

**Review**

**Subtype-selective conopeptides targeted to nicotinic receptors**

Concerted discovery and biomedical applications

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Conus peptides that are selectively targeted to different molecular isoforms of nicotinic acetylcholine receptors (nAChRs) have been identified and characterized; several have recently been shown to have significant biomedical potential. An emerging strategy for the discovery from animal biodiversity of subtype-specific ligands for ion channel families is described in this review. Characterization of the gene family encoding a set of related ligands is required for discovery using a molecular genetics approach; when discovery is guided by a knowledge of the phylogeny of the biodiverse animal lineage being used as a source of ligands, a rational, efficient scan of the library of putative ligands becomes feasible. Together, these constitute an approach to uncover subtype-specific ligands, called “concerted discovery”; this was applied to the α-conotoxins, a family of Conus peptides generally targeted to nAChRs.

Subtype-specific α-conotoxins were developed that target two groups of nAChRs, α6* and α9* α-conotoxin MI1 has become the defining ligand for identifying the α6* nAChR subtype. A synthetic analog, MI1 [E11A], further subdivides α6* nAChRs into those that contain an α4 subunit and those that do not. Importantly, these two subtypes are differentially affected by nigrostriatal damage, findings of likely relevance to the pathophysiology of Parkinson’s disease. In contrast, α-conotoxins that target α9 nAChR subtypes have potential as analgesics for the treatment of neuropathic pain that develops after nerve injury. The discovery of α9-conotoxin KgIA enabled the identification of a novel role for α9 nAChRs. Use of α9 nAChR antagonists is associated with reversal of inflammation caused by the nerve injury. Thus, subtype-specific α-conotoxins targeted to particular nAChR isoforms are not only useful for understanding the physiological role of these receptors, but can have important diagnostic and therapeutic applications as well.

Note: *Denotes the possible presence of additional subunits in the nAChR complex.

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In this review, an emerging strategy to use animal biodiversity as a source of highly specific pharmacological agents that affect ion channel function is described. Although the approach is a general one, we specifically outline the strategy for developing subtype-selective ligands for different molecular isoforms of nicotinic acetylcholine receptors (nAChRs). nAChRs are a family of ligand-gated ion channels expressed in various regions of the central nervous system and the peripheral nervous system as well as in non-neuronal tissue. These nAChRs respond to endogenous agonists including acetylcholine and choline as well as the plant alkaloid nicotine. These receptors participate in an extensive range of processes including cognitive function, motor movement, sound perception and immune function. 1 Both the strategy for discovery as well as basic science applications of subtype-selective ligands for molecular isoforms of this large and complex ion channel family, and potential new therapeutic and diagnostic possibilities will be presented in this review.

With the widespread use of gene knockout technology, the development of subtype selective pharmacological agents as complementary tools for understanding the specific functional role of individual ion channel complexes has become even more urgent. Most functional ion channels are multimeric, typically, tetrameric or pentameric, with the individual principal protein subunits generally being homologous members of ion channel gene families. 2 In most cases, a physiologically relevant ion channel complex is a heteromeric combination, with more than one gene product comprising each particular molecular isoform. The problem in using molecular genetics and gene knockout technology exclusively is that mutations in a single subunit would affect all of the different ion channel complexes that contain the mutated subunit. Since each of the different channel complexes differ in their subunit composition and physiological function, mutating or knocking out one subunit can result in a complex phenotype involving a set of several channel isoforms. Ligands that distinguish between the diverse ion channel complexes that may share one or more subunits are therefore essential for further clarification of physiological function.

nAChRs are allosteric transmembrane proteins composed of one or more α-subunits (α1–α10) either alone or in combination with one or more non-α subunits, (β-subunits (β1–β4), γ, δ or ε), that together make up the functional ligand-gated ion channel complex;
all nAChRs are believed to contain five such subunits. Various combinations of nAChR subunits show distinct anatomical location and have unique biophysical and pharmacological properties. The generation of mice that lack one or more nAChR subunits has been used to examine the function of different nAChR subtypes. At the present time, it is well established that more than one distinct nAChR subtype may contain a particular subunit. For example, there are $\alpha_2\beta_2$, $\alpha_4\beta_2$, $\alpha_5\beta_2$ and $\alpha_6\beta_2\beta_3$ nAChRs; deletion of the $\alpha_4$ nAChR subtype would eliminate all of these nAChR subtypes. This illustrates why agonists and antagonists that can differentiate between these individual receptor subtypes are urgently needed.

