

Review

Coevolution Between Three-Finger Toxins and Target Receptors

Jéssica Lopes de Oliveira  and Henrique Roman-Ramos * 

Laboratório de Biotecnologia, Postgraduate Program in Medicine, Universidade Nove de Julho (UNINOVE), São Paulo 01504-001, SP, Brazil; jessica.lo@uni9.edu.br

* Correspondence: ramoshr@uni9.pro.br

Abstract

Background: Three-finger toxins (3FTxs) are a major axis of functional diversification in advanced snake venoms, with canonical paralytic activity mediated through muscle-type nicotinic acetylcholine receptors (nAChRs) and a broader set of non-nicotinic targets. This review integrates evidence bearing on coevolution between 3FTxs and target receptors, spanning toxin origin, diversification, receptor evolution, and ecological context. **Methods:** The synthesis draws on comparative genomic and transcriptomic studies of 3FTx gene-family evolution, codon-model analyses of selection, structural characterisation of toxin–receptor interfaces, and functional assays (including receptor-mimicking peptide binding) that link sequence variation to binding and toxicity. **Results:** Across lineages, 3FTx diversification is repeatedly structured by strong constraint on the disulphide-rich scaffold with accelerated change concentrated in solvent-exposed loops, alongside birth–death dynamics and exon/segment-level innovation that expand binding specificity. On the receptor side, resistance-associated variation is most intensively characterised for the nAChR α_1 orthosteric site and includes convergent, mechanistically distinct solutions such as electrostatic repulsion and glycosylation-mediated steric interference. Within the predominantly elapid systems currently examined, integrative datasets indicate that prey-selective binding and geographically variable susceptibility can arise from modest substitutions at toxin–receptor interfaces, but they also reveal substantial taxonomic and target-specific biases. **Conclusions:** Current evidence supports adaptive diversification in both toxins and receptors, while broader evolutionary interpretations are limited by uneven sampling and the frequent lack of matched toxin and receptor variants analysed within a common evolutionary framework. Development of predictive models will require joint pipelines linking genomics, structure-informed evolutionary inference, scalable functional assays, and explicit ecological network context.

Keywords: three-finger toxins; nicotinic acetylcholine receptor; α -neurotoxins; venom resistance; molecular coevolution; birth–death evolution; mimotopes; toxin–receptor interface



Academic Editor: Michael Schubert

Received: 14 December 2025

Revised: 3 February 2026

Accepted: 12 February 2026

Published: 14 February 2026

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1. Introduction

Venom systems offer exciting possibilities for understanding the intricacies of molecular adaptation and antagonistic coevolution, but only a few toxin–receptor pairs have been detailed enough to allow robust comparative analysis. In this context, three-finger toxins (3FTxs) represent one of the most extensively studied systems. They are small, non-enzymatic proteins derived from the lymphocyte antigen-6 (Ly6)/urokinase-type plasminogen activator receptor (uPAR) superfamily that retain a conserved disulphide-stabilised “three-finger” fold whilst exhibiting extensive sequence and functional diversification of

the solvent-exposed surfaces [1–5]. Genomics, transcriptomics, and proteomics-based studies reveal that 3FTxs are the main components of the venoms of many elapids and sea snakes and that these are large multigene families with lineage-specific expansions and high venom gland expression of genera like *Acontophis*, *Bungarus*, *Dendroaspis*, *Hemachatus*, *Hoplochephalus*, *Hydrophis*, *Micrurus*, and *Naja* [6–13].

Despite their shared structural scaffold, 3FTxs have evolved to interact with muscle-type and neuronal nicotinic acetylcholine receptors (nAChRs) [14–16], muscarinic acetylcholine receptors (mAChRs) [17–21], potassium channels [22,23], acetylcholinesterase [24,25], cell membranes [26–30], and acid-sensing ion channels (ASICs) [31–35]. This combination of a conserved core and a rapidly changing surface underlies the exceptional functional breadth of 3FTxs, a textbook example of scaffold-based molecular innovation. Structural and molecular evolutionary analyses consistently show that the disulphide-bonded core is subject to strong purifying selection, whereas solvent-exposed residues in the three “fingers” loops evolve under positive selection and contribute disproportionately to the toxin–receptor interface [36,37].

Two mutually non-exclusive explanatory mechanisms have been proposed. The ASSET (Accelerated Segment Switch in Exons to alter Targeting) model, which is mainly derived from the study of three-finger toxins and other venom proteins, suggests that short exonic segments coding for key contact regions are completely replaced, thus generating “mosaic” toxins that can explore entirely new regions of sequence space whilst maintaining the overall fold [38,39]. In contrast, the RAVER (Rapid Accumulation of Variations in Exposed Residues) model, which was only developed from 3FTx data, describes a more detailed mechanism whereby adaptive point substitutions at exposed residues, particularly within loop tips and other contact surfaces, gradually accumulate, thus changing affinity and specificity without the need for extensive exon restructuring [40,41].

Whilst the mechanistic and evolutionary evidence supporting these frameworks has, until recently, been derived largely from elapid systems, reflecting both their medical prominence and the high expression of 3FTx in their venom, emerging studies of colubrid venoms now demonstrate that 3FTxs are not confined to front-fanged snakes; rather, they occur as abundant and actively evolving venom components in several rear-fanged lineages, exhibiting the same focal diversification and positive selection that characterise this toxin family across advanced snakes [42].

The best-studied 3FTx targets on the receptor side are nAChRs. Canonical α -neurotoxins interact with the muscle-type nAChR $\alpha 1$ subunit orthosteric site; therefore, they bind a small region of the extracellular domain that includes loop C and critical aromatic residues [43–46]. Comparative analyses of CHRNA1, the gene encoding the $\alpha 1$ subunit of the muscle-type nicotinic acetylcholine receptor, in various vertebrates reveal multiple independent evolutions of resistance to α -neurotoxins. These include N-linked glycosylation motifs that block toxin docking by steric hindrance, proline substitutions that change local backbone geometry, and other modifications that alter the shape or chemistry of the binding pocket. Such motifs have evolved convergently in different lineages, e.g., mammalian predators, squamate prey, and amphibians and, in most cases, they are associated with a decrease in α -neurotoxin-rich venom binding in mimotope-based assays [47–51].

These discoveries form a strong case that venom-imposed selection has been the driving force behind nAChR structural changes on numerous occasions. Simultaneously, it also acknowledges that there are limitations: not all the lineages that have been exposed to neurotoxic venom have developed resistant phenotypes, and some substitutions have taken place without obvious signs of positive selection, thus suggesting trade-offs between receptor function and resistance which will be elaborated later. Furthermore, three-finger

toxins bind with non-nicotinic targets, hence coevolution can spread to these new areas as well. For instance, muscarinic toxins such as MT1 (UniProt P81030) and MT7 (UniProt Q8QGR0), from *Dendroaspis angusticeps*, could represent the neofunctionalisation products underlying M1/M2/M3 mAChRs targeting [17,20], while toxins such as calciseptine (UniProt P22947) from *Dendroaspis polylepis polylepis* selectively target voltage-gated calcium channels [52–55].

Mambalgins broaden the scope of 3FTx–receptor interactions through their activity on ASIC1-containing channels in the central and peripheral nervous systems. Mambalgin-1 (UniProt P0DKR6) and mambalgin-2 (UniProt P0DKS3), both derived from *D. polylepis polylepis*, inhibit ASIC1a- and ASIC1b-containing channels and induce strong naloxone-insensitive analgesia in vivo. Structural and functional studies, however, indicate that mambalgin-2 binds at subunit interfaces and stabilises the channel in its non-conducting state [32–34]. A recent investigation on mambalgin-3 (UniProt C0HJB0), from *D. angusticeps*, demonstrates that toxin activity is highly dependent on both species and splice-variant, with strong inhibition of rodent ASIC1a/1b and weaker, context-dependent effects on human ASIC1b, including potentiation under mild-to-moderate extracellular acidosis [35]. These findings highlight the vast versatility of the three-finger scaffold and underscore the importance of species-specific receptor context in shaping toxin function.

Here, 3FTx–receptor interactions might be considered as a sequence of repeated arms races. On the toxin side, there is the rapid diversification of surface-exposed residues and the expansion of multigene families; thus, there could be a great variety of paralogues differing in their potency, specificity, and kinetic properties [56,57]. To counter this, on the receptor side, the convergent evolution of resistance motifs in nAChR α_1 , as well as the new examples of the targets' resistance, show that steric, electrostatic, and conformational solutions to the problem of toxin binding can be achieved repeatedly by strong selection imposed by venom, thus driving it [58].

These molecular dynamics occur within broader ecological and macroevolutionary contexts. Venoms rich in 3FTx have evolved multiple times across advanced snakes, including both elapid and colubrid lineages, and both venom composition and receptor genotypes can vary geographically, correlated with prey spectra and community structure [42,59–61]. In principle, such variation provides a basis for quantitative eco-evolutionary coupling, whereby differences in prey composition, resistance frequencies, or encounter rates could be linked to variation in selection intensity on toxin–receptor interfaces across populations or regions. Although such parameters remain sparsely characterised for most systems, this framework highlights how ecological heterogeneity can shape evolutionary trajectories without implying uniform selective regimes.

2. Evolutionary Origin and Diversification of 3FTxs

3FTxs have been identified through comprehensive studies as a specialised set of proteins which snakes evolved by modifying the Ly6/uPAR (LU) domain protein framework, and later by gene-family expansion and repeated functional divergence in different lineages [1]. A common pattern across the genomes of snakes is that innovations are made possible by (i) the structural “release” from membrane anchoring [1,37], (ii) a number of duplications and turnovers of paralogues [62,63], and (iii) the rapid diversification concentrated on solvent-exposed residues forming target-binding surfaces, though there is still a debate on the relative contribution of point substitution (RAVER) [41] versus exon/segment exchange (ASSET) [39] as main mechanisms of functional novelty. The 3FTx repertoires are not fixed; they differ substantially among elapid lineages and between marine and terrestrial radiations. Moreover, they encompass multiple independently derived functional classes, including non-canonical monomeric α -neurotoxins that have given rise to

NaV-active toxins such as Calliotoxin (UniProt P0DL82) from *Calliophis bivirgatus* [64], muscarinic toxin-like proteins that target muscarinic acetylcholine receptors [19–21], and covalent as well as noncovalent dimers whose quaternary organisation further expands functional space [65,66].

2.1. Ly6/uPAR Ancestry and Recruitment into Venom

At present, the lineage of snake venom 3FTxs can be traced to the Ly6 antigen/uPAR superfamily of metazoan proteins, which is defined by the LU domain, a small disulphide rich module of approximately 60 to 90 amino acids [1]. This domain is characterised by a conserved framework of eight to ten cysteine residues forming four to five disulphide bonds, which stabilise the characteristic “three finger” architecture comprising a central disulphide bonded core and three protruding loops. Proteins with the LU domain may be either single-domain or multidomain architectures (1–3 LU domains) and, in general, they are either secreted or cell-surface-associated, with a large number of Ly6 family proteins being glycosylphosphatidylinositol (GPI), anchored through a C-terminal hydrophobic segment which is cleaved and replaced by a GPI anchor [67–69].

Under non-venom standard conditions, the structure mentioned above supports completely different functions of receptor modulation and cell–cell interactions. For instance, LU-domain proteins serve as endogenous modulators of ligand-gated ion channels, while uPAR acts as a multi-LU receptor within proteolytic signalling networks [70,71]. In sum, these functions show that LU domains are very capable of extracellular protein–protein interactions and receptor binding; however, they are most of the time limited by membrane localisation and endogenous physiological functions [72].

2.1.1. Evolutionary Origin of Venom 3FTxs from Membrane-Anchored LU Ancestors

The large-scale comparative study of 1427 Ly6 family proteins, integrating structure-guided inference with Bayesian and maximum-likelihood phylogenetics, finds venom 3FTxs as a derived clade within toxicoferan Ly6 lineages [1]. This research establishes non-venomous “pre-3FTx” sequences as the closest sister group to venom 3FTxs.

Two key observations support this inference: first, about 86% of Ly6 proteins have a C-terminal membrane-anchoring domain (MaD), which is indicative of the superfamily’s overall membrane association; second, the reconstructed pre-3FTx condition retains this MaD, while venom 3FTxs are a derived clade characterised by MaD loss, representing a transition from membrane-bound proteins to soluble secreted toxins (Figure 1). Therefore, the central innovation is not just the sequence divergence within the LU fold, but a shift in cellular localisation and trafficking. This change allowed for expanded interaction surfaces and access to a broader range of receptor targets.

An important aspect of this evolutionary inference is that it relies on multiple lines of evidence beyond sequence similarity alone. In that study [1], structural homology was assessed using DALI, a structure-based protein comparison algorithm, and combined with phylogenetic reconstruction using ExaBayes Bayesian inference, a framework for large-scale phylogenomic analysis. In brief, these methods assign venom 3FTxs as one of the LU-domain variants and identify LU-domain proteins as the closest relatives of pre-3FTx. Taken together, and within the limits explicitly discussed, these data support the argument that venom 3FTxs originated through co-option and architectural modification of an ancestral LU-domain lineage, followed by extensive diversification of solvent-exposed residues driven by positive selection at rapidly diverging functional sites. This post-recruitment diversification, particularly pronounced in advanced caenophidian snakes, exemplifies the RAVER framework, in which functional divergence proceeds through focal adaptive substitutions at interaction surfaces while overall fold integrity is retained. [41].

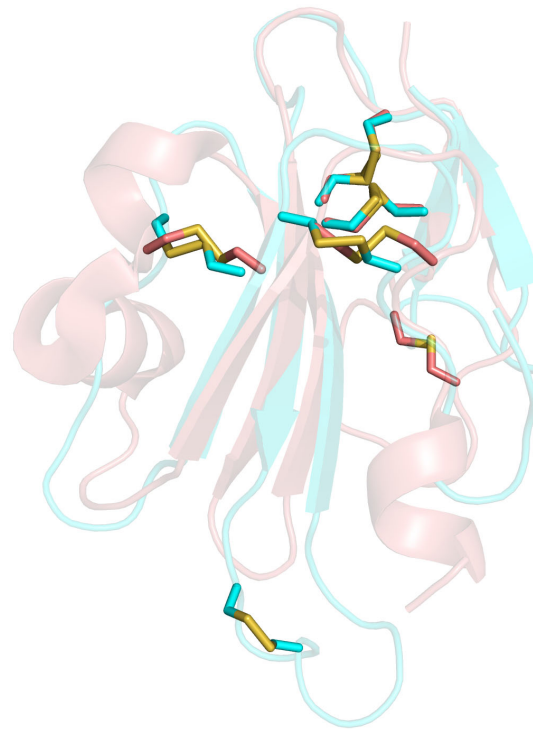


Figure 1. Evolutionary structural divergence of a soluble neurotoxin from its membrane-anchored ancestor. Shown is a superposition of the ancestral LU-domain protein CD59 (PDB 2UWR; red) and the derived long-chain α -neurotoxin, α -bungarotoxin (α BgTx—PDB 6UWZ; blue). Disulfide bonds (cysteine residues) are shown as yellow sticks. The structures are aligned on the conserved cysteine-rich core, illustrating that the central, disulphide-bonded scaffold is retained despite substantial divergence in the projecting loops. These solvent-exposed loop regions correspond to the primary sites of adaptive diversification identified by molecular evolutionary analyses, consistent with positive selection acting on toxin–receptor interaction surfaces. The comparison also highlights the loss of the C-terminal membrane-anchoring domain present in CD59 and the consequent ‘release’ of the toxin scaffold, which would have allowed rapid accumulation of surface mutations linked to receptor-subtype selectivity.

