LITERATURE REVIEW OF CELLULAR RELATEDNESS IN TOXIN AND TOXIN-LIKE NICOTINIC RECEPTOR TARGETING PROTEINS

by

Yelyzaveta Voloshyna
Bachelor of Science
George Mason University, 2020
Associate of Science
Northern Virginia Community College, 2018

Director: Dr. Nadine Kabbani, Professor
School of Systems Biology

Spring Semester 2021
George Mason University
Fairfax, VA

ABSTRACT

LITERATURE REVIEW OF CELLULAR RELATEDNESS IN TOXIN AND TOXIN-LIKE NICOTINIC RECEPTOR TARGETING PROTEINS

Yelyzaveta Voloshyna B.S.

George Mason University, 2021

Project Director: Dr. Nadine Kabbani

This project compares venomous and non-venomous snake toxins in their binding to nAChRs using a literature search and supporting the use of bioinformatics tools. A clear evolutionary functional and structural similarity is documented between venomous toxins as snake α-neurotoxins and human prototoxins as lynx. This project builds on existing knowledge in disparate fields to bridge knowledge across domains of biology from evolutionary to medical study. In the process of researching and writing this project, the author conducted a literature search and reviewed the previous work on toxins and toxin-like related proteins. This project is slated to be a reference and resource for future studies and hypothesis on toxin proteins and their evolutionary effects on nAChRs.

1. Introduction

1.1 Overview of toxins in the evolutionary context

Toxins are generally described as small molecules, proteins, or peptides that are produced within the cells of living organisms that bind to their targets, which are usually cell surface proteins such as receptors. Functionally, toxins act by impairing the normal function of cells through protein targeted pathways. As a group, biological toxins are very diverse, and scientists have classified them broadly into venom and non-venom types (i.e. endo- or exotoxins) (Kumar & Singh, 2016).

Exotoxins and endotoxins are produced by fungi or bacteria and they damage the host tissue and have an effect on their target's immune system. On the other hand, venom-derived toxins are secreted by glands in animals, like snakes, and enter the target organism via wounds. Broadly, venom consists of many types of protein as well as many types of toxins including neurotoxins and cytotoxins. Neurotoxins affect the nervous system and cytotoxins act on the cells and tissues of the whole body. A complete phylogenic tree of all existing toxins along a schematic tree of venomous organisms within the animal kingdom is presented in Figure 1. This tree reveals evolutionary relationships between venoms of various species (Kumar & Singh, 2016).

As shown in Figure 1, even the simplest of multicellular animals, such as sponges, produce toxins. *Latrunculia magnifica* Kelle sponge of a bright red color produces the small molecule actin-binding toxin latrunculin, which functions in defense against predators. This toxin is initially made in choanocytes, cells that line the interior of the sponge body and contain a central flagellum, which is surrounded by microvilli connected by a thin membrane. The toxin is then transferred to the vulnerable parts of the sponge body by archeocyte cells. To prevent self-intoxication, the sponge keeps the toxin within membrane-bound vacuoles. This process illustrates a simplex example of how a toxin is made, stored, and then released. Varied aspects of this process appear conserved across the Animal kingdom from simple to more complex venomous species (El Sayed et al., 2006).

