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Peptide and Protein Neurotoxin Toolbox in Research on Nicotinic Acetylcholine Receptors

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1. Introduction

The chapter briefly covers the history of protein and peptide neurotoxins in research on nicotinic acetylcholine receptors (nAChR). It all started with a great help of α -bungarotoxin and other similar α -neurotoxins from snake venoms in isolation from the *Torpedo* ray electric organ of the muscle-type nAChR as a first individual membrane receptor. The next contribution of α -neurotoxins was the discovery with their aid of the first neuronal nAChR in the brain now known as homooligomeric $\alpha 7$ nAChR. An overview of various α -neurotoxins (so-called three-finger toxins) is presented below showing the structural differences between them, as well as the benefits of their current application for identification and quantification of different nAChR subtypes at normal state and at various pathologies such as Alzheimer's and Parkinson's diseases, psychiatric diseases and nicotine addiction. A special emphasis is placed on the work at our institute, starting with the first detection of nAChRs as targets for the so-called weak or "non-conventional" neurotoxins. Recently, in proteomic studies of snake venoms, novel structural types have been discovered, such as covalently connected dimeric α -cobratoxin or, on the contrary - azemiopsin, the first peptide from venoms which does not contain disulfide bonds but still blocks selectively the muscle-type nAChR.

A generous source for sophisticated tools in research on nAChRs is combinatorial peptide libraries from the venoms of *Conus* marine snails. In particular, they contain α -conotoxins which not only distinguish muscle nAChRs from neuronal ones, but some of them block specifically distinct neuronal nAChR subtypes. At present, combinations of snake and snail toxins are widely used in fundamental research and in pharmacological studies.

The chapter briefly summarizes information on the spatial organization and subunit composition of different nAChR subtypes, but considers in more detail important contributions of peptide and protein neurotoxins into elucidation of the topography of the nAChR binding

sites. The information mainly came from the X-ray structures of their complexes with the acetylcholine-binding protein (AChBP), an excellent structural model of the ligand-binding domain of nAChRs. These complexes are considered as initial blocks for design of novel drugs.

2. Muscle-type, neuronal and “non-neuronal” nAChRs — Brief overview

Before considering in detail protein and peptide neurotoxins on which the Chapter is mostly focused, it is reasonable to give very shortly the information about various types of nAChR which will make easier later discussions of the specificity of one or another toxin to a particular nAChR subtype.

As mentioned in the Introduction, α -bungarotoxin made possible identification and isolation in a pure form of the nAChR from the *Torpedo* ray electric organ. Later it was found that this receptor is composed of 5 subunits arranged around the central axis along which an ion channel should be arranged (Figure 1, A). The subunits in the order of their increasing molecular masses (estimated from the SDS-gel electrophoresis) have been named α , β , γ and δ . The molecular mass of the receptor complex is around 250 kD and it should contain two α subunits and by one of the “non- α ” subunits. When nucleotide sequences of the *Torpedo* nAChR subunits and of those from mammalian muscles were established it became clear that those receptors are highly homologous. In fact, the mammalian embryonic nAChR has the same subunit stoichiometry (2 α , β , γ and δ), but in the mature form it has an ϵ subunit instead of γ . Although the relevant information at present is available in numerous biochemistry books and reviews [1-4], it should be mentioned here that nAChRs are ligand-gated ion channels: binding of a ligand (acetylcholine, nicotine or other specific agonists) will result in the channel opening and passing sodium or calcium ions will activate a variety of signaling cascades. On the other hand, binding at the same sites of competitive antagonists such as α -bungarotoxin will prevent both binding of agonists and subsequent channel opening; some so-called non-competitive antagonists, like phencyclidine, bind directly to the channel moiety but they are not discussed here.

Earlier it was thought that the ligand-binding sites of nAChRs lie within the α -subunits, hence there should be two binding sites on the muscle-type nAChRs. To-day we know that, indeed, the main contributions to binding of agonists or competitive antagonists are donated by the α -subunits. Moreover, even isolated α -subunit and its fragment in the amino-acid region 170-200 can bind α -bungarotoxin, although with lower affinity than the whole-size receptors [5,6]. However, now it is well established that the binding sites are situated at the interfaces of the α -subunits with their neighbors, and it is the variability of functional groups brought to the binding sites by less conservative “non-alpha” subunits which underlies the differences in specificity between individual nAChR subtypes [4].

What are the types and subtypes of nicotinic acetylcholine receptors? As mentioned above, binding of radioactive α -bungarotoxin to brain membranes finally brought to life the nAChR presently known as homopentameric $\alpha 7$ nAChR that is composed of five identical $\alpha 7$ -subunits. Thus, we have an example of homooligomeric receptor belonging to the family of neuronal

nAChRs. Neuronal heteromeric nAChRs are composed of two types of subunits: α and β . At present there are 9 types of neuronal α -subunits ($\alpha 2$ - $\alpha 10$) and three types of β subunits ($\beta 2$ - $\beta 4$); α and β subunits in the muscle-type receptors presumed to be $\alpha 1$ and $\beta 1$ ones. The characteristic feature of α -subunit is a vicinal disulfide between two neighboring Cys residues in the binding site (Cys192-Cys193 in the amino-acid sequence of the *Torpedo* α -subunit) which is not present in β or other “non- α ” subunits. In recent years it became clear that diverse nAChRs are very much widespread and play different functional roles also outside the neuromuscular junctions or central nervous system. In fact, neuronal nAChR subunits were found on the immune system cells, skin, lung tissue and other. The respective receptors got the name of “non-neuronal” nAChRs thus making a third group of nicotinic acetylcholine receptors (see reviews [7,8]).

Figure 1. Spatial organization of nAChRs. A – Schematic presentation of *Torpedo* nAChR, consisting of 5 subunits with the ion channel along the central axis. Two binding sites of agonists (acetylcholine and others) and competitive antagonist (α -neurotoxins from snakes, α -conotoxins from *Conus* mollusks and others) are located at the interfaces of the $\alpha 1/\gamma$ and $\alpha 1/\delta$ -subunits and marked with asterisks. B – Spatial organization of *Torpedo marmorata* nAChR derived from its cryo-electron microscopy structure. Subunits $\alpha 1$, $\beta 1$, γ and δ are colored in red, green, cyanic and blue, respectively. Three main domains of the receptor – extracellular (ECD), transmembrane (TMD), consisting of 4 α -helical fragments (M1-M4), and intracellular (ICD) are shown. C – Schematic presentation of two representatives of neuronal nAChRs – homooligomeric and heterooligomeric ones. The probable binding sites of agonists and competitive antagonist are marked with black circles.