Comprehensive phylogenetic analyses of the snake venom proteome using both Bayesian and maximum-parsimony methods also place 3FTxs as a monophyletic clade arising from the LU superfamily. They further demonstrate that 3FTx sequences have the closest sister relationships with non-venom Ly6 members like LYNX and SLURP peptides, for which the highest confidence is achieved. The authors of these studies conclude that the recruitment of 3FTx into venom represents a single event of venom acquisition that occurred prior to the diversification of caenophidian advanced snakes [73,74].

2.1.2. Genomic Neighbourhood and Synteny as Evidence for Co-Option from LU-Rich Regions

Phylogenetic inference is supported by conserved genomic context. The *Naja naja* genome has more than 30 3FTx genes that are spread over a duplicated genomic region. The region is flanked by conserved marker genes such as TOP1MT and PSCA besides several LU-family genes (like LY6E and LY6-like loci) that are in the same syntenic neighbourhood [1]. The 3FTx loci in different reptile taxa being in conserved synteny with LY6E and TOP1MT and originating from an LU-rich genomic “hotspot” without the need for transposition is a proposition that is well supported by this phylogenetic arrangement. The mechanistic aspect of this LU-rich neighbourhood is quite significant as it offers a perfect template for unequal crossing-over, tandem duplication, and recurring local rearrangements that

can lead to both copy-number expansion and infrequent architectural transitions, e.g., truncation of C-terminal membrane-anchoring segments [63,75].

Additional data from the chromosome-level assembly of *Bungarus multicinctus* agrees with this hotspot model. The study found a total of 118 toxin genes, among which there were 30 3FTx genes. Out of these, 15 toxin genes were found to be co-localized within a 13.2 Mb region on chromosome 7 [37]. The authors hypothesise that 3FTxs originated through the neofunctionalisation of a LY6E flanking paralogue, which is in line with the deduction that LU-family neighbours are the closest ancestral source. While the exact microsyntenic arrangement and directionality of the LY6E–3FTx relationship need to be determined by cross-species comparison, the corroborating evidence from different system-LU-rich neighbourhoods in both taxa, repeated local duplication, and enrichment of 3FTxs within discrete chromosomal regions supports the recruitment within a defined, ancestral LU-domain genomic landscape [1,37].

2.1.3. Stepwise Recruitment Model: Domain Architecture, Secretion, and Venom-Gland Expression

A parsimonious recruitment model integrates domain architecture, trafficking, and gene-family dynamics [1,3,37]. An ancestral LU-domain gene, typically routed through the secretory pathway but retained at the cell surface via a C-terminal membrane-anchoring element and/or GPI anchoring, undergoes a critical domain-architecture transition: loss of the membrane-anchoring segment (MaD/GPI-associated region). This transition yields a soluble LU-domain protein, subsequently subject to venom-gland-biased expression and rapid paralogue expansion and diversification. Within the phylogenomic framework described above, MaD loss functions as an enabling event that decouples the LU domain from cell-surface localisation and expands the accessible interaction space for binding to diverse extracellular targets, including ligand-gated ion channels and G protein-coupled receptors (GPCRs) [1]. Genomic organisation in *Bungarus multicinctus* supports this model: numerous 3FTx genes cluster within a single chromosomal toxin region, with origins linked to neofunctionalisation within an LU-rich neighbourhood [37].

Once recruited into the venom system, transcriptional control and transcript architecture prove readily evolvable at 3FTx loci. In *Pseudonaja textilis*, five short-chain α -neurotoxin genes encoding six isoforms each possess functional promoters (validated by CAT assay and real-time PCR), featuring a transcription initiation site, two putative TATA boxes, a CCAAT box, and consensus binding sites for AP-1, GATA-2, and C/EBP β . Alternative splicing of Pt-sntx1 generates two neurotoxin mRNA variants from a single locus, demonstrating that post-transcriptional mechanisms alone can contribute to toxin isoform diversity without additional gene duplication [76].

Promoter architecture appears remarkably conserved across 3FTx subfamilies, suggesting recruitment and subsequent diversification within a shared venom-gland regulatory context. Genomic sequencing of *Naja atra* cytotoxin 3a (GenBank U58487) and cardiotoxin-like basic protein (GenBank AJ312212) genes—both members of the 3FTx superfamily—reveals highly conserved promoter regions containing consensus transcription factor binding sites (TBP, NF-1, CACCC-binding site, Sp1, EFII), consistent with shared transcriptional mechanisms across toxin loci [77,78]. Similarly, analysis of κ -bungarotoxin genes in *Bungarus multicinctus* (GenBank Y11768 and Y11769)—which likewise encode 3FTx-family neurotoxins—reports venom-gland-specific expression and identifies shared promoter motifs (including TATA/CCAAT and multiple transcription factor binding sites such as NF-1, CACCC-binding factor, glucocorticoid response elements, LF-A1, EFII, YY1 and Sp1) [79]. Together, these findings support the inference that venom-gland expression of LU-derived secreted proteins can be achieved through stereotyped promoter architectures, whereupon duplication and coding diversification proceed under selection.

2.1.4. Functional Diversification of LU-Derived Toxins: Early Venom Recruitment and Subfamilial Radiation

LU-domain recruitment likely yielded more than canonical α -neurotoxins, which are the archetypal postsynaptic nAChR antagonists. Muscarinic toxin-like proteins BM8 (UniProt Q9PW19) and BM14 (UniProt Q8JFX7) from *Bungarus multicinctus* venom comprise 82 residues and 10 cysteines, with functional divergence between closely related isoforms associated with substitutions at positions 37–38; BM14 specifically inhibits M2 muscarinic acetylcholine receptor binding, an activity abolished by lysine modification [21]. Remarkably, the BM14 genomic locus shares virtually identical structural organisation with α -neurotoxin and cardiotoxin genes (three exons, two introns), with high intronic conservation but marked coding-sequence divergence; this pattern is interpreted as evidence that muscarinic toxin-like proteins, neurotoxins and cardiotoxins derive from a common ancestral gene and diversified functionally via coding-region evolution. Such cases support an early recruitment phase in which multiple secreted LU-derived proteins were incorporated into venom systems and subsequently partitioned by selection into distinct functional trajectories, including GPCR-active toxins [80].

2.1.5. Limitations and Unresolved Questions

Although LU-derived origin and the enabling role of membrane-anchoring domain loss are strongly supported in elapids, broader snake sampling reveals that LU/three-finger-like components may occur in phylogenetically disparate venom systems. Transcriptomic evidence from *Lachesis muta* (Viperidae) reports a “3FTx-like toxin” among highly expressed venom-gland complementary DNAs, interpreting venom evolution as dynamic, involving multiple scaffold recruitments and domain exchange among paralogues [81]. Whether such “3FTx-like” sequences represent deeply conserved homologues retained outside classical elapid radiations, independent recruitment events, or lineage-specific losses and retentions cannot be resolved without additional taxon-rich genomic and functional characterisation. Accordingly, although the LU origin of canonical snake venom 3FTxs is well supported, the timing, number, and taxonomic breadth of recruitment events, particularly outside well-sampled elapid lineages, remain open questions requiring denser genomic and population-level sampling [1,81].

2.2. Gene Duplication, Birth-and-Death Evolution and Exon Segment Dynamics

Across elapids, the genomic organisation of 3FTxs is consistent with a birth-and-death dynamic in which tandem duplication repeatedly generates new paralogues, followed by differential retention and loss. In *B. multicinctus* and *N. atra*, BAC-based genomic sampling—that is, screening and sequencing bacterial artificial chromosome (BAC) libraries composed of large-insert genomic clones (~100–120 kb)—identified multiple locally clustered duplicates (operationally defined as distinct 3FTx gene sequences recovered from the same BAC clone), with low divergence in intronic regions ($K_n < 0.05$) implying recent duplication events and ongoing local expansion [12]. In the near-chromosome-level Indian cobra (*N. naja*) assembly, most full-length 3FTx genes are physically clustered rather than dispersed: 14 of 19 full-length genes lie within a 6.3 Mb interval on chromosome 3, alongside additional 3FTx pseudogenes, directly demonstrating megabase-scale toxin-gene clustering [75]. Comparative microsynteny further places these patterns into a conserved genomic framework: the TOP1MT-linked LY6/3FTX region typically spans several megabases and is broadly conserved across vertebrates, but shows dramatic lineage-specific expansion in elapids; importantly, this snake 3FTx genomic region is explicitly mapped across multiple species including *B. multicinctus* [1]. Collectively, these datasets support the interpretation that 3FTx repertoires expand chiefly through local (tandem) duplication within constrained genomic

neighbourhoods, with paralogue turnover mediated by subsequent pseudogenisation and divergence [12].

The genomic organisation of 3FTxs in several focal systems strongly supports extensive tandem duplication and paralogue turnover [62]. In *B. multicinctus*, long-read genome assembly and annotation reveal a large toxin gene complement in which 3FTxs are concentrated within a multi-megabase toxin-gene array rather than dispersed as isolated singletons; many 3FTx copies are present as clustered neighbours. The same dataset quantifies dozens of toxin genes overall, with a substantial 3FTx complement consistent with repeated local duplication as a dominant mechanism of repertoire growth [12].

Combined evidence from cDNA and BAC-library screening in *B. multicinctus* and *N. atra* corroborates this pattern. Toxin-probe recovery of multiple genomic clones corresponding to 3FTxs and other toxin families provides direct clone-level evidence of tandem duplication, establishing it as a recurrent feature of the venom gene landscape rather than a mere inference from transcript multiplicity. Such arrangements align precisely with the expectations of a birth-and-death process, wherein new paralogues arise frequently and are subsequently maintained, neofunctionalised, or lost through pseudogenisation, yielding a dynamic family with lineage-specific expansions [12,37].

At the gene-structure level, detailed architecture is exemplified by α -neurotoxin loci in *Pseudonaja textilis* (GenBank AF204969, AF204970, AF204971, AF204972 and AF204973), where five short-chain genes encode six isoforms. Each locus comprises three exons separated by two introns; all analysed loci carry functional promoters, and at least one yields two mRNA variants via alternative splicing. This organisation directly links duplication (multiple loci encoding closely related toxins) to post-duplication regulatory and transcript-structural diversification, mechanisms that enable differential expression and functional divergence among paralogues [76].

Against this duplication-driven expansion, exon/segment dynamics (ASSET) have been proposed as an additional diversification mechanism. In *Sistrurus catenatus edwardsii*, exon-level evolution is analysed in detail and invoked to explain how novel toxin variants arise through switching of exon segments corresponding to functional surface regions, rather than solely by point substitution. This model was subsequently formalised as “accelerated segment switching in exons to alter targeting” and proposed as a potentially general mechanism contributing to venom protein diversification. However, the explanatory scope of ASSET remains debated. Critics have argued that, if invoked as a dominant driver of 3FTx diversification, extensive exon-segment replacement would be evolutionarily implausible relative to models emphasising duplication coupled with accelerated, site-specific substitution (see Section 2.3) [39,41,82].

From an empirical perspective, these alternative models make distinct predictions: a substantial contribution of ASSET would be expected to produce recurrent, boundary-constrained replacement of short exon segments and mosaic sequence architectures inconsistent with gradual point mutation alone, whereas RAVER-like diversification predicts elevated rates of adaptive substitution concentrated at exposed residues and strong phylogenetic continuity.

In synthesis, available evidence supports the occurrence of exon/segment recombination in at least some contexts, but its prevalence and quantitative contribution relative to duplication plus point mutation remain unresolved.

2.3. Patterns of Positive Selection: RAVER vs. ASSET

Across 3FTx lineages, diversification is therefore highly structured rather than uniform, reflecting strong constraints on fold stability coupled with repeated, lineage-specific remodelling of functional interaction surfaces. This structural bias sits naturally within a

birth–death framework for a multigene-family, in which duplication generates paralogue diversity and lineage-specific retention/loss determines the standing repertoire available for functional exploration [5,41,83].

The RAVER model formalises this expectation by proposing that adaptive substitutions accumulate preferentially at surface-accessible sites, particularly in loop regions, thereby tuning affinity and specificity for molecular targets without destabilising the conserved three-finger scaffold. This predicts that signatures of diversifying selection are predicted to be overrepresented in exposed residues and comparatively depleted within the buried structural core [41]. Such surface-focused enrichment is consistent with a role in target engagement, given the known geometry of 3FTx–receptor interactions, although the functional contribution of individual positively selected residues has been experimentally validated in only a subset of cases.

Several independent datasets strengthen this structure–selection coupling. In a comparative transcriptomic study across multiple Australian elapids, different α -neurotoxin classes (Types I–III) displayed heterogeneous evolutionary rates, yet non-synonymous substitutions were preferentially retained in solvent-exposed loops and other structurally permissive regions, consistent with strong constraints on the structural core and diversification of exposed toxin surfaces under positive selection [57]. This surface diversification is proposed to modulate potency and prey specificity and may delay the evolution of prey resistance, aligning with the coevolutionary logic of a RAVER-like mechanism.

Notably, the intensity and localisation of positive selection are not uniform across the 3FTx superfamily. Comparative phylogenetic analyses of *Micrurus* 3FTxs identified multiple deep clades with different diversification rates, with some experiencing extreme diversifying selection, again consistent with episodic selection acting strongly in particular lineages or functional contexts [84]. This pattern motivates a working model in which toxins involved in tight molecular recognition of discrete receptor interfaces are especially prone to repeated bursts of positive selection on exposed residues, whereas other 3FTx functional categories may show different balances between innovation and constraint once an effective mode is established [41,84].

In contrast, the ASSET hypothesis assigns a larger role to short exon-segment replacements that introduce radically altered sequence tracts and thereby reconfigure surface topology and loop conformation. Under this model, functional diversification is expected to occur through step-like transitions driven by the coordinated replacement of multiple residues within discrete exon boundaries, rather than through the gradual, site-by-site accumulation of substitutions characteristic of RAVER-like diversification. As a general mechanism across snake venom proteins, ASSET is framed as complementary to (rather than exclusive of) point substitution: accelerated point mutations “fine-tune” target specificity, whereas ASSET mediates larger functional shifts by replacing multiple important residues simultaneously [39,82].

