α-conotoxins targeting neuronal nAChRs:
Understanding molecular pharmacology and potential therapeutics

This thesis is submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

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October 2013
Declaration by the Candidate

I, Shiva Nag Kompella, declare that:

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   b) this work has not been submitted previously, in whole or part, to qualify for any other academic award;

   c) the content of the thesis is the result of work that has been carried out since the official commencement date of the approved research program;

   d) any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Signed:        Date:
Statement of contributions by others to the thesis as a whole

The collaborators for the work in this thesis include Dr. Richard Clark (School of Biomedical Sciences, The University of Queensland) for peptide synthesis and Dr. Norelle Daly (Institute for Molecular Biosciences, The University of Queensland) for NMR structural analysis in Chapter 3. Dr. Andrew Hung (RMIT University) for molecular modelling and docking simulations.

Statement of parts of the thesis submitted to qualify for the award of another degree

None

Published works by the candidate incorporated into the thesis

1. Chapter 3 Section 3.3.1:


2. Chapter 4


3. Chapter 6


Additional published works by the candidate during PhD but not incorporated into the thesis


Talks at Conferences/Meetings:


Poster presentation at Conferences/Meetings:


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<td>Isoleucine</td>
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<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half-maximal inhibitory concentration</td>
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<td>ICK</td>
<td>Inhibitory cysteine knot</td>
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<td>IP&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>inositol (1,4,5)-triphosphate receptors</td>
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<td>KCN</td>
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<td>Leu</td>
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<td>Lys</td>
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<td>MBHA</td>
<td>4-Methylbenzydrylamine</td>
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<tr>
<td>MD</td>
<td>Molecular dynamics</td>
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</table>
Mec  Mecamylamine
Met  Methionine
mg  milligrams
min  minutes
ml  milliliters
MS  Mass spectroscopy
MS-222  Tricaine methanesulphonate
nAChR  Nicotinic acetylcholine receptor
NFκB  Nuclear factor kappa beta
n  Hill slope
NHBE  normal human airway epithelial cells
NNK  4-(methylnitrosamino)-1-(3-pyridyl)-butanone
NNN  N-nitrosonornicotine
NMR  Nuclear magnetic resonance
NOE  Nuclear Overhauser Effect
NOESY  Nuclear Overhauser Effect Spectroscopy
NSCLC  Non-small cell lung carcinoma
NTD  N-terminal domain
P13K  phosphatidylinositol 3-kinase
PAC  Peripheral adenocarcinoma
PCR  Polymerase chain reaction
PDI  Protein disulfide bond isomerase
Phe  Phenylalanine
PKC  Protein kinase C
PNEC  Pulmonary neuroendocrine cells
PNS  Peripheral nervous system
PPI  Peptidyl-prolyl cis-trans isomerase
Pro  Proline
PSNL  Partial sciatic nerve ligation
RP-HPLC  Reversed-phase high performance liquid chromatography
RNA  Ribonucleic acid
RyR  Ryanodine receptors
RT-PCR  Reverse transcription polymerase chain reaction
s  Seconds
SCLC  Small cell lung carcinoma
SEM  Standard error of the mean
Ser  Serine
SNP  Single nucleotide polymorphisms
SPPS  Solid phase peptide synthesis
TFA  Trifluoroacetic acid
TH  Tyrosine hydroxylase
TIPS  Triisopropylsilane
TOCSY  Total Correlated Spectroscopy
Thr  Threonine
Trp  Tryptophan
Trt  Trityl or triphenylmethyl
Tyr  Tyrosine
Val  Valine
VDCC  voltage-dependent calcium channels
XIAP  X-liked inhibitor of apoptosis
ABSTRACT:

INTRODUCTION: Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels expressed in both central nervous system (CNS) and peripheral nervous system (PNS) and are involved in fast ACh-mediated synaptic transmission. Neuronal nAChRs are pentamers composed of a combination of alpha subunits (α\(_{2-10}\)) and beta subunits (β\(_{2-4}\)) exhibiting diverse structural and functional heterogeneity. nAChRs are shown to contribute to the physiological roles of nAChRs in neurotransmitter release and synaptic plasticity. Further, they are also implicated in various pathophysiological conditions including Alzheimer’s disease, schizophrenia, tobacco addiction and lung cancer.

α-Conotoxins, a new class of peptides that act as nAChR antagonists have been identified from the venom of predatory marine cone snails. α-Conotoxins are a class of short di-sulphide rich peptides which specifically target various nAChRs subtypes and are excellent molecular probes for identifying the physiological role of nAChR subtypes in both normal and disease states. They are defined by their characteristic cysteine framework, CCXmCXnC (Xm and Xn represent the number of non-cysteine residues), classifying them into various subclasses.

CHAPTER 3: The α3β4 subtype is shown to be involved in lung cancer, nicotine addiction and drug-abusive behaviour. Despite this, the knowledge of the pathophysiological role of α3β4 subtypes is limited by the lack of adequate subtype specific probes. To-date only five α-conotoxins inhibiting α3β4 nAChR subtype are known. Of these, α-conotoxin AuIB belonging to a unique 4/6 subclass is the only peptide shown to selectively inhibit α3β4 nAChR subtype with an IC\(_{50}\) of 2.5µM.

We report the discovery of new α4/7-conotoxin RegIIA which was isolated from Conus regius. RegIIA has a classical ω-shaped globular structure with balanced distribution of shape, charges and polarity. RegIIA specifically inhibits ACh-evoked currents of α3β2, α3β4, and α7 nAChRs isoforms. The implication of α3β4 nAChRs in various diseases such as lung
cancer and nicotine addiction along with RegIIA being the most-potent α3β4 nAChR antagonist to date led us to investigate and improve RegIIA’s selectivity profile at the α3β4 nAChR subtype. Using alanine scanning mutagenesis and modelling studies, we identified critical residues of α-conotoxin RegIIA that interact with the ACh-binding site of α3β2, α3β4 and α7 nAChRs.

CHAPTER 4: α3β2 and α7 nAChR subtypes play vital roles in various functions, such as neuronal plasticity, angiogenesis and gene regulation. α-Conotoxins targeting these receptors form excellent probes to understand their physiological roles in normal and diseases conditions. Here I describe the pharmacological properties of the novel α-conotoxin LsIA, the first peptide isolated from Conus limbus, a species of worm-hunting cone snail commonly found on the south east coast of Queensland, Australia. LsIA is an α4/7-conotoxin with the characteristic I–III and II–IV disulfide connectivity. LsIA exhibited selective and potent α7 and α3β2 nAChR subtype antagonism. In this report, I also examined the structure–function relationship of the presence of a unique N-terminal serine at position 2 and C-terminal carboxylation. Furthermore, I also investigated the pharmacological implications, involving incorporation of the α5 subunit towards the inhibition of LsIA at α3β2 nAChR subtypes.

CHAPTER 5: Novel α-conotoxins of the D-superfamily have only recently been discovered and functionally characterized. These were found to be non-competitive nAChR antagonist and are naturally dimeric, which makes their blocking mechanism more intriguing. From the venom of Conus generalis, we identified a novel αD-conotoxin GeXXA. This toxin is a disulfide-linked homodimer of a 10-Cys-containing peptide with each peptide chain made of 50 amino acid residues. Each polypeptide chain is composed of an N-terminal domain (NTD, residues 1-20) involved in dimerization and a C-terminal domain (CTD, residues 21-50). αD-GeXXA is a non-selective inhibitor of nAChR subtypes exhibiting most potency at the α7
subtype with an IC$_{50}$ of 210 nM. However, for rat and human α9α10 nAChRs, the inhibition by αD-GeXXA is irreversible, indicating the tight binding of αD-GeXXA to α9α10 nAChR. To get further insight about its inhibition mechanism, the CTD of each chain was isolated and tested against various nAChR subtypes. Contrary to native peptide, GeXXA-CTD showed selective and reversible inhibition of α9α10 nAChR subtype. This specificity was further investigated using hybrid receptors of rat and human α9 and α10 subunits. GeXXA-CTD was 10-fold less potent on human α9α10 than on rat α9α10 nAChR while similar potency was observed with ha9rα10 hybrid receptor indicating significant role of α10 subunit in inhibition. These results provide more insight into the novel blocking mechanism of α-D conotoxins.

CHAPTER 6: Conotoxins have emerged as useful leads for the development of novel therapeutic analgesics. However, the disulfide connectivity of α-conotoxins can change under oxidative and reduced conditions leading to changes in structural conformation. Exploitation of these and other peptides in research and clinical settings has been hampered by the lability of the disulfide bridges that are essential for toxin structure and activity. One solution to this problem is replacement of cysteine bridges with nonreducible dicarba linkages. We explore this approach by determining the functional characteristics of dicarba analogues of a novel analgesic α-conotoxin Vc1.1 and RgIA which is known to inhibit high voltage-activated (HVA) calcium channel currents via GABA$_B$ receptors and α9α10 nAChR subtypes. When tested, dicarba Vc1.1 and dicarba RgIA analogues showed differential activity wherein the [2,8]-dicarba analogues of both Vc1.1 and RgIA were active at HVA calcium channel current via GABA$_B$ receptors whereas the [3,16]-dicarba analogues retained its activity at α9α10 nAChR subtypes. These results provide new leads towards the elucidation of the biological target responsible for the peptide’s potent analgesic activity.
**SUMMARY:** In the past-decade, nAChRs have been identified as potential drug targets for various diseases. However, knowledge about the role of various nAChR subtypes in both physiological and pathophysiological conditions is scarce. My research describes the discovery, characterization and development of a novel α3β4 antagonist as potential tool towards understanding its role in lung cancer. Further, dicarba modification of peptides and characterization of new class of α- and αD-conotoxins provide future insights towards drug development.
CHAPTER 1

INTRODUCTION

α-Conotoxin (red) bound nicotinic acetylcholine receptor (surface representation) (generated using PyMOL Molecular Graphics System - DeLano Scientific LLC)
1.1 Nicotinic acetylcholine receptors

1.1.1 The discovery

After the initial discovery of nicotinic acetylcholine receptors (nAChR) in 1914 by Henry H Dale [1] and Otto Loewi [2], substantial progress has been made to improving our understanding of the nicotinic mechanisms brought us a step closer to ever growing knowledge hill. Numerous reviews documenting this progress have been published and focused on nAChR structure, function, physiology, pharmacological tools and potential therapeutic use [3-8]. Ironically, these advances only raised more questions about the complexity of the nervous system and neurotransmission, and the contribution of these receptors in various diseases, such as Parkinson’s disease, Alzheimer’s disease, schizophrenia, epilepsy, dementia and cancer [9, 10]. Today, the major breakthroughs in understanding nAChRs seem but a fascinating history tale that starts with the initial discovery of the chemical nature of neurotransmission by Claude Bernard, a French physiologist, in 1857. The complexity of the cholinergic transmission or role of acetylcholine (ACh) was as yet unanticipated.

In the mid-1980s, advancements in molecular biology enabled the identification and cloning of the genes that encoded the first nAChR from *Torpedo marmorata* [11]. Since then, more nAChR family members have been identified, with 16 genes encoding structurally homologous yet distinct nAChR subunits now known [12]. The muscle nAChR was primarily isolated, purified and sequenced by Jean-Pierre Changeux from the *Torpedo* electric organ [13]. Consequently, the neuromuscular junction (endplate) for the analysis of nAChR-mediated neurotransmission became widely used. This enabled physiologists to conduct various experiments to improve our understanding of the biochemical nature and physiology of neurotransmission.
nAChRs are ionotropic channels belonging to the cys-loop superfamily of the ligand-gated ion channels [3]. Other ion channels belonging to this superfamily include GABA<sub>A</sub>, glycine (GlyR), 5-HT<sub>3</sub> serotonin and zinc-activated receptors, which have similar structural features to nAChRs [12]. These features include an extracellular NH<sub>2</sub>-terminal domain (ECD), four transmembrane domains (M1-M4), and a intracellular COOH-terminal sequence (ICD) [Figure 1.1] [14]. Structural homology of human nAChRs was found to be evolutionarily linked to the ionotropic channels, dating back as far as nematodes and molluscs and to simple life forms, such as prokaryotes. [15, 16]. Along with these features, various nAChR subunits have high sequence homology with each other.

**Figure 1.1:** Structural features of *Torpedo* nAChR (PDB 2BG9 [14]). Schematic representation of (A) the pentameric structure of a *Torpedo* nAChR (B) a nAChR subunit showing various structural features (C) Cartoon representation of the three dimensional of the α-subunit of *Torpedo* nAChR generated using PyMol Molecular Graphics System (DeLano Scientific LLC.).
1.1.2 Subunit diversity

The nAChR is one of the most well-studied ligand-gated ion channel family members. This is due to various multidisciplinary studies, including genetic, protein, microscopic and structural studies, that stemmed from earlier studies on the *Torpedo* electric organ nAChR structure. Original studies made two major significant findings: 1) nAChRs from *Torpedo* have remarkable density, enabling the pseudo-crystalline studies of the receptor at 4Å resolution [14]; and 2) studies of the crystal structure of a water soluble protein, ACh binding-protein (AChBP) [17].

The 16 subunits that have been discovered are broadly classified as muscle or neuronal subtypes, based on their tissue expression. Muscle subtypes include α1, β1, δ, γ and ε subunits, and neuronal subtypes include α2–α10 and β2–β4 subunits. However, although this nomenclature is widely used and provides a simple way to class receptor subtypes, it is being discouraged by the International Union of Pharmacology [12]. This is because various studies have indicated that more than one neuronal subtype receptor is expressed in various non-neuronal tissues [18-20].

1.1.3 Receptor structure

nAChRs are pentameric structures made from various transmembrane subunit combinations. The above-mentioned subunits assemble in numerous combinations to form two distinct receptors classes, namely homomeric receptors (composed of only α subunits, such as α7) and heteromeric receptors (composed of α and β subunits, such as α4β2) [Figure 1.2]. Up until recently, only α7, α9 and α10 subunits were believed to form only homomeric receptors [21]. However, recent studies have provided evidence of the formation of α7-containing heteromeric receptors [22, 23]. The pentameric structure of these receptors was initially identified during earlier studies into the muscle nAChRs isolated from the *Torpedo* electric
organ. Only two known muscle nAChR combinations have been characterised, $\alpha_1\beta_1\delta\gamma$ and $\alpha_1\beta_1\delta\epsilon$. These were identified as fetal and adult muscle subtypes, respectively, based on their expression levels during the developmental stages [24, 25].

**Figure 1.2: nAChR subunits forming (A) homomeric and (B) heteromeric receptors.** The ACh binding site in each receptor is indicated in red triangles [26].

### 1.1.3.1 Extracellular domain/Ligand-binding domain

As mentioned earlier, nAChRs are ligand-gated ion channels and activated or opened in the presence of an endogenous agonist or ligand ACh. The ligand- or agonist-binding site exists on the extracellular domain, within the interface of two $\alpha$ subunits (in homomeric receptors) or between an $\alpha$ and $\beta$ subunit (in heteromeric receptors) in the receptor. A nomenclature of ‘agonist-binding subunit’ for $\alpha$ subunits and ‘structural subunits’ for non-$\alpha$ subunits ($\beta_1$–$\beta_4$, $\delta$, $\gamma$ and $\epsilon$ subunits), was used to indicate the presence of the cysteine loop in $\alpha$ subunits. The cysteine loop is needed to aid the formation of the functional ligand-binding domain [12]. However, this nomenclature was discontinued after it was discovered how vital the non-$\alpha$ subunits are in the binding pocket. Today, the $\alpha$-subunit interface is called the ‘principal’ or ‘$+$’ face and the $\beta$-subunit interface is called the ‘complementary’ or ‘$-$’ face [Figure 1.3] [27, 28].

Our knowledge of the N-terminal domain has developed through various binding and functional assays in combination with chemical modification and mutagenesis experiments [29-31]. Initial studies of photo affinity labelling (photolysis of covalently bound chemical
tags to the active sites of protein molecules) on muscle nAChRs using the Torpedo receptor identified key amino acid residues, such as α-Tyr 93, which constitute the agonist-binding site [32]. These studies provided an early indication of the hydrophobic nature of the agonist-binding pocket. In muscle nAChRs, this site was located between the interface of α1-δ and α1-γ subunits in fetal form, and α1-δ and α1-ε subunits in adult form [33].

More recently, major improvements in understanding the three-dimensional structure of these receptors came through X-ray crystallographic and high-resolution electron microscopy studies of the ACh binding protein (AChBP) and Torpedo receptor [17, 34]. AChBP, isolated from glial cells of molluscs (Lymnaea), is a small, water-soluble protein. Its features are structurally similar to those of the N-terminal extracellular domain of nAChRs, but it lacks any of the other characteristics of the cys-loop receptor superfamily. The binding interaction of ACh to AChBP is similar to nAChR receptor activation. As such, it has received significant attention as it may help improve our understanding of the ligand-binding domain of these proteins [35].

AChBP crystal structures revealed that the α subunit plays a major role in the agonist-binding site. The Cys-Cys pair present only within the α subunits that form the ‘principal’ face of the binding site is essential for ligand binding [Figure 1.3]. Mutation of these residues, Cys191–Cys192 (Torpedo α subunit numbering), significantly affected receptor function and assembly [36]. Furthermore, structural and simulation models revealed conformational changes in the Cys-Cys pair of up to ~ 11Å, interlocking the agonist/ligand deep within the binding pocket [37].

Along with the mobility of the Cys-Cys pair, a series of conserved aromatic amino acid residues in the ‘principal’ face contribute to the agonist-binding site. These residues are
labelled and grouped into various loops (indicated in parenthesis): Tyr93 (loop A), Trp149 and Tyr 151 (loop B), Tyr190 and Tyr198 (loop C). As the numbering indicates, the Cys-Cys pair belongs to the C-loop of the ‘positive’ interface [Figure 1.3] [38-40]. It is worth noting at this stage that among all of the identified α subunits, α5 and α10 subunits do not form the ‘principal’ face of the agonist-binding site within the receptor complex. Therefore, despite the presence of the Cys-Cys pair, these subunits do not form homomeric or heteromeric functional receptors. This may be due to the lack of conservation within the key residues. For example, substitution of Asp for Tyr at position 198 of the α5 subunit makes it inactive to the nicotinic agonist [41, 42].

Figure 1.3: Cartoon representation of the ligand binding domain between α and δ subunit of Torpedo nAChR (PDB 2BG9 [14]). Amino acids contributing by the α-subunit to the ‘principal’ or ‘+’ interface of the ACh-binding pocket (Y93, W149, Y151, Y190 and Y198) and those contributed by the β-subunit interface to the ‘complementary’ or ‘-’ interface (W54 and L108) are represented in stick, overlayed with sphere representation. The Cys191–Cys192 of α-subunit is coloured yellow. Image generated using PyMol Molecular Graphics System (DeLano Scientific LLC.).
1.1.3.2 The transmembrane domain and intracellular domain

Agonist-bound AChBP crystals significantly contributed to our understanding of the various conformational changes within the N-terminal extracellular domain and are a very valuable scientific tool [43, 44]. However, the lack of other components, such as a transmembrane domain and channel gating, hindered the extrapolation of the information to complete nAChRs.