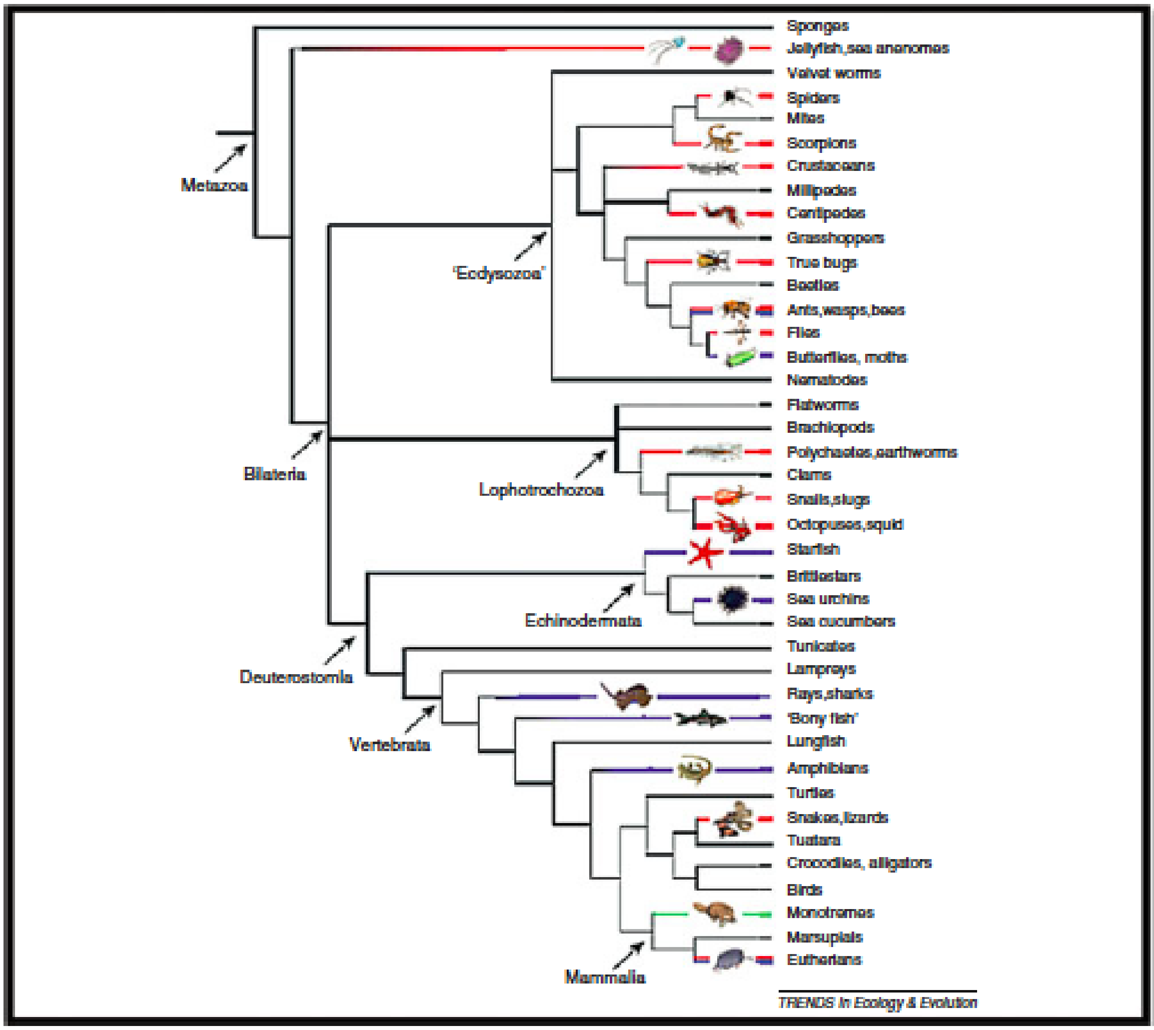

Figure 1. Evolutionary relationship between venomous animals. The figure shows the relation between the lineages of venomous animals common within the animal kingdom. Major animal lineages that contain members with venomous systems are shown by colored branches. Red-colored branches signify a venom used for predatory purposes, blue branches indicate the defensive role of venom, and green branches show lineages where venom plays role in an intraspecific competition (Kumar & Singh, 2016).

Toxins appear to co-evolve with their targets in a “predator-prey” fashion. In many cases, prey can develop resistance to the toxins of the predator, and the predator thus has to change its venom composition in order to survive. For example, mammals such as honey badgers have evolved resistance to alpha-neurotoxins from elapid snake venoms by substituting certain amino acid residues on the nicotinic acetylcholine receptor to prevent toxin binding. In turn, it is suggested, that elapid snake venom has co-evolved to bypass the honey badger’s venom resistance (Drabeck et al, 2015).

Modification in the structure of toxins may be triggered by post-transcriptional and post-translational modifications as well as recombination, gene duplication, point mutations. The formation of functionally different genes is attributed to gene duplication and functional divergence (see Figure 2, Kumar & Singh, 2016). For example, the lethal toxin β-bungarotoxin is believed to have evolved from the enzymatic toxin phospholipid A_2 (PLA_2) whose function is in digestion. With natural substitutions occurring on the PLA_2 surface, the enzyme is able to maintain its fold but changes specificity and functions (Kumar & Singh, 2016).

Another major driver for toxin evolution is species-environment interactions and the need for the organism's survival in the context of predator-prey relationship and the dynamic environment. As an example, the composition of the Malayan pitviper C. venom appears to change based on the geographic location of the snake and the variation in its prey (Daltry et al., 1996).