Structurally, the *Torpedo* nAChR is a prototype for all members of the nAChR family. First of all, it is a pentamer (composed of 5 subunits) as follows from the cryo-electron microscopy structure of the *Torpedo marmorata* receptor (see Figure 1, B). There are no structural data of this sort for any other nAChR, but their pentameric composition was presumed from computer

modeling and from some indirect data like electrophysiology analysis. At present there are no doubts that all nAChRs are indeed either pentameric homooligomers (made exclusively of 5 α -type subunits, like $\alpha 7$ nAChR, $\alpha 9$ nAChR or $\alpha 9/\alpha 10$ nAChR) or pentameric heterooligomers (composed of α and other subunits) (Figure 1, C) – for example, one of the best presented in the brain is $\alpha 4\beta 2$ nAChR [9]. As already mentioned, all nAChRs should be built similarly to *Torpedo* nAChR: namely, four transmembrane fragments M1-M4 in each subunit, the most inner ones M2 fragments lining the channel, the N-terminal extracellular fragments of each subunit together forming the ligand-binding domain excellently imitated by the X-ray structure of the acetylcholine-binding protein (AChBP) (see below). The long intracellular loops between transmembrane fragments M3 and M4 of each subunit together form the cytoplasmic (intracellular) domain.

The first and the most direct structural evidence for a common three-dimensional organization of all nAChRs came from the crystal structure of AChBP [10]. Today even more convincing are the recently solved high-resolution X-ray structures of the whole-size prokaryotic membrane proteins belonging to the same superfamily of Cys-loop ligand-gated ion channels as nAChRs [11-13]. These proteins, each composed of 5 identical subunits, do not have large cytoplasmic domains (which apparently made their crystallization much more simple than of nAChRs or other mammalian Cys-loop receptors), but in the transmembrane and ligand-binding domains they are surprisingly similar to *Torpedo* nAChR. Moreover, the same type of structure was found for a Cys-loop receptor from *Caenorhabditis elegans* [14]. Now, after having these major facts about nicotinic acetylcholine receptors, we can open our toolbox and have a closer look on protein and peptide neurotoxins.

3. Snake venom neurotoxins utilized in research on nAChRs — Primary and three-dimensional structure

The word “toolbox” in the chapter title in the first place is related to the snake venom proteins, at least historically. It was the component of *Bungarus multicinctus* venom which was found to block very efficiently the muscle-type nAChRs and could be considered as a good marker of those receptors. The history of the discovery of such a tool, namely protein neurotoxin α -bungarotoxin, is presented in a recent review [15]. There Prof. Chang shares his memories about this discovery (exactly 50 years ago!) which played such a crucial role in understanding the structure and function of both snake neurotoxins and of one of their targets, namely nAChRs. Soon after the discovery of α -bungarotoxin, similar proteins were found in other snakes, in particular in cobra venoms and the whole family got the name of α -neurotoxins (see reviews [15-17]).

3.1. α -Neurotoxins

There are two structural types of α -neurotoxins: short-chain α -neurotoxins (60-62 amino acid residues, 4 disulfide bridges) and long-chain ones (66-75 amino acid residues, 5 disulfide bonds). The first X-ray structures have been determined for the short-chain α -neurotoxins,

namely for erabutoxins a and b [18,19] (see Figure 2, A). The molecule has three loops, with a predominant β -structure, fixed in the space by 4 disulfide bridges forming a sort of a knot. This folding gave the name of “three-finger proteins” to α -neurotoxins. Later spatial structures have been determined both by NMR and X-ray crystallography for different short- and long-chain α -neurotoxins, including α -bungarotoxin [20,21]. Long-chain α -neurotoxins have the same three -finger folding as the short ones, but contain a longer C-terminal tail and an additional 5th disulfide in the central loop II (Figure 2, B). In the structures of some long-chain α -neurotoxins (α -bungarotoxin, α -cobratoxin [22] or neurotoxin I from *Naja oxiana* [23]) a short α -helical fragment was found at the tip of the loop II (see Figure 2, B).

Figure 2. Spatial structures of snake ‘three-finger’ toxins interacting with nAChRs. The ‘fingers’ are marked with Roman numbers; N-termini are labeled as well. A - erabutoxin a (PDB ID: 5EBX). B – α -bungarotoxin (1KFH); the 5th disulfide bridge in loop II is colored in magenta (contrary to all other disulfides in orange) and α -helix at tip of this loop is colored intentionally in contrast green. C – κ -bungarotoxin (1KBA); 5th disulfide bridges in loops II are colored in red. D – haditoxin (3HH7). E – dimeric α -cobratoxin (4AEA), where disulfide bridges between Cys3 from one monomer and Cys20 from the second monomer stabilize the dimeric molecule; two monomers are shown in blue and magenta, respectively. F - irditoxin (2H7Z); ‘non-conventional’ disulfides in loops I are colored in red and disulfide bond between the monomers is shown in blue. G - candoxin (1JGK); disulfide in loop I is shown in red.

One of the characteristic features of α -neurotoxins is the stability of their three-dimensional structure fixed by 4 or 5 disulfide bridges. This conclusion is supported by high similarity of spatial structures determined by NMR at different conditions (varying pH and temperatures) and by X-ray crystallography. This may be one of the crucial factors explaining high efficiency of α -neurotoxin interactions with their targets, nicotinic acetylcholine receptors. As will be

shown later, α -neurotoxins essentially preserve their conformation in complexes with the AChBP [24], with the ligand-binding domain of individual $\alpha 1$ subunit of nAChR [25] and with the chimera of AChBP and $\alpha 7$ nAChR extracellular domain [26].