The key question is therefore not whether exon/segment-scale events can occur, but whether ASSET explains 3FTx target switching and diversification relative to a predominantly RAVER-like regime of surface-biased point substitution operating on a duplication-rich gene family. The RAVER-centred critique argues that many observed patterns, particularly repeated localisation of change to exposed regions without pervasive evidence of tract-like mosaic ancestry, are more parsimoniously explained by episodic positive selection on exposed residues superimposed on birth–death dynamics, rather than by frequent short-tract exchange as a dominant driver. Establishing ASSET as a major mechanism in any locus requires explicit evidence for segment breakpoints and discordant genealogies (or gene-conversion signatures) rather than inference from segment-like patterns alone.

Both RAVER- and ASSET-like processes can be confounded by features common in toxin gene families, particularly non-allelic gene conversion among paralogues, which can inflate false positives in likelihood-based codon-model tests for positive selection. Strong claims about “selection hotspots” or apparent step-changes in surface residues should therefore be interpreted alongside explicit screening for non-tree-like processes and with genomic context whenever possible [85].

Genome- and transcriptome-enabled work in elapids provides a concrete context for how these mechanisms operate in practice. Venom gland transcriptomes and BAC-based surveys in *B. multicinctus* and *N. atra* reported 3FTx dominance in expression, identified numerous toxin gene loci, and concluded that tandem duplications substantially contributed to multigene family expansion; dN/dS analyses indicated rapid diversifying evolution in toxin families [12]. More recent chromosome-scale genomic analysis of *B. multicinctus* reported clustering of major toxin-coding genes associated with ancient local duplications, alongside a proposed origin scenario in which truncation of a GPI-anchor region in a LY6E paralogue released 3FTxs from membrane tethering, followed by expansion and mutation-driven diversification [80]. Collectively, these data reinforce a landscape in which duplication and local expansion are pervasive, creating repeated opportunities for RAVER-like fine-scale adaptation, whilst simultaneously providing the genomic substrate for exon/segment-scale innovations in specific loci [12,41,80].

Any synthesis of positive selection patterns in toxins should explicitly acknowledge ecological contingency. In an egg-eating sea snake (*Aipysurus eydouxii*) with fang loss and venom gland atrophy, the only reported 3FTx neurotoxin gene (GenBank AY559317) carried a dinucleotide deletion associated with loss of neurotoxic activity, alongside decelerated evolution in other venom components under relaxed selection [86,87]. From a population-genetic perspective, this system illustrates how shifts in ecological function can reduce or eliminate toxin-mediated fitness benefits, effectively lowering selection coefficients on venom genes and permitting mutational degradation or neutral drift. This counterpoint underscores that selective regimes on toxin loci are conditional on ecological use, and that claims of pervasive positive selection must be framed relative to functional context rather than assumed as universal defaults.

The most defensible synthesis is hierarchical. Birth–death dynamics and tandem duplication generate the paralogue substrate and lineage-specific repertoire. Within this substrate, RAVER-like selection, episodic, surface-biased, and scaffold-preserving, offers a general and repeatedly observed route to functional divergence, particularly for toxins engaged in specific molecular recognition. ASSET-like exon/segment exchange remains a plausible contributor in some loci and may mediate larger step-changes in function, but its prevalence and dominance must be evaluated locus-by-locus and lineage-by-lineage with explicit genomic and phylogenetic diagnostics rather than assumed a priori; broader discussions of accelerated toxin evolution highlight additional exon-level mechanisms that could generate major surface remodelling.

Interspecific Diversification of *Micrurus* spp. 3FTxs

Recent investigations show that diversification is highly structured among species. Phylogenetic reconstruction of eight-cysteine 3FTx across elapid snakes shows that sequences from *Micrurus* spp. are partitioned into eight distinct monophyletic clades that diverge early within the 3FTx phylogeny. Many of these *Micrurus* clades are phylogenetically closer to sequences from other elapid genera than to each other. Within this species-rich genus, patterns of molecular evolution vary markedly among lineages associated with different species groups. For example, toxins from lineages represented by species such as *M. fulvius* and *M. altirostris* exhibit very high dN/dS ratios and numerous

codons inferred to be under positive selection, whereas lineages associated with species such as *M. corallinus* and *M. tener* show substantially lower ω values and little evidence of adaptive change [84].

Structural modelling further reveals that, in rapidly evolving lineages, diversifying selection is concentrated primarily on solvent-exposed loop regions, while the disulphide-bonded core and cysteine framework remain almost perfectly conserved across the genus. When averaged across all *Micrurus* lineages, the weighted mean ω exceeds 2.4, indicating exceptionally strong diversifying selection. Taken together, these results demonstrate that species-level lineages within *Micrurus* have followed divergent evolutionary trajectories, with some undergoing repeated episodes of positive selection acting predominantly on exposed residues.

Transcriptomic analyses of individual species reinforce this picture of pronounced interspecific heterogeneity. The venom gland transcriptome of the eastern coral snake, *Micrurus fulvius*, for example, revealed 116 toxin transcripts dominated by 3FTx and PLA₂, with expression heavily skewed towards a small subset of 3FTx genes. Toxin-encoding loci exhibited substantially higher heterozygosity than non-toxin genes, and overdominance was proposed to promote gene duplication and retention [88]. Such species-specific patterns highlight that the genus encompasses both lineages in which 3FTxs dominate venom expression and others in which they are relatively reduced, and that duplication dynamics and selective pressures vary considerably among species.

Proteomic surveys across 18 *Micrurus* species corroborate these transcriptomic and phylogenetic observations. Lomonte et al. (2016) identified a clear dichotomy in venom composition, with some species producing venoms dominated by PLA₂, while others exhibit predominantly 3FTx-rich profiles [11]. *Micrurus lemniscatus carvalhoi* exemplifies the 3FTx-rich phenotype: populations from São Paulo and Rio de Janeiro express venoms overwhelmingly dominated by three-finger toxins, whereas conspecifics from the northern subspecies *M. lemniscatus helleri* and the Caatinga species *M. ibiboboca* exhibit PLA₂-predominant venoms [89].

A similar dichotomy occurs in sympatric Costa Rican species: *M. alleni* expresses a 3FTx-rich proteome, whereas *M. mosquitensis* is PLA₂-rich [90]; the recently segregated *M. yatesi* (formerly treated as a subspecies of *M. alleni*) also possesses a PLA₂-rich venom [91]. Among South American species, extreme 3FTx predominance ($\approx 95\%$ of total venom proteins) characterises the aquatic coral snake *M. surinamensis* [92] and the desert-adapted *M. tschudii tschudii* [93,94]. More balanced venom profiles are seen in *M. frontalis* and *M. spixii spixii*, which exhibit mixed proportions of 3FTx and PLA₂ [92], and in *M. clarki*, whose venom comprises roughly 48.2% 3FTx and 36.5% PLA₂ [95]. These contrasting venom phenotypes correlate with phylogenetic position and geographical distribution along a north–south axis in the Americas and can occur even among sympatric species occupying small geographic areas. Differences in toxin composition are further associated with variation in toxic activities, proteoform diversity and patterns of immunological cross-recognition. Collectively, these data indicate that diversification of 3FTx within *Micrurus* is both ancient and lineage-specific, involving multiple deeply divergent lineages, heterogeneous rates of diversifying selection and distinct venom expression regimes.

These interspecific findings reinforce the hierarchical view proposed above. Birth–death dynamics and tandem duplication have generated multiple ancient 3FTx clades within *Micrurus*, and RAVER-like positive selection acts episodically on exposed residues to drive functional divergence in particular lineages. However, the strength and localisation of diversifying selection differ among clades and species, suggesting that ecological context and prey interactions modulate the tempo of evolution. The dichotomy between PLA₂- and 3FTx-dominated venoms underscores that the contribution of 3FTx to venom function

is species-dependent. Exon/segment-scale replacements (ASSET) may still contribute to functional innovation, but phylogenetic analyses reveal no pervasive evidence for tract-like mosaic ancestry in *Micrurus* 3FTx; instead, repeated loop specific substitutions on conserved scaffolds appear to be the dominant mode of diversification. Any evaluation of RAVER- versus ASSET-like mechanisms in coral snakes must therefore consider the lineage-specific history and ecological drivers of toxin evolution, rather than assuming uniform processes across the genus.

2.4. Expansion of Three-Finger Toxins Across Snake Lineages

Substantial variation in 3FTx repertoire size and composition exists across snake radiations, with particularly rich evidence in elapids and hydrophiines. In terrestrial elapids, transcriptomic and genomic-clone-based work on *B. multicinctus* and *N. atra* demonstrates both high toxin transcriptional investment and marked differences in venom composition between closely related genera, whilst simultaneously providing genomic evidence via BAC screening for multi-copy toxin gene families, including 3FTx [12,37]. Proteomic analysis confirms these compositional differences at the protein level, with *B. multicinctus* showing balanced 3FTx and phospholipase A₂ production, whereas *N. atra* exhibits near-exclusive reliance on 3FTxs [96]. In *Bungarus* spp. specifically, genome analysis indicates that 3FTxs comprise a large, organised gene complement within a toxin-gene array, consistent with extensive lineage-specific expansion [37,80]. Chromosome-level assembly reveals that this expansion proceeds through tandem duplication, with toxin genes clustering within compact genomic regions showing evidence of both positive selection and local duplications [80]. Comparative genomic analysis of *N. naja* demonstrates that elapid 3FTx genes are distinctly organised on macrochromosomes rather than microchromosomes, with 14 of 19 full-length 3FTx genes clustered within a single 6.3 Mb chromosomal region, contrasting sharply with the microchromosomal distribution typical of viperid venom genes [75].

Marine radiations present a contrasting pattern: rather than uniformly expanding 3FTx diversity, sea snakes exhibit both conservation and lineage-specific restructuring. Early comparative transcript work on *Lapemis curtus* and *Acalyptophis peronii* reports that despite ecological divergence, 3FTx and PLA₂ sequences show relatively little variability compared with land snakes or sea kraits, interpreted as consistent with ecological streamlining and narrower prey spectra constraining toxin diversification [97]. More recent species-level studies of *Hydrophis* spp. reinforce that 3FTx dominate venom functional output whilst exhibiting “cryptic” genetic complexity. *Hydrophis cyanocinctus* venom is biochemically simple but genetically complex, with abundant 3FTx in both transcriptomic and proteomic profiles and evidence that a large fraction of toxin-coding unigenes has experienced positive selection; toxicity assays confirm that 3FTxs largely determine venom lethality in this species [9]. *Hydrophis curtus* transcriptomic analysis similarly finds that 3FTxs constitute the major venom-gland transcripts, with short- and long-chain neurotoxins being the most abundant, representing a substantial fraction of overall venom-gland expression [10,98].

Chromosome-scale sea snake genomes enable copy-number and chromosomal-context inference. These reveal that venom genes, including 3FTxs, are distributed across chromosomes with substantial quantitative variation, whilst providing explicit copy-number contrasts between closely related hydrophiines (e.g., lower versus higher 3FTx copy number in *H. curtus* relative to *H. cyanocinctus*) [99]. Notably, comparative genomic analysis demonstrates that the absolute number of functional 3FTx genes varies considerably across elapid lineages: *N. naja* possesses 19 full-length 3FTx genes with 10 pseudogenised copies, whilst *B. multicinctus* shows evidence of even greater 3FTx gene complement organisation within tandem arrays [75]. The same analysis highlights that different toxin families can expand in different hydrophiine lineages (e.g., SVMP copy-number increases in one lin-

eage), highlighting that 3FTx expansion is not monotonic and can be partially offset by expansions in other venom gene families depending on ecological context [99].

Beyond the canonical elapid-heavy distribution, 3FTx (or 3FTx-like) components are documented in broader squamate contexts. Exon-level studies explicitly analyse 3FTx evolution in a viperid systems (*Sistrurus*), indicating that LU/3FTx-like scaffolds and their diversification mechanisms, also operate in phylogenetically disparate venom systems [1,82]. The contrasting chromosomal organisation of 3FTx genes between elapids and viperids, concentrated on macrochromosomes in the former versus microchromosomes in the latter, suggests that lineage-specific genomic contexts may constrain toxin evolution trajectories [75,100]. Muscarinic toxin-like proteins identified in *Dendroaspis* and *Bungarus* provide additional evidence that the three-finger/LU scaffold is represented across diverse snake lineages [101,102], although meaningful comparisons of repertoire breadth, expression dominance, and ecological prominence across taxa remain uncertain due to extensive lineage-specific diversification and uneven venom sampling [36,103].

Across all these systems, a critical limitation remains sampling granularity: most lineages are represented by snapshots (single populations, pooled individuals, or a few exemplars), and genome-grade resolution is available for only a small subset of taxa [104–106]. This constrains the ability to integrate ecological variables with population-genetic inference, such as estimating resistance allele frequencies, spatial variation in selection coefficients, or covariance between prey community composition and toxin diversification. Taxonomically explicit conclusions are therefore robust primarily within well-sampled elapid clades (notably *Bungarus*, *Naja*, and *Hydrophis*) [107], while broader generalisations (e.g., uniform trends across Elapidae or across all marine elapids) should be treated as hypotheses pending denser genomic and population-level sampling [108].

2.5. Functional Innovation and Neofunctionalisation Within the Three-Finger Toxin Scaffold

The three-finger scaffold has supported repeated functional innovation well beyond canonical α -neurotoxins targeting muscle-type nAChR. Multiple functional “directions” are achievable within 3FTxs, including toxins acting on nicotinic and muscarinic acetylcholine receptors, as acetylcholinesterase inhibitors, as blockers of L-type calcium channels, and cytotoxic 3FTx classes. Importantly, these functions are presented not as isolated anomalies but as recurring outcomes of diversification on a robust structural platform that tolerates substantial surface variation whilst maintaining a conserved disulphide-stabilised core [5,37].

Neofunctionalisation towards voltage-gated sodium channels exemplifies this capacity. Calliotoxin (UniProt P0DL82) from *Calliophis bivirgata* targets voltage-gated sodium channels, representing a major target shift relative to classical nAChR-directed α -neurotoxins and demonstrating that 3FTx fold can be repurposed to modulate excitability via fundamentally different membrane protein targets. Evolutionary context from *Calliophis* indicates lineage-specific selection driving extreme departures from canonical functionality [64,109].

Diversification towards muscarinic receptor activities constitutes another major axis of functional innovation within the three-finger toxin scaffold [36,110,111]. Muscarinic toxins from *Dendroaspis angusticeps* were amongst the first venom-derived ligands shown to selectively target mAChRs subtypes, demonstrating how relatively modest sequence divergence on a conserved three-finger framework can yield pronounced subtype discrimination [111,112]. Subsequent work has shown that these toxins typically engage the extracellular face of mAChRs, particularly extracellular loop (ECL) determinants, rather than binding solely within the orthosteric pocket, providing a mechanistic basis for their high subtype selectivity and for diverse allosteric/functional profiles [17,20,112,113].