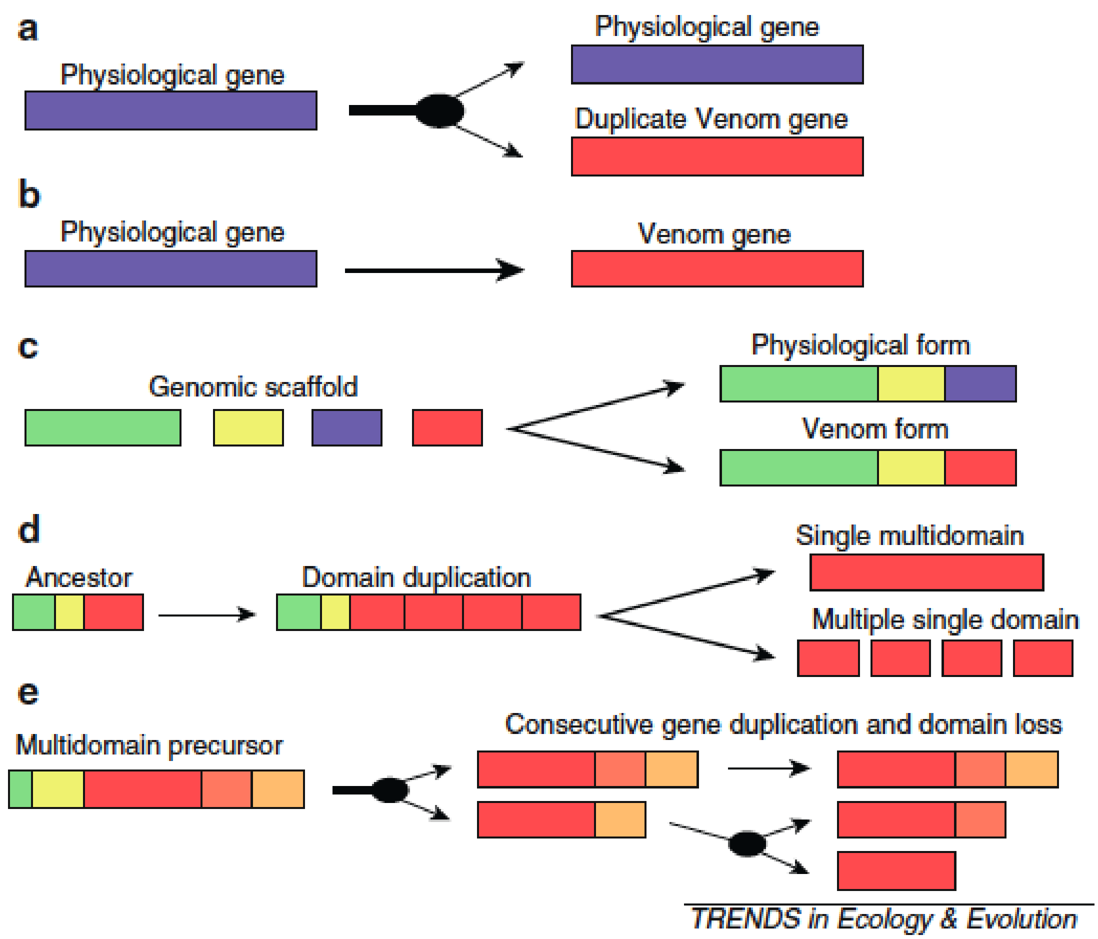

Figure 2. Mechanisms of toxin evolution. Panel (a) shows duplication of a physiological gene in the duplicate venom gene that will become a venom toxin. Panel (b) shows the modification of the physiological gene into a

venom gene. Panel (c) shows how alternative splicing plays a role in the creation of different physiological and venom toxins encoded by the same gene. Panel (d) shows how duplication of the ancestor gene domain can result in the multidomain or multiple single domain production. Panel (e) demonstrates how consecutive gene duplication and domain loss that produces multiple related venom toxins can result from the multidomain precursor gene. Black circles indicate gene duplication (Kumar & Singh, 2016).

The discovery of human prototoxins, which are non-venomous, toxin-like proteins that share similarities with snake neurotoxins in their protein structure and targets suggests that prototoxins and neurotoxins have diverged from a common ancestral gene (Miwa et al., 2019). Understanding the evolutionary origin of toxins across living organisms may lead to a better understanding of their functions and mechanisms of action. It may also uncover the common purpose of different toxins, which may be partially dictated by similarities in their molecular structure. By studying and comparing snake neurotoxins with human prototoxins, it maybe possible for scientists to develop treatments for the pathologies caused by prototoxins in humans, and to develop new toxin-based therapies based on their target site specificity.

1.2 Similarities in the molecular structure of venomous neurotoxins and non-venomous human prototoxins show convergence onto a common cellular target

Prototoxins and neurotoxins share structural similarities in the three-finger toxin (TFTx) fold. The TFTx β-fold structure of prototoxins, and elapid α-neurotoxins, is marked by cysteines that participates in the formation of disulfide cysteine bridges. Research shows that prototoxin cysteine bridges generally contain a signature motif of 10 cysteines that create a bridge that helps stabilize the β-pleated sheet fold structure (Miwa et al., 2019). One member of TFTx family is the snake derived α-bungarotoxin, a 74 amino acid, 8kDa α-neurotoxin, with a tertiary structure stabilized by four disulfide bridges, three “finger” loops, and a carboxy C-terminal tail. Two of those fingers are responsible for α-bungarotoxin binding to its target (Moise et al., 2002). Studies show that α-bungarotoxin competitively binds α7, α8, and α9 subunits of the nicotine acetylcholine receptor (nAChR) close to the agonist site in an irreversible manner thereby blocking cholinergic transmission. The presence of the aromatic residue at position 189 in the nAChR α-subunit was shown to be necessary for binding of α-bungarotoxin, but it is not required for binding the agonist, suggesting that α-bungarotoxin binds to some α subunits (Balass et al., 1997).

This similarity in the tertiary structure of α-neurotoxins and prototoxins suggests that they can act on similar targets. This was explored by research using cDNA expression library, constructed from immature granule cells that were purified from mouse cerebellum during the early postnatal days (Kuhar et al., 1993). The researchrs found that two classes of prototoxins GPI-linked and secreted are expressed. Since the TFTx fold is suggested to occur before the differentiation between venomous and non-venomous snakes (Fry et al., 2003), gene recruitment by non-toxic genes appears to be a driver in venom associated toxin production (Fry & Wüster, 2004). Within non-venomous and prototoxin expressing species, a gene duplication may have resulted in an independent specialization of the two gene copies (Loughner et al., 2016).

1.3. Common target for venomous and non-venomous toxins – nicotinic acetylcholine receptor

Venoms and prototoxins are similar in that they bind cell surface proteins such as ion channels and receptors. Neurotoxins target the nervous system, and prototoxins are expressed in the brain and the periphery. They both are found to bind to the nAChR and modulate cholinergic system activity.