**Beyond Bioprospecting: The Basis for Concerted Discovery**

We briefly outline a general strategy (which we refer to as “concerted discovery”) for developing subtype specific ligands for various nAChR subtypes. The general goal is to go beyond conventional screening, by developing related sets of ligands with divergent subtype selectivity for a targeted ion channel family. Instead of searching for one subtype-selective compound at a time, a group of candidate compounds that are biochemically and genetically related are systematically investigated, the goal being to develop a suite of ligands with divergent target selectivity. In order for such a concerted discovery approach to be successful, there are two prerequisites.

First is the starting biological material for discovery: a lineage of animals that has been reasonably successful over evolutionary time, so that it comprises a significant complement of species (Fig. 1). A phylogenetic tree for the biodiverse lineage is needed; such information is now relatively straightforward to obtain, given the modern tools available for molecular phylogeny.

A second requirement is the identification and definition of major gene families encoding products used for interacting with other animals in the environment. Such genes that are exogenously targeted to other organisms have been called “exogenes”; in contrast to most gene products, the biologically-active compounds produced from exogenes do not act endogenously within the cells or tissues of the organism that produces them, but are instead targeted to another organism. Thus, the discovery of novel ligands targeting various subtypes of nAChRs has two major starting components: a biodiverse lineage of animals, the cone snails, and an exogene family targeting the nAChR family, the $\alpha$-conotoxins, which are expressed broadly within that biodiverse animal lineage. These will be specifically discussed below.

**Discovery from the Cone Snails, a Biodiverse Lineage of Animals**

The biological rationale that underlies concerted discovery is that a lineage of animals descended from a common ancestor will likely have recruited (or possibly evolved de novo) a few gene families whose products are used to mediate interactions with animals in the environment most relevant to that lineage. However, because every species has its own particular ecological niche, such biotic interactions are species-specific. Thus, although the entire lineage may express members of a gene family, the individual exogenes encoded in the genomes of the different species in the lineage will have been subject to different selective pressures, and will therefore have diverged from each other. Because effective interactions with other animals often involves affecting the targeted animal’s nervous system, the opportunity for a diversity of neuropharmacology to evolve in a single exogene family as an animal lineage radiates is what provides raw material for concerted discovery.

In the specific example we discuss in this review, the biodiverse lineage of animals are the cone snails (genus *Conus*), comprising 500–700 different species, all of which are venomous predators (Fig. 1). Cone snails have successfully radiated in all tropical marine environments into multiple clades, correlated with prey specialization, since the eocene period. *Conus* venoms comprise one of the richest natural sources of ligands targeted to ion channels. The family of conotoxins discussed in this review, the $\alpha$-conotoxins, are generally targeted to the nAChR family of ligand-gated ion channels. Since all cone snails are venomous predators, and cholinergic neurotransmission is essential for locomotion of the prey...
of the various cone snail species, the fact that different species of *Conus* prey on different phyla already provides an obvious differential selective pressure. The diverse biological interactions of the hundreds of species of cone snails thus sets the evolutionary stage for divergent ligands targeted to different nAChR subtypes to have been generated.

**The Exogene Family: α-Conotoxins**

In traditional bio-prospecting, organisms from the wild are collected, extracts made and these are screened for pharmacological activities of interest. A major limitation is the considerable amount of material required to screen for and characterize a pharmacological activity of interest. In practice, the ability to successfully purify a bioactive compound from a crude extract so that its structural identity can be elucidated is what has limited discovery either to organisms that can be cultured, or to the discovery of compounds present at high concentrations from relatively large organisms. This traditional approach would necessarily exclude an enormous fraction of the total animal biodiversity on the planet as a source of novel pharmacologically active compounds. Most animal species are small to tiny, relatively rare and most compounds that animals produce to interact with other animals in the environment are only produced in specialized organs, and often only when needed for a specific biotic interaction.