The transmembrane domain of each nAChR subunit is made of four segments, M1–M4 [Figure 1.1]. These segments are important for channel gating and anchoring the receptor in the lipid bilayer. The four transmembrane segments are arranged inside the pentameric structure of the receptor with the M2 of all of the five subunits lining the channel pore and the M4 of all of the five subunits forming the outer surface of the receptor [Figure 1.4]. This arrangement, originally hypothesised after the initial bilayer membrane experiments with isolated M2 segments of the *Torpedo* nAChR, was authenticated only after the electron microscopic study of *Torpedo* nAChR by the Unwin group [14]. While the M2 segment is primarily involved with channel gating, M1 and M4 segments modulate receptor assembly, function and localisation. Early studies using chimeric M1 and M2 constructs reveal that M1–M2 coupling is involved in the pentameric assembly of α7 nAChR [45]. This was evaluated through simultaneous studies on muscle nAChR, and indicated that the M1 contributes to the heterodimer structure of the receptor [46].

The orientation of the M4 to the outer surface of the receptors [Figure 1.4] suggests it has a role in receptor localisation through its interactions with lipids and cholesterol in the membrane bilayer [47, 48]. Furthermore, scanning mutagenesis studies within this *Torpedo* nAChR segment altered its function and assembly [49, 50].
Figure 1.4: Surface overlayed-cartoon representation of transmembrane domains of *Torpedo* nAChR (PDB 2BG9) [14]. The M2 segment (red) of all five subunits forms the ion pore of the channel. The M1, M2 and M4 segments are represented in cyan, blue and green colour respectively. α-V255 and α-L251 residues involved in channel gating are represented in yellow spheres. Image generated using PyMol Molecular Graphics System (DeLano Scientific LLC.).

Although the ligand-binding domain and transmembrane domain are vital for nAChR function and assembly, the intracellular domain also significantly contributes to it. However, the cytoplasmic loops of M1–M2 and M3–M4 have the distinct role of signal transduction to these receptors via phosphorylation. While further validation of this function is required, recent studies suggested that potential intracellular proteins, such as Rapsyn, interact with the α9 nAChR and modulate its surface distribution [51, 52]. Mutations within the large M3–M4 intracellular loop of the α7 nAChR significantly reduced receptor function and assembly, and mutations in the α3 subunit disrupted the distribution of α3-containing receptors in the interneuronal synapse [53, 54].

1.1.4 Subunit stoichiometry of nAChRs: structural and functional implications
Sixteen nAChR subunits that assemble into homomeric or heteromeric pentamers have been identified. Heteromeric receptors exhibit a huge diversity in structure, function and receptor localisation, which can be attributed to the various possible subunit stoichiometric combinations. To date, about 30 known subunit combinations arranged in various stoichiometries have been identified. Thus, homomeric structure of a receptor provides a key advantage in structural and functional understanding of the receptor. [55].

Stoichiometry of a heteromeric receptor is defined by the identity of the 5th subunit. For example, for the \( \alpha_3\beta_2 \) receptor, the pentameric structure can assemble the 5th subunit with \( \alpha_3 \) or \( \beta_2 \), producing a simple stoichiometry of this receptor of either \( (\alpha_4)_3(\beta_2)_2 \) or \( (\alpha_4)_2(\beta_2)_3 \) [Figure 1.5]. Complex stoichiometries evolve when other subunits such as \( \alpha_5, \alpha_6 \) and \( \beta_3 \) are introduced as the 5th subunit, to form \( (\alpha_4)_2(\beta_2)_2\alpha_5 \), \( (\alpha_4)_2(\beta_2)_2\alpha_6 \) and \( \alpha_4\alpha_6(\beta_2)_2\beta_3 \) receptor combinations [Figure 1.5]. In the case of muscle nAChRs, only one subunit stoichiometry exist: \( (\alpha_1)_2\beta_1\delta\varepsilon/\gamma \) [14]. While recent studies have highlighted some of the roles played by different stoichiometric combinations [5], much is to be learned about their contribution to receptor function, pharmacology and physiology.

![Various stoichiometric combinations of a heteromeric receptor](26)

While the pure mathematical combinations of different subunits represent a complex issue, native nAChRs assemble predominantly with specific subunit stoichiometry. For example,
single-channel recording and direct biochemical studies of α4β2 nAChR, a major subtype expressed throughout central nervous system (CNS), showed assembly primarily in the (α4)2(β2)3 stoichiometry [56, 57]. However, this composition can be changed in model expression systems, such as Xenopus oocytes, via RNA injection of different subunit ratios. Recent studies on recombinant receptors using this technique showed differential agonist sensitivity based on the subunit ratio of α4 and β2 subunits used (1:10 or 10:1), along with changes in calcium permeability [58, 59]. Similar differences in affinity for both agonist (ACh) and antagonist (α-conotoxin AuIB) were observed with α3β4 nAChR stoichiometry [60]. Furthermore, recent electrophysiological studies with α5, α6 and β3 subunits revealed complex subunit stoichiometries with a unique functional role in the receptors such as receptor desensitization and gating (these concepts will be discussed in the next section). The β3 subunit was reported to extensively co-localise with the α6 subunit, while forming complex stoichiometric combinations [61].

Different α6-containing receptor stoichiometries have been implicated in the pathophysiology of neurological disorders, such as Parkinson’s disease [62]. In addition, when the α5 subunit is co-assembled with the α3β4 nAChR, calcium permeability increases [63] and nicotine-evoked dopamine release in synaptosomal preparations containing α4β2 nAChRs is affected [64]. Therefore, subunit stoichiometry plays a vital role in the pharmacological and physiological role of various nAChR subtypes.

1.1.5 Receptor function, biophysical properties and gating

Mammalian nAChRs are activated by the natural endogenous agonist ACh binding to the N-terminal extracellular domain. Upon ligand binding, the receptor undergoes rapid conformational changes which transition into channel opening. These receptors are cation
selective and modulate mono- and divalent cation influx, depolarising cell membranes and creating neuronal excitability.

Various residues within the extracellular, transmembrane and intracellular domains of the receptor contribute to the microsecond conformation changes from ligand binding to channel gating [3]. M1 and M2 are involved in the gating process, and help determine the ionic selectivity of these receptors. Mutagenesis study has shown the presence of charged glutamate and valine within M2 and a lack of proline in the intracellular domain of M1–M2 correspond with the cation permeability of nAChRs [65].

While there is no direct experimental proof that the receptor changes, the various transitions within the protein structure of the receptor are based on computational simulations from various mutational and structural studies. The current model [34] describes a rotation or torque produced by significant C-loop movement (~11 Å) when the ligand interacts with the agonist-binding site. This torque is transitioned through the receptor, influencing the orientation of the M2 that lines the channel pore. These transitions place the receptor in three structural states: closed, open and desensitised, which determine the receptor’s functional and pharmacological properties and influence various intracellular signalling cascades.

The channel-gating process involves switching from a closed or non-ligand-bound receptor state to an open state, with a change in channel pore size from ~4 Å to ~8 Å respectively [66]. This is accomplished via the transitional torque produced by the extracellular domain and transitory shift of hydrophobic residues within the M2 helices. It leads to concurrent influx of Na⁺, K⁺ and Ca²⁺ ions, which activates an array of signalling pathways.
The pore’s ion-gating function is attributed to two special properties: the near perfect arrangement of M2 helices in a radially inward tilt towards the pore axis, and symmetric orientation of hydrophobic residues α-Leu 251 and α-Val 255 that constrict to form a narrow energetic barrier during the closed state of the receptor [Figure 1.4] [67, 68]. This model was evaluated when mutational studies of these residues led to increased channel conductivity [69].

The above-mentioned studies provided the first rationale linking the physiological function of the nAChR with its biophysical properties. There are two major nAChR biophysical properties that modulate its physiological functions. Firstly, the rapid transition of the receptor between the closed and open state governs its primary role of fast synaptic transmission. The release of ACh and subsequent activation of the nAChR in presynaptic junctions modulates the release of other neurotransmitters, such as dopamine and GABA [70, 71]. Secondly, the ionic permeability of nAChRs to Ca\(^{2+}\) mediates various downstream signalling cascades [72].

The relative permeability ratio (Ca\(^{2+}/\text{Na}^+\)) of the nAChR is calculated by two methods: measuring the shift in reversal potential of nAChR-mediated current under varying calcium concentrations [73]; and calcium fluorescence imaging [74]. These techniques indicate the highest calcium permeability for homomeric α7 and heteromeric α9/α10 with a ratio of ≥10. This ratio was the least for muscle nAChRs at ~ 0.1, while all of the other heteromeric receptors exhibited a ratio of ~ 2.0 [75, 76]. While the residues that contribute to the barrier within the pore are highly conserved in various nAChR subunits, the relative permeability of calcium to sodium ions is affected by the arrangement of residues within the ion conduction path of the pore. These residues include α-Glu 262, α-Ser 266 (top end of the pore) and α-Glu 241 (at the intracellular mouth of the pore), and were identified through mutational and electrophysiological studies [77, 78]. This variance in calcium permeability changes the
physiological roles played by different nAChR subtypes [79]. The physiological implication of nAChR-mediated Ca\(^{2+}\) signalling will be discussed in detail in Section 1.6.

While nAChRs were initially identified in neuromuscular and synaptic junctions, and their primary role in fast cholinergic transmission is long established, recent discoveries showing non-synaptic nAChR expression significantly expanded the functional scope of these receptors [80]. Unlike synaptic junctions, where neurotransmitter levels are highly regulated via release and active reuptake producing the instantaneous open and closed transition of nAChR, non-neuronal nAChRs are prone to prolonged agonist application. This sustained exposure led to the identification of a third state of the receptor: desensitised.

Desensitisation is a process in which the receptor transitions from an open state to an agonist-bound non-conducting state. The transition rates between these states depend directly on the dissociation rates of the agonist. Therefore, this is dependent on the nature of the agonist bound and the subunit composition of that receptor. As α and β subunits contribute differentially to the pharmacological properties of the receptor, the same is applicable to the rate of receptor desensitisation. For example, α7 nAChRs desensitise more quickly than other subtypes. Also, incorporating the α5 subunit has been shown to change the biophysical properties of all three functional states of a receptor [63, 81].

1.1.6 nAChR-mediated Ca\(^{2+}\) signalling and modulation of neuronal growth

nAChRs exhibit strong calcium permeability, which is dependent on the receptor composition and stoichiometry [59, 76]. Subtypes such as the α7 nAChR can directly raise the cytoplasmic calcium levels due to their high permeability; however, indirect calcium influx has also been shown to occur due to nAChR-mediated depolarisation. Indirect calcium influx occurs in two
ways: through voltage-dependent calcium channels (VDCC), and being released from calcium intracellular stores, such as the endoplasmic reticulum [Figure 1.6] [82, 83].

VDCC-mediated calcium influx occurs at depolarising potentials of $>-40$ mV, which are generated by nAChR activation. Under these conditions, the initial Ca$^{2+}$ entry via nAChRs is augmented and may be functionally complementary, an association that is also observed with NMDA receptors [84].

The third process contributing to increased cytoplasmic Ca$^{2+}$ signal is through calcium induced calcium release (CICR) from intracellular stores [83]. While calcium signalling via VDCCs is significantly mediated by $\alpha_7$-, $\alpha_3$- and/or $\beta_2$-containing receptors in neuronal ganglia, $\alpha_7$ also increases transient Ca$^{2+}$ levels independently of VDCCs via CICR. Calcium-quenching experiments in neurons of substantia nigra pars compacta showed reduced cytoplasmic calcium levels when $\alpha_7$ nAChR was activated with choline [85]. Receptor antagonists were later used to show that CICR involved ryanodine (RyR) receptors and inositol (1,4,5)-triphosphate receptors (IP$_3$Rs) expressed in the endoplasmic reticulum [Figure 1.6] [82, 86]. Although the molecular mechanism behind nAChR-activated IP$_3$R-calcium release is still unclear, secondary signalling molecules, such as Ca$^{2+}$-dependent phospholipase C and Ca$^{2+}$-sensor proteins, have been implicated [87, 88]. The various mechanisms of cytoplasmic calcium influx reflect a complex and intricate functional coupling of nAChRs with RyR and IP$_3$ receptors, which leads to a sustained calcium signal. These calcium signals play a vital role in the activation of various downstream signalling processes.
Figure 1.6: nAChR-mediated Ca\(^{2+}\) signalling and modulation of neuronal growth.

Downstream signalling events upon nAChR activation are classed as immediate, interim and long-term effects. The calcium ion influx occurs through three ways: nAChR activation, VDCC activation and CICR mechanism from calcium internal stores (mediated by RyR and IP\(_3\) receptors). The Ca\(^{2+}\) influx leads to immediate cell depolarisation and activation of various kinases and proteins such as PKC, PKA and Calmodulin. This is followed by subsequent activation of downstream signalling molecules, leading to various physiological responses such as neuronal plasticity, memory, neuroprotection.

The above-discussed calcium influx is a sequential process occurring in the following order: nAChR > VDCCs > ryanodine receptor > IP\(_3\) receptors. Therefore, it is only logical for the various downstream signalling events upon nAChR activation to be sequential and classed as immediate, interim and long-term effects based on their duration and timing.
1.1.6.1 Immediate effects

nAChRs expressed in presynaptic junctions of the peripheral nervous system (PNS) and CNS mediate their primary role in fast synaptic transmission. This process not only depolarises cells, but also initiates exocytosis either directly or via VDCC activation [Figure 1.6] [89]. In addition, nAChRs and VDCCs play physiologically complimentary roles in the regulation of neurotransmitter release. In striatal synaptosomes, VDCC mediates nAChR-evoked dopamine release [90]. However [3H]noradrenaline release from hippocampal synaptosomes, triggered by α3β4 nAChR activation, is VDCC-independent [91].

1.1.6.2 Interim effects

The two major short-term implications of nAChR activation include regulation of gene expression and nAChR desensitisation. CICR-mediated calcium influx activates the IP₃ second messenger system involving various Ca²⁺-sensor proteins and kinases, such as protein kinase C (PKC), is classed as an immediate effect [Figure 1.6]. However, concurrent activation of downstream molecules, such as extracellular signal-regulated mitogen-activated protein kinase (ERK/MAPK) [Figure 1.6], that has been shown to modulate the gene expression of the tyrosine hydroxylase (TH), is defined as a short-term Ca²⁺-signalling effect. TH is a known modulator of neurotransmitter release in catecholamine-containing neurons. Prolonged nicotine exposure has been shown to promote TH mRNA expression [92]. In addition, the role of nAChRs in the regulation of c-Fos and c-Jun gene expression has long been apparent [Figure 1.6] [93].

In synaptic and non-synaptic regions, prolonged exposure of agonist leads to nAChR desensitisation. Furthermore, high intracellular calcium levels have been shown to affect various desensitisation properties, such as recovery of α7-containing nAChRs in chromaffin cells and rat hippocampal neurons [94, 95]. These changes to the nAChR’s biophysical
properties are implicated in synaptic efficacy, and corresponding changes in pathological conditions suggest a complex reciprocal relationship [81].

1.1.6.3 Long-term effects

In a complex scenario, nAChR activation and intracellular calcium influx translates into vital physiological functions, such as neuronal plasticity, memory mechanisms, neuroprotection and regulation of cell death [79]. Recent studies showed activation of other kinases, such as Ca\textsuperscript{2+}-calmodulin-dependent protein kinase (CaMPK) and cAMP response element-binding protein (CREB), was mediated via nAChR and CICR calcium influx [Figure 1.6] [96].

ERK/MAPK is an important molecule, central to various signalling cascades. Activation of the ERK/MAPK signalling cascade via nAChR activation is critical for regulating gene expression. Disrupting these kinase signalling cascades and consequent modulation of α7 nAChR expression and function has been implicated in Alzheimer’s disease [97]. Today, α7-evoked activation of phosphatidyl-inositol 3-kinase (PI3K) and ERK/MAPK is a known pathway for mediating neuroprotection and stimulating angiogenesis [Figure 1.6]. This anti-apoptotic property of nAChR signalling is implicated in various cancers and is currently a major target for novel therapeutics [98]. The nAChRs role in cancer is detailed in section 1.7.3.

The many signalling pathways mediated by direct and indirect calcium influx via nAChR-activation reflect the complexity, yet specificity, of intracellular mechanisms. While recent studies have shed light on these dynamic processes, much is yet to be proven and learnt about the exact cellular pathways mediated by various specific nAChR subtypes, to improve our understanding of their pathological implications.
1.1.8 nAChRs and diseases

Since their discovery, nAChRs have been extensively researched to build knowledge about their structure and function. However, it was only in the past few decades that their true nature and complexity has been understood. nAChR subunits have various different compositions and stoichiometries, which correspond with specific biophysical and functional properties, including tissue distribution. This suggests nAChRs may be involved in a huge range of identified and as yet unidentified health conditions. nAChRs are known to be implicated in numerous pathophysiological conditions, such as epilepsy, schizophrenia, Alzheimer’s disease, Parkinson’s disease, pain, auto-immune disease, lung cancer and nicotinic dependence, with other conditions being investigated and identified [3, 7, 99]. Each of these conditions involves dynamic cellular interactions with a specific receptor subtype. However, only three conditions will be discussed within the scope of this thesis: pain, nicotine dependence and lung cancer.

1.1.8.1 nAChRs and pain

Pain is one of the most common health conditions and affects millions of people throughout the world. It is a sensory response to nociception (a neuronal process involving noxious stimuli), can be classified as either acute (sudden onset) or chronic (prolonged persistent pain), and involves various pain pathways.

Nicotinic acetylcholine receptors have long been implicated in pain mediation, and various nicotinic compounds that produce analgesic effects have been identified [100]. The initial breakthrough came with the discovery of epibatidine which is a potent analgesic and a nAChR agonist, although this drug was discontinued due to its severe side effects [101]. This knowledge highlighted an alternative pain target to opioid receptors, which were targeted by conventional analgesics. Epibatidine is a potent agonist of α4β2 nAChRs, one of the most
abundant subtypes in the CNS, and inhibits the nociceptive signals transmitted through the dorsal horn of spinal cord [102]. The α4β2 nAChR’s role as a target for analgesics was further established with the discovery of the promising therapeutic candidate ABT-594, a potent α4β2 nAChR inhibitor that lacks the side effects of opioid-targeting drugs [103, 104]. In conjunction with α4 and β2 knockout mice studies, this paved the way for nAChRs to be novel therapeutic target in pharmaceutical industry [105] and various compounds that target α4β2 nAChRs are currently in clinical trials [5].

While, α4β2 nAChR subtype is a prominent candidate, recent study showed various other nAChR subtypes with α7, α3, α9, α10, β2 and β4 subunits, to be involved in pain pathways [106]. This study re-established the role of the α4β2 nAChR subtype in the analgesic efficacy of various compounds. However, it also suggested that the α3β2 and α3β4 subtypes have a significant role in the analgesia by these compounds. While this study excludes the role of the α7 nAChR subtype in pain alleviation, other studies contradict this idea [107, 108]. These other studies showed that α7 nAChR subtype activation induces anti-nociceptive in acute pain model and anti-inflammatory effects in peritoneal macrophages. This was collaborated by further studies in which α7 knockout mice exhibited increased hyperalgesia (an exaggerated response to pain stimulus) and pain inflammation [109].

A new mechanism of nAChR-mediated pain modulation via receptor desensitisation has also been proposed, and involves direct activation of these receptors. Incorporating auxiliary α5 subunits in α4β2 and α3β4, increases receptor desensitisation [110].
Finally, α9-containing receptor inhibition by a novel class of conotoxins showed significant analgesic effects in peripheral neuropathy pain models [111, 112], and as a result, α9 has also been proposed as novel target for analgesic drugs. Conotoxins and their analgesic properties will be discussed in detail in the Chapters 6.