3.2. Dimeric three-finger neurotoxins

First of all, we should mention here κ -bungarotoxins and several homologous neurotoxins which are dimers, but do not have covalent intermolecular bonds between monomers [27]. Each monomer is very similar to a typical long-chain α -neurotoxin: the same additional 5th disulfide at the tip of the central loop II, but a slightly shorter C-terminal tail (total number of amino acid residues 66 but not 75 as in α -bungarotoxin) (see Figure 2, C). The molecular targets of κ -bungarotoxins are neuronal nAChRs, but contrary to α -neurotoxins they have high affinity to neuronal $\alpha 3\beta 2$ nAChR [28]. Interestingly, it was established about 20 years ago that there is one common property of α -neurotoxins and κ -neurotoxins, namely the additional disulfide in the loop II is essential for recognition of neuronal nAChRs. It was found that selective reduction of that disulfide and subsequent alkylation or removal of the respective cysteines in both types of toxins abolished their high affinity binding to $\alpha 7$ and $\alpha 3\beta 2$ nAChRs, respectively (without decreasing the affinity of long-chain α -neurotoxins to muscle-type nAChRs [29,30]). On the other hand, introduction of additional disulfide into the central loop of short-chain α -neurotoxins considerably increased their affinity for $\alpha 7$ nAChR [31,32].

It is not yet absolutely clear why κ -bungarotoxins have preference for heteromeric nAChRs. There was a hypothesis that an important role in selectivity of κ -bungarotoxins towards $\alpha 3\beta 2$ nAChRs belongs to the residue Lys26 [24]. However, its introduction to α -neurotoxin having a high affinity for $\alpha 7$ nAChRs only decreased considerably binding to this receptor but did not bring any affinity for $\alpha 3\beta 2$ nAChRs [32]. Apparently, dimerization as such is important to force a protein, composed of two classical α -neurotoxins, to recognize a heteromeric neuronal nAChRs as can be seen on the example of other recently discovered dimeric neurotoxins.

One toxin, haditoxin from the King cobra venom [33] looks very similar to κ -bungarotoxin. Haditoxin is a non-covalent dimer composed of two short-chain α -neurotoxins, rather than of long-chain ones, and the monomers adopt a topological arrangement (Figure 2, D) reminiscent of that observed earlier for monomers in κ -bungarotoxin. Haditoxin can block not only muscle-type nAChRs, as typically observed for short-chain α -neurotoxins, but surprisingly it also blocks homooligomeric $\alpha 7$ and heterooligomeric $\alpha 3\beta 2$ nAChRs. This finding appears to be in contradiction with the earlier found necessity of the additional disulfide in the central loop for recognition of neuronal nAChRs. However, it should be kept in mind that blocking of neuronal nAChRs by haditoxin was observed only at very high toxin concentrations [33]. It should be also mentioned that, strictly speaking, haditoxin cannot be assigned to classical short-chain α -neurotoxins because its homology to erabutoxin is only 50%, whereas it is 75-80% with the muscarinic toxin-like proteins (MTLP) having different targets [34].

Novel types of dimeric α -neurotoxins were recently discovered: contrary to κ -bungarotoxin or haditoxin, these are covalently bound where two molecules of α -cobratoxin are connected by two intermolecular disulfide bonds [35]. Before describing a biological activity of this new tool, it should be mentioned that such intermolecular disulfide is the first case of this post-

translational modification found for the whole huge family of three-finger toxins. Dimeric α -cobratoxin retained, although at a lower level, the capacity to block $\alpha 7$ and muscle-type nAChRs and in addition acquired the ability to block $\alpha 3\beta 2$ nAChR - again, with lower potency than did κ -bungarotoxin [35]. Interestingly, selective reduction of the disulfides in the loop II of dimeric α -cobratoxin abolished its activity against $\alpha 7$ nAChR. It could be expected in view of earlier described similar modification of α -cobratoxin itself, but this chemical modification even increased the affinity for $\alpha 3\beta 2$ nAChR [36]. Since dimeric α -cobratoxin is present in the *Naja kaouthia* cobra venom only in minute amounts (0.01% in crude venom, as compared to 10% for α -cobratoxin itself or to 0.1% for κ -bungarotoxin), unequivocal localization of intermolecular disulfides by chemical means could not be done. Fortunately, dimeric α -cobratoxin has been recently crystallized (Figure 2, E) and the high-resolution X-ray structure revealed the disposition of the intermolecular disulfide bridges: the disulfide Cys3-Cys20 or Cys3'-Cys20' in each monomer is not formed, but Cys3 of one monomer finds Cys20' of another monomer, while Cys3' of the latter makes a disulfide with Cys20 of the former [36].

As will be shown later, the main contribution to binding of α -neurotoxins both to nAChRs and to their models comes from the tip of the central loop II of α -neurotoxins. In dimeric α -cobratoxin the two tips are in close proximity and computer modeling showed impossibility of docking such a structure to AChBP, suggesting that some conformational changes should occur in the dimeric α -cobratoxin to ensure its binding observed in radioligand and electrophysiology experiments [36].

The discovery of dimeric α -cobratoxin was followed by finding another three-fingered toxin where monomers are connected by a disulfide bridge [37]. It was irditoxin isolated from Colubrid snake *Boiga irregularis*. In contrast to dimeric α -cobratoxin present in venom in minor amounts, irditoxin is a main component of boiga venom. Again, strictly speaking, irditoxin is neither a short- nor a long-chain α -neurotoxin: the monomers forming this toxin belong to non-conventional toxin type (see below) and each monomer contains an extra cysteine residue forming one disulfide bridge between two monomers (or protomers). None of these cysteines is present in classical α -neurotoxins. In the first protomer, the additional cysteine is located in loop I whereas in the second protomer it is in loop II. The three-dimensional structure of irditoxin [37] (see Figure 2, F) shows that the central loops II of the two protomers are oriented in a similar way as the central loops of dimeric α -cobratoxin (Figure 2, E).

3.3. Weak (non-conventional) three-fingered neurotoxins

A characteristic feature of this group of three-fingered toxins is the presence of additional disulfide bridge not in the central loop II, as in long-chain α -neurotoxins or in κ -bungarotoxins, but in the N- terminal loop I. Some representatives of this group were known long ago, but many of them did not have a strong toxicity (that is why their name was "weak toxins") and their targets were unknown. At present this group of toxins, consisting of 62-68 amino acid residues, is quite well investigated and has a more general name "non-conventional neurotoxins" [38]. The toxicities for the most of group members are very low (5-80 mg/kg) in contrast to classical α -neurotoxin with toxicities in the range from 0.04 to 0.3 mg/kg. However, some very potent toxins (like γ -bungarotoxin with LD50 of 0.15 mg/kg) are also included in the

group of non-conventional toxins. Since, as mentioned above, molecular targets of weak (non-conventional) toxins for a long time were unknown, an important step in this field was the work [39] where was discovered that weak toxin (WTX) from *Naja kaouthia* cobra venom interacted with micromolar affinity with the $\alpha 7$ and muscle-type nAChRs, the binding being practically irreversible. Later it was found [40] that candoxin (Figure 2, G), another non-conventional toxin, interacted both with $\alpha 7$ and muscle-type nAChR with high affinity. An interesting feature of candoxin is that its attachment to the muscle-type receptor was easily reversible. Even more impressing species specificity was reported for denmotoxin, a non-conventional toxin isolated from Colubrid snake *Boiga dendrophila*: it was able to interact irreversibly and with high affinity with chick muscle nAChR, but only with low affinity with mouse receptors [41].