Advances in molecular genetics and molecular phylogeny now make it possible to access bioactive compounds that are rare and produced by very small organisms. One key that makes this possible are technological advances in molecular genetics. Concerted discovery requires characterizing gene families that encode the candidate molecules present in a crude extract so that its structural identity can be elucidated. This is what has limited discovery either to organisms that can be cultured, or to the discovery of compounds present at high concentrations from relatively large organisms. This traditional approach would necessarily exclude an enormous fraction of the total animal biodiversity on the planet as a source of novel pharmacologically active compounds. Most animal species are small to tiny, relatively rare and most compounds that animals produce to interact with other animals in the environment are only produced in specialized organs, and often only when needed for a specific biotic interaction.

One particular gene family expressed in *Conus* venom ducts, the α-conotoxins are the major *Conus* exogene family that interact with the nAChR family of ion channels. This *Conus* peptide gene family has been the source of many subtype specific ligands for the different nAChR subtypes. As is true of all *Conus* peptide families, the initial translation product of genes that encode the α-conotoxins is a prepro-peptide precursor (examples in Fig. 2). Each family or superfamily can be identified by two defining characteristics: the conserved signal sequence, as well as the pattern of Cys residues in the mature toxin sequence, as is illustrated (Fig. 2) for the α-conotoxin precursors. In addition, the α-conotoxin gene has an intron in the preproteptide region; there is a highly conserved sequence in this intron that borders the mature toxin region. The conserved signal sequence and the conserved intron sequence can be used to design PCR primers to deduce new α-conopeptide sequences using either cDNA libraries or genomic DNA.

The ability to determine peptide sequences by PCR methods through the α-conotoxin genes that encode them has resulted in a revolutionary change in the number of α-conotoxins accessible for analysis. Instead of having to accumulate large amounts of venom from every *Conus* species of interest, followed by biochemical purification of each individual α-conotoxin, a single PCR reaction reveals the sequences of most, if not all α-conotoxins encoded in the genome of a *Conus* species. The number that can be accessed from each species varies considerably, and in some cases can exceed over 25 different α-conotoxin sequences from a single *Conus*. As has been repeatedly observed for *Conus* venom peptides, there is virtually no interspecific molecular overlap between mature conotoxin sequences, even when closely related *Conus* species are compared. The facile discovery of α-conotoxin sequences by molecular genetics has therefore created an ever-increasing inventory of α-conotoxins (>1500) that in principle, can be evaluated for nAChR subtype selectivity; a random approach to determining the specificity of these peptides, would require considerable resources and time. However, by a judicious combination of molecular genetics, peptide chemistry and molecular phylogeny, an ordered exploration of the subtype selectivity of this large inventory of α-conotoxin sequences has been achieved in the last decade and has yielded a significant number of useful subtype-specific ligands for individual nAChR isoforms.

**Overview of Targets and Ligands: Clades of nAChR Subunits, Clades of Cone Snails and their α-Conotoxins**

A considerable body of evidence has accumulated suggesting that most α-conotoxins interact with two adjacent nAChR subunits, with determinants for potency and specificity on both subunits. Because α-conotoxins are generally competitive nAChR antagonists (but not always—see refs. 9 and 10), the α-conotoxin binding site is believed to overlap with the agonist binding site, and lie between two adjacent nAChR subunits.

It is useful to take a broad overview of nAChR subunit phylogeny. Based on the presence of a characteristic loop containing two vicinal cysteine residues, the “cys loop”, the subunits are conventionally divided into two broad classes: α (that have a cys loop) and non-α.
subunits (that do not have the cys loop). However, when the entire sequences of the subunits are compared, the phylogenetic tree of subunits that can be constructed leads to a picture that is somewhat different; a summary of the phylogeny done by several investigators is shown in Table 1.\textsuperscript{11,12} The α subunits clearly fall into two very divergent classes, a small group that can assemble to form functional receptor complexes that comprise only α subunits (and in some cases, homomeric pentamers), are evolutionarily distant from all other subunits. It has been postulated that the primordial nAChR receptor was most similar to these nAChR subunits (clade 1 in Table 1). In mammals, the α7, α9, and α10 subunits therefore belong to a very divergent clade from all other mammalian nAChR subunits (in other organisms, such as \textit{C. elegans}, this clade is much larger). We refer to these as “the ancestral-type α subunits.”