Although complete target deconvolution and physiological roles remain unresolved for all muscarinic-like venom proteins, the recurring identification of muscarinic-toxin(-like) 3FTxs in elapids, including the discovery of muscarinic toxin-like proteins BM8 and BM14 in *Bungarus multicinctus*, supports the view that muscarinic-related functional space is repeatedly accessible to the three-finger scaffold across venom systems [21,101].

Quaternary-structure innovation further expands 3FTx functional repertoire. The synergistic-type toxin (UniProt P0DQP2) structure from *Dendroaspis jamesoni jamesoni* is a homodimer stabilised by an interchain disulphide bond, with an extensive dimer interface involving loops II and III. This is significant for neofunctionalisation because dimerisation can (i) remodel the effective binding surface, (ii) alter avidity and geometry for receptor engagement, and (iii) create emergent properties unavailable to monomeric toxins, even without substantial changes to monomer fold. Such structural mechanisms provide an additional layer of evolvability, supplementing point substitutions and exon/segment exchange by enabling higher-order structural novelty [5,65].

Genome- and transcriptome-scale studies further reveal that innovation is intertwined with duplication. *Bungarus multicinctus* is particularly informative: the presence of numerous 3FTx genes, including both canonical and neglected subfamilies, coupled with evidence for differential selective regimes across toxin classes (adaptive evolution in neurotoxins versus stronger purifying selection in cytotoxins), supports a model in which duplication generates a substrate for experimentation whilst selection partitions paralogues into distinct mechanistic niches [37,80]. In sea snakes, the dominance of 3FTxs in expression and toxicity, alongside evidence for positive selection on toxin-coding unigenes, indicates ongoing adaptive tuning even within comparatively streamlined repertoires [9,97,98].

A coherent framework emerges: the three-finger scaffold is structurally robust and thus permissive to extensive sequence exploration; gene-family expansion via tandem duplication supplies paralogue diversity; selection concentrates on exposed loop residues (RAVER-like dynamics) to tune target engagement; and, in some cases, exon/segment exchange or changes in oligomeric state facilitate discrete jumps in functionality.

3. Structural and Functional Basis of 3FTx–Receptor Interactions

3.1. Canonical α -Neurotoxins and Muscle-Type nAChR $\alpha 1$

Canonical α -neurotoxins include several structural classes within the three-finger toxin (3FTx) fold. All share a conserved, disulphide-rich core that supports three β -sheet loops (“fingers”), but the classes differ in loop lengths, disulphide patterns and terminal regions, and these differences shape receptor subtype selectivity [2,5]. In a high-resolution model for long-chain α -neurotoxin binding, the crystal structure of the pentameric α -cobratoxin (Cbtx—UniProt P01391), from *N. kaouthia*, in complex with acetylcholine-binding protein (AChBP), shows that long-chain toxins have an extended loop II featuring the Trp²⁵–Cys²⁶–Asp²⁷–Ala²⁸–Phe²⁹–Cys³⁰ segment and a distinctive fifth disulphide bond (Cys²⁶–Cys³⁰) that closes loop II into a small, constrained loop [114] (Figure 2).

The same loop-II feature is also seen in α -bungarotoxin (α BgTx—UniProt P60615), which carries the closely matching Trp²⁸–Cys²⁹–Asp³⁰–Ala³¹–Phe³²–Cys³³ segment and an equivalent loop-closing disulphide (Cys²⁹–Cys³³). This reinforces that a disulphide-closed loop II is a consistent hallmark of long-chain α -neurotoxins. By contrast, short-chain α -neurotoxins lack this loop-II disulphide and tend to differ in the relative size and shape of loops I and II, which in turn affects binding mode and receptor preference in comparative analyses [41,114,115] (Figure 1).

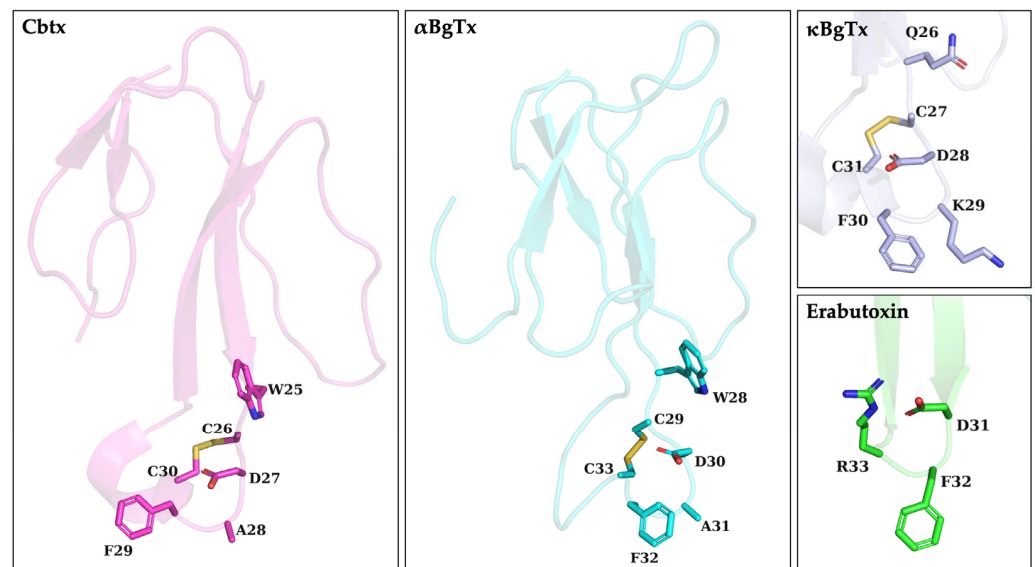


Figure 2. Loop II architecture in representative 3FTx neurotoxins. Long-chain α -neurotoxins (Cbtx, α BgTx) feature an extended loop II with a conserved WCDAFC motif and a fifth, constraining disulphide bond, whereas short-chain α -neurotoxins (Erabutoxin—UniProt P60775—*Laticauda semifasciata*) lack this cyclising bond and κ -neurotoxins (κ BgTx—UniProt P01398—*Bungarus multicinctus*) display alternative loop configurations. Loop II corresponds to a major toxin–receptor contact surface and is consistently identified as a hotspot of positive selection, linking these structural differences directly to adaptive diversification under the RAVER framework. These architectural variations illustrate how conserved scaffolds support lineage-specific functional optimisation. Structures were rendered in Open-Source PyMOL (v3.1.0) using PDB entries 1YI5 (Cbtx), 6UWZ (α BgTx), 1KBA (κ BgTx), and 1QKD (Erabutoxin).

κ -type neurotoxins (κ -neurotoxins) are another well-recognised class of nAChR-targeting 3FTx, but they stand apart from the classical α -neurotoxins in both structure and functionality. κ -Bungarotoxin (UniProt P01398) is a 66-residue, cysteine-rich peptide (10 cysteines, consistent with five disulphide bonds) [116] and, in solution, it forms a homodimer rather than remaining monomeric [117]. Functionally, κ -neurotoxins show a clear preference for neuronal nAChR subtypes that include $\alpha 3$ or $\alpha 4$ subunits (for example, $\alpha 3\beta 2$ and $\alpha 4\beta 2$), rather than the muscle-type receptor. Within the Cbtx–AChBP structural framework, κ -neurotoxins have been described as diverging from long-chain α -neurotoxins in two main ways: they carry a different residue at the position that corresponds to Cbtx Trp²⁵, and they have a much shorter C-terminal tail. Both features have been proposed to help shift binding towards neuronal nAChR subtypes and away from the muscle-type receptor [114] (Figure 1).

Muscle-type nAChRs are pentameric ligand-gated ion channels whose extracellular domain (ECD) presents orthosteric agonist-binding pockets at subunit interfaces; the binding cavity is bordered by “principal” (α -subunit) and “complementary” (adjacent subunit) components, operationally defined in AChBP as the interface between a principal face containing loop C and a complementary face containing loop F [114]. This principal/complementary architecture is conserved in muscle-type receptors and supports the classic localisation of snake α -neurotoxin binding to $\alpha 1$ -containing subunit interfaces with neighbouring non- α subunits [49]. In the context of a structure-guided resistance analysis, the identified α -neurotoxin binding site on nAChR $\alpha 1$ is described as comprising loop C (principal side) together with the Cys-loop and loop F (complementary-side elements), with additional interaction surfaces involving neighbouring δ and γ subunits, consistent with α -toxin engagement across the $\alpha 1$ -adjacent-subunit interface rather than

binding to an isolated $\alpha 1$ surface [49]. This interface localisation is also consistent with structure-informed pairwise interaction analyses that place short-chain α -neurotoxin loop-II determinants in proximity to α -subunit residues in loop-C region (vicinity of Tyr¹⁹⁰) and to complementary-face residues contributed by neighbouring subunits [114].

Within this conserved receptor architecture, α -neurotoxins bind by sterically occluding the orthosteric pocket and stabilising characteristic conformations of the pocket-lining loops. In the Cbtx–AChBP complex [114], Cbtx is oriented such that loop II penetrates deeply into the ligand-binding pocket at the subunit interface, while loop I and the C-terminus provide additional contact points; loop III contributes comparatively few direct contacts in this particular complex. The interface is extensive (20–25% of the toxin's accessible surface), multipoint, and includes a mixture of hydrophobic, hydrogen-bonding and electrostatic interactions, with four positively charged toxin residues among the 18 residues contributing to the buried interface.

Residue-level mapping in the complex identifies loop-II residues Trp²⁵, Asp²⁷, Ala²⁸, Phe²⁹, Ser³¹, Ile³² and Arg³³ among those within contact distance of the pocket, together with loop-I residues (e.g., Thr⁶, Pro⁷, Ile⁹) and C-terminal residues (e.g., Phe⁶⁵, Arg⁶⁸) that engage principally loop-C residues on the principal face and selected residues on the complementary face. On the receptor side (AChBP numbering), toxin contacts cluster in the loop-C region (e.g., Ser¹⁸²–Glu¹⁹³, including Tyr¹⁸⁵, Cys¹⁸⁷ and Tyr¹⁹²) and in complementary-face residues (including residues contributed by loop F and adjacent structural elements), with the structure explicitly tabulating contacts such as Asp²⁷–Tyr¹⁸⁵, Phe²⁹–Tyr¹⁸⁵/Tyr¹⁹² and Arg³³–Cys¹⁸⁷/Tyr¹⁹² on the principal face, and additional interactions involving complementary-face residues (e.g., Trp⁵³, Arg¹⁰⁴) [114].

Importantly, the same study integrates these structural observations with mutational data: residues including Trp²⁵, Asp²⁷ and Arg³³ affect binding to both muscle-type (α_1)₂ $\beta\gamma\delta$ and neuronal α_7 receptors, whereas Phe²⁹, Arg³⁶ and Phe⁶⁵ were implicated in shaping receptor-subtype specificity, thereby providing an explicit structure–function bridge between contact geometry and functional discrimination [114]. At the receptor level, loop-C mobility and repositioning of loop C and loop F by up to ~10 Å upon toxin binding are described as part of the antagonist-bound conformation, consistent with α -neurotoxin binding stabilising an “uncapped” pocket state distinct from agonist-bound conformations. Complementing this AChBP-based template, receptor-side modifications that project directly into the toxin–receptor interface can dominate binding outcomes: for example, glycosylation at the centre of the α -neurotoxin/nAChR interface is highlighted as a mechanism by which a bulky glycan can sterically interfere with α -neurotoxin binding to α_1 -containing sites [49,118], with structural interpretation explicitly referencing a carbohydrate chain occupying the toxin–receptor interface complex and thereby impeding productive toxin engagement [49]. Together, these data support a mechanistic picture in which canonical α -neurotoxins exploit a conserved orthosteric-site architecture, mainly via loop-II insertion and loop-C engagement, while class-specific loop geometry, terminal segments, and receptor-side microfeatures (including glycosylation and local residue substitutions) tune binding strength and receptor-subtype specificity [49,114,115,118].

3.2. Non-Classical Three-Finger Toxins: Muscarinic, Na_v-Targeting and Synergistic Toxins

Non-classical venom phenotypes, including muscarinic receptor-active 3FTx, Na_v-modulating toxins, and dimeric toxin architectures, materially broaden the space of potential antagonistic interactions by recruiting additional neurophysiological targets. In contrast to the comparatively well-resolved nAChR– α -neurotoxin axis, these systems are currently supported chiefly by toxin-side evidence (pharmacology, electrophysiology, and/or structure–function mapping), whereas receptor-side evolutionary corroboration remains limited. In particular, for the interfaces discussed below, phylogenetically paired

datasets demonstrating naturally occurring receptor sequence variation (mapped to the toxin–interaction surface) that measurably alters susceptibility to homologous toxins are largely lacking. We therefore treat these as asymmetric or presently unresolved coevolutionary scenarios, emphasising toxin-side innovation while delineating the evidentiary requirements for strict reciprocal, arms-race inference. Operationally, under a geographic mosaic framework, strict arms-race coevolution requires evidence beyond toxin-side neofunctionalisation, specifically, recurrent target-side molecular change at interaction-relevant residues and, ideally, matched geographical covariance consistent with reciprocal selection [119].

Against this evidentiary backdrop, the 3FTx fold provides a useful model of scaffold plasticity, having been repeatedly adapted to engage structurally disparate receptor classes rather than being confined to nicotinic signalling [2,5,41]. Muscarinic toxin-like proteins (MTLPs) exemplify GPCR targeting within the three-finger fold: in *Bungarus multicinctus*, two MTLPs (BM8 and BM14) were isolated and shown to differ sharply in receptor binding, with BM14 binding the M2 mAChR with high affinity, whereas BM8 does not bind detectably [21]. The same study identified a minimal sequence feature plausibly underpinning this functional divergence; BM14 carries two additional lysine residues at positions 37 and 38 (in loop II), absent from BM8, implicating localised changes in surface charge and loop-II chemistry as determinants of muscarinic receptor recognition within an otherwise conserved 3FTx fold.

A distinct axis of target innovation is illustrated by callitoxin from the long-glanded blue coral snake (*Calliophis bivirgatus*) which modulates skeletal muscle Na_v1.4 by shifting activation to more hyperpolarised potentials and impairing inactivation, thereby facilitating excitability and producing spastic paralysis; this mode of action is framed as convergent with Na_v-targeting toxins from phylogenetically distant venomous lineages [64]. Callitoxin is further characterised as a 3FTx closely related to elapid cytotoxins and to *Dendroaspis* MT3 (UniProt P81031), indicating that relatively modest sequence divergence within the three-finger scaffold can yield radical changes in molecular target class and physiological output.

Quaternary structure provides a third route to non-classical diversification with direct implications for engagement geometry and subtype breadth. Haditoxin (UniProt A8N286), from *Ophiophagus hannah* [120], antagonises both muscle ($\alpha\beta\gamma\delta$) and multiple neuronal (α_7 , $\alpha_3\beta_2$, $\alpha_4\beta_2$) nAChR subtypes, displays the highest affinity for α_7 , and adopts a high-resolution (~1.5 Å) homodimeric structure that is non-covalent in solution. Notably, haditoxin combines a κ -neurotoxin-like quaternary arrangement with monomeric subunits resembling curaremimetic short-chain α -neurotoxins, underscoring that oligomeric assembly can be decoupled from monomer class and can expand receptor subtype range in ways not predictable from short- versus long-chain monomer criteria alone.