3.4. Three-finger snake neurotoxins having other targets than nicotinic acetylcholine receptors

Before considering in detail the mechanisms of interactions between α -neurotoxins and nAChRs and describing their earlier and current roles of tools, it is appropriate to say a few words about the whole family of three-finger proteins from snake venoms (see reviews [16,17]). They all have the same “three-finger” fold but are decorated with quite different functionally active amino-acid residue and, as a result, attack distinct targets. For example, in the preceding paragraph we considered WTX from *Naja kaouthia* venom which blocked nicotinic acetylcholine receptors. Its very low toxicity allowed testing of its behavioural activity on rats which suggested action on muscarinic acetylcholine receptors [42]. Indeed, subsequent radioligand analyses revealed the WTX interaction with the different subtypes of muscarinic acetylcholine receptors [43]. It should be noted here that we have a dualism of action for this group of the three-finger proteins from snake venom: namely, blocking of one acetylcholine receptor (the nicotinic one) belonging to the family of ligand-gated ion channels and another acetylcholine receptor, the muscarinic one which is a member of the superfamily of G-protein-coupled receptors (GPCR).

Much more strong effects on muscarinic acetylcholine receptors exert so-called muscarinic neurotoxins isolated from the green mamba *Dendroaspis angusticeps* [44-46]. Structurally these proteins are of the same type as short-chain α -neurotoxins. Interestingly, they can distinguish different subtypes (M1-M7) of muscarinic acetylcholine receptors and on some of them exert not the inhibitory, but the potentiating effects. There is not yet much information about how muscarinic toxins recognize their targets. A large series of mutations was performed both on the muscarinic toxin MT7 and on the M1 muscarinic receptor and the results of this pair-wise mutagenesis, analyzed by computer modelling, indicated that all three loops I-III should be involved in the interaction and the main binding site for this allosteric modulator is located in the extracellular loops of the receptor [46].

There are also several three-finger proteins from snake venoms (calciceptin, FS2) blocking Ca^{2+} channels [47,48]. We should also mention here fasciculin, a three-finger protein with 4 disulfides, targeting the acetylcholinesterase. Interestingly, the X-ray structures of fasciculin

in complex with acetylcholinesterases were the first examples presenting a three-finger toxin bound to its biological target [49,50].

One of the most well-represented groups in the snake venoms are so-called cytotoxins (some of them were earlier called cardiotoxins) which apparently do not have a single well-defined target but disrupt the cell membranes thus inducing a multitude of effects (see reviews [51,52]). As a result of proteomic studies new three-finger proteins are being found in the snake venoms, and one of the minor components in the *Naja kaouthia* cobra venom was identified as a glycosylated cytotoxin I [53]. This post-translational modification, for the first time discovered for the family of three-finger toxins, considerably decreased the cytotoxicity of this protein, whereas enzymatic deglycosylation restored it to the level of cytotoxin I activity [53]. Another really a minor component of that venom (less than 0.01% in the crude venom) was a dimer of cytotoxin and α -cobratoxin connected by two intermolecular disulfide bridges which revealed a weak activity against neuronal nicotinic acetylcholine receptors [35].

We also would like to mention here the recent discovery of three-finger neurotoxins which interact with another group of GPCR, namely with the adrenoreceptors [54,55]. These toxins are most similar to muscarinic toxins and were also isolated from the eastern green mamba *Dendroaspis angusticeps*. One such toxin (α -Da1a) has a very high affinity (0.35 nM) for the α_1 adrenoreceptor, while another one (α -Da1b) has a lower affinity but is more selective towards α_2 types [55]. Interestingly, these toxins are considered as possible drugs against prostate hypertrophy.

Although it is not the topic of the present review, it is appropriate to mention here that there are three-finger proteins in nervous and immune system of mammals and insects belonging to the Ly6 family and some of them bind to nicotinic acetylcholine receptors and regulate their functioning *in vivo* (see [56-59] and recent publications from our institute [60-63]).

3.5. Peptides from snake venoms acting on nicotinic acetylcholine receptors

Such peptides are not as numerous as α -neurotoxins or non-conventional toxins targeting different subtypes of nAChR. Until recently the only group was that of waglerins isolated from the venom of South Asian snake *Tropidolaemus wagleri* which consist of 22-24 amino acids and contain one disulfide bridge [64,65]. These toxins bind with high affinity to muscle-type nAChR [66]. Interestingly, waglerins can distinguish embryonic ($\alpha_1\beta_1\gamma\delta$) and "mature" ($\alpha_1\beta_1\epsilon\delta$) muscle-type nAChR: waglerin-1 efficiently blocks the ϵ -containing form, but not the γ -form of this receptor [67]. While snake venom α -neurotoxins bind with practically equal efficiency to the two binding sites (formed by two α -subunits with their non- α neighbors) in the muscle-type nAChRs, waglerin-1 binds 2100-fold more tightly to the α - ϵ than to the α - δ binding site of the mouse nAChR [68]. Several amino acid residues in the nAChR subunits participating in waglerin binding were identified by site directed mutagenesis [69], namely Asp59 and Asp173 were shown to be important for waglerin binding at both sites. On the other hand, the disulfide in waglerin was found to be essential for its activity, as well as several residues in its N-terminal part of the amino acid sequence [70].

A new peptide was recently found in the snake venom possessing a capacity to block muscle-type nAChR [71]. It is azemiopsin, isolated from the *Azemiops feae* viper venom, which consists of 21 amino acid residues. By the chain length azemiopsin is similar to waglerins and, moreover, shares with them a homologous C-terminal fragment. However, it possesses a unique structural feature: contrary to all earlier known proteins and peptides from the venoms of snakes or poisonous *Conus* mollusks (see below), whose structure is fixed by one or several S–S-bonds, azemiopsin contains no disulfides. It dose-dependently blocked acetylcholine-induced currents in *Xenopus* oocytes heterologously expressing human muscle nAChR, and was more potent against the adult ($\alpha_1\beta_1\epsilon\delta$) than the fetal ($\alpha_1\beta_1\gamma\delta$) form. Ala-scanning and analysis of competition with α -bungarotoxin for binding to *Torpedo* nAChR resulted in identification of the azemiopsin residues essential for its activity which in general were found to be different from those responsible for the waglerin activity [71].