The remaining nAChR subunits are more closely aligned to the conventional nAChR subunit nomenclature; these can be divided into α and non-α subunit clades. The α subunits all form a monophyletic clade, (except that included in this clade is the β4 subunit, which is more closely allied to α subunits despite the lack of the cys-loop). All the non-α subunits form another monophyletic clade. There are also pairs of subunits indicated in Table 1 that are more closely related to each other than to any other subunits in the clade (e.g., the α3 and α6 subunits are such a closely-related pair).

Thus, in general, the α-conotoxins target either an interface between two α subunits or the interface between an α subunit and a non-α subunit (Fig. 3). The divergence of the “ancestral” α-subtypes (i.e., α7, α9, and α10) suggests that ligands that target these types of receptors are likely to be correspondingly less-related to the ligands that target other types of nAChR’s, with α and non-α subunits at the binding interface.

In order to find candidates within the α-conotoxin family that target these various types of nAChRs, it is useful to understand the phylogenetic structure of the biodiverse lineage in which the α-conotoxin family is expressed. This is shown in Figure 4—although only a fraction of the total number of species are shown, it is obvious that distinct clades can be defined: species within a clade are more closely related to each other than to any \textit{Conus} species that belongs to a different clade. Thus, the biodiverse lineage that gave rise to the α-conotoxin family has its own phylogenetic structure, and this confers an underlying organization based on evolutionary history for the peptide toxins expressed in \textit{Conus} venoms as well.

**Investigating the α-Conotoxin Family Guided by Molecular Phylogeny**

The original α-conotoxins were purified from fish-hunting cone snails; most of these belong to \textit{Pionoconus}, one of the clades of \textit{Conus}, (indicated by the red arrow in Fig. 4). The very first α-conotoxin that was characterized in detail with regard to subtype selectivity was α-conotoxin MII from the venom of \textit{Conus magus} (Fig. 5). It was established that this peptide was highly specific for the muscle subtype of the nAChR, with a strong preference (104-fold) for the interface between the α7 and δ subunit.\textsuperscript{13} Additional peptides from species in the same clade were obtained, some by purification from venom (such as α-conotoxin SIA), with the majority deduced by molecular genetics and the analysis of the sequences of their encoding α-conotoxin genes (three examples, Mn1.4, Cn1.1, Sm1.1, are shown in Fig. 5). All of these peptides appear to have the same specificity: they are targeted to the muscle subtype of the nAChR. Thus, peptides in the clade indicated by the red arrow in Figure 4 yielded mainly α-conotoxins targeted to the muscle subtype. Exceptions were when more than one peptide was present in the venom. Thus, a second venom peptide in the α-conotoxin family from \textit{Conus magus}, α-conotoxin MII (which will be discussed further below) proved not to be targeted to the muscle subtype.
In order to characterize additional α-conotoxins targeted to neuronal nAChRs, other clades of Conus were analyzed; Figures 4 and 5 illustrate the basic strategy. The analysis of sequences from clades indicated by the blue arrows in Figure 4 yielded peptides that did not inhibit the muscle subtype of the nAChR, but were targeted to various neuronal subtypes. Examples are α-conotoxin AuIB from the mollusc-hunting species Conus aulicus, which preferentially targets the α3β4 neuronal subtype\(^\text{14}\) (as is illustrated in Fig. 5) and α-conotoxin PIA from Conus purpurascens (a fish-hunting species, but in a different clade from Pionoconus); this peptide prefers the α6β2* subtype.\(^\text{15}\)