1.1.8.2 nAChR signalling and lung cancer

The pathophysiology of lung cancer has studied for almost half a century. Divided into two major types: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), it is the second most frequent and leading cause of cancer-related deaths worldwide. NSCLC accounts for 80% of all lung cancer cases [113] and smoking, specifically tobacco intake, has been a documented risk factor for lung cancer since the 1950s [114, 115]. Nicotine, polycyclic aromatic hydrocarbons and nicotine-derived metabolites, such as 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (NNK) and N-nitrosonornicotine (NNN), are among the carcinogens in tobacco smoke that are primary agents that initiate cancer [116]. Tobacco-related tumorigenesis is caused either via the genocentric model, in which genomic interaction of nicotine and carcinogenic nitrosamines leads to DNA adduct formation and mutation [117], or the biochemical model, in which tobacco-derived nicotine and nitrosamines act as potent nAChR agonists [118] and mediate the cell signalling pathways of growth, proliferation [119] and apoptosis [120]. The biochemical model also leads to various biological effects, including nicotine addiction and dependence [121].

1.1.8.2.1 Role of α3 subunit

Following the Schuller group’s first report in 1989, which implicated nAChRs in the regulation of cancer cell growth [119], many studies started exploring the role of nAChRs in cancer development and progression. In 1998, Schuller’s group conducted radiolabelled binding assay studies using [125I]α-BTX and [3H]EB. These studies conclusively showed that
NNK and NNN, tobacco-specific carcinogenic nitrosamines, were potent nAChR agonists [118]. At this point, concentrations of these nitrosamines were known to be 5000–10,000 times greater than nicotine in tobacco smoke, with the NNN concentration two–three times that of NNK [117].

The binding assay experiments in this study also revealed the selective binding profile of these nitrosamines. NNK was found to be a high-affinity ligand of the α-BTX-sensitive α7 nAChR subtype, while NNN is selective to the α4/α3 nAChR subtype, classified as an EB-sensitive neuronal nAChR [Figure 1.7]. Also, the proliferation assay, which used the incorporation of [3H]thymidine, identified that NNK’s binding to the α7 nAChR subtype is a potent mitogenic stimulus. However, NNN and nicotine showed no effect in the proliferation assay. These results, along with previous studies showing apoptotic inhibition of lung cancer cells by nicotine, provided strong evidence that nAChRs are involved in tumour progression [120].

Consecutively, NNK and nicotine were also shown to have a direct biochemical effect on rapid Akt activation in normal human airway epithelial cells (NHBE) [122]. Serine/Threonine kinase Akt is a key regulator of cell cycle process. Its activation leads to various signalling cascades involved in cell proliferation and apoptosis [123]. This was confirmed by Dennis’ group, which demonstrated constitutive activation of Akt/protein kinase B in 90% of NSCLC cell lines [124] and increased phosphorylation of downstream Akt substrates, such as MDM2, mTOR and NFκB [Figure 1.7] [125]. This is known to promote cellular survival, resistance to chemotherapy and radiation, and NFκB-dependent apoptotic inhibition.
Figure 1.7: nAChR mediated signalling pathway involved in lung cancer and their cells of origin. A summarised model of the various cellular signalling molecules involved in SCLC and NSCLC, mediated by the activation of α7 and heteromeric α3-containing receptor such as αβ34 nAChRs. Activation of α7 nAChR by NNK or nicotine has shown to enhance cell proliferation via β-Arrestin-SRC pathways involving the activation of Raf1 and ERK family of kinases. Whereas, α7 and α3-containing receptor mediated activation of Akt, contribute to the anti-apoptotic properties of NNK, NNN and Nicotine.

- kinases; - transcription factors; - IAP proteins

In 2003, Dennis’ group provided the first evidence that nAChR stimulation causes Akt overexpression in NSCLCs [122]. It also conclusively provided the first indication that α3 antagonists are potential therapeutic drugs towards NSCLC. Treatment with NNK and nicotine, led to rapid activation of Akt in NHBEs. This action was attenuated in the presence
of the α3/α4 antagonist DHβE, but not the α7 nAChR antagonists α-BTX and MLA, which showed no effect. Further, Akt phosphorylation increases in NHBEs were observed in the presence of an α3-specific agonist, α-ATX [126]. This supports the idea that nicotine causes α3-mediated Akt activation. This was later confirmed by Arredondo’s study, in which mecamylamine (Mec), a nAChR antagonist effectively abolished NNN’s pathobiologic effects in BEP2D cells [127].

1.1.8.2.2 Physiological to biochemical translation: cellular difference mediated by α7 and α3 subunits

SCLC and NSCLC exhibit different physiological profiles as well as different major histological types. NSCLCs show constitutive Akt activation, increased NFκB-dependent cellular survival and inhibition of apoptosis, mediated by α3/α4 nAChR subunits and due primarily to the agonist effects of tobacco carcinogens NNN and nicotine. Concurrent studies show that NNK’s tumorigenic effects are mediated by the α7 nAChR subtype predominantly in SCLC, but also in NSCLC [128, 129]. Molecular studies show that α7 nAChR expression in SCLC and pulmonary neuroendocrine cells (PNEC) is high [128], which is consistent with α7 nAChR expression being upregulated as a result of chronic NNK exposure [130]. These results, in conjunction with previous binding studies, show tumorigenesis in SCLC and PNECs is mediated via the α7 nAChR subtype, while both α7 and heteromeric α3 nAChR subtypes are involved in NSCLC and peripheral adenocarcinoma (PAC) [118, 129].

An in vitro proliferation assay of PNECs or SCLC in the presence of nicotine or NNK induced a serotonergic autocrine loop, which was abolished when an α7 nAChR antagonist or serotonin uptake inhibitor was added [131, 132]. This autocrine response involved the activation of cellular regulators the serine/threonine kinase RAF1, protein kinase C (PKC), mitogen-activated kinases ERK1 and ERK2, and transcription factors FOS, JUN and MYC.
Along with studies that show pharmacological inhibition of these regulators block the proliferation and apoptosis response identified α7 as the key regulator of cell proliferation [133, 134]. However in NSCLC, NNK-induced cell proliferation activated signal transducers, transcription 1 (STAT1), NFκB, GATA3, β-arrestin-SRC, and ERK1 and ERK2 [Figure 1.7] [127, 135]. While, NNK’s anti-apoptotic activity in SCLC was modulated by activating Bcl-2, in NSCLC, E2F1-mediated upregulation of survivin and X-liked inhibitor of apoptosis (XIAP) contributed to nicotine-induced apoptotic inhibition [Figure 1.7] [134, 136]. However, these results are yet to identify the role of IAP (Inhibitors of Apoptosis) proteins (survivin and XIAP) as characteristic signalling proteins underlying the anti-apoptotic effect in NSCLC.

1.1.8.2.3 Targeting the α3β4 nAChR: unlocking novel therapeutics for NSCLC

Previous studies have identified nicotine and its derivatives as potent nAChR agonists and highlighted their regulatory role in cancer cell apoptosis [118, 120]. Recent candidate–gene analysis and genome-wide association studies (GWAS) have also identified gene clusters and single nucleotide polymorphisms (SNP) encoding α3, α5 and β4 nAChR subunits associated with lung cancer [118, 137]. These studies showed the existence of CHRNA5/A3/B4 gene cluster overexpression and other specific nAChR subunits in lung cancer tissue [138, 139]. An important transcription factor, ASCL1, was shown to play a key regulatory role in the initiation and development of SCLC, and the overexpression of the CHRNA5/A3/B4 gene cluster [140, 141]. These studies suggest a strong correlation between the overexpression α3, α5 and β4 subunits and lung cancer.

NNK and NNN are leading carcinogens involved in the development of lung cancer in smokers. Along with nicotine, these nitrosamines have also been shown to be strong nAChR agonists and easily displace the endogenous nAChR agonist, ACh [118]. Binding studies have
shown that NNK is a selective α7 nAChR agonist, and NNN is a selective agonist of α3/α4-containing nAChRs [118]. Subsequent studies also showed that expression and activation of signalling molecules involved in cell proliferation and the apoptotic inhibition of NSCLC increased in the presence of NNN and NNK. This response was abolished when an α7 or α3 nAChR antagonist was added [124, 136]. More recent GWAS also suggest α3β4 nAChRs are key modulators of cell proliferation and apoptotic inhibition of NSCLC [137]. However, a lack of adequate molecular α3β4-specific probes has limited the study of the physiological role of this receptor subtype in disease progression.
1.2. Conotoxins

The venom of various animals, such as snakes, spiders and molluscs, has long attracted the attention of scientists for their ability to kill or paralyse their prey, almost instantly. Most venomous animals target invertebrates, with few targeting vertebrates. The venom of animals that do target vertebrates has been found to be dangerous – even fatal – to humans. Various bioactive peptides, called conotoxins, in the venom bind selectively and potently to different ion channel receptors. This makes them biomedically and pharmacologically significant [142].

**Figure 1.8: Predatory marine cone snails (A) Conus regius [143] and (B) Conus victoriae.**

Predatory marine snails of the genus *Conus* from the *Conidae* superfamily, is one of the largest and most diverse genera, with around 500 snail species [Figure 1.8]. Few snails in this genus prey on vertebrates like fish. Their venom, which includes thousands of conotoxins, also targets different ion channels in humans. α-Conotoxins, a conotoxin subclass found in this venom, are short, disulphide-rich peptides that selectively target different nAChR subtype isoforms [144].

1.2.1 Venom apparatus and biosynthesis

The vast diversity in conotoxin structure and sequence corresponds to the complex venom apparatus and biosynthesis process in cone snails. The cone snails’ general venom apparatus
includes a long, convoluted tubular gland that synthesises venom, a muscular bulb that moves the venom, and the proboscis, which helps deliver the venom and consists of various hollow, harpoon-shaped radula teeth [Figure 1.9] [143, 145].

![Figure 1.9: Schematic representation of cone snail's venom apparatus representing the main parts](image)

The conotoxin diversity in venom extends from cone snail gene and proteome level to post-translational modifications. Interestingly, thousands of peptides in the venom are generated from a relatively small number of gene families [147]. The genes encoding conotoxins translate into a precursor protein molecule consisting of three distinct segments: N-terminal signal sequence, a pro-peptide region and the mature toxin sequence. The N-terminal signal sequence and pro-peptide region are highly conserved, however, hypermutation is observed in the mature toxin sequence [147, 148].

After the gene is translated, various enzymes are responsible for the production of the mature toxin. These include protein disulphide isomerase (PDI), peptidyl-prolyl cis-trans isomerase (PPI), cysteine-rich protease Tex31 and immunoglobulin-binding protein (BiP). Recent studies have identified various PDI isoforms that contribute to the conotoxins diversity at proteomic level [149]. In addition to this variability in the mature toxin sequence, mature peptides can undergo post-translational modifications, such as C-terminal amidation and
proline hydroxylation, which confers a unique functional activity compared with unmodified sequences [150, 151].

Due to their potential pharmacological significance, it is vital to find a way to effectively and accurately synthesise conotoxins in vitro and characterise their structure and function. Understanding the diverse post-transcriptional and post-translational regulation of cone snail venom biosynthesis will provide important information that will help to improve in vitro synthesis of complex conotoxins.

1.2.2 Classification and nomenclature

The venom repertoire of cone snails is mainly constituted of disulphide-rich conopeptides and are categorised into various superfamilies and classified by their structural and functional features, such as cysteine pattern and pharmacology. Figure 1.10 illustrates some of these superfamilies. This classification is also used to name conotoxins.

The first nomenclature guidelines were introduced in 1985 and updated several years later [152]. They outline that conotoxins that have been functionally characterised should be labelled with (in the order listed below):

- a Greek letter indicating their pharmacological target
- one (uppercase) or two letters (second letter is lowercase) from the Conus species from which the peptide was isolated
- a Roman numeral indicating the cysteine framework pattern
- an uppercase letter representing the peptide variant discovered in respective cone species.

For example, α-RgIA represents a conotoxin isolated from Conus regius with cysteine framework I that targets ACh receptors.
Figure 1.10: Classification of conotoxins. The classification of conotoxins into various superfamilies is based on their cysteine pattern and pharmacology. Subclass α- and αD-conotoxins discussed in the following chapter of the thesis are boxed in red. Na⁺ – voltage gated sodium channel; K⁺ – voltage gated potassium channel; Ca²⁺ – voltage gated calcium channel; nAChR – nicotinic acetyl choline receptors [153].

There has been a significant increase in the number of conotoxins discovered from complex venom repertoire due to recent advances in protein and molecular techniques such as high-performance liquid chromatography (HPLC) and reverse transcription polymerase chain reaction (RT-PCR) [154]. The pharmacology of some of these peptides is unknown, so they are labelled differently from those that have been functionally characterised. They are labelled with:
• one or two letters (both lowercase) from the *Conus* species from which the peptide was isolated

• an Arabic numeral representing the cysteine framework

• a lowercase letter representing the peptide variant.

For example, before its pharmacological characterisation, α-RgIA was represented as rg1a.

1.2.3 Conotoxin structural diversity

Along with high sequence hypervariability, conotoxins show structural diversity. The disulphide framework of different conotoxin classes translates these peptides into specific, rigid, three-dimensional structures [155]. These structures play an important role in determining pharmacological properties of a peptide, including its potency and selectivity [156]. In the past decade, nuclear magnetic resonance (NMR) has enabled to determine the three-dimensional structures of many conotoxins with characteristic cysteine frameworks [157]. For example, α-conotoxins of the A superfamily of conopeptides, fold into a ω-shaped structure with a characteristic α-helix secondary structure [Figure 1.11(A)], whereas, χ-conotoxin MrIA of the T superfamily is dominated by β-helix secondary structures [Figure 1.11(B)] [157, 158]. Conotoxins with three or more disulphide bonds, such as ω-conotoxin MVIIA, have complex cysteine knot structures [Figure 1.11(C)] [159]. α-Conotoxins of the D superfamily are composed of five disulphide bonds and exhibit dimerization that yields complex three-dimensional structures [160]. These characteristic folds reveal critical residues that interact with the ion channel and determine the pharmacological activity of conotoxins.
Figure 1.11: Structural diversity among conotoxins. Cartoon representation of the three dimensional structures of conotoxins (A) α-Vc1.1 (PDB 2H8S) [161] (B) χ-MrIA (PDB 2EW4) [162] (C) ω-MVIIA (PDB 1MVI) [163]. α-helics are shown in blue, β-sheets in red and disulphide bonds are shown in yellow. Images generated using PyMol Molecular Graphics System (DeLano Scientific LLC.).

Though rich in cysteines, these residues are buried within the conotoxin’s three-dimensional structure, exposing a hydrophobic patch at the surface [164]. The surface-exposed residues contribute to overall net charge of the conotoxin. This charge distribution influences the peptide’s selectivity and potency for its pharmacological target. For example, α-conotoxins with net positive charge target muscle nAChR subtypes, and those with neutral or negative net charge target neuronal nAChR subtypes [165].

1.2.4 Conotoxin structure–activity relationship

Conotoxin diversity enables them to identify various isoforms of ion channels, making them excellent probes to study different receptors. α-Conotoxins are excellent examples of this, because they specifically and potently target nAChR subtypes [166]. This has increased our understanding of the physiological role each receptor subtype plays in normal and disease states. In this thesis, I will limit my discussion to α- and αD-conotoxins.

α-Conotoxins range from 12–25 amino acids in length and belong to the A superfamily of conopeptides. They are characterised with the CCX_nCX_mC cysteine framework, where n and
$m$ represent the number of amino acids and indicate the subclass of a peptide. Most native $\alpha$-conotoxins exhibit I–III and II–IV disulphide connectivity, yielding a $\omega$-shaped, three-dimensional globular conformation [155]. Although, $\alpha$-conotoxins exhibit sequence hypervariability, conserved residues are observed among various $\alpha$-conotoxins. Table 1.1 lists some of the $\alpha$-conotoxins that target neuronal nAChR subtypes. Residues in loop1 (residues between Cys II and III) are highly conserved, with most peptides showing a $–$SXPA$–$ motif.

**Table 1.1: Sequence alignment of $\alpha$-conotoxins targeting neuronal nAChR subtypes.**

<table>
<thead>
<tr>
<th>$\alpha$-Conotoxin</th>
<th>Sequence</th>
<th>nAChR selectivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ls1a</td>
<td>GCCSNPACRVNPNIC*</td>
<td>$\alpha3\beta2\approx\alpha7&gt;\alpha3\alpha5\beta2$</td>
<td>[167]</td>
</tr>
<tr>
<td>GID</td>
<td>IRDyCCSNPACRVNNOHVC#</td>
<td>$\alpha7\approx\alpha3\beta2&gt;\alpha4\beta2$</td>
<td>[164]</td>
</tr>
<tr>
<td>ArIB</td>
<td>DECCSNPACRVNNPHVCRRR#</td>
<td>$\alpha7\approx\alpha6\beta2^&gt;\alpha3\beta2$</td>
<td>[168]</td>
</tr>
<tr>
<td>RegIIA</td>
<td>GCCSHPACNVPNPHIC*</td>
<td>$\alpha3\beta2&gt;\alpha3\beta4\approx\alpha7$</td>
<td>[169]</td>
</tr>
<tr>
<td>OmIA</td>
<td>GCCSHPACNVNNPHICG*</td>
<td>$\alpha3\beta2&gt;\alpha7&gt;\alpha6\beta2^$</td>
<td>[170]</td>
</tr>
<tr>
<td>GIC</td>
<td>GCCSHPACAGNNQHIC*</td>
<td>$\alpha3\beta2\approx\alpha6\beta2^*&gt;\alpha7$</td>
<td>[171]</td>
</tr>
<tr>
<td>AuIB</td>
<td>GCCSYPPCFATNPD- C*</td>
<td>$\alpha3\beta4$</td>
<td>[172]</td>
</tr>
<tr>
<td>RgIA</td>
<td>GCCSDPRCRYR- CR#</td>
<td>$\alpha9\alpha10&gt;&gt;\alpha7$</td>
<td>[173]</td>
</tr>
</tbody>
</table>

* amidated C-terminus; # carboxylated C-terminus; ^ receptors containing the subunits; conserved cysteine framework is indicated in yellow.

Significant interaction between these loop1 residues and the principal face of the receptor was identified using X-ray studies of AChBP, ImI, [A10L,D14K]PnIA [Figure 1.12] and [A10L]TxIA co-crystal structures [174, 175]. These studies identified that loop1 has a role in the peptide’s secondary structure and affinity, and loop2 residues interact with the complementary side of the receptor, contributing to the $\alpha$-conotoxin’s subtype selectivity [Figure 1.12] [156].
1.2.4.1 Use of alanine scanning mutagenesis

To date, most mutational studies have involved replacing various non-cysteine residues with the inert residue alanine. These experiments provided vital information about the various α-conotoxin residues that interact with receptors, and in combination with modelling simulation studies, they have helped to determine α-conotoxin pharmacological selectivity [166]. For example, loop2 residues (11–15) in Vc1.1 have been shown to be important for its activity at α9α10 nAChR subtypes. Alanine mutation of these residues causes significant loss in activity at this subtype [177]. In RgIA, similar experiments showed an approximate 1500-fold loss in activity for [R9A]RgIA at the α9α10 subtype. Surprisingly, the opposite effect was seen at the
α7 subtype, with an approximate 5-fold increase in activity [178]. This mutation was homologous with that of α4/3-conotoxin ImI, which selectively inhibits α7 and α3β2 nAChR subtypes [179].