4. α -Conotoxins, peptides from poisonous marine snails *Conus*, acting on nicotinic acetylcholine receptors

Historically, snake venom α -neurotoxins were the first extremely important tools which made possible “digging out” in a purified form the first representative of the nAChR family, namely the muscle-type receptor from the *Torpedo* ray electric organ. Then, in the early 80^s, the peptide toxins were discovered in the marine mollusk *Conus geographus* venom which caused postsynaptic inhibition at the neuromuscular junction in frog and got the name of conotoxins [72]. The following studies brought to life a tremendous number of so-called conotoxins or conopeptides from different species of *Conus* snails. The number of *Conus* species living in different seas and oceans is about 1000 and the available data show that the venom of each species should contain in excess of 1000 conopeptides. Thus, *Conus* mollusks provide researchers with huge combinatorial libraries of peptides. The main task of slowly moving *Conus* mollusks is to immobilize their preys (small fishes, worms etc.), that is why their venoms contain a variety of peptides paralyzing the nervous systems of their targets. Evolutionary each *Conus* species is adjusted to a particular area and a distinct food source, hence the individuality of each venom. There are several types of conotoxins differing in their targets: α -conotoxins block nAChRs, μ -conotoxins are acting on Na⁺-channels, κ -conotoxins interact with K⁺-channels, ω -conotoxins block specifically certain Ca²⁺-channels and one of such ω -conotoxins became a very potent analgesic (trade name Ziconotide or Prialt; see more about these and many other conotoxins and conopeptides in recent reviews [73–75]). The number of discovered conotoxins is rapidly increasing because nowadays they appear not so much due to isolation from *Conus* venoms (usually available only in minute amounts) but due to deciphering mRNAs obtained from the venom glands.

Since this chapter is devoted to neurotoxic proteins and peptides interacting with nicotinic acetylcholine receptors, below we will consider only those conotoxins which target these receptors. The major group is α -conotoxins, competitive antagonists of nAChRs. They have 12–19 amino-acid residues, as a rule amidated C-terminus and two disulfide bonds between Cys residues C¹–C³ and C²–C⁴ (see Table). There are also several other groups of conotoxins

acting on nAChRs (ψ -, α A-, α A_S-, α C-, α S- and α D), but they are not numerous, are not as widely used as α -conotoxins and will not be considered here.

Toxin	<i>Conus species</i>	Amino acid sequence ¹	Selectivity
3/5 α -conotoxins			
Gl	<i>C. geographus</i>	ECCNPACGRHYSC*	α 1 β 1 γ / ϵ δ
MI	<i>C. magus</i>	GRCCHPACGKNYSC*	α 1 β 1 γ / ϵ δ
SIA	<i>C. striatus</i>	YCCHPACGKNFDC*	α 1 β 1 γ / ϵ δ
4/3 α -conotoxins			
ImI	<i>C. imperialis</i>	GCCSDPRCAWRC*	α 7, α 9 α 10; α 3 β 2; α 3 β 4
RglA	<i>C. regius</i>	GCCSDPRCRYRCR	α 9 α 10
4/4 α -conotoxins			
BulA	<i>C. bullatus</i>	GCCSTPPCAVLYC*	α 3(α 6) β 2, α 3(α 6) β 4
4/6 α -conotoxins			
AulB	<i>C. aulicus</i>	GCCSYPPCFATNPDC*	α 3 β 4
4/7 α -conotoxins			
PnlA	<i>C. pennaceus</i>	GCCSLPPCAANNPDYC*	α 3 β 2
PnlB	<i>C. pennaceus</i>	GCCSLPPCALSNPDYC*	α 7; α 3 β 4
MII	<i>C. magus</i>	GCCSNPVCHLEHSNLC*	α 3 β 2(β 3); α 6-containing
Vc1.1	<i>C. victoriae</i>	GCCSDPRCNYDHPEIC*	α 9 α 10; α 3 β 4, α 3(α 5) β 2
TxIA	<i>C. textile</i>	GCCSRPPCIANNPDLC*	α 3 β 2
ArIB	<i>C. arenatus</i>	DECCSNPACRVNPHVCRRR	α 7, α 6 α 3 β 2 β 3, α 3 β 2

¹ Scheme of disulfide closing for naturally-occurring α -conotoxins –

* indicates an amidated C-terminus; the names of α -conotoxins typed in italics mean that their structures were identified in cDNA libraries.

Table 1. Most studied members of naturally-occurring α -conotoxins.

α -Conotoxins are structurally subdivided into subgroups depending on the number of amino acid residues between the C²–C³ and C³–C⁴ cysteines (see Table) forming the first and second loops, respectively. This structural feature affects the α -conotoxin specificity to particular nAChR subtypes. All at present known 3/5 α -conotoxins are potent blockers of muscle type nAChRs (and conventionally can be called ‘muscle’ α -conotoxins). The members of other subgroups (4/3, 4/4, 4/6, 4/7) act on various neuronal nAChR subtypes (and can be called ‘neuronal’ α -conotoxins). It is very rare when naturally occurring neuronal α -conotoxin blocks specifically only one neuronal nAChR subtype, usually neuronal α -conotoxins interact with two or more nAChR subtypes (see Table).

Most of muscle 3/5 α -conotoxins can discriminate species-specifically two binding sites on muscle or *Torpedo* nAChRs. For example, α -conotoxins MI, GI or SIA have up to 10000 times higher affinity for $\alpha 1/\delta$ - over $\alpha 1/\gamma$ site in muscle nAChR [76,77]; in contrast to more effective binding of these peptides, although not with such a great difference, to $\alpha 1/\gamma$ site in *Torpedo* receptor [78,79].