For α-conotoxin peptides specific for nAChR complexes composed of the Clade I subunits in Table 1 (the ancestral type),
it was discovered a very distant clade, indicated by the green arrow (known as *Stephanoconus*) was enriched in peptides that target nAChRs composed of “ancestral” subunits. Examples are shown in Figure 5. It is notable that the peptides from the *Stephanoconus* clade have a characteristically different spacing between cysteine subunits, particularly when compared with the α-conopeptides from the *Pionoconus* clade (these groups of peptides are known as the α4/3 and the α3/5 subfamilies, respectively). Peptides highly selective either for the αβ subtype or for the αβγδ subtype were discovered from the *Stephanoconus* clade.\(^{16}\) Thus, discrete subfamilies of α-conopeptides (e.g., the α3/5 and the α4/3 subfamilies) are enriched in specific clades within the genus Conus, such as *Pionoconus* and *Stephanoconus*, represented by the red and green arrows in Figure 4. What this study reveals is that different clades of cone snails have targeting specificity of their α-conopeptides that is highly biased. Thus, sampling peptides from different clades of *Conus* was what made it possible to discover α-conotoxins highly selective for different nAChR subtypes.

The applications of such subtype-specific ligands for nAChRs will be discussed in the following sections of this review. The availability of highly selective α-conotoxins targeted to two groups of nAChRs has had special biomedical significance. These are the α-conotoxins targeted to the α6* nAChRs and α5* nAChRs.

**The α6* nAChR Subtype and Parkinson’s Disease**

nAChRs containing the α6* subunit are found predominantly in catecholaminergic and visual pathways. The former includes the dopaminergic nigrostriatal pathway implicated in the pathophysiology of Parkinson’s disease.\(^{17}\) Parkinson’s disease is a movement disorder characterized by widespread neurodegenerative changes with particularly prominent losses of dopamine-producing cells in the nigrostriatal pathway. Early symptoms of Parkinson’s disease, which include tremor, rigidity, slowed movement and impaired balance and coordination, are subtle and may occur gradually. There are currently no imaging or blood tests available to diagnose this disorder. Notably, epidemiological studies have repeatedly demonstrated an approximately 50% reduced incidence of Parkinson’s disease in smokers. The apparent protective effect of smoking directly correlates with both the duration of smoking and quantity of cigarettes smoked. Furthermore, the protective effect is reduced when smoking is stopped.\(^{18}\) Thus there is pharmaceutical interest in drugs that target nAChRs for the diagnosis, treatment and possible prevention of this condition.\(^{18}\)

Stimulation of presynaptic striatal nAChRs results in dopamine release.\(^{19}\) The use of nAChR subtype selective ligands coupled with nAChR subunit deletion mice has helped identify receptor subtypes that mediate dopamine release.\(^{20}\) These studies combined with immunoprecipitation data implicate two classes of nAChRs that underlie nicotine-stimulated dopamine release: α-conotoxin MII sensitive nAChRs, the αββ3 and ααββ3 subtypes and α-conotoxin MII insensitive nAChRs, the αβ2 and ααβ2 subtypes. Although in rodents, only 30–40% of nicotine-stimulated dopamine release is modulated by α6* nAChRs, in primates approximately 75% of release being mediated through α6* (α-conotoxin MII-sensitive) subtypes.\(^{21}\) These latter findings suggest that this subtype may play a major role in nAChR mediated dopaminergic activity in striatum.

Striatal nAChRs are also implicated in the addictive effects of nicotine. Studies using fast scan cyclic voltammetry and receptor subunit knockout mice have indicated that there are two populations of dopaminergic fibers; α5* (non-α6) and α6* nAChRs play distinct roles in neurons that modulate tonic vs. phasic firing.\(^{22}\) α6* nAChRs play a predominant role in dopamine release in nucleus accumbens, a collection of neurons involved in reward.\(^{23}\)

As noted above, the α6* subunit is structurally closely related to the α3 subunit and thus the initial difficulty was to develop ligands that were able to distinguish between the α6* and α5* nAChRs. The most selective native toxin was identified from the purple cone, *Conus purpureascens*. α-conotoxin PIA was found to be 75-fold selective for α6* over α5* nAChRs.\(^{15}\) This definitively showed that the α-conotoxin template was capable of discriminating between these closely related receptor subtypes (α3 and α6 subunits have approximately 80% identity in the ligand-binding extracellular domain).