Alanine scanning mutagenesis not only contributed to the molecular basis for various conotoxins’ antagonism of their respective nAChR subtypes, but also significantly improved our understanding of these peptides’ subtype selectivity. Furthermore, alanine mutation improved α-conotoxin potency and selectivity. For example, E11A mutation in α-conotoxin MII improved its selectivity and potency towards α6-containing nAChRs [180]. In Vc1.1, N9A mutation increased potency at α9α10 nAChR subtypes about 10-fold [177].

1.2.5 Conotoxin re-engineering

α-Conotoxin use in research and clinical settings has been hampered because of their peptidic nature, which makes them susceptible to natural degradation, such as proteolytic attack, or disruptions of their disulphide connectivity. These problems drastically affect their bioavailability and half-life. In vitro chemical synthesis of conotoxins allows them to be chemically modified to improve their structure and ability to be used in research and clinical settings.

1.2.5.1 Dicarba modification

Various strategies to improve α-conotoxin stability have been implemented, such as cyclisation and selenocysteine modification [181, 182]. Recent studies have shown that replacing the cysteine bridges (S–S motif) with non-reducible dicarba links (CH₂–CH₂, CH=CH groups) [183] is a novel way to improve stability and resistance to peptide degradation and scrambling [184]. In addition, this modification maintains the pharmacological activity of α-conotoxins [185, 186].
1.2.6 Conotoxins: potential therapeutics

That conotoxins selectively and potently inhibit various ion channels has put them at the forefront of novel drug development. Several conotoxins are, and have been, tested clinically to treat a range of health conditions, including neuropathic pain and benign prostatic hyperplasia [187]. For example, \( \omega \)-Conotoxin MVIIA, isolated from *Conus magus*, inhibits N-type calcium channels [188] and is an FDA-approved drug for pain relief. Peptides undergoing clinical trials include \( \chi \)-MrIA (a non-competitive neuronal noradrenaline receptor inhibitor) to treat benign prostatic hyperplasia symptoms [189], and conantokin-G (a selective NMDA receptor inhibitor) to manage epilepsy [190].

Recent studies have also identified nAChR subtypes that are involved in various health conditions, such as schizophrenia (\( \alpha 7 \) and \( \alpha 4\beta 2 \)), Parkinson’s disease (\( \alpha 6 \)-containing receptors), Alzheimer’s disease (\( \alpha 7 \) and \( \alpha 4\beta 2 \)), lung cancer (\( \alpha 3\beta 4 \)) and pain (\( \alpha 4\beta 2 \)). These discoveries have provided new therapeutic applications for \( \alpha \)-conotoxins that target neuronal nAChRs [40, 99, 144, 191].
1.3. References


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CHAPTER 2

MATERIALS AND METHODS

This chapter describes the protocols followed for the synthesis, and structural and functional characterisation of conotoxins.

2.1 Materials

Rink amide methylbenzhydrylamine (MBHA) resin (Novabiochem), O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), N-(9-fluorenyl) methoxycarbonyl (Fmoc), dimethylformamide (DMF), N,N'-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), ether, HPLC grade acetonitrile (ACN), potassium cyanide (KCN), ninhydrin, tricaine methanesulphonate (MS-222), acetylcholine (ACh), hydrogen fluoride (HF).

2.2 Peptide synthesis

2.2.1 Solid-phase peptide synthesis (SPPS)

Chemical synthesis using SPPS involves building a peptide chain that is covalently attached to a solid resin support. This concept was introduced by Merrifield in 1963 [1]. Stepwise assembly of the amino acids on the resin uses one of two types of chemistry, depending on the N-terminal protecting group of amino acids - 9-fluorenylmethoxycarbonyl (Fmoc) and tert-butyloxy carbonyl (t-Boc). Along with one of these orthogonal protecting groups, side chain protecting groups are also present on amino acids which prevent any intermediate reactions during peptide elongation.

Peptide elongation involves two basic steps: N-terminal deprotection and a coupling reaction. These steps are where the major differences between Fmoc and Boc peptide synthesis are seen. In Boc chemistry, the deprotection step uses an acid, such as TFA, whereas Fmoc chemistry uses a base, such as piperidine. The two also require different reactions to cleave the peptide from the solid resin. Boc needs HF, while Fmoc uses TFA [1, 2]. Because Fmoc
uses low-hazard reagents, it is safer than Boc, which is why many scientists prefer it. However, both methods have specific advantages, and the type of SPPS method used depends on the final peptide needed.

2.2.2 Fmoc SPPS

All peptide analogues discussed in Chapter 3 were synthesised using Fmoc chemistry. The various steps involved in this method are detailed below. Fmoc peptide synthesis was carried out in collaboration with Dr Richard Clark (The University of Queensland, Brisbane).

Our collaborators provided the peptides described in other chapters.

2.2.2.1 Peptide assembly

The basic apparatus for SPPS includes a reaction vessel with a sintered glass filter and tap at the bottom, which is connected to a vacuum line via a solvent trap [Figure 2.1]. The filter supports the resin upon which the peptides are assembled. The tap allows solvents to be removed from the vessel.

![Figure 2.1: The basic apparatus set up needed for solid-phase peptide synthesis.](image)
All peptide analogues were assembled on rink amide MBHA resin (Novabiochem; 0.7 mmol·g\(^{-1}\)) using HBTU-mediated manual SPSS with an *in situ* neutralisation procedure for Fmoc chemistry [3]. The amount of resin needed for each reaction was calculated based on the scale of peptide synthesised in mmoles and the resin’s substitution value (provided with resin).

\[
\text{Mass of resin} = \frac{\text{scale in mmoles}}{\text{substitution value}}
\]

All peptide analogues were synthesised at 0.25 mmoles scale, where 460 mg of rink amide MBHA resin (substitution value of 0.54) was used, calculated using the above formula. The resin was allowed to swell overnight, soaked in DMF, and then transferred into the reaction vessel.

Each amino acid assembly cycle consisted of Fmoc deprotection with 20% piperidine in DMF, followed by Fmoc amino acid coupling using HBTU and DIPEA in DMF. A two-fold excess of Fmoc amino acids was used in the coupling reactions. Each coupling reaction used 2 mmoles of each amino acid. The amino acids are activated by dissolving them in 2 mL of 0.5 M of HBTU/DMF solution followed by the addition of 174 µL of DIPEA. When DIPEA was added, the amino acid mixture was immediately added to the resin and left for 10–15 min to allow for efficient coupling.

A Kaiser Ninhydrin test was carried out to determine the coupling percentage [4]. The Kaiser Ninhydrin test detects the amount of free amine present on the resin, indicated by the solution turning purple. In this test, 3–5 mg of the resin was removed, washed with 50% DCM/methanol and then air dried. To this dried resin, 2 drops of 76% w/w phenol in ethanol, 4 drops of 0.2 mM KCN in pyridine and 2 drops of 0.28 M ninhydrin in ethanol was added.
The sample was incubated at 100 °C for 5 min, and then 2.8 mL of 60% ethanol in water was added to it before it was briefly centrifuged to settle the resin at the bottom. The solution’s absorbance at 570 nm, was then calculated against the reagent blank using ultraviolet-visible spectrophotometry. The coupling % was calculated using the following equation:

\[
\% \text{ coupling} = 100 \times \left(1 - \frac{A_{570} \times 200}{SV \times \text{mass of resin}}\right)
\]

where, \(A_{570}\) is the absorbance value obtained at 570 nm and SV is the substitution value of the resin.

Values > 99% indicated a successful coupling reaction, and peptide assembly continued with the addition of the next amino acid [4]. However, the ninhydrin test cannot be used to determine the coupling efficiency of an amino acid after proline. This is due to lack of primary amines at the N-termini of proline. Under these conditions, the coupling reaction is generally either repeated or an Isatin test can be performed to check for coupling.

In the Isatin test, 5 drops of Isatin were dissolved in 3% n-butanol and 2 drops of 10% acetic acid were added to the dry resin, before the solution was incubated for 5 min at 100 °C. Yellow or colourless resin indicated efficient coupling, whereas a blue or grey-green colour represented poor coupling [5].

2.2.2.2 Peptide cleavage

When the last amino acid was coupled, the N-terminal Fmoc group was removed using the usual deprotection procedure and washed with DMF. Peptide cleavage from the dried resin (0.4 g) was achieved by treating it with 100 mL of a chemical cocktail composed of TFA, TIPS and water (95:2.5:2.5 TFA:TIPS:water v/v/v). TIPS and water within the cocktail are scavengers that prevent the modification of unprotected side-chains. The reaction proceeded at room temperature (20–23 °C) for 2.5 h. The TFA was then evaporated (not completely
dried) and the peptide was precipitated with 30 mL of ice-cold ether. The peptide was then extracted using 50% buffer A/B (Buffer A: H2O/0.05% TFA; Buffer B: 90% CH3CN/10%H2O/0.045% TFA) using a separating funnel. Any residual ether was removed and the peptide was lyophilised [6].

2.2.2.3 Directed disulphide formation

All peptides were synthesised in globular conformation (I–III; II–IV disulphide connectivity) through Fmoc – Cys(Acm) – OH coupling at positions 2 and 8 of the amino acid sequence (I–III disulphide bond). The scavengers in the chemical cocktail used for cleaving the peptide do not deprotect the Acm group on I–III Cys residues.

Crude peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Phenomenex C18 column using a gradient of 0–80% B in 80 min, with the eluent monitored at 215/280 nm. These conditions were used in subsequent purification steps unless stated otherwise. Electrospray mass spectroscopy (ES-MS) confirmed the molecular mass of the fractions collected. Those fractions displaying the correct molecular mass of linear peptide were pooled and lyophilised for oxidation.

Linear peptides were oxidised in two steps [Figure 2.2]. First, they were dissolved in 0.1 m NH4HCO3 (pH 8.2) at a concentration of 0.3 mg/mL, and stirred overnight at room temperature. This created the II–IV disulphide bond. The Acm protecting groups were stable under these conditions. The [II–IV]-disulphide peptides were purified using RP-HPLC, confirmed with ES-MS and then lyophilised.

Second, the second disulphide bond (I–III) was formed using the oxidation by iodine method. The peptides were dissolved (1 mg/mL) in buffer A (H2O/0.05% TFA) before iodine in
Acetonitrile was added until the solution turned orange/yellow. The reaction was incubated for 5 min at 37 °C. Excess iodine was destroyed by adding sodium ascorbate. The oxidised peptides were then purified by RP-HPLC using a gradient of 0–80% buffer B over 160 min. Analytical RP-HPLC and ES-MS confirmed the synthesised peptides’ purity and molecular mass [7].

**Figure 2.2:** Directed disulphide formation of α-conotoxins using orthogonal cysteine protecting groups.
2.2.3 NMR spectroscopy

Dr Richard Clark (School of Biomedical Sciences, The University of Queensland) carried out all NMR spectroscopy experiments.

$^1$H NMR was done to determine the successful folding of all peptide analogues outlined in Chapter 3. NMR data for all peptides were recorded on Bruker Avance 500- and 600-MHz spectrometers, with samples of > 95% purity dissolved in 90% H$_2$O and 10% D$_2$O (Cambridge Isotope laboratories, Massachusetts, USA).

Two-dimensional NMR experiments included total correlation spectroscopy (TOCSY) and nuclear overhauser effect spectroscopy (NOESY) recorded at 280 K. Spectra were analysed using Topspin 1.3 (Bruker) and Sparky software. Most spectra were recorded at pH 3.5. Sequence-specific resonance assignment was carried out for each peptide’s spectra in collaboration with Dr Norelle Daly (Institute for Molecular Biosciences, The University of Queensland) [8].

2.3 Electrophysiological recordings in Xenopus oocytes

*Xenopus laevis* frogs were anesthetised using MS-222 (1.3 g/L) solution before oocytes were surgically extracted. The oocytes were then incubated with collagenase (3 mg/mL) dissolved in OR2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$ and 5 mM HEPES, pH 7.4) for 1–2 h (until the vitelline membrane surrounding the oocytes was digested). The collagenase was then removed by washing the oocytes with OR2 buffer.

Oocytes at stages V–VI (larger in size) with distinct animal (dark) and vegetal pole (white) were selected for mRNA injection [Figure 2.3]. cDNAs encoding the rat $\alpha$3, $\alpha$4, $\alpha$9, $\alpha$10, $\beta$2 and $\beta$4 nAChR subunits and human $\alpha$7 nAChR subunit, subcloned into the oocyte expression
vector pT7TS, were used for the mRNA preparation using the mMESSAGE mMACHINE Kit (Ambion Inc, USA). All oocytes were injected with 25 ng of cRNA for α9 and α10, and 5 ng of cRNA for all other subunits. They were then kept at 18 °C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES, pH 7.4) supplemented with 50 mg/L gentamicin and 100 µg/units/mL penicillin streptomycin for 2–5 days before recording [Figure 2.3].

Membrane currents from Xenopus oocytes were recorded using two-electrode voltage clamp (virtual ground circuit) with either a GeneClamp 500B amplifier (Molecular Devices) or an automated workstation with eight channels in parallel, including drug delivery and online analysis (OpusXpress™ 6000A workstation, Axon Instruments Inc.) [Figure 2.3]. Voltage-recording and current-injecting electrodes were pulled from borosilicate glass (GC150T-7.5, Harvard Apparatus Ltd) and had resistances of 0.3–1.5 MΩ when filled with 3 M KCl [9].

Figure 2.3: Schematic representation of the various steps involved in two-electrode voltage-clamp studies using Xenopus laevis oocytes.
All recordings were made at room temperature using a bath solution of ND96, as described above. During recordings, oocytes were perfused continuously at a rate of 2 mL/min, and peptides were incubated for 300 s before the ACh was added. ACh (200 µM for α7 and 50 µM for all other nAChR subtypes) was applied for 2 s at 2 mL/min, with 180–240 s washout periods between applications. Cells were voltage-clamped at a holding potential of −80 mV. Data were filtered at 10 Hz and sampled at 500 Hz [10].

Desensitisation experiments were carried out using 50 µM ACh applied for 30 s, followed by a 300 s washout. The onset ($k_{on}$) and recovery ($k_{off}$) from block by the peptide was measured by bath applying the peptide at 2 mL/min for 5 min, followed by washout. On-rate kinetics were carried out with ACh + peptide pulse applied at the indicated time intervals. ACh pulses during recovery from block contained no peptide. The percentage response or percentage inhibition was obtained by averaging the peak amplitude of three control responses directly before exposure to the peptide.

2.4 Data analysis

Concentration–response curves for antagonists were fitted by unweighted nonlinear regression to the logistic equation:

$$E_x = E_{max} X^n_{H}/(X^n_{H} + IC_{50}^{n_{H}})$$

where $E_x$ is the response, $X$ is the antagonist concentration, $E_{max}$ is the maximal response, $n_H$ is the slope factor and $IC_{50}$ is the antagonist concentration that gives 50% inhibition of the maximal response.

All electrophysiological data were pooled (n = 4–8 for each data point) and represent arithmetic means ± standard error of the fit.
The rates of onset and recovery from block during peptide washout were obtained from exponential fits to the data using GraphPad Prism 5. The $k_i$ for the peptide was then calculated using the equation:

$$k_i = k_{\text{off}} / k_{\text{on}}.$$

Computation was done using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

2.5 References


CHAPTER 3

α-Conotoxin RegIIA targeting α3β4 nAChR:

unlocking novel therapeutics towards lung cancer

Conus regius

http://www.coneshell.net
3.1 INTRODUCTION

3.1.1 Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChR) are ligand-gated ion channels expressed in the central nervous system (CNS) and peripheral nervous system (PNS) [1, 2]. They are pentameric receptors composed of a combination of alpha subunits (α2–10) and beta subunits (β2–4). nAChRs exhibit a diverse structural and functional heterogeneity through formation of various heteromeric (e.g. α3β2 and α4β2) and homomeric isoforms (formed by only α7 and α9 subunits) [3]. Their physiological role is well understood and they are known to modulate pre- and post-synaptic transmission in the CNS, and visceral and somatic sensory transmission in the PNS [4-6].

nAChRs have been implicated in various pathophysiological conditions, including Alzheimer’s disease, schizophrenia, tobacco addiction and lung cancer [2]. Despite considerable progress in understanding these pathological conditions, knowledge of the distribution and neurophysiological role of individual receptor subtypes is limited by the lack of adequate isoform-specific probes [7].

3.1.2 The pathophysiological role of the α3β4 nAChR subtype

Following the first report by Schuller’s group in 1989, which suggested that nAChRs may have a role in regulating cancer cell growth [8], many studies were initiated to investigate the role of nAChRs in cancer development and progression [9]. Two reports identified nicotine and its derivatives as potent nAChR agonists and their regulatory role in cancer cell apoptosis [10, 11]. Genome-wide association studies (GWAS) led to the identification of various single
nucleotide polymorphisms (SNP) within a gene cluster encoding α3, α5 and β4 nAChR subunits, and these SNPs are associated with lung cancer [9, 12]. In addition, the α3β4 subtype has been shown to be involved in nicotine addiction and drug-abuse [13]. However, knowledge of the distribution and physiological role of the α3β4 subtype is still limited. The development of a α3β4 subtype-selective inhibitor, which could help develop drugs to treat cancer and nicotine addiction, is very desirable.

3.1.3 α-Conotoxins targeting neuronal nAChRs

Conotoxins are bioactive peptides isolated from the venom of cone snails belonging to the genus Conus [14, 15]. α-Conotoxins, a class of short disulphide-rich peptides from this venom [16], specifically target various nAChR subtypes and are excellent molecular probes for identifying the physiological role of nAChR subtypes in normal and disease states [17]. To date, many α-conotoxins have been characterised, and their structural and functional properties are well documented in various reviews and online databases (http://www.conoserver.org) [14, 18-22]. While it is evident that α-conotoxins, such as ImII and MII, exhibit selective inhibitory activity at α7 [23] and α3β2 [24] nAChR subtypes respectively, most known peptides target multiple subtypes [25]. Mutagenesis experiments have therefore become an important tool to improve the selectivity and potency of these peptides [26]. Furthermore, α-conotoxin AuIB is the only peptide known to selectively target the α3β4 nAChR subtype with an IC50 of 2.5 µM [27].

Here, I describe the discovery, and biochemical, biophysical and functional characterisation, of RegIIA. This α4/7-conotoxin was isolated from the venom of Conus regius, a worm-hunting cone snail species that inhabits the Western Atlantic Ocean. It is one of the most
potent α3β4 nAChR antagonists, but does not inhibit the α4β2 subtype. This selectivity profile makes RegIIA a prospective probe for studying nicotine addiction processes.

RegIIA has a classical α-conotoxin globular structure (ω-shaped fold) indicating that it has an exquisite balance of shape, charges, and polarity exposed on its surface to enable it to potently block the α3β4 nAChR. The pathophysiological association of α3β4 nAChRs in various diseases such as lung cancer and nicotine addiction, along with RegIIA being one of the most potent known α3β4 nAChR antagonists, led us to investigate and improve RegIIA’s selectivity profile at the α3β4 nAChR subtype. Using alanine scanning mutagenesis and modelling studies, we identified critical residues of α-conotoxin RegIIA that interact with the ACh-binding site of α3β2, α3β4 and α7 nAChRs.
3.2 AIMS

3.2.1 Characterisation of α-conotoxin RegIIA isolated from *Conus regius*.

— To determine the pharmacological profile of RegIIA using two-electrode voltage-clamp technique in *Xenopus* oocytes expressing recombinant nAChR subtypes.