“Mutagenesis” studies of α -conotoxins (in fact not the mutagenesis as such, but substitutions of amino acid residues by solid-phase peptide synthesis) gave information about those residues which are the basis of the high affinity and selectivity to a particular receptor or receptor subgroup. For example, the crucial role of Arg9 in α -conotoxin GI, as well as of Pro6 and Tyr12 in α -conotoxin MI for discriminating the $\alpha 1/\gamma$ - and $\alpha 1/\delta$ -sites was revealed [80-82]. Interestingly, Arg9 proved important for a neuronal 4/3 α -conotoxin RgIA for its $\alpha 9\alpha 10$ nAChR specificity [83]. Similar “mutagenesis” studies resulting in revelation of residues crucial for activity were done also for many other α -conotoxins (ImI, PnIA, MII, GID, Vc1.1, AuIB) [84-89].

Like in the analysis of interactions between different nAChR types and snake venom neurotoxins, when much efforts has been spent by many laboratories to establish the topography of their binding, similar studies have been undertaken to elucidate the mechanism of nAChR recognition by α -conotoxins. Among them were above-mentioned multiple substitutions in the amino acid sequences of naturally occurring α -conotoxins, making their structures more rigid, syntheses of radioactive, fluorescent and photoactivatable derivatives. Combination with mutagenesis of the receptor subunits (pair-wise mutagenesis) gave information about possible contact points between α -neurotoxins and nAChRs, as well as between α -conotoxins and nAChRs. The relevant information can be found in numerous reviews (see, for example, [90-92]), but will not be considered in detail here, because this chapter contains a special section where crystal structures of α -neurotoxins and α -conotoxins in complexes with the relevant biological targets will be discussed.

5. Three-dimensional structures of peptide and protein neurotoxins in complexes with the nicotinic receptor models and fragments

It was already mentioned that the crystal structure of the acetylcholine-binding protein (AChBP) provided an impressing jump in the structural analysis of not only nicotinic acetylcholine receptors but of all other members of the Cys-loop receptor family. This water-soluble protein was found to modulated synaptic transmission in glia of *Lymnaea stagnalis* fresh-water mollusk and was purified using affinity chromatography on a column with the attached α -bungarotoxin [93]. Sufficient amounts of AChBP were obtained by heterologous expression and the crystal structure was determined at 2.7 Å resolution [10]. This structure clearly showed that AChBP is an excellent structural model of N-terminal ligand-binding domains of all nAChRs: crystal AChBP was in a pentameric state, similarly to the whole-size nAChRs. In spite of low homology with the amino-acid sequences of extracellular domains of nAChR subunits (not more than 25%), AChBP contains all those amino acid residues which earlier in receptor studies were found essential for interacting with the cholinergic agonists and antagonist. The

AChBP crystal structure revealed that such residues are all clustered in the middle of AChBP, at the interfaces between its subunits (or protomers). At present, the X-ray structures of several molluscan AChBPs are known (from *Lymanaea stagnalis*, *Aplysia californica*, *Bulinus truncatus*), as well of their complexes with a wide variety of agonists and antagonists which gave quite a detailed picture of the respective binding sites in these AChBPs and of their contacts with ligands. Biochemical data and computer modeling show convincingly that these structures shed light on the receptor binding sites *per se* and on the ligand disposition in the binding sites of muscle and neuronal nAChRs. The relevant information can be found in recent original papers [94-97] and reviews [98,99], and below we will consider in detail only the crystal structures of complexes with protein and peptide neurotoxins.

Interestingly, the first AChBP crystal structure in complex with a competitive antagonist was that of *Lymanaea stagnalis* AChBP with bound α -cobratoxin [24]. (In parentheses it may be mentioned that later more structures were solved for the *Aplysia californica* AChBP complexes, but *L. stagnalis* AChBP has a much higher affinity for α -neurotoxins than AChBPs from other species). First of all, X-ray analysis revealed 5 α -cobratoxin molecules attached at the interfaces between 5 identical subunits (or protomers) of AChBP (Figure 3, A). The major role in the organization of the binding site is played by aromatic residues (so-called “aromatic box”) of AChBP. Long before crystallographic studies, protein chemistry and mutagenesis revealed that these aromatic residues were important for binding different agonists and antagonists to diverse muscle-type and neuronal nAChRs. It was proposed that the binding sites are formed by three fragments (A, B, C) of polypeptide chain of one subunit and by three fragments (D, E, F) of the polypeptide chain of the other one on which these aromatic residues are located (see review [100]). The first three fragments in real receptors are on the α -subunits and form the main (principal) binding surface, while the last three are on non- α -subunits and compose the complementary binding surface. In the case of homopentameric receptors like $\alpha 7$ ones, the A-C loops are on the “front surface” of one $\alpha 7$ -subunit and D-F on the “back surface” of the neighboring identical subunit. In general, the X-ray structure of the AChBP complex with α -cobratoxin is in accord with the earlier ideas on the α -neurotoxin binding to nAChRs formulated on the basis of chemical modification of α -neurotoxins, their mutagenesis, photoaffinity labeling and mutagenesis of receptors (see reviews [101,102]). Indeed, there is a multipoint binding of α -cobratoxin and the major role, as earlier shown by “wet biochemistry” methods, is played by the toxin central loop II.

The comparison with the NMR and X-ray structures for α -neurotoxins revealed that α -cobratoxin did not need to change its conformation dramatically to be accommodated in the binding region of AChBP. On the contrary, the AChBP loop C containing the disulfide between the neighboring cysteines (which is also a characteristic feature of all nAChR α -subunits) had to move to periphery up to 10 Å from the position which it occupied in the AChBP containing no bound ligand. (This movement should be supplemented with essential changes in conformation of loop F from complementary AChBP protomer.) Moreover, the earlier solved structure of AChBP with such agonist as nicotine revealed that, when agonist comes to the binding site, loop C embraces it and moves closer to the central axis of the molecule [94]. At present there are many crystal structures of various AChBPs in complexes with versatile

specific or nonselective agonists and antagonists of the muscle-type and neuronal nAChRs and it appears to be a general rule: antagonists versus agonists induce movements of the loop C in the opposite directions.