These examples therefore primarily substantiate toxin-side functional diversification and target-class expansion, while reciprocal target-side adaptation remains to be demonstrated for these specific interfaces.

Collectively, these muscarinic-, Na_v- and dimerisation-based innovations show that a shared three-finger scaffold can be re-parameterised through localised loop chemistry (e.g., loop-II charge), altered electrostatic landscapes, and higher-order assembly to yield qualitatively distinct receptor interactions, thereby substantiating toxin-side functional diversification and target-class expansion and outlining putative selective pressures on molecular targets [5,21,41,64,120]. At present, however, the principal constraint on coevolutionary inference across these non-canonical axes is the paucity of lineage-resolved receptor sequence–function datasets required to demonstrate reciprocal target-side counteradaptation.

3.3. Mapping Positively Selected Residues onto Toxin–Receptor Interfaces

A central approach for integrating evolutionary inference with molecular mechanism in 3FTx biology is the identification of positively selected sites (PSS) using

codon-based models (dN/dS frameworks) and subsequent projection of those sites onto three-dimensional toxin structures and toxin–receptor complexes [115]. In a broad comparative analysis across functional classes, selection analyses demonstrated that multiple α -neurotoxin types evolved under strong diversifying selection and advanced the RAVER framework to explain how adaptive point substitutions are concentrated in exposed residues, permitting rapid functional divergence while preserving the structurally constrained disulphide-rich core [41]. This model is structurally testable because canonical receptor engagement is mediated predominantly by solvent-exposed loop surfaces (especially loop II, supplemented by loop I and C-terminal segments in long-chain toxins) that directly participate in binding-pocket occlusion [41,114]. Accordingly, structure-mapped selection analyses, including combined codon-model inference and mapping onto toxin structures and exemplar toxin–receptor complexes, report enrichment of positively selected sites at or adjacent to interaction interfaces [115]. These approaches operationalise a mechanistic hypothesis: if receptor diversification alters the geometry or electrostatics of the orthosteric site, toxin adaptation should preferentially accumulate in loop-exposed positions that directly contact loop C and complementary-face elements of the receptor, rather than within the core β -sheets that maintain the three-finger scaffold. While these patterns strongly implicate surface residues in functional diversification, direct experimental confirmation through mutagenesis or binding assays has so far been obtained for only a fraction of inferred sites.

Concrete structure–function examples help to anchor this mapping logic. In the Cbtx–AChBP crystal structure, a defined set of contact residues in loop II (Trp²⁵, Asp²⁷, Phe²⁹, Arg³³) and the C-terminus (e.g., Phe⁶⁵) lies within the binding pocket and is explicitly cross-referenced to mutational datasets in which substitutions in either toxin or receptor reduce affinity by multi-fold, thereby identifying functional “hot spots” that are also physically embedded in the interface. In parallel, receptor-side mechanisms such as interface-proximal glycosylation provide a structurally interpretable route by which selection on receptor surface features could reduce toxin binding without necessarily perturbing endogenous acetylcholine signalling, because the glycan projects directly into the toxin–receptor contact zone [49,118].

Adaptive evolution in 3FTx–receptor systems is concentrated in exposed loop residues, particularly loop II and adjacent surfaces. This surface-focused pattern is consistent with strong positive selection acting on toxin–receptor interfaces and is compatible with scenarios in which receptor variability imposes shifting selective pressures. Selection analyses, functional mapping, and structural validation all support this mechanism, consistent with RAVER-like diversification.

4. Molecular Evolution of Target Receptors and Toxin Resistance

4.1. Structural Determinants of α -Neurotoxin Binding to nAChR

The muscle-type nAChR presents an orthosteric ligand-binding site in the ECD at subunit interfaces. In the canonical loop nomenclature used for nicotinic ligand-binding domains, the principal face (provided by the α subunit) contributes loops A–C, whereas the complementary face (provided by the adjacent non- α subunit) contributes loops D–F; together these elements shape the binding pocket accessed by acetylcholine and competitively occupied by α -neurotoxins. A prominent architectural feature is the flexible loop C “lid”, which carries the vicinal cysteines characteristic of nicotinic receptors and which undergoes conformational changes upon ligand engagement, thereby contributing to both ligand recognition and gating-linked rearrangements [5]. Within this framework, α -neurotoxins bind at or near the same orthosteric pocket that normally accommodates acetylcholine, and their affinity and specificity reflect a balance between (i) conserved

receptor chemistry required for cholinergic signalling and (ii) toxin surface features that exploit those conserved receptor determinants [5,49].

High-resolution and structure-guided approaches have established that α -neurotoxin recognition is dominated by a set of conserved receptor residues that define aromatic and “proline” subsites within the orthosteric pocket, together with loop C positioning that determines accessibility. In a crystallographic model of an α -neurotoxin–receptor surrogate complex, the loop C aromatic residue Phe¹⁸⁹ makes direct van der Waals contact with the conserved toxin disulphide in loop II (Cys²⁶–Cys³⁰), highlighting loop C as a key determinant of toxin engagement [121]. The same structural analysis emphasises that substitutions at the loop C–adjacent interface can perturb toxin binding by altering local packing and aromatic complementarity (for example, changes at Lys¹⁸⁵ and neighbouring positions that remodel the pocket), thereby modulating affinity without wholesale disruption of the ECD fold [121]. Complementary functional and comparative analyses further support a two-subsite view of the toxin-binding region: an aromatic subsite and a proline-associated subsite in which Pro¹⁹⁴ is repeatedly implicated in resistance-linked variation, consistent with a binding mode in which loop C and its immediate flanking residues couple steric fit to receptor–toxin complementarity [49]. Beyond side-chain packing, electrostatic complementarity is also central: α -neurotoxins tend to present strongly positive surfaces, whereas the susceptible receptor pocket includes negatively charged features that facilitate docking; thus, alterations that reduce this complementarity can measurably weaken binding [48]. Finally, glycan-mediated effects provide a structurally explicit route to resistance: introduction of N-linked glycosylation sequons in or near loop C can place bulky glycans adjacent to the binding pocket, physically occluding toxin access or altering loop C positioning, an effect supported by both structural inference and receptor-engineering experiments [50,121].

4.2. Convergent Evolution of Molecular Resistance in Vertebrate nAChR α_1 -Subunit

Comparative work across vertebrates supports the view that molecular resistance to α -neurotoxins has evolved repeatedly via a limited set of mutational “solutions” concentrated in the α_1 orthosteric region. Across studies, three recurrent motif classes stand out. First, the repeated origin of N-glycosylation sequons (Asn—any—Ser/Thr) at or near loop C introduces steric hindrance within the binding pocket; such sequons recur in resistant lineages and can be sufficient to reduce α -neurotoxin effects when engineered into susceptible receptor contexts [49,50]. Second, the repeated appearance of positively charged residues (Arg/Lys) at key positions within the orthosteric site can impede binding by diminishing favourable electrostatic interactions, or even creating electrostatic repulsion against positively charged toxins [47,48]. Third, proline-associated changes, either substitutions of proline residues implicated in the “proline subsite” or other structurally influential replacements affecting loop C conformation, recur as candidate resistance determinants, consistent with a mechanism in which subtle backbone remodelling reduces toxin complementarity while retaining acetylcholine responsiveness [47,49].

Macroevolutionary sampling indicates that these solutions have arisen independently in multiple ecological contexts where elapid α -neurotoxins impose strong selection. Among mammals, phylogenetic analyses identify independent origins of resistance-associated amino acid replacements in the toxin-binding site, including convergent changes in honey badgers, hedgehogs and pigs, alongside distinct glycosylation-based modifications inferred in venom-resistant mongooses, together implying repeated evolution through different biochemical routes acting at homologous receptor sites [122]. In reptiles, broad sampling within Australian squamates demonstrates that resistance motifs are neither singular nor phylogenetically confined: across Australian skinks, multiple independent lineages exhibit toxin-site changes

including N-glycosylation motifs, proline substitutions, arginine insertions, shifts in receptor electrochemical state, and even novel cysteines, with functional and modelling analyses supporting the interpretation that these changes impede neurotoxin binding [47]. Similarly, targeted work in Australian agamids shows that a specific N-glycosylation motif (Asn¹⁸⁷–Val¹⁸⁸–Thr¹⁸⁹) mediates steric resistance in *Pogona vitticeps*, while a wider survey across agamid diversity finds this mechanism absent outside *Pogona*, indicating that even under comparable predatory regimes, resistance can be sharply clade-restricted [123]. Patterns in snakes themselves reinforce that resistance is not purely a prey phenomenon: resistance-associated modifications in nAChR orthosteric regions have been investigated in elapids, including glycosylation-linked effects at key toxin-binding positions, suggesting within these lineages that receptor-mediated resistance both enables and is reshaped by venom evolution [50]. Together, these data argue for repeated, convergent evolution of receptor modifications at a small number of structurally privileged positions, with ecological exposure to α -neurotoxic venoms providing the selective context for recurrence.

4.3. Mechanistic Classes of Resistance: Steric, Electrostatic and Conformational

At a mechanistic level, resistance to α -neurotoxins can be usefully organised into steric, electrostatic and conformational classes, focusing on structural modifications at the toxin–receptor interface that directly alter binding affinity or accessibility. This classification does not preclude additional layers of resistance, such as modulation of receptor expression levels, changes in subunit stoichiometry, or compensatory downstream signalling, but reflects the mechanisms for which direct molecular and functional evidence is currently strongest. Within this framework, steric resistance is best exemplified by the repeated evolution of N-linked glycosylation motifs within or immediately adjacent to loop C: such motifs can place a branched glycan close to the orthosteric pocket, impeding toxin approach and/or preventing the snug loop C–toxin packing required for high-affinity binding. The functional sufficiency of this mechanism is supported by receptor-engineering experiments, in which glycosylation at key positions (notably around Asn¹⁸⁹ and nearby loop C residues) reduces susceptibility to short-chain α -neurotoxins, and by comparative evidence from prey and predator lineages in which loop C-adjacent glycosylation motifs track resistance phenotypes [47,50,123].

Electrostatic resistance is supported by direct binding assays demonstrating that replacement of negatively charged residues with positively charged residues (e.g., charge reversal to lysine) can reduce α -neurotoxin binding via electrostatic repulsion. This mechanism acts by altering the local electrostatic landscape of the orthosteric site, thereby disfavoured productive toxin engagement without requiring gross structural rearrangement. Electrostatic resistance has convergently evolved multiple times within snakes and is also functionally supported for arginine-associated resistance in mammalian exemplars [48]. As with steric mechanisms, this class captures direct effects on toxin–receptor interaction; regulatory mechanisms operating at the level of receptor abundance or signalling may further modulate susceptibility but are presently less well resolved for α -neurotoxin systems.

Conformational resistance is most consistently linked to proline-centred changes in the orthosteric pocket: because proline strongly constrains backbone geometry, substitutions affecting proline-associated elements (including positions implicated in the “proline subsite”) can remodel loop C and its neighbouring structural context, thereby diminishing toxin complementarity while preserving the essential architecture required for acetylcholine binding and receptor activation; comparative analyses in vertebrates and within-species radiations identify such proline-associated changes as recurrent correlates of resistance [47,49]. In practice, resistant lineages may accumulate combinations of these changes (for example, coupling glycosylation-mediated steric effects with charge-based repulsion), potentially producing synergistic reductions in toxin affinity.

4.4. Constraints and Trade-Offs in Receptor Evolution

Despite strong and repeated selection imposed by α -neurotoxins, receptor-mediated resistance is unevenly distributed, implying substantial constraints on feasible evolutionary trajectories at nAChR $\alpha 1$. The orthosteric binding site must retain high-fidelity cholinergic signalling at the neuromuscular junction, and much of its architecture is conserved because it underpins acetylcholine recognition and gating-linked conformational changes; consequently, only a narrow subset of substitutions at surface-exposed, toxin-contacting positions is likely to provide resistance without compromising receptor function [40]. Experimental work that measures acetylcholine-evoked currents while manipulating toxin-site segments supports this constraint: resistance-conferring modifications (such as glycosylation within the α subunit ligand-binding segment) can reduce toxin inhibition while preserving measurable acetylcholine-induced responses, illustrating that successful resistance often involves “surgical” changes that decouple toxin binding from physiological activation [50]. Ecological and comparative patterns further suggest that resistance is favoured only in some lineages even under exposure: in Australian agamids, glycosylation-based resistance appears restricted to *Pogona* and is argued to reflect a compensatory response in a morphologically vulnerable lineage with limited escape capability, whereas other agamids, despite encountering neurotoxic elapids, lack comparable binding resistance and instead possess alternative defensive traits (speed, defensive spines, arboreality) that may reduce the net selective benefit of receptor modification [123]. Likewise, resistance can be lost when selective regimes change: in pythons, patterns consistent with strong electrostatic resistance followed by subsequent loss under reduced predatory pressure support the idea that resistance mutations may carry costs (or be disfavoured when benefits wane), leading to evolutionary reversals [124]. Even where resistance evolves repeatedly within a regional radiation, it remains patchy: among skinks, only a subset of lineages exhibit resistance motifs, indicating that lineage-specific ecology, exposure intensity, and the compatibility of particular mutations with receptor function jointly shape the realised distribution of resistance across taxa [47]. Overall, receptor evolution in this system reflects an interplay between strong selection for toxin evasion and stringent functional constraint on the neuromuscular signalling apparatus, producing convergent reuse of a limited mutational repertoire and frequent heterogeneity in resistance outcomes across sympatric lineages.

5. Integrated Evidence for Coevolution Between Three-Finger Toxins and Receptors

Direct tests of coevolution between 3FTxs and their targets integrate (i) ecologically realistic venom/toxin phenotypes, (ii) interspecific (and sometimes intraspecific) receptor sequence variation at orthosteric sites, and (iii) quantitative functional assays linking toxin–receptor molecular complementarity to selective contexts. In the 3FTx–muscle-type nAChR $\alpha 1$ system, mimotope panels coupled to high-throughput binding assays enable explicit tests of prey- and predator-associated binding patterns, moving beyond sequence evolution inference alone [51,125,126].

5.1. Mimotope-Based Binding and Prey-Selectivity Assays

Mimotopes are short synthetic peptides capturing key determinants of the nAChR $\alpha 1$ orthosteric site recognised by α -neurotoxins, typically focusing on loop C and adjacent residues (~187–200, species-specific numbering). Design choices preserve binding-relevant chemistry whilst improving stability and assay compatibility: cysteine-to-serine substitutions mitigate oxidative instability, and N-terminal biotinylation enables immobilisation on streptavidin-coated sensors. These peptides provide a standardised, evolutionarily comparable proxy for the dominant toxin-binding surface, enabling systematic cross-taxon sampling impractical for native receptors [51,125,126].

Mimotope assays employ bio-layer interferometry (BLI) for label-free, real-time binding monitoring. In Afro-Asian elapids, broad BLI screens against mimotopes representing amphibian, lizard, bird, rodent, and snake prey revealed strong prey-associated binding differences structured by elapid clade and geography, consistent with local adaptive tuning of α -neurotoxin activity [126]. Binding profiles varied among *Naja* clades, with pronounced lizard-mimotope binding in some non-*Naja* elapids and population-level divergence within *O. hannah* across localities, demonstrating that prey-selectivity reflects lineage-specific ecological opportunity rather than a universal optimum.

In *Micrurus*, large-scale BLI datasets across multiple venoms revealed binding patterns structured primarily by phylogeny rather than prey categories alone. A key coevolutionary signal emerged: consistently reduced binding to snake-derived mimotopes, supporting the hypothesis that snake prey have evolved nAChR α_1 changes diminishing α -neurotoxin susceptibility [125]. Mechanistically, recurrent substitutions (e.g., Trp187Ser) contribute to reduced binding, illustrating how mimotope panels identify specific resistance determinants from macro-patterns [125]. However, mimotope-derived kinetic parameters require comparative interpretation rather than direct receptor affinity surrogates, as peptide-level simplifications inevitably alter binding kinetics [125,126].

5.2. Coevolution in Specific Predator–Prey Systems

Several systems demonstrate coherent alignment between venom phenotype, target-site variation (or mimotope proxies), and ecological interaction. Among Afro-Asian elapids, prey-class mimotope panels reveal lineage-structured prey-selective binding, with geographic structuring with *O. hannah*, indicating variable selective environments even within genera [126]. Subsequent high-throughput applications of this assay framework across additional Afro-Asian and African elapids further demonstrate that α -neurotoxin binding to nAChR orthosteric sites differs systematically among amphibian-, reptile-, avian- and mammal-representative mimotopes, consistent with ecological diet shaping toxin–receptor interactions rather than neutral divergence alone [127,128].

In marine *Hydrophis*, integrated genomic and multiomic analyses reveal closely related sea snakes differing substantially in venom architecture, including 3FTx gene copy number (e.g., 20 vs. 10 copies) and venom-gland expression profiles. These differences align with trophic specialisation; structural modelling predicts that highly expressed 3FTx variants interact differentially with nAChR α -subunit domains from diet-representative prey, consistent with receptor-mediated ecological matching driving toxin divergence [56,99]. Functional binding assays using prey-specific nAChR mimotopes further support this interpretation, demonstrating that aquatic elapid venoms show non-uniform affinities across prey classes reflective of their feeding ecology [128].

The *Micrurus* radiation exemplifies divergent coevolutionary trajectories. Venom dichotomy, alternative investment in toxin families with rapid diversification, favours postsynaptic α -neurotoxin function in some lineages. BLI data confirm non-uniform α -neurotoxin binding across *Micrurus* venoms, with recurrently reduced snake-mimotope binding consistent with counteradaptation [125]. Comparable reductions in orthosteric-site binding have now been documented across multiple elapid lineages, reinforcing the inference that prey or predator receptor modification can recurrently shape venom functional space where snakes interact with other venomous or resistant taxa [49,127].

Rear-fanged colubrids demonstrate extreme functional partitioning: closely related 3FTxs show sharply contrasting lizard versus mammal toxicity profiles, indicating strong selection for prey-specific efficacy [100]. Although outside Elapidae, these patterns parallel prey-selective binding trends observed in elapid α -neurotoxins and provide a broader

comparative framework for understanding how ecological specialisation can drive fine-scale functional divergence within homologous toxin scaffolds.

Predator-side resistance provides a further level of integration. In mammals that prey on spitting cobras, resistance to α neurotoxins arises at the muscle type α -1 nAChR through a substitution that replaces a neutral aromatic tryptophan with a positively charged arginine at position 187, thereby introducing electrostatic repulsion at the binding site and consistent with long-term antagonistic coevolution [48]. Comparative analyses reveal that analogous charge-altering substitutions and glycosylation motifs disrupting α -neurotoxin binding have evolved convergently in multiple vertebrate lineages exposed to elapid venoms, further supporting repeated ecological selection on the same molecular interface [49,58,129].

Primate susceptibility profiling reveals non-random phylogenetic resistance distribution, with elevated resistance in Afro-Asian primates driven by a few amino acid changes, supporting reciprocal arms-race dynamics in regions of historical elapid sympatry [51]. Similar patterns of geographically structured resistance have been identified in other elapid-exposed vertebrates, reinforcing the conclusion that prolonged ecological interaction with neurotoxic elapids can leave a detectable molecular signature at venom target sites [58,129].

Collectively, these systems demonstrate how repeated ecological interactions between elapids and their prey or predators drive convergent and lineage-specific modifications at a shared molecular interface, linking venom phenotype, receptor evolution and functional outcome. This integration is summarised in Table 1 and Figure 3, which together synthesise comparative and experimentally validated evidence for reduced α -neurotoxin binding across representative elapid-exposed vertebrates. By juxtaposing ecological context, experimental approach and residue-level effects at the muscle-type nAChR α -1 orthosteric site, these summaries provide a concise framework for visualising recurrent molecular solutions arising during antagonistic coevolution.

Table 1. Ecological and functional correlates of resistance to elapid α -neurotoxins in exposed vertebrates. Summary of representative predator–prey systems illustrating how prolonged ecological interaction with neurotoxic elapids is associated with reduced binding of α -neurotoxins at the nicotinic acetylcholine receptor.

Elapid–Counterparty Interaction	Ecological Interaction	Analyte Tested	Toxin Class Implicated	Mechanistic Effect on Binding	Experimental Approach	Ref.
Homininae vs. <i>Naja</i> spp.	Long-term sympatry and primate predation	Whole venoms	α -Neurotoxins (3FTx; inferred driver)	Reduced venom binding due to combined electrostatic and steric effects	Comparative BLI venom-binding assays across primate mimotopes; phylogenetic analysis	[51]
Honey badger (<i>Mellivora capensis</i>) vs. African elapids	Repeated ecological exposure to elapid venoms	Whole venoms	α -Neurotoxins (3FTx; inferred driver)	Altered electrostatic landscape reduces venom α -neurotoxin affinity	Comparative sequence analyses linked to venom-binding resistance patterns	[48]
Mongoose (<i>Herpestidae</i>) vs. α BgTx	Frequent predation on elapids	Purified toxin	α -Bungarotoxin	Steric obstruction abolishes toxin access to the binding interface	α -Bungarotoxin binding assays using receptor fragments and mutational analysis	[130,131]
Australian elapids vs. Australian skinks	Chronic predation by neurotoxic elapids	Purified toxins	α -Neurotoxins	Reduced toxin binding without compromising acetylcholine sensitivity	Functional receptor assays with purified toxins	[47]

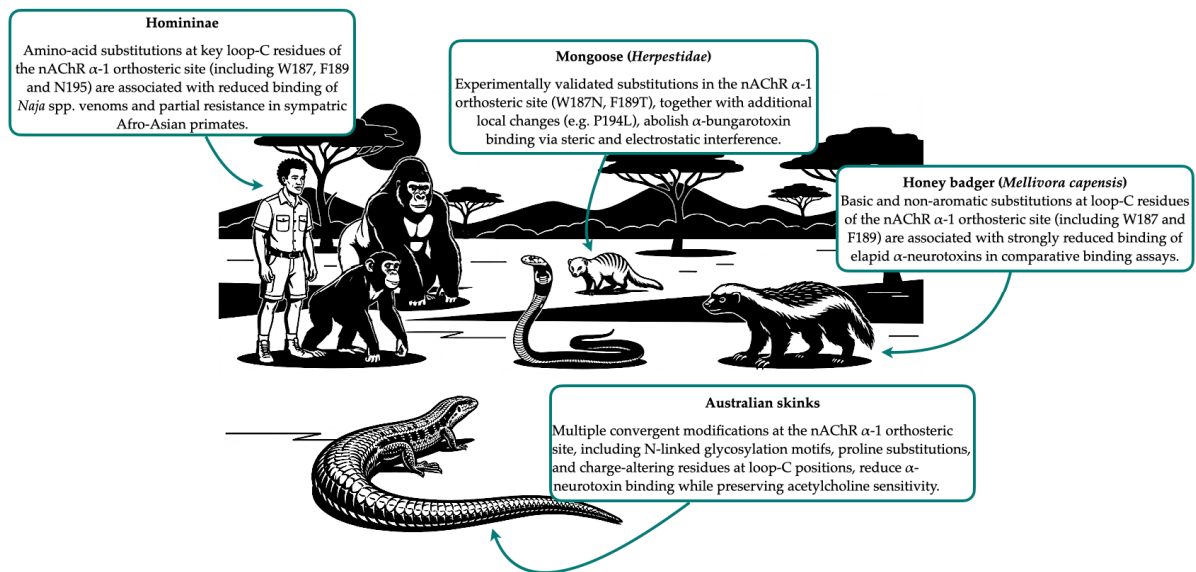


Figure 3. Convergent molecular adaptations at the nicotinic acetylcholine receptor underlying resistance to elapid α -neurotoxins. Schematic illustration of vertebrate taxa exposed to neurotoxic elapid venoms showing modifications at the muscle-type nAChR α -1 orthosteric site (loop C) that reduce toxin binding. In Afro-Asian primates (Homininae), amino-acid substitutions at key loop-C positions (including residues Trp¹⁸⁷, Phe¹⁸⁹ and Asn¹⁹⁵) are associated with reduced binding of *Naja* spp. venoms and partial resistance in sympatric lineages [51]. In mongooses (*Herpestidae*), experimentally validated substitutions at the orthosteric site (Trp187Asn, Phe189Thr), together with additional local changes (e.g., Pro194Leu), abolish α -BgTx binding through steric and electrostatic interference [130,131]. In the honey badger (*Mellivora capensis*), basic and non-aromatic substitutions at loop-C residues (including residues Trp¹⁸⁷ and Phe¹⁸⁹) are associated with strongly reduced binding of elapid α -neurotoxins in comparative binding assays [48]. Australian skinks exhibit multiple convergent modifications at the nAChR α -1 orthosteric site, including N-linked glycosylation motifs, proline substitutions and charge-altering residues, which reduce α -neurotoxin binding while preserving acetylcholine sensitivity [47].

5.3. Toxin Diversification Shaped by Receptor Variability

Receptor variability acts as a moving, structured target channelling toxin diversification. Comparative analyses demonstrate that long-chain α -neurotoxin interfaces experience sustained adaptive change in toxin residues, whereas homologous receptor segments show weaker positive selection signatures, reflecting asymmetric coevolution where rapidly evolving toxin gene families explore sequence space more readily than pleiotropic, functionally constrained receptors [115].

This asymmetry aligns empirically with elevated venom gene evolutionary rates in sea snakes and phylogenetically structured *Micrurus* binding outcomes tracking receptor-state differences across prey clades [99,125]. Consistently reduced α -neurotoxin binding to snake mimotopes, coupled with identified substitutions, exemplifies how discrete receptor changes reshape the functional landscape experienced by toxins, favouring compensatory evolution [125].

These findings support a coevolutionary framework: (i) receptor variation at structurally privileged loop C positions creates discrete compatibility classes for orthosteric binding, (ii) toxin gene-family diversification supplies standing variation to exploit or restore compatibility, and (iii) ecological filtering (diet, prey turnover, geography) determines favoured toxin–receptor matches [56,99,115,126].

Substantial uncertainties persist. Mimotope panels interrogate limited receptor surfaces without capturing conformational or assembly-dependent effects, and prey receptor sampling remains sparse relative to toxin diversity. Expanding paired venom–receptor sam-

pling within characterised interaction networks, particularly snake–snake interactions in *Micrurus* and predator resistance systems in mammals and primates, will distinguish arms-race dynamics from ecological fitting to pre-existing receptor diversity [48,51,115,125,126].

6. Ecological and Macroevolutionary Context of Three-Finger Toxin–Receptor Interactions

6.1. Venom Phenotype Convergence and Ecological Filtering

At the macroevolutionary scale, a consistent result emerging from comparative venom phenomics is that snakes have repeatedly converged on a limited set of venom “solutions”, despite the vast combinatorial space available to multi-gene toxin repertoires. A particularly clear expression of this principle is the finding that most among-species variance in venom composition can be captured by a low-dimensional phenotypic space dominated by a small number of toxin families, with convergence towards recurrent formulations over deep time rather than continuous exploration of novel compositional regimes [132]. In this framing, 3FTx-rich venoms are not simply an idiosyncrasy of a particular lineage, but one repeatedly occupied region of venom space in which selective regimes favour rapid incapacitation via high-affinity engagement of conserved neuromuscular targets; importantly, convergence at the phenotypic level can arise even when the underlying genetic routes differ (e.g., lineage-specific expansions, losses, and regulatory shifts), because selection acts on functional outputs (capture success, handling time, prey escape probability) rather than on shared molecular histories per se.

Within this constrained venom space, ecological filtering provides a mechanistic bridge between macroevolutionary convergence and toxin–receptor coevolution. Comparative transcriptomics across phylogenetically diverse Australian elapids indicates that species preferentially express different classes of α -neurotoxic 3FTx (types I–III), interpreted as a trophic signal consistent with divergence in feeding ecologies; yet, across toxin types, substitutions accumulate in broadly similar structural regions (predominantly exposed loops), supporting the view that ecological filtering can repeatedly channel adaptive change towards a restricted set of functionally permissive sites on the 3FTx scaffold [57]. At the within-species level, an integrated toxin-expression, functional, and evolutionary analysis of a generalist rear-fanged snake demonstrates a different but complementary outcome of ecological filtering: the coexistence of distinct prey-selective 3FTx paralogues in a single venom, with one toxin showing high lethality towards lizards but not mammals, and a second showing the reverse pattern [100]. Such bimodal prey-selective toxicity illustrates how selection imposed by heterogeneous diets can favour retention of multiple functionally differentiated toxin lineages, rather than a single “generalist” solution, thereby expanding the set of receptor variants that toxins must effectively engage [100]. Beyond elapids, an -omics synthesis of rear-fanged colubrid venoms emphasises that while several toxin families are broadly distributed across “colubrid” venoms, 3FTxs appear close to restricted to Colubridae sensu stricto, and that the strongest opportunities for novel venom components may lie in lineages with specialised diets, again highlighting ecological filtering as a major constraint on which toxin families dominate and, by extension, which receptor systems are placed under the strongest reciprocal selection [133].

Marine elapids offer an additional axis along which ecological filtering can stabilise (or erode) 3FTx-dominated solutions. In *H. cyanocinctus*, integrated transcriptomic/proteomic profiling characterises a venom that is biochemically simple but genetically complex, with venom lethality largely attributable to 3FTxs and with evidence of pervasive positive selection across toxin-coding genes, consistent with strong selective pressure in a marine predatory context where rapid immobilisation of fast-moving prey is advantageous [9]. At broader phylogenetic scale across sea snakes, toxin gene expression and evolution have

been linked to ecological variables, including a reported relationship between dietary breadth and toxin diversity, suggesting that community-level prey availability can filter venom repertoires and thereby modulate the intensity and direction of selection on receptor targets [97]. Conversely, the egg-eating sea snake *Aipysurus eydouxii* exemplifies how a shift in trophic niche can “put the brakes” on venom evolution, plausibly via relaxed selection on prey-immobilising toxins when prey do not require rapid neuromuscular incapacitation [87]. Taken together, these patterns indicate that 3FTx–receptor coevolution is expected to be most pronounced where ecological filtering favours sustained reliance on α -neurotoxic 3FTx, and attenuated where venom phenotypes shift towards other toxin families or where trophic specialisation reduces the selective premium on receptor-targeting neurotoxins [87,132].

6.2. Spatial Heterogeneity and Community Context

Spatial heterogeneity, arising from restricted gene flow, phylogeographic structure, and geographic variation in prey communities, creates the conditions under which locally divergent venom phenotypes (and, potentially, local resistance) can emerge, thereby generating the raw material for geographically variable coevolutionary dynamics. A particularly direct illustration is provided by *Laticauda colubrina*, for which proteomic characterisation of Balinese venom is described as divergent from prior reports from other localities, with stark geographic differences proposed to reflect trophic adaptation (notably involving eel prey) and reinforced by behavioural site fidelity that would limit homogenising gene flow among distant populations [134]. In this setting, spatial variation in venom composition is not merely descriptive: it implies spatial variation in the dominant molecular interactions (e.g., relative reliance on short-chain neurotoxins versus other lethal components), and thus geographic differences in which receptor variants are most consequential targets of selection [134].

A second, conceptually important case concerns New World coral snakes. Comparative proteomics across *Micrurus* highlights a widely discussed phenotypic dichotomy in which venoms tend to be dominated either by presynaptic PLA₂ toxins or by postsynaptic 3FTxs, with an overall, but imperfect, phylogeographic pattern previously proposed along a North–South axis, and additional locality sampling revealing a more complex distribution than anticipated [43,89]. Because these alternative phenotypes emphasise different physiological targets (and hence different axes of prey susceptibility), the geographic mosaic of 3FTx- versus PLA₂-dominant *Micrurus* venoms suggests that the opportunity for strong 3FTx–nAChR coevolution will itself be spatially structured, with some regions experiencing sustained selection on receptor orthosteric-site variation while others may experience weaker or qualitatively different selective regimes [43]. Functionally explicit evidence for fine-scale heterogeneity also emerges from prey-selectivity assay work on α -neurotoxic venoms, which reports both interspecific patterns and intraspecific (including geographic) differences in venom activity, demonstrating that ecologically relevant variation can occur on relatively short spatial and evolutionary scales [126]. In aggregate, these studies support a view in which coevolutionary signal is unlikely to be uniform across a species’ range: instead, it should covary with (i) the local dominance of 3FTxs among venom components, (ii) the identity and relative abundance of prey species in local communities, and (iii) the extent to which population structure allows divergence to accumulate in both venom repertoires and receptor targets [89,126,134].

Evidence bearing on spatial variation in resistance is necessarily more fragmentary in most systems, but comparative receptor sequencing across broad radiations can still reveal strong heterogeneity in exposure and selection. In Australian skinks, multiple independent origins of α -neurotoxin resistance motifs are inferred across diverse lineages, with

functional testing supporting resistance in some taxa but not others; the patchy distribution is interpreted in the context of differential vulnerability and ecology (e.g., defensive morphology, locomotor capacity, arboreality) that would modulate realised exposure to α -neurotoxic predators [47]. Although this dataset is not a population-genetic test of “hotspots” and “coldspots” in the strict geographic-mosaic sense, it does demonstrate that even within a single continental fauna, receptor evolution can be highly non-uniform, consistent with spatially and ecologically variable encounter regimes shaping the strength of reciprocal selection [47]. The community context is therefore not a backdrop but a determinant of coevolutionary detectability: in sympatric assemblages containing multiple venomous predators and multiple potential prey (each with different degrees of exposure and alternative defences), selection on both toxin portfolios and receptor binding surfaces is expected to be mediated by interaction strength, not simply by co-occurrence [47,126].

6.3. Coevolution in Multi-Species Networks Versus Pairwise Arms Races

The classical pairwise arms-race model treats coevolution as reciprocal escalation between a focal predator and a focal prey, predicting tight coupling between toxin innovation and resistance evolution. In contrast, diffuse coevolution in multi-species networks arises when predators exploit multiple prey types and/or prey experience multiple venomous predators, distributing selection across interacting partners and potentially favouring (i) broader-spectrum venom strategies, (ii) modular toxin repertoires targeting different prey subsets, and (iii) patchy or clade-restricted evolution of resistance where exposure is intense and alternative defences are insufficient. Several 3FTx systems in the corpus align more naturally with the network view than with strict pairwise escalation. The demonstration that a single generalist snake can maintain distinct prey-selective 3FTx paralogues with opposing toxicity profiles (lizard-biased versus mammal-biased) is, in effect, a microcosm of diffuse selection: toxins diversify to cover heterogeneous prey receptor landscapes rather than tracking a single prey lineage [100]. Likewise, macroevolutionary convergence of venom phenotypes towards a small set of family-dominated formulations suggests that, over long timescales, selection may often operate through recurrent ecological roles (prey guilds, habitats, prey mobility) rather than through persistent, exclusive pairwise antagonisms [132].

Network effects are even more explicit when resistance evolution is considered in predators and prey embedded in communities of venomous snakes. In pythonids, resistance to α -neurotoxins mediated by electrostatic charge repulsion is analysed in a phylogenetic and ecological context that explicitly invokes sympatry with particular α -neurotoxic lineages (e.g., cobras and snake-eating king cobras), as well as secondary loss of resistance plausibly associated with reduced predation pressure following ontogenetic increases in body size and shifts in vulnerability [124]. This illustrates two core predictions of diffuse coevolution: resistance evolution may be concentrated in the most-exposed or most-vulnerable lineages, and can be lost when exposure regimes change, even if venomous snakes remain present in the broader region [124]. Similarly, across Australian skinks, repeated origins of resistance motifs coexist with many lineages lacking such changes, consistent with uneven selection intensity among sympatric taxa due to ecological and behavioural differences that alter encounter rates and outcomes [47]. In combination, these findings caution against treating 3FTx-nAChR coevolution as a uniform, globally coupled process: instead, the most robust expectation is a heterogeneous landscape in which recurrent ecological settings favour 3FTx-rich venoms (creating repeated opportunities for selection on receptor binding sites), but resistance evolves idiosyncratically where exposure is strong and constraints permit, and may be absent where interaction strength is low or alternative defences reduce the payoff of molecular resistance [47,100,124,132].

7. Methodological Perspectives and Limitations

Inference of coevolution between three-finger toxins (3FTxs) and their targets integrates heterogeneous evidence streams, each with distinct identifiability constraints. The strongest support for reciprocal adaptation derives from concordance between (i) codon-model signals of lineage- or site-restricted diversification in toxin families, (ii) mechanistically interpretable receptor residue variation within orthosteric binding interfaces, and (iii) functional assays demonstrating that these sequence differences measurably shift toxin–receptor binding or toxicity consistent with ecological expectations [10,41,48,97,100,122,123]. Methodological limitations arise not only from familiar statistical pitfalls, but also from scale mismatches (e.g., deep-time toxin diversification versus shallow receptor sampling) and from the fact that gene-family evolution, population structure, and ecological turnover can each generate patterns mimicking, or obscuring, reciprocal selection [10,39,41,97].

7.1. *dN/dS-Based Tests of Selection and Their Interpretation*

Codon-model approaches formalise selection inference via the ratio of nonsynonymous to synonymous substitution rates ($\omega = dN/dS$), but interpretability depends critically on the hierarchical level at which heterogeneity is modelled. Global ω estimated across an alignment usefully rejects neutral evolution but poorly describes proteins with strong spatial and temporal constraint heterogeneity, as localised adaptive change becomes diluted by conserved sites [135,136]. This is particularly acute for 3FTxs, where disulphide-rich scaffolds remain conserved whilst diversification concentrates in solvent-exposed loop residues; consequently, alignment-wide mean ω can remain <1 even when specific sites experience diversifying selection in particular clades or ecological contexts [39,41,100]. The RAVER framework anticipates this pattern and motivates site, branch, and clade models over global summaries [41].

Best practice employs model comparisons separating background constraint from episodic or site-restricted adaptation. Studies commonly implement nested likelihood models with multiple-testing correction, and tie statistical inference to mechanistic plausibility by mapping positively selected sites onto structural features and solvent-accessibility analyses, privileging surface-localisation patterns over isolated p -values [41]. This is especially important for 3FTxs because alignment uncertainty concentrates in loop regions most likely harbouring adaptive substitutions; small indel-placement differences alter site homology and hence apparent $\omega > 1$ clustering [39,41]. Best practice therefore emphasises robustness to alignment choices, tree uncertainty, and model specification, asking whether supported sites fall in chemically and structurally plausible interaction surfaces rather than maximising “significant sites”.

Power and identifiability constraints also shape the recurring toxin–receptor asymmetry. Large toxin multigene families provide many paralogous lineages and substantial sequence divergence, yielding strong site- or branch-site signals when sampling spans relevant clades [10,41]. Conversely, receptor genes often exhibit functional constraint and subtle adaptive trajectories: resistance can hinge on few substitutions within the binding interface whilst remaining receptor regions stabilise under pleiotropic constraint [48,122,123]. Formal dN/dS tests may fail to reject $\omega \leq 1$ even when receptor variability has clear functional consequences, because selection concentrates on few sites and taxon sampling remains sparse relative to adaptive timescales. The honey badger system exemplifies both promise and fragility: convergent CHRNA1 changes received phylogenetic testing, but functional interpretation remained incomplete when receptor variants were not experimentally assayed across all implicated lineages. In aggregate, ω -based analyses are most informative as hypothesis generators reconciled with structural localisation and functional directionality, rather than as stand-alone coevolution adjudicators.

7.2. Cophylogeny, Ancestral Reconstruction and Network Approaches

Joint phylogenetic approaches detect whether evolutionary histories of interacting molecules exhibit congruence beyond chance expectation, providing formal scaffolds for matching toxin and receptor evolutionary change. Classical cophylogeny analyses compare trees and evaluate congruence via event-based reconciliation (cospeciation, duplication, host-switching) or topological similarity measures [137–139]. In principle, analogous logic applies to 3FTx and receptor phylogenies, asking whether toxin clades preferentially track receptor clades or resistance-associated motifs. However, the venom–receptor problem departs from textbook cophylogeny: toxin phylogenies reflect extensive duplication and birth–death dynamics, whilst receptors are typically single-copy (or low-copy) loci with strong pleiotropic constraint [41,122]. These asymmetries mean apparent congruence may reflect shared species history rather than reciprocal adaptation, whilst genuine reciprocal adaptation may be obscured by non-comparable gene-family and species trees. The most defensible near-term application focuses on specific toxin subfamilies with clear target annotation and receptor regions with established functional relevance, rather than whole-family reconciliation.

Ancestral sequence reconstruction (ASR) offers a complementary approach by converting inferred historical intermediates into experimental objects. The logic is straightforward: infer ancestral states under explicit evolutionary models, synthesise ancestral proteins, and assay function to determine whether inferred trajectories imply directional shifts in binding, toxicity, or resistance [140]. Limited but instructive applications already exist—for example, parsimony-based inference of ancestral nAChR residues in mammalian lineages implicated in resistance [122]. Extending to fuller ASR, reconstructing ancestral toxins within focal clades and ancestral receptor variants within interacting prey/predator sets, could provide direct temporal-ordering tests (e.g., whether resistance motifs predate toxin diversification) [140]. Principal limitations are familiar: deep-node uncertainty, indel sensitivity in loop-rich toxins, and the risk that resurrected single proteins fail capturing multi-component venom phenotypes and physiological receptor context [39,41,140]. ASR is therefore best framed as generating discriminating functional predictions tested across plausible ancestral ensembles, rather than deterministic historical reconstruction [140].

Ecological network approaches move beyond pairwise arms-race narratives by embedding molecular interaction data within multi-species food webs and community assemblages. Formal descriptors of interaction structure (modularity, nestedness, partner diversity) and statistical frameworks link interaction patterns to trait evolution and phylogenetic structure [141]. For 3FTx–receptor systems, realistic networks treat venom phenotypes as interacting with distributed prey receptors, such that toxin selection becomes diffuse and mediated by prey community composition rather than single canonical prey lineages [10,97,141]. Several studies motivate this perspective, particularly those linking venom gene diversity or conservation to diet breadth or ecological context, yet explicit coupling of molecular coevolution analyses with network inference remains largely prospective for 3FTx systems [10,97].

7.3. Integrating Structural, Functional and Ecological Data

The most informative inferences arise when molecular evolution is interpreted through mechanistic structure and confronted with functional assays in ecological frames. Structural information discriminates between statistically supported substitutions and those plausibly modulating receptor engagement, particularly for proteins where adaptive change clusters in solvent-exposed loops [39,41,100]. This can be accomplished through functional assays, binding kinetics, electrophysiology, in vivo toxicity, anchor sequence and structure in phenotypic directionality [125,126]. Representative integrative strategy: (i) detect

selection or rapid diversification in toxin lineages using codon models, (ii) map candidate sites to structural surfaces engaged in target binding, and (iii) test whether toxins differentially bind receptor-derived mimotopes or variants consistent with hypothesised prey specificity [41,100,125,126]. Such integration reduces the risk that statistical artefacts (alignment-driven calls, unmodelled gene conversion, and unrecognised paralogy) are mistaken for adaptation, because mechanistic and functional coherence become additional inference filters.

Concrete examples illustrate both strengths and current bottlenecks. *Spilotes sulphureus* explicitly combines venom-gland transcriptomics, proteomics, evolution, and structural modelling, demonstrating that taxon-specific venom effects emerge from coordinated 3FTx sequence/structure and expression variation rather than single data types in isolation [100]. Mechanistically interpretable resistance with minimal molecular changes illustrates this power: electrostatic charge-reversal substitutions in nAChR reduce α -neurotoxin binding, validating structurally motivated binding-interface hypotheses [48]. The bearded dragon nAChR N-glycosylation motif, coupled with comparative agamid evidence and functional venom interrogation, shows how post-translational modification integrates resistance inference—whilst underscoring that receptor adaptation may escape conventional ω -based tests focusing solely on amino-acid substitutions [123]. At broader scales, venom gene diversity/conservation explicitly related to diet breadth or niche provides templates for ecological integration, with clear recognition that correlation does not itself evidence reciprocal molecular adaptation [10,97].

Despite advances, principal limitations remain (i) sparse, uneven sampling (few populations, limited prey/predator pairs, restricted receptor diversity) and (ii) incomplete functional coverage (many inferred variants never assayed in matched combinations), and (iii) there are scale disconnects between microevolutionary processes (population selection) and macroevolutionary patterns (gene-family diversification) [10,41,97,100]. Most promising directions tighten interlayer coupling: structural modelling constrained by experimental interfaces; evolutionary models informed by solvent exposure or interface annotation; systematic ancestral reconstruction of matched toxin–receptor pairs; explicit prey–predator embedding in network models discriminating pairwise from diffuse coevolution [39,41,140,141]. The essential analytical components already exist—codon models, mechanistic hypotheses, scalable binding assays—and the next methodological gains will likely come from designing studies treating these as single pipelines rather than parallel, loosely connected evidence streams.

8. Knowledge Gaps and Future Directions

8.1. Under-Sampled Taxa and Targets

Although three-finger toxins (3FTxs) are recognised as a major axis of venom diversification and functional innovation, the empirical landscape remains conspicuously uneven across snake and prey lineages. At the predator level, the densest mechanistic and coevolution-oriented datasets cluster around elapids, particularly systems where α -neurotoxins and muscle-type nAChR $\alpha 1$ can be assayed using tractable binding platforms, whilst broad caenophidian diversity remains thinly characterised in terms of 3FTx repertoires, toxin–receptor specificity, and ecological contexts generating consistent directional selection [142]. Even where transcriptomic surveys extend across rear-fanged colubroids and viperids, they explicitly highlight disproportionate focus on front-fanged lineages, motivating deeper, lineage-rich reconstructions of toxin family histories and convergences across advanced snakes [142].

Within rear-fanged snakes, the imbalance is twofold: (i) many taxa remain sparsely sampled at sequence and proteoform levels, and (ii) few studies explicitly connect 3FTx di-

versification to prey-relevant function. Nevertheless, proof-of-principle exists for tractable integration. Prey-linked divergence among colubrine 3FTx genes has been inferred from venom gland transcriptomes and structural prediction, with duplication and selection concentrated on residues hypothesised to influence prey target interactions [42]. In a generalist rear-fanged predator (*Spilotes sulphureus*), integration of venom gene expression, prey assays, and structural modelling revealed two abundant 3FTxs with contrasting prey-selective lethality, implicating heightened sequence variability within surface-exposed loops [100]. These studies demonstrate that even “non-model” colubroids yield tractable coevolutionary inferences when ecological and functional data couple with evolutionary analysis [42,100].

Parallel gaps exist in other ecologically distinctive or methodologically challenging radiations. *Atractaspis* exemplifies this: despite recognition of sarafotoxins, a transcriptomic survey of *A. aterrima* revealed a lineage historically neglected yet unexpectedly rich in 3FTx diversity under positive selection, implying substantial unexplored toxin and target evolution opportunity [143]. In marine systems, reference-quality genomes of *H. cyanocinctus* and *H. curtus* reveal not simply diversification but lineage-specific losses and 3FTx gene dosage effects, with authors explicitly linking genomic changes to prey preference divergence, underscoring the need to connect 3FTx gene-family dynamics to shifting trophic regimes [99].

Asymmetries in prey sampling are equally acute. Most work on resistance and specificity centres on the vertebrate muscle nAChR $\alpha 1$ orthosteric site, largely because it is experimentally accessible and directly linked to the canonical paralytic α -neurotoxin phenotype [125,126]. High-throughput bio-layer interferometry (BLI) platforms now enable taxonomically flexible testing using receptor-mimicking peptides spanning amphibian, lizard, snake, bird, and rodent targets, revealing prey-selective patterns and intraspecific/geographical binding differences [126]. A broad *Micrurus* survey against taxon-specific mimotopes detected pronounced potency heterogeneity and a characteristic orthosteric-site substitution (tryptophan $\rightarrow$ serine) associated with reduced snake susceptibility, explicitly interpreted as consistent with Red Queen dynamics [125]. Yet these advances also expose major gaps: most prey clades remain underrepresented at population-level receptor sequencing and functional testing, and coevolutionary inference remains dominated by a single receptor class and binding region [125,126]. However, expanding prey taxon coverage rapidly reshapes mechanistic inference: reptile resistance can involve distinct molecular routes, including charge-reversal substitutions generating electrostatic repulsion [48] and N-glycosylation-mediated mechanisms proposed as distinctive resistance traits [123]. Broader prey sampling is therefore a prerequisite for discovering true resistance diversity and constraints on receptor evolution, not merely incremental completeness [48,123].

Finally, an important, and still weakly integrated, frontier concerns 3FTx targets beyond muscle nAChR $\alpha 1$. Foundational work emphasises that 3FTx functional diversification extends to diverse receptors and ion channels via focal surface mutation and exonic segment exchange [5]. However, the coevolutionary literature remains disproportionately anchored to α -neurotoxins, with non-classical target work comparatively fragmented. Muscarinic toxin-like proteins from *B. multicinctus* include paralogues with distinct conformations and receptor subtype interactions, with binding determinants hinging on a few key-position substitutions [21]. In *C. bivirgatus*, 3FTx evolution links to lineage-specific functional shifts consistent with sodium-channel targeting, highlighting novel prey-immobilisation strategies [109]. What remains absent is a coherent, comparative framework treating non-canonical targets as coevolutionary arenas on par with nAChR $\alpha 1$, mapping target diversity in prey, quantifying toxin cross-reactivity, and identifying whether resistance evolves via parallel structural principles across receptor families [5,21,109].

8.2. From Species-Level Comparisons to Population Genomics

A pervasive limitation is that many evolutionary and functional studies remain effectively species-level, often treating one (or few) individuals, transcriptomes, or pooled venoms as representative of species' toxin repertoires and ecological interactions. This is not trivial: venom phenotypes and receptor genotypes vary within species across geography, ontogeny, and prey communities; sparse sampling can blur or invert apparent selection directionality. The strongest coevolutionary claims increasingly emerge from studies detecting intraspecific variation or revealing phylogeographic complexity inconsistent with species-level typologies. Comparative *Micrurus* proteomics highlight a continent-scale “3FTx/PLA₂ venom dichotomy” “general, but imperfect” along a North–South axis, with multi-locality sampling revealing that phylogeny alone does not explain phenotype and toxin arsenals exhibit substantial evolvability [89]. Afro-Asian elapid BLI assays detect not only prey-selective patterns but “intraspecific, and geographical differences” in α -neurotoxic binding, directly demonstrating functionally relevant venom variation below species level [126]. These results imply contemporary selection operates on standing variation in toxin expression and toxin–receptor affinity landscapes, with local ecology shaping realised venom efficacy [89,126].

Transitioning to a population-genomics understanding requires shifts in sampling design and analytical integration. For venoms, this combines dense geographic sampling with high-resolution proteomics and transcriptomics, distinguishing expression variation, gene copy-number differences, and allelic diversity; for receptors, it requires population-level sequencing of relevant regions and functional testing of locally prevalent variants against local venoms. Several especially tractable “bridge systems” exist where foundational resources enable high-impact population sampling. *Micrurus* is a clear candidate: extensive work documents broad nAChR α 1 orthosteric-site binding heterogeneity [125] whilst independent proteomics reveals complex phylogeographic venom-phenotype structure with clinical and evolutionary consequences [89]. Afro-Asian elapid radiations (notably *Naja* and *Ophiophagus*) are similarly attractive because prey-selective binding quantified in multi-target frameworks already incorporates multiple geographic locales [126]. Rear-fanged colubroids, though historically underrepresented, now support integrated designs linking prey assays to toxin evolution and structural modelling, promising for local-adaptation studies [42,100]. Marine lineages connect population-genomic patterns to macroevolution: sea snake genomes implicate lineage-specific 3FTx gene loss and dosage effects as prey-preference correlates, motivating population tests of whether genomic patterns track ecological gradients and prey turnover [99].

Methodologically, the opportunity space is broad but demands careful attention to scale. Genome-wide resequencing identifies demographic structure and enables toxin/receptor loci selection scans, but interpretation requires parallel venom composition and toxin–receptor phenotyping [89,126]. Conversely, BLI platforms provide high-throughput, target-flexible binding quantification, but mapping binding variation to fitness requires explicit ecological grounding: prey availability, encounter rates, and whether assayed targets capture dominant pathophysiological mechanisms [125,126]. Sampling logistics, venom compositional complexity, and multi-locus selection statistics remain substantial constraints, but only population-level designs directly test whether coevolution proceeds through recurrent sweeps, local polymorphism, or mosaic dynamics [89,125,126].

8.3. Toward Predictive Models of Toxin–Receptor Coevolution

The field aspires to move from retrospective inference toward predictive, mechanistically grounded models forecasting toxin and receptor evolutionary trajectories. Concrete reasons for cautious optimism exist. First, resistance mechanisms increasingly resolve to

interpretable biophysical principles. Charge-reversal substitutions in nAChR $\alpha 1$ generate electrostatic repulsion against positively charged α -neurotoxins, evolved convergently multiple times [48]. Glycosylation-associated mechanisms recur in vertebrate resistance, with evidence motivating lineage-specific hypotheses about N-glycosylation contributions to reptilian toxin insensitivity [123]. These mechanistic classes (electrostatic, steric, post-translational) provide feature sets linking genotype to binding phenotype and predator–prey outcomes [48,123]. Second, the experimental landscape is increasingly quantifiable. High-throughput BLI platforms provide scalable binding data across venoms and taxon-specific receptor mimotopes, exposing prey–target effects and intraspecific/geographic variation essential for building predictive frameworks [125,126]. Third, 3FTx evolutionary plasticity is reconstructed using integrative toolkits incorporating machine learning: AlphaFold2 and protein language models (ProtT5), combined with synteny and phylogenetics, infer deep history and functional opportunity space in the 3FTx gene superfamily [1]. Related work uses structural prediction to interpret lineage-specific toxin diversification and link sequence evolution to target interactions [42,100].

Near-term prediction is likely modular and data-driven. One tractable approach models toxin–receptor binding and cross-reactivity as functions of (i) surface electrostatics, (ii) shape complementarity at interaction-prone loops, and (iii) local flexibility modulating induced fit, coupled to evolutionary models incorporating site-specific constraint and receptor-region functional indispensability. Critically, such models must accommodate intrinsic asymmetry: toxins diversify via duplication and neofunctionalisation, whilst receptors are typically constrained by essential physiological roles, potentially limiting resistance to structurally permissible solutions [1,48].

A parallel, increasingly realistic direction uses machine learning to predict binding and resistance effects. Supervised models trained on curated toxin–mimotope binding data could predict phenotypic consequences of receptor substitutions or forecast which toxin motifs retain potency across prey clades [125,126]. Unsupervised embedding approaches, already used in 3FTx evolutionary reconstruction, may identify latent sequence-space structure correlating with target class or functional mode [1].

However, substantial prerequisites and limitations remain, and any predictive programme must be anchored in rigorous experimental and phylogenetic validation. The most immediate constraint is scarcity of comprehensive, standardised training data spanning diverse toxin clades, receptor variants, and ecologically realistic contexts; current binding datasets are dominated by a single receptor family and often rely on mimotopes rather than full complexes [125,126]. Additionally, non-canonical targets (muscarinic receptors and sodium channels) remain underrepresented in coevolutionary datasets, despite clear 3FTx functional diversification evidence [5,21,109]. Models built primarily on α -neurotoxin–nAChR $\alpha 1$ interactions risk overfitting and misgeneralisation to other target classes. Finally, predictive inference fails if ecology is ignored: genomic and evolutionary signals require interpretation via prey communities, dietary breadth, and selective landscapes from trophic transitions and toxin repertoire shifts, including 3FTx gene loss or dosage effects tracking ecological divergence [99]. The most robust predictive models explicitly integrate structural biophysics, evolutionary constraint, and ecological context, treating prediction as falsifiable hypothesis generation rather than mechanistic experimentation substitution [1,99,125,126].

9. Conclusions

Three-finger toxins and molecular targets constitute an unusually powerful coevolution system at the interface of genotype, molecular mechanism, and ecological interaction. Across caenophidian snakes, the 3FTx scaffold has repeatedly redeployed through duplication, domain modification, and neofunctionalisation, generating a repertoire extending

beyond canonical α -neurotoxins to diverse receptor and ion-channel targets [1,5]. Even within rear-fanged lineages and historically neglected radiations, available evidence indicates 3FTx diversification can link tightly to prey function under strong positive selection, reinforcing predator–prey antagonism as persistent molecular innovation engines. Conversely, marine systems reveal non-uniform trajectories: lineage-specific 3FTx gene loss and dosage effects track ecological divergence and prey preference shifts, highlighting that coevolutionary dynamics include both gain and loss of toxin functions depending on trophic opportunity and constraint.

The muscle-type nAChR $\alpha 1$ receptor remains the most intensively studied interaction locus, justifiably so: its orthosteric site drives paralysis and admits flexible binding assays revealing prey selectivity, phylogenetic structure, and subspecies geographic venom-efficacy variation. However, resistance is not monolithic. Mechanistically distinct routes, electrostatic repulsion and glycosylation-associated traits, demonstrate convergent but biophysically separable solutions to similar ecological problems. The growing ability to map mechanisms onto comparative datasets and quantify binding phenotypes at scale provides foundations for integrating structural, functional, and ecological information into coherent narratives.

The central challenge, and opportunity, lies in bridging scales. Progress depends on expanding taxonomic coverage across predators and prey, moving beyond species-level generalisations toward population-genomic and functional-genomic designs, and building predictive frameworks combining biophysical realism with evolutionary constraint and ecology. Integration requires genuinely multidisciplinary workflows: dense geographic sampling allied to high-resolution venomomics; population-level receptor sequencing aligned with functional assays; phylogenetic models accommodating gene-family turnover and constraint; and machine learning approaches anchored in experimentally validated interaction data. If these components integrate, 3FTx–receptor systems will remain premier models for molecular coevolution and may become one of the first venom systems in which evolutionary trajectories and functional outcomes are predictable with experimentally testable precision.

Author Contributions: Conceptualisation, J.L.d.O. and H.R.-R.; methodology, J.L.d.O. and H.R.-R.; validation, J.L.d.O. and H.R.-R.; formal analysis, J.L.d.O. and H.R.-R.; investigation, J.L.d.O. and H.R.-R.; resources, H.R.-R.; data curation, J.L.d.O. and H.R.-R.; writing—original draft preparation, J.L.d.O. and H.R.-R.; writing—review and editing, J.L.d.O. and H.R.-R.; supervision, H.R.-R.; project administration, H.R.-R.; funding acquisition, H.R.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the São Paulo Research Foundation (FAPESP, grant no. 2017/18398-1) and was partly financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil; Finance code 001). JLO was supported by a Ph.D. scholarship from CAPES (grant nos. 88887.155632/2025-00).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analysed in this study.

Acknowledgments: The authors thank the Universidade Nove de Julho (UNINOVE) for institutional support.

Conflicts of Interest: The authors declare no conflicts of interest.

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