3.2.2 Alanine scan mutagenesis.

— To understand the molecular mechanism and critical residues that determine RegIIA’s specific nAChR subtype selectivity.

3.2.3 Significance

— In conjunction with modelling simulation being done by our collaborators, this study could provide a detailed understanding of RegIIA’s molecular pharmacology at the α3β4 and α3β2 nAChR subtypes.

— This study could also provide valuable information to aid the future design and development of α3β4-selective drugs to treat lung cancer and nicotine addiction.
3.3 RESULTS

3.3.1 Selective α-conotoxin RegIIA inhibition of recombinant nAChR subtypes

RegIIA selectivity was examined by inhibiting ACh-evoked currents mediated by various nAChRs subtypes expressed in *Xenopus* oocytes. ACh was applied at 5 min intervals and the corresponding membrane currents were assessed. The peptide was bath incubated for 5 mins before co-application of ACh and the peptide. Synthetic RegIIA (1 µM) completely inhibited ACh-evoked current amplitude produced by α3β4, α3β2 and α7 nAChRs [Figure 3.1]. However, RegIIA did not inhibit muscle (αβγδ) or α4β2 nAChRs (n = 4–5). RegIIA (1 µM) inhibited only 20 ± 5% of ACh-evoked current amplitude of α9α10 nAChR. Concentration–response curves of RegIIA display the order of selectivity and their corresponding IC50 values for α3β2 (11 nM) > α3β4 (47 nM) > α7 (61 nM) [Figure 3.2 and Table 3.1].

Figure 3.1: Concentration-dependent RegIIA inhibition of ACh-evoked current amplitude mediated by (A) α3β2, (B) α3β4 and (C) α7 nAChRs expressed in oocytes.
Figure 3.2: Selectivity of α-conotoxin RegIIA inhibition of nAChR subtypes. RegIIA (1 µM) completely inhibited α3β2, α3β4 and α7 receptors, and inhibited the α9α10 receptor by ~20%. Concentration–response curves for RegIIA inhibition gave IC$_{50}$ of 16 nM for α3β2 (●), 48 nM for α3β4 (▲) and 51 nM for α7 (□). All data represents mean ± SEM; n = 4–7.

Table 3.1: α-Conotoxin RegIIA inhibition of recombinant nAChR subunits expressed in Xenopus oocytes.

<table>
<thead>
<tr>
<th>nAChR subtype</th>
<th>IC$_{50}$ (95% CI)</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>α3β2</td>
<td>10.7 nM (8.8–12.9)</td>
<td>−1.1 ± 0.1</td>
</tr>
<tr>
<td>α3β4</td>
<td>47.3 nM (39.5–56.6)</td>
<td>−1.5 ± 0.2</td>
</tr>
<tr>
<td>α7</td>
<td>61.2 nM (47.8–78.4)</td>
<td>−1.2 ± 0.2</td>
</tr>
<tr>
<td>α9α10</td>
<td>&gt;1 µM</td>
<td></td>
</tr>
<tr>
<td>α4β2</td>
<td>&gt;1 µM</td>
<td></td>
</tr>
<tr>
<td>αβδγ</td>
<td>&gt;1 µM</td>
<td></td>
</tr>
</tbody>
</table>

RegIIA (1 µM) had no effect on α4β2 or αβγδ, and inhibited α9α10 by only 20%. All data represents n = 4–7.
3.3.2 Directed peptide synthesis of α-conotoxin RegIIA analogues and NMR

α-Conotoxins have a conserved Cys-framework. Their three-dimensional structure is dominated by a helical structure. In native α-conotoxins, I–III and II–IV disulfide connectivity is dominant and folds the peptide into globular conformation [28, 29]. However, α-conotoxin disulfide connectivity can change upon oxidation and reduction to form ribbon (I–IV and II–III disulfide bonds) or beads (I–II and III–IV disulfide bonds). These conformations play significant roles in potency and specificity of α-conotoxins at nAChRs [24, 30].

Under normal conditions of solid-phase peptide synthesis, the percentage of a peptide in a single conformation varies, which can be undesirable. This problem was solved by using amino acids with stable protective groups (Acm) under normal oxidative conditions (0.1 M NH₄HCO₃, pH 8.2). The use of Cys-Acm amino acids at positions 1 and 3 and a two-step oxidation procedure, yielded the alanine mutants in a globular conformation (I–III and II–IV disulfide bonds), shown by HPLC. This was confirmed by 2D NMR (COSY and NOESY), which showed the negative 1H shift values between the amino acids 3 and 7 positions and indicated the presence of α-helix secondary structure [Figure 3.3].
3.3.3 Inhibition of nAChR subtypes by α-conotoxin RegIIA analogues:

To understand the RegIIA structure–activity relationship at nAChRs, RegIIA analogues were tested on α3β2, α3β4 and α7 nAChR subtypes at a concentration of 300 nM [Figure 3.4]. [H14A]RegIIA showed complete loss in activity at all of the above-mentioned nAChR subtypes. At α7 nAChR subtype, no noticeable change was seen for [P13A]RegIIA inhibition, whereas inhibition by all other analogues was significantly reduced or completely lost [Figure 3.4(B)]. In contrast, no change in inhibition of the α3β4 nAChR subtype by RegIIA analogues was observed except for [N9A]RegIIA which completely lost its activity at α3β4. Furthermore, [N11A]RegIIA and [N12A]RegIIA selectivity for α3β4 nAChR subtypes improved. Their inhibition of the α3β2 nAChR subtype was significantly reduced (by approximately 50%), whereas no change was observed at α3β4 nAChR subtype [Figure 3.4(B)]. This was apparent in the concentration–response curves for [N11A]RegIIA and
[N12A]RegIIA, which showed the inhibition curve for the α3β2 (red line) nAChR subtype shifted toward the right [Figure 3.5]. The IC$_{50}$ values for [N11A]RegIIA and [N12A]RegIIA at the α3β2 nAChR subtype was 115.9 nM (95% CI 62.7 – 214.1; $n_H = –0.9 \pm 0.22$) and 278 nM (95% CI 153.4 – 503.8; $n_H = –1.1 \pm 0.25$) respectively, and at the α3β4 nAChR subtype is 51.6 nM (95% CI 44.2 – 60.1; $n_H = –2.0 \pm 0.21$) and 112 nM (95% CI 92.1 – 136.2; $n_H = –1.7 \pm 0.32$), respectively [Table 3.2].

Figure 3.4. RegIIA and alanine analogue (300 nM) inhibition of various nAChR subtypes expressed in *Xenopus* oocytes. (A) Bar graph of inhibition of nAChR subtypes by RegIIA and its analogues. Data represents mean ± SEM, n = 4–6. (B) Two-way ANOVA scatter plot illustrating the loss of activity of the RegIIA analogues (300 nM) relative to wild-type RegIIA at various nAChR subtypes. [H14A]RegIIA completely lost its activity at α3β2, α3β4 and α7 nAChRs. [N9A]RegIIA was more selective for the α3β2 subtype than RegIIA was. [N11A]RegIIA and [N12A]RegIIA selectivity for the α3β4 nAChR subtype significantly improved. All analogues, except [P13A]RegIIA, significantly lost activity at the α7 nAChR subtype. *** p < 0.001, * p < 0.05; n = 4–6.
Figure 3.5: [N11A]RegIIA and [N12A]RegIIA exhibiting improved selectivity at α3β4 nAChR subtypes (A) Superimposed traces showing ACh-evoked current inhibition of the α3β2 nAChR subtype by 100 nM (i) RegIIA, (ii) [N11A]RegIIA and (iii) [N12A]RegIIA. (B) Concentration–response curves for (ii) [N11A]RegIIA and (iii) [N12A]RegIIA inhibition of the α3β4 nAChR (black line, open symbols) and α3β2 nAChR subtypes (red line, closed symbols). [N11A]RegIIA and [N12A]RegIIA shifted the curve to the right for the α3β2 (red line) nAChR subtype, giving an IC₅₀ value of 116 nM and 278 nM, respectively. All data represents mean ± SEM; n = 4–6.
Table 3.2: RegIIA and analogues inhibition of nAChR subtypes.

<table>
<thead>
<tr>
<th></th>
<th>α3β4</th>
<th></th>
<th>α3β2</th>
<th></th>
<th>α7</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (95% Cl)</td>
<td>n&lt;sub&gt;H&lt;/sub&gt;</td>
<td>IC₅₀ (95% Cl)</td>
<td>n&lt;sub&gt;H&lt;/sub&gt;</td>
<td>IC₅₀ (95% Cl)</td>
<td>n&lt;sub&gt;H&lt;/sub&gt;</td>
</tr>
<tr>
<td>RegIIA</td>
<td>47.3 nM (39.5–56.6)</td>
<td>−1.5 ± 0.2</td>
<td>10.7 nM (8.8–12.9)</td>
<td>−1.1 ± 0.1</td>
<td>61.2 nM (47.8–78.4)</td>
<td>−1.2 ± 0.2</td>
</tr>
<tr>
<td>[N11A]RegIIA</td>
<td>51.6 nM (44.2–60.1)</td>
<td>−2.0 ± 0.2</td>
<td>115.9 nM (62.7–214.1)</td>
<td>−0.9 ± 0.2</td>
<td>ND</td>
<td>−</td>
</tr>
<tr>
<td>[N12A]RegIIA</td>
<td>112 nM (92.1–136.2)</td>
<td>−1.7 ± 0.3</td>
<td>278 nM (153.4–503.8)</td>
<td>−1.1 ± 0.2</td>
<td>ND</td>
<td>−</td>
</tr>
<tr>
<td>[N11A,N12A]RegIIA</td>
<td>370 nM (3.09–442.3)</td>
<td>−1.7 ± 0.2</td>
<td>9.87 µM (7.9–12.4)</td>
<td>−1.5 ± 0.3</td>
<td>21.5 µM (17.1–27.0)</td>
<td>−3.1 ± 0.8</td>
</tr>
</tbody>
</table>

IC₅₀ values with 95% CI and Hill slope (n<sub>H</sub>) obtained from concentration–response curves for RegIIA and analogues at α3β2, α3β4 and α7 nAChR subtypes. All data represent mean of n = 4–6 experiments. ND – not determined.
3.3.4 Double mutant [N11A,N12A]RegIIA selectively inhibits α3β4 nAChR subtype

Double mutant [N11A,N12A]RegIIA was synthesised to better understand the cumulative effect of the two residues on nAChR activity. When tested, [N11A,N12A]RegIIA inhibited the α3β4 nAChR subtype with an IC$_{50}$ of 370 nM (95% CI 3.09 – 442.3; $n_H = -1.7 ± 0.2$) and a 7-fold decrease in potency compared with RegIIA [Figure 3.6]. However, at the α3β2 and α7 nAChR subtypes, potency decreased by approximately 1,000-fold (IC$_{50}$ = 9.9 µM) and 360-fold (IC$_{50}$ = 21.5 µM), respectively [Table 3.2]. This indicates an approximate 27-fold change in selectivity at the α3β2 nAChR subtype, and an approximate 58-fold change at the α7 nAChR subtype.

![Figure 3.6: [N11A,N12A]RegIIA inhibition of α3β2 and α3β4 nAChR compared with that of wild-type RegIIA.](image)

Concentration–response curve of [N11A,N12A] RegIIA gave an IC$_{50}$ of 370 nM at α3β4 (▲) and 9.9 µM at α3β2 (■), with an approximate 27-fold change in selectivity. All data represents mean ± SEM; n = 4–6.
3.4 DISCUSSION

3.4.1 *Conus regius*: characterisation of novel α-conotoxin RegIIA

Since 1994, when the first α-conotoxin ImI was discovered in the worm-hunting *Conus imperialis* and found to target neuronal nAChRs, numerous α-conotoxins have been identified and functionally characterised [14, 31]. Most native α-conotoxins have a ω-shaped, three-dimensional globular conformation, which results from the two disulfide bonds (I–III and II–IV) in the peptide’s characteristic cysteine framework CCXₙCXₘC (classified as cysteine framework I) [29]. The number of amino acids indicated by $n$ and $m$ designate the subclass of the peptide. RegIIA belongs to the α4/7 subclass and exhibits the classical ω-shaped globular structure, with balanced shape, charges and polarity.

Today, various peptides have been identified from the venom of *C. regius*, a Western Atlantic worm-hunting cone snail species that belongs to various superfamilies, of which 8 belong to the A-superfamily with the cysteine framework I [32]. The conotoxin composition of *C. regius* venom gained special importance due to the identification of α-conotoxin RgIA, which selectively targets the α9α10 nAChR subtype and high voltage-activated (HVA) calcium channel currents via GABA$_B$ receptors [33]. α-Conotoxin RegIIA, also isolated from *C. regius*, potently inhibits the α3β4 nAChR subtype [34]. HVA calcium channels modulated by GABA$_B$ receptors are involved in pain pathways and the α3β4 nAChR subtype is implicated in lung cancer pathophysiology. This makes the discovery of RgIA and RegIIA very significant and puts them at the forefront of potential new therapeutics.
3.4.2 Alanine mutagenesis reveals the pharmacological role of the –NNP– motif in RegIIA

RegIIA exhibits high homology to various known peptides [Table 3.8], with two main features: an –SHPA– conserved sequence and –NNP– motif. While the –SHPA– conserved sequence is observed in the peptides of different subclasses that target various nAChR subtypes, the –NNP– motif has been specifically observed in peptides that inhibit α3β2 and α7 nAChR subtypes, such as OmIA [35], EpI [36], PnIA [37], TxIA [38] and ArIIB [39]. This observation corroborates with the most important finding of this study – the alanine mutation of the –NNP– motif significantly affected the peptide’s inhibitory activity at α3β2 and α7 nAChR subtypes. Additionally, the [N11A,N12A] RegIIA analogue is the most potent of the α3β4 nAChR subtype-selective peptides.

Alanine mutations at other positions also provided vital information about the structure–function relationship between α-conotoxins and neuronal nAChRs. Asparagine at the ninth position is critical for RegIIA inhibition of the α7 and α3β4 nAChRs, because alanine mutation of this residue completely abolished RegIIA inhibition of both nAChR subtypes.

3.4.3 Molecular modelling and molecular dynamics reveal structural topology of α3β2 and α3β4 nAChRs and residues that interact with RegIIA

To elucidate the molecular mechanism of RegIIA’s inhibition of the α3β4 nAChR subtype, an alanine scan mutagenesis protocol was used. This technique is well established, and in conjunction with atomistic molecular dynamics (MD) simulations, it has previously enabled spectacular progress in the field of molecular pharmacology [26, 40]. X-ray and NMR studies of AChBP–α-conotoxin complexes, and the support of synthetic analogues, promoted the
understanding of ligand–receptor interactions [41-44]. The extracellular N-terminal domain (ECD) of β2 and β4 nAChR subunits, to which α-conotoxins bind, exhibited a high sequence homology (70%). Recent MD simulation studies also reveal a well-preserved structural topology of α3β2 and α3β4 nAChRs. However, the ACh-binding pocket interface between the α and β subunits was larger in α3β2 than α3β4 nAChR subtypes [45]. This difference could signify a distinct shift in [N11A] and [N12A]RegIIA selectivity for α3β2 and α3β4, even though both of these residues primarily interact with the α3(+) interface.

To elucidate the structure–function relationship of the alanine scan mutagenesis results, our collaborators carried out atomistic MD simulations of [N11A,N12A]RegIIA. The results indicated that the double mutant induced a significant loss in contact Y92, S149, Y189, Y196 residues of the α3 subunit, and W57, and F119 residues of the β2 subunit induced by the double mutant. A comprehensive receptor mutagenesis study of various α-conotoxins (MII, GID and PnIA) that inhibit the α3β2 nAChR indicated that the β2 subunit pharmacophore, comprised of T59, E61, V111, F119 and L121 residues, has a significant role in ligand binding [46].

A more recent molecular docking study of α-conotoxin GIC and the ECD of human α3β2 and α3β4 nAChR subtypes revealed that all three subunits have residues that interact with the conotoxin (α3 subunit: Y92, Y150, Y189 and Y196; β2 subunit: W57, V111, F119 and L121; β4 subunit: W57, I111, L119 (Q119 in rat β4) and L121). It is also interesting to note that the α3Y196 and β2F119 residues of the α3β2-GIC model were more closely located than the α3Y196 and β4L119 residues of the α3β4-GIC model [45]. These results are consistent with the MD simulations displaying the loss of pairwise contacts between wt RegIIA and [N11A,N12A]RegIIA at α3β2 compared to α3β4 nAChR subtypes. This study provides the
first experimental evidence into the molecular pharmacological difference between an α-conotoxin and these two nAChR subtypes at structural and functional levels.

3.5 SUMMARY AND CONCLUSION

The CHRNA3, CHRNA4 and CHRNA5 gene clusters encoding the α3, β4 and α5 nAChR subunits gained significant importance in recent years. This may be due to the recent genetic and physiological studies implicating that the α3β4 nAChR has a direct functional role in the pathophysiology of lung cancer and nicotine addiction [9, 12]. While the α3β4 nAChR was initially identified in ganglia, recent studies show it is also distributed throughout the CNS and other tissues, such as the interpeduncular nucleus and medial habenula [47, 48].

Identifying and successfully synthesising [N11A,N12A]RegIIA, a α3β4 nAChR subtype-selective antagonist, could help to decipher the physiological role of this receptor. Our study also extends the understanding of RegIIA interactions with various nAChR subtypes and elucidated the key residues on the toxin and receptor binding sites. This information is invaluable in the design and development of α3β4-selective drugs to treat lung cancer and nicotine addiction.
Table 3.3: Sequence alignment of α-conotoxins targeting various nAChR subtypes.

<table>
<thead>
<tr>
<th>α-Conotoxin</th>
<th>Sequence</th>
<th>nAChR selectivity</th>
<th>IC$_{50}$ for α3β4 nAChR inhibition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RegIIA</td>
<td>GCCSHPACNVNPHIC*</td>
<td>α3β2&gt;α3β4&gt;α7</td>
<td>50 nM</td>
<td>[34]</td>
</tr>
<tr>
<td>OmIA</td>
<td>GCCSHPACNVNPHICG*</td>
<td>α3β2&gt;α7&gt;α6β2</td>
<td>-</td>
<td>[49]</td>
</tr>
<tr>
<td>GIC</td>
<td>GCCSHPACAGNNQHIC*</td>
<td>α3β2&gt;α6β2&gt;α7</td>
<td>-</td>
<td>[50]</td>
</tr>
<tr>
<td>PeIA</td>
<td>GCCSHPACSVNHPELC*</td>
<td>α9a10&gt;α3β2&gt;α6β2*&gt;α3β4&gt;α7</td>
<td>480 nM</td>
<td>[51]</td>
</tr>
<tr>
<td>Mr1.1</td>
<td>GCCSHPACSVNPDCI*</td>
<td>α3β2&gt;α3β4&gt;α7</td>
<td>1400 nM</td>
<td>[52]</td>
</tr>
<tr>
<td>Ls1a</td>
<td>SGCCSNPVACRVPNNPNC*</td>
<td>α3β2&gt;α7</td>
<td>-</td>
<td>[53]</td>
</tr>
<tr>
<td>AuIB</td>
<td>GCCSYPPCFATNPDC*</td>
<td>α3β4</td>
<td>2500 nM</td>
<td>[54]</td>
</tr>
<tr>
<td>BuIA</td>
<td>GCCSTPPCAVLY---C*</td>
<td>β2*&gt;β4*</td>
<td>28 nM</td>
<td>[55]</td>
</tr>
<tr>
<td>PIA</td>
<td>RDPCCSNPVCTVHNQPQC*</td>
<td>α6β2*&gt;α6β4=α3β2&gt;α3β4</td>
<td>520 nM</td>
<td>[56]</td>
</tr>
<tr>
<td>ArIB</td>
<td>DECCSNPACRVNPVHVCRRR*</td>
<td>α7=α6β2*&gt;α3β2</td>
<td>-</td>
<td>[57]</td>
</tr>
</tbody>
</table>

Amino acids homologous to RegIIA are labelled with grey background. Peptides inhibiting the α3β4 nAChR subtype (orange) and their corresponding IC$_{50}$ values are shown. The conserved cysteine framework is highlighted in yellow.
3.6 References


characterization of alpha-conotoxin LsIA with potent activity at nicotinic acetylcholine receptors, *Biochemical pharmacology*.


characterization of alpha-conotoxin LsIA with potent activity at nicotinic acetylcholine receptors, *Biochemical pharmacology* 86, 791-799.