5.1. X-ray structure of the extracellular domain of muscle nAChR $\alpha 1$ subunit in complex with α -bungarotoxin

Until now we were considering the X-ray and Electron microscopy structures of closely related but independent objects of studies: acetylcholine binding proteins and *Torpedo* nAChR. It should be emphasized that the structures of bound cholinergic agonists and antagonists until recently were available only for their complexes with AChBPs. That is why when researchers wished to analyze in three-dimensions the interactions of agonists or antagonists with the muscle-type or neuronal nAChRs, they had to rely on computer modeling. Fortunately, one of the bridges between the AChBPs and nAChRs spatial structures has been recently open: the X-ray structure has been determined for the α -bungarotoxin complex with heterologously expressed ligand-binding domain of mouse muscle nAChR $\alpha 1$ subunit [25]. Many laboratories have earlier tried, with the aid of heterologous expression, to obtain ligand-binding domains of $\alpha 1$ or $\alpha 7$ subunits as individual proteins and to determine their three-dimensional structure. Although in certain cases those proteins could bind α -bungarotoxin with relatively high affinity (but not with the nanomolar constants as intact receptors) [103-106], in no case the proteins could be crystallized. In view of the above-said, the work [25] is clearly a breakthrough. Using random mutagenesis, the authors have chosen a protein with a low tendency to aggregation. In spite of its having the mutation of Trp149 (localized in loop B and known to be important for binding agonists and antagonists), the protein could bind α -bungarotoxin. It was namely the complex of α -bungarotoxin rather than the free domain which was successfully crystallized. (Thus, in addition to helping isolate the *Torpedo* nAChR and *L. stagnalis* AChBP, α -neurotoxins played again an important role, this time in crystallization of the nAChR subunit ligand-binding domain.) The structure of the complex has been solved at a very high resolution (1.94 Å) (see Figure 3, B).

Although this domain is a monomer, its spatial structure is very similar to an AChBP protomer in a pentameric complex. A molecule of bound α -bungarotoxin occupies the position similar to that of α -cobratoxin in complex with *L. stagnalis* AChBP (compare Figure 3, A and B). It should be emphasized that in the complex with $\alpha 1$ domain, α -bungarotoxin utilized for interaction only the principal side, while α -cobratoxin in complex with pentameric AChBP has contacts with both principal and complementary sides at the subunit interface. However, instead of this, α -bungarotoxin forms contacts with the sugar moiety present in the nAChR domain but absent in AChBPs.

5.2. X-ray structure of α -bungarotoxin with a chimera of *L. stagnalis* AChBP/ligand-binding domain of the human $\alpha 7$ subunit

This work can be considered as a further development of the recent breakthrough in the analysis of ligand binding domains of nAChRs when an important step was done in ascending from models to true receptors. The authors of [107] managed to substitute about

Figure 3. Crystal structures of the AChBP/nAChR domain-toxin complexes. Top and side views are in upper and lower lines, respectively. A - α -Cobratoxin bound to *L. stagnalis* AChBP (PDB ID: 1YI5); toxins and proteins are shown in blue and green. B - α -Bungarotoxin bound to the N-terminal domain of nAChR $\alpha 1$ subunit (2QC1); toxin and subunit are shown in blue and red; the sugar moiety presented in this complex were excluded for clarity. C - α -Bungarotoxin bound to the chimeric protein composed of N-terminal domain of nAChR $\alpha 7$ subunit and *L. stagnalis* AChBP (4HQP); toxins and chimeras are shown in blue and magenta.

70% of the amino-acid residues in *L. stagnalis*AChBP (not touching the less hydrophobic Cys-loop) for residues of the $\alpha 7$ subunit and crystallized this protein in free form and in complex with epibatidine, an potent but nonselective nAChR agonist[108]. The observed pentaoligomeric structure can be considered as the closest proximation to the 3D structure of the ligand-binding domain of the true $\alpha 7$ nAChR. Practically the same $\alpha 7$ /AChBP chimera has been used to crystallize a complex with α -bungarotoxin [26]. Again, it was a pentaoligomer with 5 attached α -bungarotoxin molecules (see Figure 3, C). In general, disposition of α -bungarotoxin is very close to what was observed for α -bungarotoxin in complex with the $\alpha 1$ domain or for α -cobratoxin complex with the *L. stagnalis* AChBP (compare with Figure 3, B and A). Basing on the high-resolution structure of the $\alpha 7$ /AChBP- α -bungarotoxin chimera, the authors designed a series of $\alpha 7$ nAChR mutants and from the analysis of their activities and efficiency of α -bungarotoxin binding collected a very detailed information about the intermolecular interactions which ensure the high affinity for α -bungarotoxin binding [26]. In particular, they not only confirmed the role of the “aromatic box”, but also revealed the importance of amino-acid residues which in the amino acid sequence are direct neighbors of those aromatic residues.

5.3. X-ray structure structures of AChBP complexes with α -conotoxins

The first X-ray structure of the AChBP complex with α -conotoxin [109] has been solved soon after elucidation of the X-ray structure of the *L. stagnalis* AChBP complex with α -cobratoxin.

Figure 4. Spatial organization of complexes of α -conotoxins from different groups and *A. californica* AChBP derived from their crystal structures. Only two adjacent monomers of AChBP colored in cyan and yellow for clarity in side views are presented. All α -conotoxins are shown in magenta. A – complex with α -conotoxin PnIA[A10L, D14K] variant from 4/7 α -conotoxin group (PDB ID: 2BR8). B – complex with α -conotoxin Iml from 4/3 α -conotoxin group (2C9T). C – complex with α -conotoxin BulA from 4/4 α -conotoxin group (4EZ1).

In this case it was another AChBP, namely the protein from the marine mollusk *Aplysia californica* [110]. First of all, contrary to the α -cobratoxin complex, it was a high-resolution (2.4 Å) structure and, secondly, it was the first X-ray structure for a representative of the huge conotoxin library in complex with a biological target. The crystals were raised for the complex of α -conotoxin PnIA analog having two substitutions ([A10L] and [D14K]) which had high affinity both for *L. stagnalis* and *A. californica* AChBPs and potently inhibited acetylcholine-induced currents in $\alpha 7$ nAChRs expressed in oocytes [109].

Hydrophobic contacts were found to play the major role in the interaction of α -conotoxin PnIA[A10L, D14K] with *A. californica* AChBP (Figure 4, A). As in other AChBP complexes with agonists or antagonists, at the principal side the contacts are formed mainly by highly conserved aromatic amino acid residues - Trp145, Tyr186, Tyr193. At the complementary side the contributions are from aliphatic residues (Val106, Met114, Ile116). It should be stressed again that loop C in the complex with α -conotoxin moves to the periphery of the AChBP molecule by more than 10 Å, as compared with its disposition in the “apo” form of *A. californica* AChBP. A similar shift was also observed, as mentioned above, for the α -cobratoxin complex [24], as well as for the majority of AChBP complexes with other antagonists (see reviews [98,111,112]). Thus, the conclusion that the most obvious distinction between the first steps in the binding modes of agonists versus antagonists is the induced movement of the loop C (to the central axis for the former and outwards for the latter) appears to be correct. However, there are some deviations from this trend: for example, strychnine is an antagonist both of the nAChRs and glycine receptors, but in the case of its complex with the *A. californica* AChBP, the loop C shift to the periphery was only very slight [113]. The changes in the disposition of the loop C were not pronounced also for AChBP complexes with partial agonists [96].