Nicotinic acetylcholine receptors are pentameric transmembrane proteins that respond to ligand neurotransmitter acetylcholine (see Figure 3, Nirthanan et al., 2016). They are found in the central nervous system and the peripheral nervous system where they play role in sympathetic and parasympathetic system activation, as well as, skeletal muscular contractions. They work by regulating cellular signaling and also play a role in the immune system responses, memory, and reproduction (Wessler & Kirkpatrick, 2017; Meizel, 2004). Assembled channels have a protein molecular weight mass of ~290 kDa, with several ligand binding sites located at the interface between two different α subunits with either δ or γ subunit (see Figure 3b). Upon agonist binding, the nAChR changes conformation to open a central channel pore for cation conductance. α-neurotoxins bind to the nAChR irreversibly and prevent the agonist from binding (Squire, 2003). By binding to nAChRs, snake venoms inhibit the ion flow into the cell (Nirthanan et al., 2016). Due to the generally almost irreversible binding pattern of those toxins, the only way to get rid of them is by degrading the toxin-bound target receptor and replacing it with a newly synthesized one.

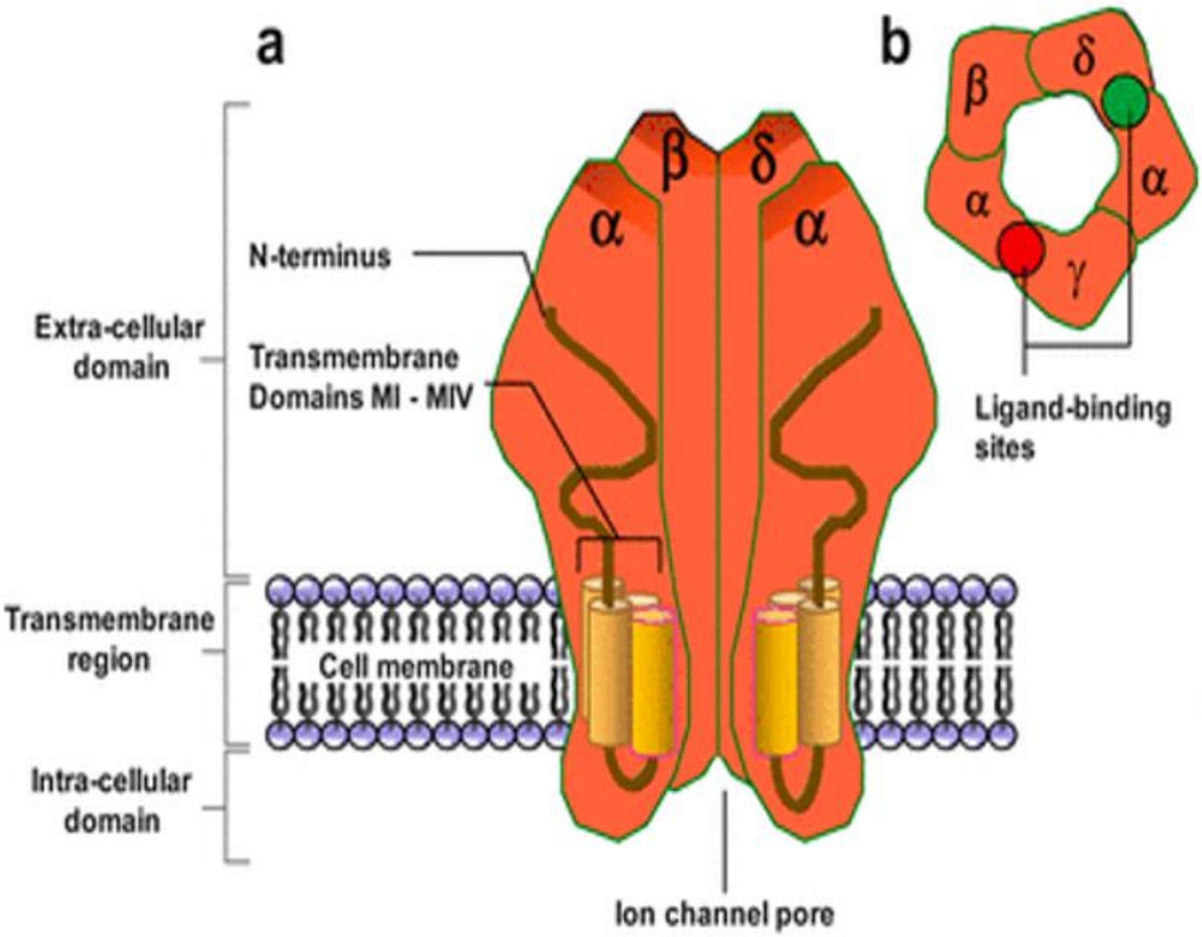

Figure 3. The structure of a muscular nAChR. Panel (a) shows the general composition of the nAChR with relation to its position in the membrane and its extra-cellular domain, transmembrane region, and intra-cellular domain. The γ subunit is not shown. Panel (b) shows the view of nAChR from the top, the association of its five subunits, and its ligand-binding sites (Nirthanan et al., 2016).

As previously mentioned snake α-bungarotoxin binds almost irreversibly to nAChRs, different prototoxins on the other hand, seem to have a varied affinity to their nAChR targets (Kessler et al., 2017). It has also been suggested that prototoxins may play an allosteric regulatory function at the nAChR site (Miwa et al., 2019) The variability of prototoxins can be explained by the differences in the binding specificity and localization or expression between the different members (Miwa et al., 2006).

