending signals to the CNS the pallid bat may have a mechanism similar to the grasshopper mouse (Rowe 2013). In the grasshopper mouse Nav1.7 is opened by scorpion venom but Nav1.8 is blocked by venom. Nav1.7 is primarily responsible for action potential generation while Nav1.8 is responsible for action potential propagation. By shutting down Nav1.8 any depolarization or

calcium entry at the cell body would be irrelevant from a pain sensation perspective as there would be no way to transmit the signal to the CNS. While the most parsimonious solution might at first appear for a venom resistant species to have a mutation preventing any depolarization or calcium entry this tactic is confounded by the vast array of molecules present in scorpion venom. With some scorpions having up to hundreds of individual proteins (Casewell et al.) in their venom an organism would need a copious number of mutations to be fully resistant to the venom. By taking a signal blocking route, however, the animal would only need one or a few mutations, enough for venom to block the signal it is trying to generate. In this way the problem of venom resistance is simplified to only a few, or even one, gene as opposed to dozens or hundreds.

Another advantage to allowing the venom to depolarize the soma while blocking the signal is one of sensitization and desensitization. Scorpion envenomation is marked by an acute initial pain phase which then gives way to a longer term burning sensation along with hyperalgesia, allodynia and systemic dysfunction (Review: (Casewell et al.)) While dealing with the initial pain is crucial for prey capture and consumption, a predator which is later gripped by debilitating pain would itself make an easy meal for one of its predators. Therefore there must be some mechanisms in place to deal with the longer lasting effects of envenomation.

Calcium mediated desensitization has been extensively studied as possible routes for treatment of chronic pain, focusing especially on TRPV1, TRPA1, and TRPM8 (Gordon-Shaag, Zagotta and Gordon; Rohács et al.; Julius; Wang et al.; Touska et al.; Vyklicky et al.). If the pallid bat employs a similar signal blocking strategy as the grasshopper mouse then stimulating the

cell body while the block is in effect would activate desensitization pathways to eliminate the lingering effects of scorpion envenomation.

For the final part of our study we looked at signs of positive selection in gleaning bat SCN9A and SCN10A to explore possible mechanisms for a signal blocking approach by deactivating the VGSCs involved in action potential initiation and propagation even in the presence of a high somatic calcium level.

Positive Selection

We analyzed two genes for positive selection using PAML. Of the two genes tested only SCN9A (Schematic 1) showed signs of significant selection with some residues in SCN10A approaching significance (Schematic 2). SCN9A is known as the primary target for *C. sculpturatus* venom and is crucial for action potential initiation (Maertens et al.; Moraes et al.; Rowe et al.). Interestingly one of the sites identified as being under positive selection, E772, is in a known venom binding region and the mutation seen in 2 of the 3 scorpion hunting bats tested is very similar to known mutations which reduce toxin binding in a related VGSC (Cestèle et al.). E1218 is not in a known venom binding region and the substitution is a change from a glutamic to an aspartic acid however it is in an extracellular region which may come into contact with a toxin. While both are acidic amino acids with similar chemical properties a similar substitution in (VGSC) was enough to completely abolish toxin binding affinity and, oddly, make (VGSC) susceptible to the insect specific toxin from that scorpion (Gur et al.). The absence of a corresponding mutation in *O. hemprichii* may be due to alternate venom resistance mechanisms. However the genome of *O. hemprichii* is of low quality and needed to be assembled to *Myotis lucifugus* genome in order to get a partial assembly. For this reason the

assembly of *O. hemprichii* may be incorrect in places where the assembler biased the reference genome over the raw reads. The sites detected as under selection in the C-terminus cytoplasmic tail were only detected in *M. lyra* and could be the result of poor sequencing depth for the *M. lyra* genome at that point.

There were some residues in SCN10A in gleaning bats (Schematic 2) which approached the threshold for significance and may contribute to venom resistance by altering gating kinetics or ion channel regulation. SCN10A has higher sequence diversity between species than SCN9A, a feature which can often confound positive selection analyses. While no signs of positive selection was detected by PAML. It is interesting to note that at the residues in SCN10A important for venom binding in the grasshopper mouse **Q**VS**E**K (**E**VS**Q**K in house mouse) is **E**VG**H**T in *O. hemprichii*. It is unknown if this mutation alters venom function in *O. hemprichii* but given the importance of this area in venom binding it merits further research.

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