In parallel, a structure/function analysis of α-conotoxin MII was performed in an attempt to generate ligands with preference for α6* nAChRs. Intercysteine residues were individually mutated to alanine and the resulting analogs were tested against α6β2 and α5β2 nAChRs. The native peptide, α-conotoxin MII favored binding to α6β2β3 by 5.6-fold. Several analogs including S4A, N5A, E11A, N14A and L15A had a more favorable α6β2β3 to α5β2 ratio that exceeded 20-fold. By combining mutants in pairs and triplets new analogs were generated, some of which were more than 1000-fold

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**Figure 5. Target selectivity of various α-conotoxins.** The α-conopeptide sequences discussed in the text are shown with their target selectivity for different nAChR subtypes. Thus, α5/6 nAChRs are selectively targeted to two groups of nAChRs has had special biomedical significance. These are the α-conotoxins targeted to the α6* nAChRs and α5* nAChRs.
two sites with approximately 43% of the sites being the higher affinity site. This higher affinity site was preferentially lost with α4 nAChR subunit deletion. These findings, coupled with previous studies using subunit selective antibodies suggested that the higher affinity site represented the α6α4β2β3 subtype. Interestingly, the α6α4β2β3 subtype was also the one preferentially lost with nigrostriatal damage, suggesting that this latter subtype may have a distinct functional role in the nigrostriatal pathway.

These studies were next extended to monkey striatum. In this species, MII [E11A] also discriminated between two binding sites, one with an IC50 of 9.7 fM and the other with an IC50 of 51 pM. Thus two distinct receptor populations were identified with greater than 5000-fold difference in affinity between the two α6β2* nAChR subtypes. Monkeys were then administered MPTP to induce nigrostriatal damage, and the experiments were repeated to study the resulting nAChR receptor changes. In contrast, to unlesioned animals, competition with MII [E11A] was now monophasic with an apparent selective loss of the highest affinity, binding site which most likely represents the α6α4β2β3 subtype.

The critical experiment was then performed in human post-mortem tissue in individuals without and with moderate Parkinson’s disease. In control human striatum, MII [E11A] discriminated between two populations of α-conotoxin MII sensitive receptors with a greater than 500-fold difference in IC505 between the two sites, similar to results seen in monkey and mouse. In addition, similar to mouse and monkey, the highest affinity MII [E11A] binding site was selectively lost in the striatum of cases with moderate Parkinson’s disease (Fig. 6). The implications of the results are two-fold:

1) There are selective dopaminergic neuron populations that are more sensitive to neurodegeneration induced either by treatment with the neurotoxin MPTP or by the pathophysiological process of Parkinson’s disease.

2) The α-conotoxin analog MII [E11A] effectively discriminates between two populations of α6β2* nAChR subtypes and the receptor population bound with highest affinity by MII [E11A] is contained in those neurons selectively lost with nigrostriatal damage. Thus, the putative α6α4β2β3 nAChR may represent a molecular marker for detection of the pathophysiology of Parkinson’s disease. Early detection of Parkinson’s disease is problematic in that >50% of nigrostriatal neurons and >80% of striatal dopamine are lost before clinical symptoms are observed. As there are no laboratory diagnoses for Parkinson’s disease, a biochemical method of early detection is urgently needed. MII [E11A] or derivatives thereof may potentially serve as the basis of novel imaging agents for such early detection.18,21

The α6β2* nAChR Subtype and Neuropathic Pain

Alpha-conotoxins also reveal a novel approach to the treatment of neuropathic pain.32 Current analgesics work through a limited
number of pharmacological mechanisms. Because of this, conditions such as chronic neuropathic pain are often only partially treated. One area of interests is neuronal nAChRs. Much work has focused on targeting CNS $\alpha_4\beta_2$ nAChRs. However, peripherally located nAChRs also may serve as therapeutic targets.33