CHAPTER 4

Characterisation of LsIA:

First $\alpha$-conotoxin isolated from *Conus limpusi*
4.1 INTRODUCTION

α-Conotoxins are a class of bioactive peptides isolated from the venom of cone snails belonging to the genus *Conus*. They inhibit various nAChR subtypes with a high degree of specificity and potency. This unique pharmacological profile has led to the development of α-conotoxins as novel molecular probes for the physiological study of nAChR subtypes and drugs to treat various pathological conditions involving cholinergic mechanisms of action [1]. The pharmacological profile of an α-conotoxin is determined by the peptide’s structure and sequence. α-Conotoxins of the α4/7 and α4/3 subclass, defined by their characteristic cysteine framework, inhibit neuronal nAChR subtypes almost exclusively [2].

nAChRs are pentameric transmembrane ion channels. They are constituted by various nAChR subunits, which classifies them into homomeric receptors, such as α7 or α9, or heteromeric receptors, such as α4β2 or α3β2 [3]. nAChRs primarily mediate fast synaptic transmissions in the CNS and PNS. They also modulate cholinergic transmission in non-neuronal cells, mediating subtype-specific physiological functions [4].

α3β2 and α7 nAChR subtypes play vital roles in various functions, such as neuronal plasticity, angiogenesis and gene regulation [5]. They also are involved in various pathophysiological conditions, such as schizophrenia, Alzheimer’s disease and myasthenia gravis [6].

Here I describe the pharmacological properties of the novel α-conotoxin LsIA, the first peptide isolated from *Conus limpui*, a species of worm-hunting cone snail commonly found on the south east coast of Queensland, Australia. LsIA is an α4/7-conotoxin with the characteristic I–III and II–IV disulfide connectivity. LsIA exhibited selective and potent α7 and α3β2 nAChR subtype antagonism. In this report, I also examined the structure–function relationship of the presence of a unique N-terminal serine at position 2 and C-terminal
Furthermore, I also investigated the pharmacological implications, involving incorporation of the α5 subunit towards the inhibition of LsIA at α3β2 nAChR subtypes.

4.2 AIMS

4.2.1 Characterisation of LsIA.

— To functionally characterise LsIA – the first α-conotoxin isolated from *Conus limpusi*.

— To identify the role the N-terminal sequence and C-terminal carboxylation play towards the pharmacological profile of LsIA.

4.2.2 Significance

— To identify and understand the pharmacological profile of novel α-conotoxins at nAChR.

— To understand the structure–functional relationship of unique α-conotoxin features.

— To understand the functional implication of α5 subunit in conotoxin pharmacology.
4.3 RESULTS

4.3.1 LsIA inhibition of recombinant nAChR subtypes

I determined LsIA potency and selectivity at neuronal nAChRs by examining its effect on ACh-evoked currents mediated by different nAChR subunit combinations expressed in *Xenopus* oocytes. ACh was applied every 5 min and ACh-evoked membrane currents were assessed. LsIA (1 µM) completely inhibited ACh-evoked current amplitude mediated by α7, α3β2 and α3α5β2 nAChR subtypes. However, LsIA (3 µM) inhibited α3β4 nAChR-mediated currents by only 40 ± 5% (n = 6) and had no effect on α9α10 and α4-containing nAChR subtypes (n = 4–8) [Figure 4.1].

![Figure 4.1: α-Conotoxin LsIA selectivity for various nAChR subunit combinations expressed in *Xenopus* oocytes. (A) Superimposed traces of ACh-evoked currents in the absence (control) and presence of various LsIA concentrations at α7, α3β2 and α3α5β2 nAChR subtypes. (B) Concentration–response curves for LsIA inhibition of different nAChR subtypes gave IC$_{50}$ values of 10.3 nM (95% CI, 8.8–12.1 nM; nH = –1.3 ± 0.1) at α3β2; 31.2 nM (95% CI, 26.1–37.3 nM; nH = –1.1 ± 0.1) at α3α5β2; and 10.1 nM (95% CI, 8.7–11.6 nM; nH = –2.1 ± 0.4) at α7 subtypes. LsIA (1 µM) completely inhibited ACh-evoked currents mediated by α3β2, α3α5β2 and α7 nAChRs. Data represents mean ± SEM, n = 3–6.](image-url)
LsIA reversibly inhibited α3α5β2 about three times less potently (IC50 = 31.2 nM) than it did the α3β2 subtype (IC50 = 10.3 nM). α5 subunit expression was confirmed by desensitisation experiments described in the Material and methods section. As shown in a previous study [7], the time course response of the α3α5β2 nAChR subtype showed notably faster desensitisation of ACh-evoked currents compared with those of the α3β2 nAChR subtype [Figure 4.2 Inset]. Concentration–response curves for LsIA inhibition of ACh-evoked currents at different nAChR subtypes and their corresponding IC50 value exhibit the following selectivity sequence: α7 (10.1 nM) ≅ α3β2 (10.3 nM) > α3α5β2 (31.2 nM) [Figure 4.1].

The on- and off-rates (k_on and k_off) for nAChR inhibition by the peptide were obtained from the time course of responses in the presence and upon washout of the peptide [Figure 4.2 and 4.3 and Table 4.1]. LsIA (10 nM) recovered more slowly from block at the α7 nAChR subtype than at the α3β2 and α3α5β2 nAChR subtypes, giving a k_i of 1.47 x 10^{-9} M [Figure 4.3 and Table 4.1]. This k_i value is 6.8-fold lower than IC50. A similar trend was observed at the α3α5β2 subtype, which had a k_i value 3.3-fold lower than IC50 [Table 4.1].
Figure 4.2: Kinetics of LsIA inhibition of peak ACh-evoked current amplitude as a function of time. Onset (filled bar) of LsIA (30 nM) block and recovery (open bar) upon washout at α3β2 (A) and α3α5β2 (B) nAChR subtypes. *Inset:* Representative ACh (50 µM)-evoked currents in oocytes expressing (A) α3β2 and (B) α3α5β2 nAChR subtypes.
Figure 4.3: Kinetics of α-conotoxin LsIA block and recovery at the α7 nAChR subtype. (A) Representative ACh-evoked currents in the absence and presence of LsIA in oocytes expressing α7 nAChR. LsIA (10 nM) was bath applied for 5 min before washout. Responses to a 1-s pulse of ACh (200 µM) and toxin for on-rate, and ACh alone for off-rate, kinetics was measured at various time intervals. C is the ACh control response before the toxin was applied. (B) LsIA inhibition of peak ACh-evoked current amplitude as a function of time. Onset (filled bar) of block by LsIA (10 nM) and recovery (open bar) upon washout at the α7 nAChR subtype.
<table>
<thead>
<tr>
<th>α-Conotoxin LsIA’s kinetic constants for blocking nAChR subtypes&lt;sup&gt;a&lt;/sup&gt;.</th>
<th>( k_{\text{on}} ) (min&lt;sup&gt;−1&lt;/sup&gt; M&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>( k_{\text{off}} ) (min&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>( k_i ) (M)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α&lt;sub&gt;3&lt;/sub&gt;β&lt;sub&gt;2&lt;/sub&gt;</td>
<td>6.93 (4.99–8.87) x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.052 (0.910–1.194)</td>
<td>15.2 x 10&lt;sup&gt;−9&lt;/sup&gt;</td>
<td>10.3 x 10&lt;sup&gt;−9&lt;/sup&gt;</td>
</tr>
<tr>
<td>α&lt;sub&gt;3&lt;/sub&gt;α&lt;sub&gt;5&lt;/sub&gt;β&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.26 (0.28–2.24) x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>1.205 (0.766–1.644)</td>
<td>9.6 x 10&lt;sup&gt;−9&lt;/sup&gt;</td>
<td>31.2 x 10&lt;sup&gt;−9&lt;/sup&gt;</td>
</tr>
<tr>
<td>α&lt;sub&gt;7&lt;/sub&gt;</td>
<td>1.35 (1.17–1.53) x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>0.199 (0.182–0.215)</td>
<td>1.47 x 10&lt;sup&gt;−9&lt;/sup&gt;</td>
<td>10.1 x 10&lt;sup&gt;−9&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers in parentheses are 95% CI. Mean of data from n = 6–8 experiments.
4.3.2 Influence of C-terminal carboxylation and N-terminal truncation of LsIA

Conotoxins typically have a high degree of post-translational modification [8, 9], with C-terminal amidation shown to influence structure and enhance target specificity [9]. Native α-conotoxin LsIA also undergoes C-terminal amidation. Our collaborators synthesised a C-terminally carboxylated analogue of LsIA (LsIA#) to examine its activity at different nAChR subtypes. Interestingly, LsIA# was ~three-times less potent at the α7 subtype and ~three-fold more potent at the α3β2 subtype [Figure 4.4].

![Graph A](image1.png)

**Figure 4.4**: Influence of N-terminus truncation and C-terminus carboxylation of LsIA on ACh-evoked current inhibition at the α7 (A) and α3β2 (B) nAChR subtypes. Concentration–response curves for LsIA#, [Δ1]LsIA and [Δ1–2]LsIA inhibition gave IC$_{50}$ values of 30.7 nM (95% CI, 21.7–43.4 nM; $n_H = -1.5 \pm 0.4$), 23 nM (95% CI, 19.3–27.5 nM; $n_H = -2.0 \pm 0.3$) and 44.1 nM (95% CI, 37.1–52.4 nM; $n_H = -2.5 \pm 0.4$) at α7, respectively; and 3.3 nM (95% CI, 2.2–5.1 nM; $n_H = -1.0 \pm 0.2$), 56.2 nM (95% CI, 40.4–78.2 nM; $n_H = -1.0 \pm 0.2$) and 92.4 nM (95% CI, 69.3–123.3 nM; $n_H = -0.9 \pm 0.1$) at α3β2, respectively. The broken line represents the carboxylated LsIA (LsIA#) concentration–response curve. Data represents means ± SEM, n = 4–7.
α-Conotoxin LsIA contains a unique N-terminal serine residue. Truncation of the four residue N-terminal tail of α-GID has been shown to influence activity at neuronal nAChRs [10]. Our collaborators synthesised two truncated analogues, [Δ1]LsIA (lacking the serine at position 1) and [Δ1-2]LsIA (lacking the serine at position 1 and glycine at position 2). [Δ1]LsIA- and [Δ1-2]LsIA-amidated peptides were two-fold and four-fold less potent at the α7 subtype than LsIA, respectively [Figure 4.4 and Table 4.2]. They were also five-fold and nine-fold less potent at the α3β2 subtype than LsIA, respectively [Figure 4.4 and Table 4.2]. These results indicate that the N-terminal sequence plays a major role in LsIA potency.

Table 4.2: Half-maximal inhibitory concentrations (IC₅₀) and Hill slope (nᵢ) values from concentration–response curves for LsIA and its analogues at the α3β2 and α7 nAChR subtypes.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC₅₀ (nM)</th>
<th>nᵢ</th>
<th>IC₅₀ (nM)</th>
<th>nᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td>LsIA</td>
<td>10.3 (8.8–12.1)</td>
<td>–1.3 ± 0.1</td>
<td>10.1 (8.7–11.6)</td>
<td>–2.1 ± 0.4</td>
</tr>
<tr>
<td>[Δ1] LsIA</td>
<td>56.2 (40.4–78.2)</td>
<td>–1.0 ± 0.2</td>
<td>23 (19.3–27.5)</td>
<td>–2.0 ± 0.4</td>
</tr>
<tr>
<td>[Δ1–2] LsIA</td>
<td>92.4 (69.3–123.3)</td>
<td>–0.9 ± 0.1</td>
<td>44.1 (37.1–52.4)</td>
<td>–2.5 ± 0.3</td>
</tr>
<tr>
<td>LsIA#</td>
<td>3.3 (2.2–5.1)</td>
<td>–1.0 ± 0.2</td>
<td>30.7 (21.7–43.4)</td>
<td>–1.5 ± 0.4</td>
</tr>
</tbody>
</table>

a Numbers in parentheses are 95% CI. Mean of data from n = 5–8 experiments.
4.4 DISCUSSION

α-Conotoxins evolved as selective probes that target nicotinic receptors, parallel with the phylogenetic evolution of marine cone snails belonging to the genus Conus. This corresponds with the structural similarities between various conotoxins, including conserved cysteine framework and residues. The cysteine framework of α-conotoxins (CC-C-C) divides the peptide sequence into two loops, from which they are classified into various subclasses. A high-sequence homology or conserved residues are observed across various α-conotoxins [Table 4.3].

Table 4.3: Sequence alignment of LsIA and α-conotoxins.

<table>
<thead>
<tr>
<th>α-Conotoxin</th>
<th>Sequence</th>
<th>nAChR selectivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ls1a</td>
<td>SGCCSNPACRVNNPNIC*</td>
<td>α3β2≈a7&gt;α3α5β2</td>
<td>This study</td>
</tr>
<tr>
<td>GID</td>
<td>IRDγCCSNPACRVMNOHV#</td>
<td>α7≈a3β2&gt;α4β2</td>
<td>[10]</td>
</tr>
<tr>
<td>ArIA</td>
<td>IRDECCSNPACRVMNOHVCR#</td>
<td>a7&gt;a3β2</td>
<td>[11]</td>
</tr>
<tr>
<td>ArIB</td>
<td>DECCSNPACRVMNOHVCR#</td>
<td>a7≈α6β2*&gt;α3β2</td>
<td>[11]</td>
</tr>
<tr>
<td>MII</td>
<td>GCCSNPVCHELHSNL*</td>
<td>α3β2≈α6β2*&gt;α6β4</td>
<td>[12]</td>
</tr>
<tr>
<td>RegIIA</td>
<td>GCCSHPACNVNNPHIC*</td>
<td>α3β2&gt;α3β4&gt;α7</td>
<td>[13]</td>
</tr>
<tr>
<td>OmIA</td>
<td>GCCSHPACNVNNPHICG*</td>
<td>α3β2&gt;α7&gt;α6β2*</td>
<td>[14]</td>
</tr>
<tr>
<td>GIC</td>
<td>GCCSHPACAGNNQHIC*</td>
<td>α3β2≈α6β2*&gt;α7</td>
<td>[15]</td>
</tr>
<tr>
<td>Mr1.1</td>
<td>GCCSHPACSVNPDIC*</td>
<td>α3β2&gt;α3β4&gt;α7</td>
<td>[16]</td>
</tr>
<tr>
<td>PnIA</td>
<td>GCCSLLPPCAANNPDY*</td>
<td>α3β2&gt;a7</td>
<td>[17]</td>
</tr>
<tr>
<td>AuIB</td>
<td>GCCSYPPCFATNPDC*</td>
<td>α3β4</td>
<td>[18]</td>
</tr>
<tr>
<td>BuIA</td>
<td>GCCSTPPCAVLY---C*</td>
<td>β2*&gt;β4*</td>
<td>[19]</td>
</tr>
<tr>
<td>RgIA</td>
<td>GCCSDPRCRYR---CR#</td>
<td>a9a10&gt;α7</td>
<td>[20]</td>
</tr>
</tbody>
</table>

* denotes an amidated C-terminus; #, free carboxyl C-terminus; γ, γ-carboxyglutamate. Cysteine residues are highlighted yellow. Residues homologous to LsIA are highlighted grey.

Previous studies that aimed to understand the molecular interactions involved in α-conotoxin subtype-selective antagonism of nACHRs used X-ray studies of co-crystal AChBP and ImI structures, [A10L,D14K]PnIA and [A10L]TxIA. They revealed significant interaction between loop 1 (residues between Cys II and III) and the principal face of the receptor [21, 22]. The loop 2 residues of the peptide were shown to majorly interact with the complementary side of the receptor. These studies identified the general role of loop1 in
peptide affinity, while loop2 contributes to the subtype selectivity of the α-conotoxin. Also, structurally, loop1 plays a vital role in the secondary structure of the peptide (presence of α-helix). NMR studies revealed significant disruption of the peptide's classic ω-globular structure upon mutagenesis [23]. This was also indicated through the presence of conserved residues within loop1 of various subclasses of α-conotoxins [Table 4.3]. AChBP co-crystal studies also showed significant hydrogen bonding and van der Waals interactions between the Ser-, Pro- and Gly-conserved residues of loop1 in almost all α-conotoxins and the receptor binding pocket [21].

α-Conotoxin LsIA is a new peptide isolated from C. limpusi that potently inhibits nAChRs. It is an α4/7 conotoxin and exhibits equipotent inhibition at α3β2 and α7 subtypes. Pairwise sequence alignment shows strong homology between LsIA and previously characterised α-conotoxins that target the α3β2 and α7 nAChR subtypes.

LsIA exhibits two sequence motifs, namely –SXPA– and –NNP–. These motifs are also found among OmIA, RegIIA, Mr1.1 and ArIB conotoxins, which have a similar pharmacological profile to LsIA [Table 4.3]. The structural and functional significance of the –SXPA– motif within loop1 of conotoxins was discussed earlier.

Mutation of Ser and Pro residues to Ala in α-conotoxin GID led to significant activity loss at α7 and α7/α3β2 nAChR subtypes, respectively [24]. A similar interaction between LsIA and α7 and α3β2 nAChR subtypes is expected.

Along with conserved motifs, LsIA exhibits a unique N-terminal Ser residue. In this study, I examined the functional implication of the N-terminal sequence, including the most common post-transcriptional modification found in the α-conotoxins: C-terminal amidation. The
influence of the N-terminal sequence was previously examined in α-conotoxin GID. Initial studies with [Δ1-4]GID showed significant activity loss at the α4β2 nAChR subtype, but no change in IC₅₀ was observed at α7 and α3β2 nAChR subtypes [10]. Interestingly, a noticeable loss in activity at α7 and α3β2 subtypes was observed when the first three residues were deleted ([Δ1-3]GID). However, [Δ1] and [Δ1-2]GID truncation only affected α3β2 inhibition [24].