2. Biological Significance

Natural selection, as the process of differential survival and reproduction of organisms based on their adaptability, where “each slight variation of a trait, if useful, is preserved”, is one mechanism that drives evolution (Darwin, 1859). In order to survive in the dynamic environment, snake venom serves an important role in immobilizing the prey and protecting the snake from dangerous predators. α-neurotoxin TFTxs are the most abundant non-enzymatic group of snake venom proteins and they act by binding to the nAChR at the prey’s neuromuscular junction (Nirthanan et al., 2016). The prey injected with a α-neurotoxin TFTx venom exhibits rapid skeletal muscle paralysis leading to death from respiratory failure. The composition of snake venom is also suggested to be correlated with a snake’s diet (Barua & Mikheyev, 2020). Previous research provides evidence that predation on other reptiles by venomous snakes is associated with peptide toxins of low molecular weight. (Davies & Arbuckle, 2019). Also, it has been suggested that venoms consisting predominantly of TFTxs have replaced the venoms of an enzymatic kind, including snake venom serine protease (SVSP), as a major and widespread component in elapid venoms (see Figure 4, Barua & Mikheyev, 2019). Various changes in prey diversity and environmental conditions of the snakes may lead to changes in the expression of different toxin families. This variation may produce a broad range of phenotypic effects, which may lead to speciation. For example, the changes in toxin expression may influence mating behaviors. It has been suggested that snakes exhibit mating preference towards the snakes of the opposite gender with a similar venom composition, but more research is needed to test this hypothesis. Another consequence of differential expression of toxin families may involve indirect ecological changes related to the food searching styles, differences in everyday behaviors, and the choices of habitat. These suggested consequences could ultimately result in reproductive isolation, which would confirm the possibility of new species formation. Overall, it is suggested that the snake venom evolved with both the changes in the environment and the evolution of the prey. Due to these changes, some snakes may have become extinct if they could not keep up with the dynamic environment. Therefore, the term adaptive radiation is used in this context (Barua & Mikheyev, 2020).

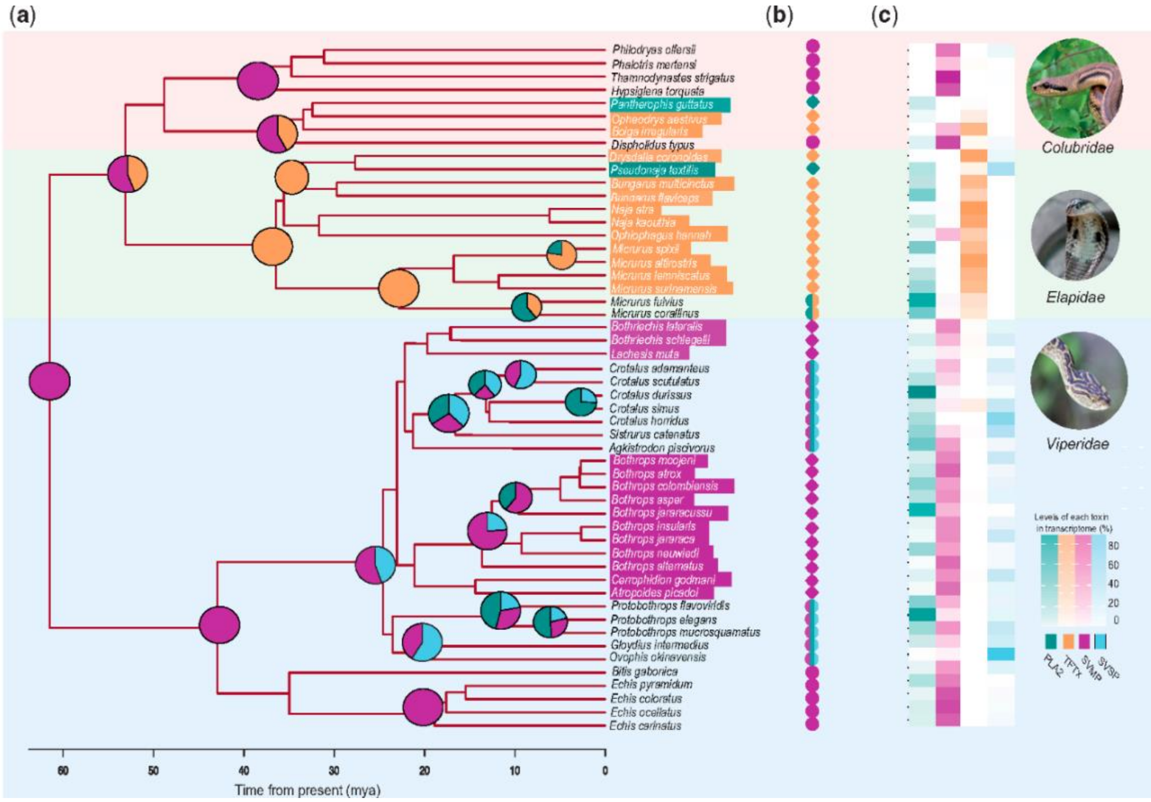

Figure 4. Evolution of venom toxins. Panel (a) Pie charts at selected nodes represent ancestral state reconstruction (ASR) of the four major toxins TFTx, SVMP, SVSP, and PLA2. Panel (b) demonstrates common selective regimes. Panel (c) indicates the relative abundance of venom toxin in snakes of indicated toxin families (Barua & Mikheyev, 2019).