Using a cDNA approach, Livett and coworkers isolated a novel $\alpha$-conotoxin from $C$. victoriae. Intriguingly, Vc1.1 significantly reduced the mechanical hyperalgesia in two models of peripheral neuropathy, the chronic constriction injury model and partial nerve ligation model.34,35 Investigation of the activity of Vc1.1 indicated that it is a potent antagonist of $\alpha_9\alpha_{10}$ nAChRs.36-38 This was among the first evidence to suggest that $\alpha_9^*$ nAChRs might play a role in the treatment of pain. A post-translationally modified analog, Vc1a was not found to be analgesic.38 Interestingly, however, both Vc1.1 and Vc1a lead to functional nerve recovery following nerve injury.40

One possible mechanism for producing analgesia is the block of nAChRs on nerves. However, $\alpha_9$ and $\alpha_{10}$ subunits have a very restrictive tissue distribution that may not include sensory neurons. An alternative possibility is that the target is immune cells where a number of nAChR subtypes, including $\alpha_9$ and $\alpha_{10}$ are expressed.41 Although immune cells express nAChRs the function of nAChRs in immune tissues is poorly understood.42 Development of novel conotoxin antagonists that discriminate among receptor subtypes present in immune cells may help resolve this problem. The historically defined role of $\alpha_9\alpha_{10}$ nAChRs is in the inner ear where these nAChRs mediate synaptic transmission between efferent olivocochlear fibers and cochlear hair cells.43,44 $\alpha$-conotoxin PeIA isolated from $Conus$ pergrandis was the first nAChR antagonist to be able to discriminate between $\alpha_9\alpha_{10}$ nAChRs and homomeric $\alpha_7$ nAChRs.45 However its selectivity for $\alpha_9\alpha_{10}$ is limited. To remedy this problem, the concerted discovery approach described in the sections above was taken to obtaining novel peptides and the conotoxin RgIA was isolated. This $\alpha$-conotoxin is highly selective for the $\alpha_9\alpha_{10}$ nAChR.46,47

The selectivity of $\alpha$-conotoxin RgIA allowed us to more stringently evaluate a potential role of $\alpha_9\alpha_{10}$ nAChRs in the analgesic effect of $\alpha$-conotoxins. The intramuscular administration of RgIA was shown to be analgesic in the chronic constriction injury nerve model.36 Interestingly, both RgIA and Vc1.1 also substantially reduced the accumulation of immune cells (macrophages and T-cells) at the site of nerve injury. In addition as with Vc1.1, repeated daily administration of RgIA produced a stable decline in injury-induced mechanical hypersensitivity36 (Fig. 7).

The effect on immune cells suggests an intriguing mechanistic possibility. Injury to a peripheral nerve leads to inflammation and an increased number of cholinergic cells at the site of injury. The inflammatory process induced by such changes, is reversed by administering an $\alpha_9\alpha_{10}$ antagonist.48 This reduction of inflammation may result in subsequent recovery of nerve function.

Thus, the availability of $\alpha_9^*$-selective conotoxins has opened new avenues in pain-related research. In addition, the conotoxin reversal of inflammation also hints that block of $\alpha_9^*$ nAChRs also has potential in the treatment of inflammatory disorders.

Discussion

The high subtype-selectivity of $\alpha$-conotoxins is based on the fact that the binding site for these peptides straddles two different

![Figure 7](https://example.com/figure7.png)
Subtype-selective conopeptides targeted to nicotinic receptors: concerted discovery and biomedical applications

...subunits; in effect, for high affinity, a particular combination of adjacent subunits is required. This principle was established early by the pioneering study of Sine and co-workers13 on the muscle-subtype-specific α-conotoxin MI, and has generally been verified by subsequent structure/function studies on α-conotoxins.

The work that we have reviewed here has demonstrated that the selectivity of α-conotoxins is potentially even greater: the results with α-conotoxin MII [E11A] demonstrate that these peptides can potentially detect the presence or absence of subunits not directly at the α-conotoxin binding site. Thus, α-conotoxin MII has determinants on both the α- and β-subunits and structure/function studies suggest that the binding site for this peptide is at the interface between α- and β-subunits. However, the discovery of the [E11A] MII analog that detects the presence of an α4 subunit, which is presumably not directly at the α-conotoxin binding site implies that the non-adjacent α4 subunit causes conformational alterations at the binding site, altering the affinity of MII [E11A]. If α-conotoxin binding is sensitive to non-adjacent subunits even more refined targeting of these peptides (or analogs of the peptides) to nAChR subtypes becomes feasible.