Another interesting feature of AChBP complexes was for the first time observed with partial agonists: in distinct binding sites within a pentameric AChBP molecule these compounds

had different orientations [96]. Such multiplicity was first thought to be inherent only in partial agonists, but later altering dispositions in the 5 AChBP binding sites were observed for the complexes of such alkaloid antagonists as strychnine and d-tubocurarine [113]. Moreover, in several binding sites two alkaloid molecules managed to be accommodated simultaneously [113].

Variations of the ligand orientation in the binding sites of AChBPs and nAChRs are of undoubted interest. In the *A. californica* AChBP complex, all 5 bound α -conotoxin PnIA[A10L, D14K] molecules had the same conformation and orientation. This was also true for the later solved structures of α -conotoxin ImI complexes [114,115] (see Figure 4, B). These structures (very similar to those of α -conotoxin PnIA[A10L, D14K]) confirmed that, although bound α -conotoxin PnIA analog had two substitutions and was in this respect “unnatural α -conotoxin”, the X-ray structure of its complex correctly revealed the structural principles of the α -conotoxin-AChBP recognition. Fine adjustments of such a recognition were brought to light by the structure of *A. californica* AChBP complex with the α -conotoxin TxIA[A10L] [116]. In general, the structure of this complex was very similar to those of α -conotoxin PnIA[A10L, D14K] or α -conotoxin ImI, but with a noticeable difference: this α -conotoxin derivative occupied exactly the same region as the two above-mentioned α -conotoxins, but it was turned around the central axis by about 20 degrees. The authors proposed that such rotation reflects certain differences in the selectivity of this particular α -conotoxin [116]. The latest published structure of the AChBP complex with α -conotoxin (November 2013) is announced by the Protein Data Bank (PDB) the structure with ID - 4EZ1. This is a complex of *A. californica* AChBP with α -conotoxin BuIA [117]. Despite the fact that α -conotoxin BuIA is a member of other subgroup of α -conotoxins (4/4) its position and orientation in the complex with AChBP (Figure 4, C) very close to that of both α -conotoxin PnIA analog (4/7 subgroup) and α -conotoxin ImI (4/3 subgroup) (compare Figure 4, A, B and C). In any case, from the four solved X-ray structures for AChBP complexes with α -conotoxins it followed that some variations in their attachment are possible. It might be expected that variations may be even more pronounced when α -conotoxins interact with true nAChRs, especially with heterooligomeric ones having different subunit interfaces.

Indeed, interpretation of the cross-linking of photoactivatable derivative of α -conotoxin GI to *Torpedo californica* nAChR in terms of the model built on the basis of the X-ray structure of the AChBP complex with α -conotoxin PnIA[A10L, D14K], suggested that for bound α -conotoxin two orientations are possible where the disposition of photoactivatable group differs by about 90 degrees [118]. Later a similar situation was demonstrated for an agonist, namely for the photoactivatable derivative of epibatidine [119]. This compound was shown to bind to only one site in the *T. californica* nAChR, but to 2 sites in the neuronal $\alpha 4 \beta 2$ nAChR which presumes two different dispositions of the bound ligand [119]. Naturally, cross-linking is not such a direct evidence as the X-ray structure, but the latter are available only for the AChBP complexes and the multiplicity of alkaloid antagonist orientations in the frames of one AChBP molecule [113] has been already mentioned.

6. Summary

In this chapter we tried to briefly present almost a 50-year history of using protein and peptide neurotoxins in fundamental and practical studies of nicotinic acetylcholine receptors (nAChRs). It was shown that the discovery of α -neurotoxins in the snake venoms was an extremely important step which made possible identification and isolation in individual form of the first nAChR from the *Torpedo* ray electric organ. Many laboratories comprehensively analyzed this receptor and it soon became clear that it is an appropriate model for nAChRs of all classes, namely muscle, neuronal and the so-called “non-neuronal” ones. Later, in addition to the three-finger α -neurotoxins, new shorter and smaller but not less efficient tools were found: namely, among a huge family of various peptides in the venoms of marine *Conus* mollusks, one particular group happened to be invaluable for research on nAChRs. Here we speak about α -conotoxins which not only discriminate the muscle-type from neuronal nAChRs, but some of them even are selective towards a particular neuronal nAChR subtype. One should not think that the discovery of α -conotoxins put the α -neurotoxins into archives. First of all, even to-day α -bungarotoxin and its radioactive and fluorescent derivatives are the most reliable tools for identification and measuring the levels of the functional $\alpha 7$ nAChRs. Secondly, α -neurotoxins played another leading role a decade ago helping to purify the acetylcholine-binding protein (AChBP). The discovery and the X-ray structure of this protein, an ideal model for the ligand-binding domains of all nAChRs, was the major breakthrough in elucidating the three-dimensional structure of nAChRs and especially of their ligand-binding site topography. Our chapter also presented the data on the crystal structures of AChBP complexes both with α -neurotoxins and α -conotoxins that gave information about the topography of their interactions with the key residues in the binding site, thus providing a basis for new drug design. The next step was the establishment of the crystal structures of α -neurotoxins with chimera of AChBP and $\alpha 7$ nAChR ligand-binding domain, which can be considered as a good mimic of the true $\alpha 7$ receptor, as well as the X-ray structure of the α -bungarotoxin complex with a mutated nAChR $\alpha 1$ subunit extracellular domain. In this chapter we were not discussing the bacterial pentameric ligand-gated ion channels (belonging to the same family as nAChRs), but at present not only high-resolution X-ray structures are available for them, but also for their complexes with different ligands. In particular, one of such receptors (ELIC) happened to be a close analog of the mammalian GABA-A receptors. We might hope that one day high resolution structures become available for nAChRs or their homologs in complexes with α -neurotoxins and/or α -conotoxins, to which the chapter is devoted. It will give new life to these still invaluable tools in fundamental research on nAChRs and in numerous practical applications.

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