Based on evolutionary structural relatedness and common targeting of the nAChR, snake derived toxin and protoxins appear to have many overlapping properties. However, since humans do not secrete prototoxins, how endogenous prototoxins operate through nAChR regulation is not clear and raises an important question: If human prototoxins and snake α-neurotoxins have evolved from a common ancestral gene, why have humans preserved prototoxins? One could speculate that based on the snakes' information and the given similarities, there should be an important function that prototoxins play in humans (either directly or indirectly). On the other hand, it is tempting to speculate on the nature of pathologies that may arise from dysregulation of prototoxin genes within humans and other mammals. Research on the prototoxin lynx1 using knockout mice of the lynx1 gene demonstrates a role for this protein in nicotine mediated neuroprotective effects in the brain. Aging lynx1 knockouts where also shown to to suffer from progressive neural degeneration (Miwa et al., 2006).

Past research has discovered multiple members of the prototoxin genes that arise from the y6/uPAR superfamily and include lynx1 and lynx2 prototoxins that seem to play an important role in the regulation of the nAChRs and are thus potential targets for pharmacological treatments (Miwa et al., 2019). A tree of the molecular evolutionary relationship between neurotoxic snake venoms and human prototoxins, as well as members of other selected toxin families that are not considered in this research, is presented in Figure 5.

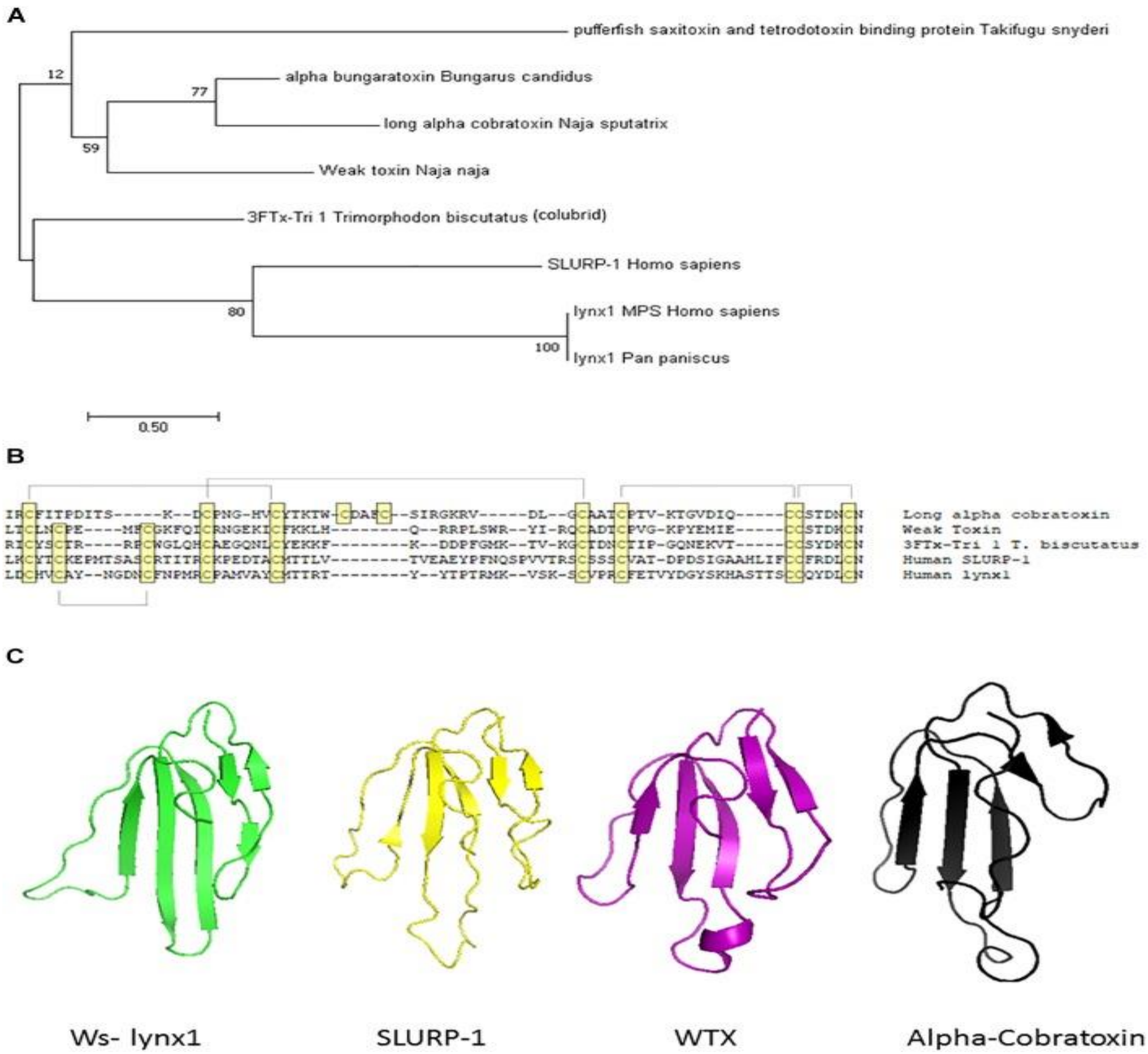

Figure 5. Evolutional relationship between different toxins and their structural similarities. Panel (A) shows the bootstrap consensus tree of molecular evolutionary relationship. Panel (B) indicates the amino acid comparison of selected toxin family members. Panel (C) demonstrates a structural comparison of selected toxin family members (Miwa et al., 2019)

There are certainly similarities between the structures of the studied toxins, and investigating these similarities even further enables scientists to use the available snake toxins to understand human prototoxins and their mechanisms of action. Moreover, toxins provide a natural template for peptide-based drug design in the context of various human diseases.