To develop subtype-specific α-conotoxins for the individual nAChR subtypes, it has been productive to carry out discovery on a fairly broad front. Although our focus has necessarily been on a single family of Conus peptides, the α-conotoxins, an important feature for the discovery of highly subtype selective ligands was to examine the biodiverse animal lineage broadly; different clades of Conus species have evolved peptides with quite different nAChR subtype-selectivity. This has been illustrated by showing that one clade of fish-hunting Conus are highly enriched in peptides that can selectively target the muscle subtype while another clade of Conus species that specialize in feeding on amphinomid worms produce a subfamily of α-conotoxins, the α4/5 subfamily whose venoms are enriched in peptides targeted to the nAChR subtypes exclusively formed from α-subunits (the “all α” nAChRs). Thus, exploring a single gene family across a broad phylogenetic range within a biodiverse lineage, has made discovery of divergent subtype-specific ligands targeting one ion channel family, such as the nAChRs, possible.

As has been detailed in the sections above, several α-conotoxins may have biomedical utility (for Parkinson’s disease and neuropathic pain, in the specific examples given). From these and other biomedically important conotoxins, some general insights have emerged relevant to future biomedical applications.

For Conus peptides targeted to ion channels, the fact that ion channels are highly conserved, and that the different subtypes of most ion channel families emerged early in evolution has provided the high potential of these peptides for neuropharmacological applications. The characteristic differences between different subtypes of ion channels are apparently conserved, even as diverse nervous systems have evolved. Thus, as Conus peptides evolve in Conus species to target a particular ion channel subtype, there is a fair likelihood that the mammalian homolog of the physiologically-relevant target will be the highest affinity target in the mammalian nervous system. The potency and selectivity of each peptide for individual homologs of its preferred target (e.g., a homologous human nAChR subtype) is a function of both evolutionary distance and chance. The binding affinity of any given Conus peptide for a homolog of the physiologically-relevant target may change drastically if an important amino acid determinant for affinity is mutated to an unacceptable amino acid residue; in general however, the more closely related the taxon, the more likely that the affinity for the homolog will not differ greatly from the physiologically relevant, native target. For this reason, peptides that target particular nAChR subtypes can retain their very high affinity and selectivity for human receptor subtypes even though they have obviously not been directly selected for a mammalian receptor subtype.

A second consideration is that although the proteins may be highly structurally conserved over evolution, their expression patterns are not. Thus, a particular molecular isoform of a nAChR may be used for one purpose in one group of animals, and for a different physiological purpose by another lineage. The conotoxin that has been developed into the commercial drug Prialt, ω-conotoxin MIIVA comes from a fish-hunting cone snail, and when injected into fish, causes paralysis. It would appear that this peptide helps the fish-hunting cone snail species from which it was derived, Conus magus, capture its fish prey. Thus, the target of the peptide, an α2β-containing voltage-gated Ca-channel subtype, is apparently expressed at the neuromuscular junction of the teleost fish prey of Conus magus. However, when the peptide is injected into mammals, no paralysis is observed; thus, in mammals, this Ca-channel is not a major subtype at the neuromuscular junction. The basis for the therapeutic application of the peptide is that this voltage-gated Ca-channel subtype is found in the dorsal horn of the spinal cord, where pain fibers synapse with spinal cord neurons. Because of the difference in expression patterns, the peptide causes paralysis in fish, but is an effective analgesic drug for human patients.

The difference in expression combined with conservation of the target structure provides the general rationale for potential biomedical applications. The example given above of α-conotoxin RgIA is probably parallel: most likely, an α4/5-type nAChR homolog may be important for capture of the fireworm prey of Conus regius (although little is known at the present time about the specific distribution of different ion channel subtypes in polychaete worms). The peptide’s potential for neuropathic pain is enabled because the mammalian homolog is not expressed at neuromuscular junctions.

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