A similar trend was observed in LsIA, where truncation of first and second residues lead to considerable loss in activity at the α7 and α3β2 nAChR subtypes [Figure 4.4 and Table 4.2]. This activity loss was more prominent at the α3β2 subtype, with ~5-fold reduction, than at the α7 subtype, which only showed ~2-fold less activity, with [Δ1]LsIA. This effect was further amplified for [Δ1-2]LsIA, which triggered ~9-fold and ~4-fold activity loss at α3β2 and α7 nAChR subtypes, respectively. Surprisingly, the most striking effect on peptide function was observed when the C-terminus was carboxylated (LsIA#). This modification led to a ~3-fold increase in potency at the α3β2 nAChR subtype, but caused an opposite effect at the α7 nAChR subtype, with a ~3-fold activity loss.

In α-conotoxin GID, C-terminus carboxylation only influenced peptide activity at the α3β2 nAChR subtype [24]. One explanation for this is the formation of intra-molecular salt bridges or hydrogen bonds when the free C-terminus carboxyl group is incorporated into LsIA.

In this study, I report a difference in pharmacological activity of LsIA at α5-containing nAChRs, with LsIA being 3-fold less potent at the α3α5β2 subtype than at the α3β2 subtype. Unlike the α3 subunit, the α5 subunit is an auxiliary subunit that does not form functional receptors when expressed with β2 or β4 [25]. However, its role as the fifth subunit in nAChR pharmacology and physiology has recently gained prominence, because it influences
conductance [26], agonist sensitivity [27] and ion permeability [7]. Previous studies of α-conotoxin Vc1.1 at α5-containing nAChRs showed no significant IC$_{50}$ change [28]. Interestingly, kinetic analysis of LsIA inhibition revealed faster onset of block ($k_{on}$) at the α3α5β2 subtype than at the α3β2 subtype, which contributes to the 3-fold lower $k_i$ value at α3α5β2. No significant difference in $k_{off}$ was observed between the α3α5β2 and α3β2 nAChR subtypes. LsIA off-rate kinetics was slower at the α7 subtype than at the α3β2 and α3α5β2 subtypes. Also, a significant ~ 7-fold difference was observed in the $k_i$ and IC$_{50}$ values at the α7 subtype. This difference could be due to a higher Hill slope ($n_H$) of −2.1 at the α7 subtype than at the other subtypes, indicating positive peptide binding cooperativity.

4.5 SUMMARY AND CONCLUSION

LsIA is the first peptide isolated from the venom of $C. limbusi$. Structure–activity data collected in this study indicates N-terminal and C-terminal sequences have unique and specific roles in the pharmacology of this peptide. The N-terminal sequence (Ser1 and Gly2 residues) contributes to nAChR binding affinity, while C-terminus modification imparts subtype selectivity. While a number of α-conotoxins that potently inhibit α3β2 and α7 nAChR subtypes exist [Table 4.3], I report differences between α-conotoxin LsIA’s pharmacology and kinetics of inhibition in the presence and absence of the auxiliary α5 subunit. In conclusion, this study provides vital information to improve our understanding of nAChR inhibition and aid development of novel analogues with improved subtype selectivity.
4.6 References


CHAPTER 5

Novel αD-conotoxin GeXXA from Conus generalis reveals unique nAChR binding mechanism

http://www.coneshell.net

Conus generalis
5.1 INTRODUCTION

As mentioned earlier (Chapter 1, Section 2), various bioactive peptides have been identified from the venom of cone snails belonging to the genus *Conus*, labelled conotoxins. The disulphide-rich peptides targeting nAChRs are grouped into various superfamilies, based on their structural and functional properties: the A- (α- and αA-conotoxins), M- (ψ-conotoxins), S- (αS-conotoxins) and C-conotoxins (αC-conotoxins) [1].

Conotoxins from the D-superfamily were only recently discovered and functionally characterised [2, 3]. First isolated from the venom *C. vexillum*, three novel αD-conotoxins, αD-VxXIIA, αD-VxXIIB and αD-VxXIIC, were functionally characterised as potent inhibitors of α7- and β2-containing neuronal nAChRs [2]. Kauferstein *et al.* (2009) identified and analysed two new αD-conopeptides from the vermivorous snails *Conus mustilinus* (αD–M) and *Conus capitaneus* (αD–Cp). These peptides have a pharmacological profile similar to that of αD-VxXIIA with a 72% sequence homology [Table 5.1] [4].

In the present study, we identified novel αD-conotoxin GeXXA from the venom of *Conus generalis*. Functional characterisation revealed αD-GeXXA non-selectively inhibits nAChRs, an action not previously identified in αD-conotoxins, which usually exhibit characteristic subtype selectivity. We also report the first synthesis of a functionally active monomeric αD-GeXXA isoform.
Table 5.1: Sequence alignment of αD-conotoxin GeXXA with other, previously discovered αD-conotoxins.

<table>
<thead>
<tr>
<th>αD-Conotoxin</th>
<th>Sequence</th>
<th>nAChR selectivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GeXXA</td>
<td>DVH-RPCQSVRPGRVWGKCCITLRCSTMCCARADCTCVYHTWRGHGCSCV-</td>
<td>α7&gt;α3*&gt;αβδγ</td>
<td>This study</td>
</tr>
<tr>
<td>Ms20.3</td>
<td>DVR--EQQVTPTGSSWGKCCMTRCMCTMCARSGCTCVYHWRRGHCSCPG</td>
<td>α7&gt;β2*</td>
<td>[4, 5]</td>
</tr>
<tr>
<td>Cp20.3</td>
<td>EVQ--EQQVTPTGSSWGKCCMTRCMCTMCARSGCTCVYHWRRGHCSCPG</td>
<td>α7&gt;β2*</td>
<td>[4, 5]</td>
</tr>
<tr>
<td>VxXXA</td>
<td>D--EQQ VTPTGSKWGRCCCLNRCPGMCPPASHCYCVYHWRGRGHGCSC--</td>
<td>α7&gt;α3β2</td>
<td>[2]</td>
</tr>
<tr>
<td>VxXXB</td>
<td>DDE-SECIINRTDSPWGRCCRTMCGSMCCPRNGCTCVYHWRRGHCSCPG</td>
<td>α7&gt;β2*&gt;αβδγ</td>
<td>[2]</td>
</tr>
<tr>
<td>VxXXC</td>
<td>DLR--QCTRNPSTWGRCCLNPCCGFCCPRSGCTCAYNWRRGIYSC--</td>
<td>α7&gt;β2*</td>
<td>[2]</td>
</tr>
</tbody>
</table>
5.2 AIMS

5.2.1 Characterisation of αD-conotoxin GeXXA isolated from *Conus generalis*

— To determine the pharmacological potency and selectivity of dimeric and monomeric GeXXA at recombinant nAChR subtypes expressed in *Xenopus* oocytes using the two-electrode voltage clamp technique.

5.2.2 Receptor hybrid studies

— To identify the site where dimeric and monomeric αD-conotoxin GeXXAs bind with nAChRs, and the molecular mechanism behind their inhibition of nAChRs.

5.2.3 Significance

— This study, in conjunction with modelling simulations our collaborators are doing, could shed light on the molecular mechanism behind the αD-conotoxins interaction with nAChR subtypes.

— αD-conotoxins represent a novel class of nAChR-inhibiting peptides as potential neurophysiological tools and drug therapeutics. This study could also provide valuable information that could aid novel peptide design and the development of future drugs.
5.3 RESULTS

5.3.1 Concentration-dependent inhibition of α3-containing nAChRs by αD-conotoxin GeXXA

To examine the pharmacological activity of αD-conotoxin GeXXA at nAChRs, it was tested on ACh-evoked currents mediated by different nAChR subtypes expressed in *Xenopus* oocytes. GeXXA (1 µM) had no or little effect on α4β2 and α4β4 subtypes, but inhibited α3β4 and muscle (α1β1εγ) by 70–80%, and inhibited α9α10, α7 and α3β2 subtypes completely [Figure 5.1(A)].

Concentration–response curves showed GeXXA was more selective for α3-containing than α4-containing nAChR subtypes [Figure 5.1(B)]. It was most potent at the α7 nAChR subtype, with an IC$_{50}$ of 210 nM (95% CI, 174–253) and Hill slope ($n_H$) of −1.6 ± 0.2. The IC$_{50}$ (95% CI) and Hill slope values from concentration–response curves for the inhibition of various nAChR subtypes are summarised in Table 5.2.

**Table 5.2: Pharmacological profile of dimeric αD-conotoxin GeXXA inhibition of various nAChRs.**

<table>
<thead>
<tr>
<th>nAChR subtype</th>
<th>IC$_{50}$ (95% CI)</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>α7</td>
<td>210 nM (174–253)</td>
<td>−1.6 ± 0.2</td>
</tr>
<tr>
<td>α3β2</td>
<td>498 nM (407–609)</td>
<td>&gt; −1.0</td>
</tr>
<tr>
<td>α3β4</td>
<td>614 nM (491–768)</td>
<td>−2.1 ± 0.4</td>
</tr>
<tr>
<td>α4β2</td>
<td>&gt; 3 µM</td>
<td>–</td>
</tr>
<tr>
<td>α4β4</td>
<td>&gt; 3 µM</td>
<td>−0.9 ± 0.2</td>
</tr>
<tr>
<td>αβδγ</td>
<td>743 nM (606–911)</td>
<td>−1.6 ± 0.2</td>
</tr>
</tbody>
</table>

All data points indicate mean ± SEM; n = 4–7.
Figure 5.1: Dimeric αD-conotoxin GeXXA inhibition of various nAChR subtypes. (A) Superimposed current traces obtained in the absence (control) and presence of 300 nM of dimeric αD-conotoxin GeXXA inhibition at the (i) α7 nAChR subtype and (ii) α3β2 nAChR subtype. (B) Concentration–response curves obtained for dimeric αD-conotoxin GeXXA inhibition of nAChR subtypes. Dimeric GeXXA (3 µM) non-selectively inhibited all nAChR subtypes, except α4β2. The highest potency of inhibition was observed at the α7 nAChR subtype. The IC$_{50}$ (95% CI) and Hill slope ($n_H$) values from concentration–response curves are summarised in Table 5.2. All data points represent the mean ± SEM; n = 4–7.
5.3.2 Monomeric αD-conotoxin GeXXA selectively inhibits the α9α10 nAChR subtype

We describe for the first time monomeric αD-conotoxin GeXXA inhibition of nAChRs. When tested on various nAChR subtypes, 1 µM of monomeric GeXXA completely inhibited the α9α10 nAChR subtype, but had little or no activity at other nAChR subtypes. This antagonism of α9α10 was also reversible [Figure 5.2(A)], unlike dimeric αD-conotoxin GeXXA which irreversibly inhibited α9α10 subtype [data not shown]. The concentration–response curve obtained for the monomeric GeXXA at α9α10 nAChR gave an IC50 of 198 nM (95% CI, 164–238; nH = −1.7 ± 0.3) [Figure 5.2(B)].

Figure 5.2: α9α10 hybrid nAChR inhibition by monomeric αD-conotoxin GeXXA. (A) Superimposed ACh-evoked currents obtained in the absence (control) and presence of 1 µM of monomeric αD-conotoxin GeXXA at human and rat α9α10 nAChR subtype. (B) Monomeric αD-conotoxin GeXXA was 10-fold less potent at the human α9α10 nAChR subtype than at the rat α9α10 nAChR subtype, whereas no change was observed at the hybrid hα9rα10 receptor. All data points indicate mean ± SEM; n = 4–7.
5.3.3 α9α10 hybrid nAChR studies reveal the site of monomeric αD-conotoxin GeXXA binding

The difference between dimeric and monomeric αD-conotoxin GeXXA inhibition of the α9α10 nAChR subtype was further investigated on hybrid receptors using human and rat α9 and α10 nAChR subunits. When initially tested on human α9α10, monomeric α-D GeXXA was 10-fold less potent compared with rat α9α10. However, similar potency was observed on the hybrid hα9rα10 receptor to that of rat α9α10 subtype [Figure 5.2(B)].

Concentration–response curves for monomeric αD-conotoxin GeXXA inhibition of rat, hybrid and human α9α10 nAChR subtypes gave IC₅₀ values of 198 nM (95% CI, 164–238; n_H = –1.7 ± 0.3), 224 nM (95% CI, 194–258; n_H = –1.4 ± 0.1) and 2.02 μM (95% CI, 1.82–2.25; n_H = –1.7 ± 0.1), respectively. All data points represent the mean ± SEM; n = 4–7.
5.4 DISCUSSION AND CONCLUSION

Conotoxins are invaluable pharmacological tools, each with unique structural and functional features. αA-Conotoxins consist of up to 30 amino acid residues and generally characterised by their competitive antagonism of muscle nAChRs, while ψ-Conotoxins are known to be non-competitive muscle nAChR antagonists [6].

While there have been significant advances in developing our understanding of nAChRs, much of their physiological role and pharmacology of these unique peptides on nAChRs is still unknown.

D-superfamily conotoxins consist of 45–50 amino acid residues and have characteristic 10 cysteine residue framework [Table 5.1] [5]. They have various structural and functional features. First, several post-translational modifications, such as carboxylation and hydroxylation, are seen in native peptides. Second, native peptides occur as homodimers connected by disulphide bonds [4]. αD-conotoxin GeXXA is a disulphide-linked homodimer of two identical peptides and consists of 50 amino acid residues with 10 cysteines [Figure 5.3].

![Figure 5.3: The sequence and disulphide linkage of αD-conotoxin GeXXA. For clarity, only the N-terminal sequence of the second subunit is shown.](image-url)
Previous pharmacologically characterised αD-conotoxins were shown to be non-competitive muscle and neuronal nAChR antagonists, with selectivity for α7- and β2-containing receptors [2]. However, even though each GeXXA peptide chain shared high homology with other known αD-conotoxins [Table 5.1], αD-GeXXA exhibited concentration-dependent inhibition of various nAChR subtypes, with selectivity for α7 and α3-containing nAChRs. αD-GeXXA inhibits α7 (IC$_{50}$ 210 nM), α3β2 (IC$_{50}$ 498 nM), α3β4 (IC$_{50}$ 614 nM) and muscle (α1β1δγ) (IC$_{50}$ 743 nM) nAChR subtypes, and weakly inhibits α4β2 and α4β4 nAChR subtypes (IC$_{50}$ > 3 µM). However, its inhibition of both rat and human α9α10 nAChR subtypes was irreversible, indicating that it binds tightly to these receptors.

The crystal structure of native αD-GeXXA [contributed to this study by collaborators] provided further insight into its unique pharmacological activity and mechanisms of action. Structures at 1.5 Å resolution revealed an N-terminal domain (NTD, residues 1–20) and a C-terminal domain (CTD, residues 21–50) in each peptide chain, arranged in an approximately 2-fold symmetric architecture. An interchain disulphide bond between Cys6 of one chain and Cys18 of another chain within the NTD facilitates αD-GeXXA dimerisation [Figure 5.4].

Figure 5.4: The crystal structure of αD-conotoxin GeXXA. The NTDs of two GeXXA subunits are shown in green and light green. The CTDs are represented in orange and light orange. The disulphide bonds are colored yellow. [Provided to this study by collaborators].

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We report the first synthesis of αD-GeXXA’s C-terminal domain (CTD) and its activity. The isolated CTD adopts a canonical inhibitory cysteine knot (ICK) disulphide linkage. When tested on various nAChR subtypes, αD-GeXXA-CTD selectively and reversibly antagonises the rat α9α10 nAChR subtype, with an IC₅₀ of 198 nM, but is 10-fold less potent at the human α9α10 nAChR subtype (IC₅₀ 2.02 µM). Hybrid receptor studies indicate that the α10 nAChR subunit contributes to the difference between αD-GeXXA’s potency for human and rat α9α10 nAChR subtype.

These results indicate that the α10 nAChR subunit is a major binding site for αD-conotoxin GeXXA. Peptide sequence alignment revealed residue differences between the extracellular domains of mature human and rat α10 subunits [Figure 5.5]. Molecular dynamics simulations [contributed to this study by collaborators] indicate a two-site binding interaction between αD-GeXXA and the His7 of the α10 subunit. Recently, similar hybrid-receptor studies of the α9α10 nAChR subtype found that α-conotoxin Vc1.1 and RgIA preferentially bound to the α10α9 pocket [3, 7]. These findings are also supported by electrophysiological data that show Hill slope values > 1 for αD-GeXXA interaction with various nAChRs, which indicates a positive cooperative binding mode. The Hill equation has been extensively used to aid understanding of the complex pharmacokinetic and pharmacodynamic models of drug–receptor interaction [8-10].

This study is the first to report monomeric αD-conotoxin activity, and gives insight into αD-conotoxin GeXXA’s novel binding mechanism at nAChRs.
Figure 5.5: Pairwise sequence alignment of the ACh-binding site of the mature α10 subunit peptide. This alignment reveals the difference between human and rat residues (background white). The mature α10 subunit peptide lacks the signal sequence (first 25 residues). Peptide sequences were obtained from the NCBI Protein online database.
5.5 References


CHAPTER 6

Dicarba modification of α-conotoxins exhibits differential selectivity

for nAChRs and GABA_B receptors

Conus victoriae
6.1 INTRODUCTION

6.1.1 Conotoxins - *Conus victoriae* and *Conus regius*

α-Conotoxins in the venom of the marine cone snails, are short disulphide-bonded peptides ranging from 12–30 amino acids. These peptides specifically target nAChR subtypes and have potential therapeutic applications. *Conus victoriae* and *Conus regius* venoms gained a special interest, due to identification of two novel peptides, α-conotoxins Vc1.1 and RgIA, respectively. These peptides have exhibited analgesic properties in rat neuropathic pain models [1, 2].

α-Conotoxins Vc1.1 and RgIA belong to the cysteine framework I family (CCXₙCXₘC) and exist in a globular conformation with I–III and II–IV disulphide connectivity in native form [3]. Both of these peptides were first identified using a cDNA approach [4, 5]. Vc1.1 is 16 amino acids in length and belongs to the α4/7 subclass of conotoxins. Like most conotoxins, Vc1.1 exhibited various post-translational modifications, including C-terminus amidation, hydroxylation of Pro6 and γ-carboxylation of Glu14 residues. This modified peptide, called vc1a, was identified in the venom of *Conus victoriae* and is the native form of Vc1.1 [6]. On the other hand, RgIA is an α4/3 conotoxin, consisting of 12 amino acids, and lacks post-translational modification.

6.1.2 The molecular mechanism of analgesia

The involvement of nAChRs in pain pathways is now well established [7]. However, the role of nAChR subtypes in pain pathways and the molecular mechanism of these pathways are yet to be elucidated. Various small molecule compounds acting as α4β2 and α7 nAChR antagonists are the primary leads for pain therapeutics [8]. However, the identification of α-conotoxins as nAChR subtype-selective probes has led to the development of novel drugs to
treat neuropathic pain [9]. A major breakthrough came with the discovery of α-conotoxins Vc1.1 and RgIA, which have acute and cumulative anti-nociceptive effects in chronic constriction nerve injury (CCI) and partial sciatic nerve ligation (PSNL) rat models of neuropathic pain [1, 2]. Subcutaneous or intramuscular administration of these peptides alleviated mechanical hyperalgesia and allodynia. This effect lasted up to 24 h post-administration without the rats developing a tolerance to the drug. In addition, Vc1.1’s analgesic effects lasted up to one week after treatment was ceased [10].

The pharmacological profiles of Vc1.1 and RgIA were only established three years after their analgesic properties were identified. Initial electrophysiological studies using synthetic Vc1.1 confirmed the α3β4 nAChR subtype is its pharmacological target, with an IC$_{50}$ of 3 µM [11]. However, concurrent studies revealed Vc1.1 and RgIA also potently inhibit the α9α10 nAChR subtype, with an IC$_{50}$ of 64 nM and 5 nM, respectively [4, 12]. These studies suggested the α9α10 nAChR subtype as a novel therapeutic target for neuropathic pain.