A water-soluble variant of lynx1 prototoxin called ws-lynx1 was found to elicit a non-competitive and concentration-specific effect on nAChRs α7, α3β2, and α4β2 by interaction with residues on two of the "finger loops". However ws-lynx1 effect differs from endogenous lynx1, which is anchored to the plasma membrane by GPI. Endogenous GPI-linked lynx1 was found to cause the desensitization of the nAChRs and caused the target receptor to have a lower affinity for the agonist. Lynx1 was also found to alter nAChR opening, expression, and desensitization, which may contribute nicotine addiction (Miwa et al., 2019).

Due to high levels of lynx1 expression in the hippocampus, cerebellum, and cortex, it has been suggested to also play a role in the negative regulation of nAChR-dependent cortical and hippocampal plasticity. Indeed, lynx1 knockout mice (lynx1KO) are found to perform better at learning and memory tasks, and studies suggest that lynx1 is neuroprotective in Alzheimer's Disease by competing with $A\beta_{1\text{-}42}$ for nAChR target. Lynx2 was shown to also be expressed in the hippocampus however, unlike lynx1, which appears localized to CA3, lynx 2 is abundant in CA1 as well as the prefrontal cortex and the amygdala, suggesting a role for this lynx in emotional control. This has been supported by studies using lynx2 knockout mice that show an increase in anxiety related experimental behaviors (Miwa et al., 2019). Anxiety arises from the reaction to a perceived threat. Venomous snakes release toxins in response to a threat as a self-protection mechanism. The results of this study suggest that the release of prototoxin lynx2 may be a part of the self-protection mechanism in non-venomous mammals, further suggesting the possibility of threat pathways being conserved between these venomous and non-venomous species.

Goals, Hypotheses, and Future Directions

3.1. Project goals

The goal of this project is to compare venomous and non-venomous snake toxins in their binding to nAChRs using literature search and supporting use of bioinformatics tools. A clear evolutionary functional and structural similarity is documented between venomous toxins as snake α-neurotoxins and human prototoxins as lynx. This project will build on existing knowledge in disparate fields to bridge knowledge across domains of biology from evolutionary to medical study. In doing so, I will define key pathways and mechanisms that enable parallel, convergence, and possible divergence across protein toxin actions and targeting in various species.

3.2. Hypotheses

Specific Hypothesis 1. Due to structural similarity between snake toxins and human prototoxins that contain TFTx fold and their targeting of the nAChR, snake toxins and prototoxins drive common signaling pathways in cells.

Specific Hypothesis 2. Due to their evolutionary relatedness, venomous toxins and human prototoxins are likely to share common gene regulatory networks across species.

Specific Hypothesis 3. Species-specific adaptation to key environmental factors enables diversity and divergence in proteins with toxin-like properties.

3.3. Future Directions

Specific Direction 1. Homologs and orthologs of toxin and toxin like related proteins will be identified based on similarities between the sequences of snake neurotoxins, human prototoxins, and sequence alignments.

Specific Direction 2. Important areas of the proteins based on overlapping parts of the sequences will be examined and their potential role in the toxin action, as well as its effect on the nAChRs, will be studied.

Specific Direction 3. Semi-quantitative analysis of toxin and toxin like relatedness will be conducted.

The following public databases and tools as well as others will be used:

a. BLAST

BLAST is an NIH-developed searching tool that finds alignment between biological sequences. It can compare the protein sequences to the databases of sequences and shows the statistical significance of the provided protein alignments (McGinnis, 2004).

b. UniProt

UniProt is a database for protein sequence and functional information developed by the European Bioinformatics Institute, the Swiss Institute of Bioinformatics, and the Protein Information Resource. UniProt can provide annotations on protein names, sequences, their functions, catalytic residues, expression pattern, location, and sites of significant domains, cofactor and ion binding sites, protein-protein interactions, and protein variants (The UniProt Consortium, 2017).

c. SignalP v. 5.0

SignalP is a powerful tool for signal peptide predictions. Signal peptides are the short sequences of amino acids in the N-terminus of many newly synthesized proteins and they serve as a ZIP code to where those proteins are going to go after translation (Armentos et al., 2019).

d. NCBI Protein Database

NCBI protein database is an integrated collection of protein sequences pulled from several scholarly sources (NCBI, 2016, pp.7-19).

e. RCSB PDB Structural Database

Research Bioinformatics Protein Data Bank(RCSB PDB) is a free repository of archive information about shapes of proteins and their assembly (Burley et al., 2019).

f. T3DB

The Toxin and Toxin Target Database (T3DB) is an open bioinformatics resource that includes information about toxins and their targets. This database enables researchers to obtain the mechanisms of particular toxins and their target proteins (Wishart et al., 2015).

g. PANTHER

Protein Analysis Through Evolutionary Relationships (PANTHER) Classification System is a resource for protein classification from the evolutionary and functional standpoints (Mi et al., 2019).

4. References

Armenteros J, Tsirigos K, Sønderby C, Petersen T, Winther O, Brunak S, Heijne G, Nielsen H. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nature Biotechnology. 2019. 37:420-423.

Balass M, Katchalski-Katzir E, Fuchs S. The α-bungarotoxin binding site on the nicotinic acetylcholine receptor: Analysis using a phage–epitope library. Proceedings of the National Academy of Sciences. 1997. 94 (12) 6054-6058.

Barua A, Mikheyev A. Many Options, Few Solutions: Over 60 My Snakes Converged on a Few Optimal Venom Formulations. Molecular Biology and Evolution. 2019. 36(9):1964–1974.

Barua A, Mikheyev A. Toxin expression in snake venom evolves rapidly with constant shifts in evolutionary rates. Proceedings of the Royal Society B: Biological Sciences. 2020. 287:1-10.

Burley S, et al. RCSB Protein Data Bank: Biological macromolecular structures enabling research and education in fundamental biology, biomedicine, biotechnology and energy. Nucleic Acids Research. 2019. 47:464-474.

Daltry J, Wuster W, Thorpe R. Diet and snake venom evolution. Nature. 1996. 379:537–540.

Darwin C. (1859). *On the origin of species by means of natural selection, or, the preservation of favoured races in the struggle for life*. London: J. Murray.

Davies E, Arbuckle K. Coevolution of Snake Venom Toxic Activities and Diet: Evidence that Ecological Generalism Favours Toxicological Diversity. Toxins. 2019, 11(12):711.

Drabeck D, Dean A, Jansa S. Why the honey badger don't care: Convergent evolution of venom-targeted nicotinic acetylcholine receptors in mammals that survive venomous snake bites. Toxicon. 2015. 99:68-72.

El Sayed K, Youssef D, Marchetti D. Bioactive natural and semisynthetic latrunculins. Journal of Natural Products. 2006. 69(2):219-223.

Fry B, Lumsden N, Wüster W, Wickramaratna J, Hodgson W, Kini R. Issolation of a neurotoxin (alpha-colubritoxin) from a nonvenomous colubrid: Evidence for early origin of venom in snakes. Journal of Molecular Evolution. 2003. 57(4):446-452.

Fry B, Wüster W. Assembling an arsenal: Origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences. Molecular Biology and Evolution. 2004. 21(5):870-883.

Kessler P, Marchot P, Silva M, Servent D. The three-finger toxin fold: a multifunctional structural scaffold able to modulate cholinergic functions. Journal of Neurochemistry. 2017. 142:7-18.

Kuhar S, Feng L, Vidan S, Ross M, Hatten M, Heintz N. Changing patterns of gene expression define four stages of cerebellar granule neuron differentiation. Development. 1993. 117(1):97-104.

Kumar R, Singh B. (2016). *Evolution of Toxin. In: Protein Toxins in Modeling Biochemistry.* Springer Briefs in Biochemistry and Molecular Biology. Springer, Cham. 113-131.

Loughner C, Bruford E, McAndrews M, Delp E, Swamynathan S, Swamynathan S. Organization, evolution and functions of the human and mouse Ly6/uPAR family genes. Human Genomics. 2016.10:1-19.

McGinnis S, Madden T. BLAST: At the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Research. 2004. 32:20-25.

Meizel S. The sperm, a neuron with a tail: ‘neuronal’ receptors in mammalian sperm. Biological Reviews. 2004. 79:713-732.

Mi H, Muruganujan A, Ebert D, Huang X, Thomas P. PANTHER version 14: More genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. Nucleic Acids Research. 2019. 47:419-426.

Miwa J, Anderson K, Hoffman K. Roles of Endogenous Mammalian Neurotoxin-Like Proteins in Modulating Nicotinic Acetylcholine Receptor Function to Influence Complex Biological Processes. Frontiers in Pharmacology. 2019. 10:1-12.

Miwa et al. The Prototoxin lynx1 Acts on Nicotinic Acetylcholine Receptors to Balance Neuronal Activity and Survival In Vivo. Neuron. 2006. 51(5):587-600.

Moise L, Piserchio A, Basus VJ, Hawrot E. NMR structural analysis of alpha-bungarotoxin and its complex with the principal alpha-neurotoxin-binding sequence on the alpha 7 subunit of a neuronal nicotinic acetylcholine receptor. The Journal of Biological Chemistry. 2002. 277 (14):12406–12417.

NCBI Resource Coordinators. Database resources of the National Center for Biotechnology Information. Nucleic Acids Research. 2016. 44:7-19.

Nirthanan S, Awal W, Niranjan N. Snake α-Neurotoxins and the Nicotinic Acetylcholine Receptor. (2016) In: Gopalakrishnakone P, Inagaki H, Mukherjee A, Rahmy T, Vogel CW. Snake Venoms. *Toxinology*. Springer, Dordrecht.1-39.

Squire L. (2003). *Fundamental neuroscience*. Amsterdam: Academic Press. 1426.

The UniProt Consortium. UniProt: the universal protein knowledgebase. Nucleic Acids Research. 2017. 45:158-169.

Wessler I, Kirkpatrick C. Non-neuronal acetylcholine involved in reproduction in mammals and honeybees. Journal of Neurochemistry. 2017.142:144-150.

Wishart D, Arndt D, Pon A, Sajed T, Guo A, Djoumbou Y, Knox C, Wilson M, Liang Y, Grant J, Liu Y, Goldansaz S, Rappaport S. T3DB: the toxic exposome database. Nucleic Acids Research. 2015. 43:928-934.