Recent studies have also uncovered a new biological target for both of these peptides. Vc1.1 and RgIA inhibit high voltage-activated (HVA) calcium channel currents via GABA$_B$ receptor activation, which suggests these α-conotoxins may mediate a novel pain pathway [13-15].

6.1.3 The α9α10 nAChR subtype: expression and function

Of the various nAChR subunits, only the α7 and α9 subunits form homopentamers, and the α10 subunit assembles only with the α9 subunit to form functional receptors [16]. Co-assembly with the α10 subunit also significantly increases the expression of α9α10 nAChRs [17]. α9 and α10 subunits are expressed in various tissues, such as skin, dorsal root ganglia (DRG), pars tuberalis of the pituitary gland, and cochlear hair cells [10]. In the auditory
system, which is composed of cochlear hair cells, α9-containing receptors mediate the synaptic transmission, and therefore modulate auditory stimuli [18]. In skin, α9 and α10 subunits regulate the cell-adhesion properties of keratinocytes and modulate wound-healing (re-epithelialisation) [19, 20]. While α9 and α10 subunits are implicated in cholinergic signalling in the above-mentioned tissues, their precise subunit stoichiometry is yet to be determined. Although α9 can form a functional homomeric receptor, the expression of α10 subunit was found to be necessary for synaptic transmission in cochlear cells [21]. Furthermore, Plazas et al. (2005) showed that α9α10 when recombinantly expressed in oocytes, stoichiometry of (α9)2(α10)3 was observed [22].

6.1.4 α-Conotoxin drug development: limitations and strategies

The nature of the compound is an important aspect of drug development, because it determines the drug’s side effects. While the peptidic nature of α-conotoxins is a unique advantage for drug development, it is also their Achilles’ heel. α-Conotoxins are susceptible to natural peptidic degradation, which drastically affects their bioavailability and half-life. The functional impediment of α-conotoxins via peptidic-degradation can result from a proteolytic attack on the N- and/or the C-terminus of the peptide, or from disruption (scrambling) of the disulphide linkage within the α-conotoxin.

Various strategies to improve α-conotoxin stability have been implemented, such as cyclisation and selenocysteine modification. Peptide cyclisation has been shown to counter the proteolytic degradation problem in Vc1.1, RgIA and other peptides [23, 24]. The α-conotoxin three-dimensional structure is dominated by a helical structure shaped by a conserved disulphide framework. The most predominant I–III and II–IV disulphide connectivity is found in native α-conotoxins and folds the peptide into globular conformation. However, the disulphide connectivity of α-conotoxins can interchange under oxidative and
reduced conditions to form ribbon (I–IV and II–III disulphide bonds) or bead (I–II and III–IV disulphide bonds) conformations.

Peptide exploitation in research and clinical settings has also been hampered by the lability of the disulphide bridges that are essential for conotoxin structure and activity [25]. Alteration of disulphide linkage by methods such as selenocysteine modification broke new ground in drug development [26]. Replacing the cysteine bridges with non-reducible dicarba links has also been identified as a novel solution to the lability problem. It was shown to significantly improve α-conotoxin ImI stability, while its functional activity remained comparable with that of the native peptide.

Here, I explore the functional implications of this approach on novel analgesic α-conotoxin Vc1.1 and RgIA, which inhibit HVA calcium channel currents via GABA$_B$ receptor activation and α9α10 and α3β4 nAChR subtypes.
6.2 AIMS

6.2.1 Characterisation of dicarba modified $\alpha$-conotoxins Vc1.1 and RgIA.

- To functionally characterise regioselective dicarba analogues of $\alpha$-conotoxins Vc1.1 and RgIA using two-electrode voltage-clamp technique in *Xenopus* oocytes expressing recombinant nAChR subtypes.

6.2.2 Significance

- This study, in conjunction with structural studies by our collaborators, could provide a detailed understanding of the functional implications of dicarba modification on the Vc1.1 and RgIA activity at nAChRs.

- It could also provide valuable information about the development of dicarba peptides as novel drugs to treat neuropathic pain.
6.3 RESULTS

6.3.2 Regioselective dicarba Vc1.1 analogues exhibit differential activity at α9α10 nAChR subtypes

The functional effects of dicarba modification on Vc1.1 was tested on recombinant α9α10 and α3β4 nAChR subtypes heterogeneously expressed in Xenopus oocytes using the two-electrode voltage-clamp technique. When tested, cis- and trans-[3,16]-dicarba Vc1.1 analogues (3 μM) inhibited the α9α10 nAChR subtype by 24% and 54%, respectively, but the [2,8]-dicarba Vc1.1 analogue showed no inhibitory effects [Figure 6.1(A)]. A similar trend was observed for these peptides at the α3β4 nAChR subtype [Figure 6.1(B)].

![Figure 6.1: Percentage inhibition of ACh-evoked currents by dicarba Vc1.1 analogues (3 μM) at α9α10 (A) and α3β4 (B) nAChR subtypes. All data represent the mean ± SEM; n ≥ 3.](image)

A concentration–response analysis gave an IC$_{50}$ of 2.8 μM (95% CI, 2.0–4.1 μM) and a Hill slope of $-1.3 ± 0.3$ (n = 4) for trans-[3,16]-dicarba Vc1.1. The cis isomer was ~ five-fold less active, with an IC$_{50}$ of 12.5 μM (95% CI, 5.6–27.9 μM) and a Hill slope of $-0.8 ± 0.2$ (n = 4) [Figure 6.2(C)].
Figure 6.2: Dicarba Vc1.1 analogues concentration-dependent inhibition of ACh-evoked currents at the α9α10 nAChR subtype. Superimposed ACh (50 µM)-evoked current traces mediated by (A) α9α10 and (B) α3β4 nAChR subtypes in the absence (control) and presence of 3 µM of [3,16]-trans-dicarba Vc1.1 and [3,16]-cis-dicarba Vc1.1. (C) Concentration–response curves for Vc1.1 and dicarba Vc1.1 analogue inhibition of the α9α10 nAChR subtype. All data represents mean ± SEM; n ≥ 4.

6.3.2 Dicarba modification of RgIA confers similar pharmacological effects to those of Vc1.1

In this study, each of the peptides was compared with native RgIA on α9α10 and α7 nAChRs expressed in *Xenopus* oocytes. Native RgIA reversibly inhibited ACh-evoked currents mediated by α9α10 and α7 nAChRs in a concentration-dependent manner, with an IC$_{50}$ of 5.5 nM ($n_H = -1.3$) and 3.3 µM ($n_H = -0.9$), respectively [4].
At the α9α10 nAChR subtype, cis- and trans-[3,12]-dicarba RgIA analogues (3 µM each) inhibited ACh-evoked currents by 35.4 ± 1.5% and 31.5 ± 2.1% (n = 3), respectively [Figure 6.3(A)(i)]. The cis isomer exhibited an IC50 of 1.15 µM (95% CI, 0.84–1.55 µM) and a Hill slope of −1.9 ± 0.4 (n = 3). The trans isomer was 1.3-fold less active, with an IC50 of 1.47 µM (95% CI, 1.01–2.15 µM) and a Hill slope of −1.2 ± 0.2 (n = 3) [Figure 6.3(B)].

In contrast, at the α7 nAChR subtype, cis-[3,12]-dicarba RgIA inhibition of ACh-evoked currents was similar to that of native RgIA, with an IC50 of 3.73 µM (95% CI, 1.42–9.82 µM) and a Hill slope of −1.6 ± 0.6 [Figure 6.3(B)]. The trans-isomer was inactive at α7 when tested at a concentration of 3 µM [Figure 6.3(A)(ii)]. Replacing the [2,8]-cystine bridge significantly abolished nAChR activity, with both the cis- and trans-[2,8]-dicarba RgIA inactive when tested at 10 µM at the α9α10 nAChR subtype [data not shown].
Figure 6.3: [3,12]-Dicarba RgIA analogue inhibition of ACh-evoked currents at rat α9α10 and human α7 nAChRs expressed in *Xenopus* oocytes. (A) (i) Superimposed ACh (50 µM)-evoked currents mediated by α9α10 nAChRs in the absence (control) and presence of 3 µM each of *cis*-[3,16]-dicarba RgIA and *trans*-[3,16]-dicarba RgIA. (ii) Superimposed ACh (200 µM)-evoked currents at α7 nAChRs in the absence (control) and presence of 3 µM each of *cis*-[3,16]-dicarba RgIA and *trans*-[3,16]-dicarba RgIA. (B) Concentration–response curves for *cis*-[3,12]-dicarba RgIA gave an IC₅₀ of 1.15 µM (95% CI, 0.84–1.55 µM) and a Hill slope of −1.9 ± 0.4 at the α9α10 nAChR. *trans*-[3,12]-dicarba RgIA gave an IC₅₀ of 1.47 µM (95% CI, 1.01–2.15 µM) and a Hill slope of −1.2 ± 0.2 at the α9α10 nAChR. At the α7 nAChR subtype, *cis*-[3,12]-dicarba RgIA had an IC₅₀ of 3.73 µM (95% CI, 1.42–9.82 µM) and a Hill slope of −1.6 ± 0.6, but *trans*-[3,12]-Dicarba RgIA was inactive. All data represent mean ± SEM; n ≥ 3.
6.4 DISCUSSION

6.4.1 Dicarba modification imparts differential pharmacological selectivity to \( \alpha \)-conotoxins Vc1.1 and RgIA

Dicarba modification of the disulphide bridge imparted differential and significant changes to the pharmacological profile of \( \alpha \)-conotoxins Vc1.1 and RgIA. Dicarba modification of the Cys2–Cys8 disulphide bond in Vc1.1 and RgIA, led to complete loss of activity at nicotinic receptors. Both peptides also lost appreciable activity at the \( \alpha 9\alpha 10 \) nAChR subtype when the Cys3–Cys16 disulphide bond in Vc1.1 and Cys3–Cys12 disulphide bond in RgIA were replaced with dicarba linkage. The [3, 12]-dicarba RgIA analogues are more potent than the corresponding dicarba Vc1.1 analogues, and their IC\(_{50}\) change was more prominent than the native peptide. NMR structural analysis [experiments carried out by our collaborators; data not shown] of these peptides shows a clear perturbation of the three dimensional structure of [2,8]-trans-dicarba Vc1.1 and RgIA analogues, which contributes to its loss of activity at nAChR subtypes.

While previous point mutation studies of Vc1.1 and RgIA revealed the key residues interacting with the \( \alpha 9\alpha 10 \) nAChR subtype, this study and molecular dynamics simulations [experiments conducted by our collaborators; data not shown] propose significant interaction between the peptides’ disulphide bonds and ACh-binding pocket. Our collaborators’ molecular docking studies revealed disulphide stacking interaction between the Cys2–Cys8 bond and disulphide of the C-loop of the principal subunit. This interaction was shown to be important for \( \alpha 4/7 \) conotoxin PnIA[A4L,D14K] bound to the AChBP [27]. This model is corroborated by the complete loss of activity of the [2,8]-dicarba-modified Vc1.1 and RgIA peptides. It also explains the significant variance in peptide function, whereas NMR studies [conducted by our collaborators] show no significant perturbation except for trans-
[2,8]-dicarba Vc1.1. Furthermore, when tested, these peptide analogues exhibited opposite effects on their corresponding biological targets. [2,8]-Dicarba Vc1.1 analogues inhibited HVA calcium channel currents via GABA<sub>B</sub> receptor activation in rat DRG neurons, but [3,16]-dicarba Vc1.1 peptides were inactive [results published] [28]. Similar results were also observed with RgIA dicarba analogues [experiments conducted by our collaborators; data not shown]. These results suggest that the CysII–CysIV disulphide bond may interact with GABA<sub>B</sub> receptors in a similar way to that in which the CysI–CysIII bond interact with nAChRs.

6.4.2 Molecular determinants of Vc1.1 and RgIA pharmacological selectivity

Unlike other nAChR subtypes, α9α10 has very unique pharmacological properties, which are yet to be fully understood. Various compounds, such as nicotine, cytisine and epibatine, which are agonists of α7 and other heteromeric receptors, antagonise the α9α10 nAChR subtype [10]. Furthermore, inhibition of α9α10 nAChR by non-cholinergic antagonists, such as strychnine (glycine receptor antagonist), represents a distinctive and mixed pharmacological property for this receptor subtype [17].

Vc1.1 emerged as novel drug to treat neuropathic pain after various studies showed it’s cumulative and long-lasting alleviation of hyperalgesia and allodynia. However, drug development of Vc1.1 was stopped at phase 2A of the human clinical trials, because potencies at rat and human α9α10 nAChR subtype inhibition differed significantly [29]. Various studies that aimed to explain the molecular determinants of Vc1.1 and RgIA identification of their pharmacological target, the α9α10 nAChR subtype, were later conducted [3]. Point mutational analyses of RgIA revealed that region 5–7 interacts with the α9α10 binding pocket [30]. Similar studies using Vc1.1 showed that as well as region 5–7, a second region, 11–15, is needed for Vc1.1 activity at the α9α10 subtype [31]. This study also showed significantly
increased potency at rat and human α9α10 subtypes when Asn9 was changed to hydrophobic residues in Vc1.1 [31].

These studies had two significant effects on the development of Vc1.1 and RgIA as drug leads. Firstly, they elucidated the molecular mechanism of inhibition at α9α10, providing vital information about the structure–function relationship between these two α-conotoxin classes. Secondly, peptide analogues developed in these studies with increased activity at α9α10 subtype provided new lead compounds to aid in drug development.

**6.5 SUMMARY AND CONCLUSION**

α-Conotoxins are subtype-selective nAChR antagonists and established leads for drugs to treat various pain conditions [23]. Here, I have outlined various studies that contributed to our understanding of the molecular pharmacology of Vc1.1 and RgIA at α9α10 and GABA_B receptors. These studies have significantly progressed development of these novel α-conotoxins as pain therapeutics. I have also successfully identified the potential use of dicarba modification of these peptides to improve their bioavailability and half-life, and maintain their biological activity. My results provide vital information about the molecular mechanism of peptide inhibition of the α9α10 nAChR subtype. Together with previous mutational studies, synthesis and functionally characterisation of new dicarba analogues that aim to improve peptide potency are being carried out.
6.6 References


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CHAPTER 7

Conclusion & future directions
Nicotinic research has significantly grown over the past 30 years. [1-3]. However, there are still numerous challenges and questions to be answered that are related to these ligand-gated channels [4]. There has also been a prominent shift in the direction of research into nAChRs. While previous studies focused on nAChR structure and function, present research aims to understand the physiological roles played by various nAChR subtypes [5, 6]. Today, nAChR subtypes are implicated in numerous neuronal and non-neuronal diseases, which further indicate their vital physiological role [1, 7].

α-Conotoxins, a diverse group of peptides that act as subtype-specific nAChR antagonists, are the ideal tool to identify the role played by nAChRs in various physio- and pathophysiological processes. Developments in molecular techniques and peptide chemistry have exponentially increased the number of α-conotoxins being discovered [8]. However, out of the 700 cone snail species, each with 100s of α-conotoxins, only 0.1% have been functionally characterised to date [9, 10]. There are also various challenges in the clinical exploitation of these peptides. In this thesis, the collaborative studies identify and tackle some of the pressing issues related to the therapeutic development of conotoxins, such as nAChR subtype-specific ligand potency and selectivity, and α-conotoxin stability under physiological conditions.

α-Conotoxin AuIB is the only peptide known to selectively target the α3β4 nAChR subtype with an IC₅₀ of 2.5 µM [11]. In this thesis, I successfully characterised a novel α4/7 conotoxin RegIIA, isolated from the venom of Conus regius. This peptide, although active at α3β2 and α7 nAChRs, potently inhibited the α3β4 nAChR subtype, making it a significant discovery. RegIIA is only the fifth α-conotoxin known to target the α3β4 nAChR subtype. Other characterised α-conotoxins known to inhibit the α3β4 nAChR subtype are BuIA, AuIB, PIA and PeIA [12]. Unlike α-conotoxin AuIB, which belongs to a unique α4/6 subclass and exhibits distinct sequence, RegIIA and other α-conotoxins exhibit high-sequence homology.
This made RegIIA an ideal candidate to help us identify and understand the molecular determinants governing its α3β4 nAChR subtype antagonism. Using alanine scanning mutagenesis, I not only identified the critical residues that interact with each of the nAChR subtypes, but also successfully synthesised an analogue that potently and selectively targets the α3β4 nAChR subtype. This will be invaluable in deciphering the physiological role played by the α3β4 nAChR subtype, and for the design and development of α3β4-selective drugs to treat lung cancer and nicotine addiction.

In this thesis, I characterised another α-conotoxin, LsIA, isolated from Conus limpusi. This peptide was of special interest, since it contained a unique N-terminal serine amino acid in addition to a glycine, which is found in almost all conotoxins. To date, very few α-conotoxins have been found to exhibit this N-terminal sequence [12]. Therefore, I examined the significance of this sequence, including C-terminus carboxylation, on LsIA’s functional activity. While the truncated analogues caused LsIA’s loss in activity at both of its pharmacological targets, which was similar to previous studies with α-conotoxin GID [13], C-terminal carboxylation changed selectivity between the α3β2 and α7 nAChR subtypes. I also extended this study to identify the role the α5 nAChR subunit plays in α-conotoxin pharmacology. Interestingly, while α5 subunit incorporation into the α3β2 nAChR subtype decreased LsIA’s IC₅₀ three-fold, a faster onset of block (k_on) was observed at the α3α5β2 nAChR subtype. Together, these results present a novel strategy for the development of novel analogues with improved subtype selectivity and suggest a pharmacological role played by the auxiliary α5 subunit in the inhibition of nAChRs by α-conotoxins.

I also functionally characterised a novel conotoxin, GeXXA, which belongs to the D-superfamily. αD-conotoxins are unique homo-dimeric peptides that non-competitively target nAChRs. However, very few peptides from this family have been characterised. αD-GeXXA
selectively inhibits α7 and α3-containing nAChRs and irreversibly inhibits α9α10 nAChR subtype. This is the first report of monomeric αD-conotoxin activity, selectively inhibiting the α9α10 nAChR subtype. These results present a novel binding mechanism for an αD-conotoxin and provide future insights for the development of new nAChR antagonists.

Finally, in a collaborative study, I examined the functional implications of dicarba modification of the disulphide bonds in α-conotoxins Vc1.1 and RglA. Both of these peptides exhibit analgesic properties and inhibit α9α10 nAChR and N-type calcium channel currents via GABA\(_B\) receptor activation [14]. However, the lability of the disulphide bond has hampered clinical exploitation of these and other α-conotoxins.

Dicarba modification of the disulphide bonds has been shown to improve peptide stability [15, 16]. I identified a significant reduction in activity for Vc1.1 and RglA at the α9α10 nAChR subtype. However, interestingly, regioselective I–III and II–IV dicarba analogues exhibited differential selectivity towards their biological targets. These results suggest that the CysI–CysIII disulphide bond of Vc1.1 and RglA interacts with α9α10 nAChR subtype receptors. This may help us establish a novel strategy for developing subtype-selective nAChR antagonists and new leads for stable drugs to treat various pain conditions.

Overall, the studies outlined in this thesis have provided crucial information to improve our understanding of the molecular pharmacology and potential therapeutic use of α-conotoxins.
References:


