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Commentary

Toxin insights into nicotinic acetylcholine receptors

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Abbreviations:
AChBP, acetylcholine binding protein
CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid
Cbtx, α-cobratoxin
PnIA, α-conotoxin PnIA
ImI, α-conotoxin ImI
MLA, methyllycaconitine
PEG, polyethylene glycol
EPI, epibatidine
LOB, α-lobeline
nAChR, nicotinic acetylcholine receptor

ABSTRACT

Venomous species have evolved cocktails of bioactive peptides to facilitate prey capture. Given their often exquisite potency and target selectivity, venom peptides provide unique biochemical tools for probing the function of membrane proteins at the molecular level. In the field of the nicotinic acetylcholine receptors (nAChRs), the subtype specific snake α-neurotoxins and cone snail α-conotoxins have been widely used to probe receptor structure and function in native tissues and recombinant systems. However, only recently has it been possible to generate an accurate molecular view of these nAChR–toxin interactions. Crystal structures of AChBP, a homologue of the nAChR ligand binding domain, have now been solved in complex with α-cobratoxin, α-conotoxin PnIA and α-conotoxin ImI. The orientation of all three toxins in the ACh binding site confirms many of the predictions obtained from mutagenesis and docking simulations on homology models of mammalian nAChR. The precise understanding of the molecular determinants of these complexes is expected to contribute to the development of more selective nAChR modulators. In this commentary, we review the structural data on nAChR–toxin interactions and discuss their implications for the design of novel ligands acting at the nAChR.

1. Introduction

The nicotinic acetylcholine receptors (nAChRs) are non-selective pentameric cation channels that open in response to the binding of the neurotransmitter acetylcholine (ACh) [1]. They belong to the ligand-gated ion channel family (LGIC) that also includes the $GABA_A$ and $GABA_C$, glycine and $5\text{-}HT_3$ receptors. Based on different localisation and pharmacology, the nAChRs are divided into two distinct classes [2]. The muscle nAChR is the major neurotransmitter receptor at the neuromuscular junction and comprises two α1, one β1, one δ and one γ subunits. In contrast, the neuronal nAChRs are

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found in the nervous system and comprise five α subunits (α7, α9 or α10) or a combination of α and β subunits (e.g. α4β2, α6α3β). The multiple possible combinations provided by the latter group greatly expands the potential pharmacology of nAChRs, making the identification and role of each subunit in native tissue only possible with the development subtype selective probes. The nicotinic receptors are responsible for the nicotine addiction associated with tobacco smoking, and abnormal functions of these receptors result in a number of disorders and diseases, called channelopathies [3]. In addition, several nAChR subtypes found in the brain have been implicated in epilepsy and neurodegenerative diseases [4]. Therefore, the nAChRs represent attractive therapeutic targets.

A high percentage of sequence identity exists among the 12 known neuronal nAChR subunits, especially when only the ACh binding site is considered [5]. This conservation of sequence explains, at least in part, why it has been difficult to obtain subtype selective small molecules, particularly agonists that act deep within the conserved ACh binding pocket. However, selective peptide antagonists from snake or cone snail venoms provide a rich array of highly selective pharmacological tools able to discriminate amongst many of the different nAChR subunit combinations [6]. They often show high selectivity toward a particular mammalian nAChR subtypes through specific interactions with residues located outside the conserved ACh binding site [7]. Understand the specific chemistry directing this specificity could hold the key for the design of novel and selective drugs. However, the lack of atomic resolution structure of nAChRs has hampered this approach.

With the recent determination of several crystal structures of snake and cone snail toxins in complex with AChBP, a protein homologue of the nAChR ligand binding domain, a target pharmacophore approach is now possible [8–10]. Indeed, it is now possible to dissect the complex toxin-receptor at the atomic level and to compare the molecular interactions used by different toxins to achieve their high affinity binding. This information can be analysed to determine the minimum ligand pharmacophore required for binding [11]. Based on this structural information, a rational approach to engineer novel ligands that make complementary interactions with residue unique to a particular nAChR subtype is then possible. This task appears feasible, since predictions from models of the same toxins in complex with nAChRs are in good agreement with the AChBP–toxins crystal data, revealing that mammalian nAChR models based on AChBP are valid targets for the docking of ligands and the screening of virtual libraries [11–13].

In addition to the ligand binding modes, an analysis of all AChBP structures available to date reveals how the conformations observed in the crystals may relate to the resting (closed state), activated (open state) and desensitised (closed state) conformations of the nAChR. The nAChRs are described as allosteric proteins that spontaneously transition between a series of conformational states in a ligand dependent manner [14]. According to this allosteric model, the antagonist ligands including neurotoxins would stabilise the resting state, while the agonist molecules would trap the receptor in its open state [15]. The desensitised state is then reached after prolonged exposure to agonists. From an analysis of the AChBP structures, it is predicted that the nAChR structures of the desensitised state do not differ significantly, at least in the ligand binding domain, from the agonist-bound states of the nAChR, while the antagonist-bound structures appear clearly different.

2. Animal peptide toxins as tools to probe the nAChR structure and function

Venomous animals such as snakes or cone snails inject a potent mixture of mostly peptides and proteins into their prey to paralyse muscles and disrupt key functions such as the capacity to escape [16]. Given its key physiological role, is not surprising that nicotinic acetylcholine receptors are a major target of venom peptides. For instance, it has been shown that every cone snail species examined to date had at least one nAChR antagonist in its venom [17]. These conotoxins belong to the α- or ψ-class, depending on their competitive or non-competitive mode of action, respectively [6]. The largest group are the α-conotoxins that target muscle nAChRs and/or specific subtypes of neuronal nAChRs in mammals [17]. While a muscle nAChR selective antagonist is an obvious weapon to paralyse a prey, the neuronally active α-conotoxins discovered more recently have proved most interesting. Indeed, they can distinguish amongst different subunit arrangements and therefore represent valuable pharmacological tools to study the electrophysiological properties of nAChR subtypes, their distribution in native tissues, and may even have potential as drug leads [18–20]. Reflecting the number of residues present in each of the two loops formed by the disulfide bonds, these peptides are called 3/5, 4/3, 4/4 or 4/7 α-conotoxins. Such conotoxin frameworks are often closely associated with specific α-conotoxin selectivity. For instance, the 3/5 α-conotoxins (e.g. GI, MI, SI) have high affinity for the muscle nAChR, but no apparent activity at the neuronal nAChRs. In contrast, 4/3 α-conotoxins (ImI and ImII) block selectively the neuronal α7 nAChR, but seem to use different binding sites to exert their similar antagonist activity [21]. The 4/4 α-conotoxins (BuIA) have only recently been discovered and these potently block several nAChR subtypes, with the highest potency for α3- and chimeric α6-containing nAChRs [22]. Finally, the 4/7 α-conotoxins provide the most diverse pharmacology, with some selective for muscle (EI), neuronal α7 (PnIB, A10L-PnIA), α3β2 (PnIA, GIC), α3β2α6 (MII, PIA) or α3β4 (AuIB) nAChRs [20]. A selective α-conotoxin for α4β2, the most represented nAChR subtype in the brain, would represent a very useful tool, but is yet to be discovered. Many structures of α-conotoxins have been determined using mainly NMR but also crystallographic methods [23]. Interestingly, the neuronal 4/7 α-conotoxins share a common fold comprising a short disulfide bond stabilised helix and a conserved proline, despite having highly divergent sequences.

Similarly to the α-conotoxins, the postsynaptic curaremimetic toxins isolated from Elapid and Hydrophiid snakes bind to the nAChR with high affinity and selectivity [24–26]. The short chain toxins are 60–62 residue peptides with four

disulfide bonds that only bind muscle nAChR with high affinity, while the long chain toxins (66–74 residues and five disulfide bonds) bind potently to both the muscle and α7 neuronal nAChR subtypes. Historically, the long chain neurotoxin α-bungarotoxin isolated from the banded krait snake (*Bungarus multicinctus*) venom was the first toxin used to characterise the nAChR [27,28]. Nowadays, this toxin is still widely used as a purification, affinity labelling and radioligand tool. A similar toxin isolated from a cobra *naja naja siamensis* (α-cobratoxin), has also been extensively used to probe the nAChR [29,30]. Structures of long chain neurotoxins have been obtained both by NMR and crystallographic methods and revealed a common ''three-fingered'' fold [31]. Interestingly, while the overall secondary structure is dominated by β strands, the tip of loop II forms a small α-helix that contains most of the important residues for nAChR binding. Structures of α-neurotoxins were also elucidated in complex with small nAChR mimotope peptides, giving insights into the molecular mechanism involved for toxin-receptor recognitions [32–34].

Despite of their larger size, long chain α-neurotoxins were believed to act like the smaller α-conotoxins, with the tip of loop II mimicking the small helix seen in conotoxins, allowing them to share a same binding site on the nAChR [35]. This hypothesis was supported by competitive binding and mutagenesis data that showed overlap of nAChR determinants for snake and cone snail toxins, and toxin mutants that identified equivalent residues as most important for binding [36]. Thanks to the AChBP–toxins crystal structures, we can now directly compare α-conotoxin and α-neurotoxin binding sites and understand the many differences, as well as the similarities in their binding mode.

3. AChBP-toxin crystal structures

The acetylcholine binding protein (AChBP), first isolated from the mollusc *Lymnaea stagnalis*, has become the established model for the ligand binding domain of nAChRs since its structure was solved in 2001 by Sixma and colleagues [37]. The AChBP crystal structure elegantly revealed the three-dimensional organization of the ACh binding site at 2.7 Å, rationalising 50 years of biochemical data [37]. Since this pioneering work, the structures of AChBP from two other mollusc species as well as in complex with various ligands have become available, increasing the interest for this protein [38,39].

AChBP was recently co-crystallised with snake and cone snail toxins. First, Bourne et al. determined the crystal structure of α-cobratoxin in complex with *L. stagnalis* AChBP [9]. Five toxin molecules were found deeply inserted in all five possible AChBP subunit interfaces, with a ~45° angle relative to the median axis of the AChBP ring. Changes in loop C and F conformations were associated with α-cobratoxin binding, as compared to HEPES- or agonist-bound AChBP structures. In particular, loop C is stabilised in a more ''open'' conformation (Fig. 3A) resembling that of the ligand-free AChBP structures (Fig. 3F and G). The toxin interacts mainly through three distinct anchoring points, loop I, the tip of loop II, and the C-terminus (Fig. 1A). The contacts established appear essentially hydrophobic and aromatic in nature, with toxin residues F29 and R33 of α-cobratoxin filling the ACh pocket, interacting with W53, Y185, Y192 and C187 (Table 1). Additional contacts are made between toxin residues I5-D8 (tip of loop I) and F65 (C-terminus) and AChBP residues T184, A191 and E190.

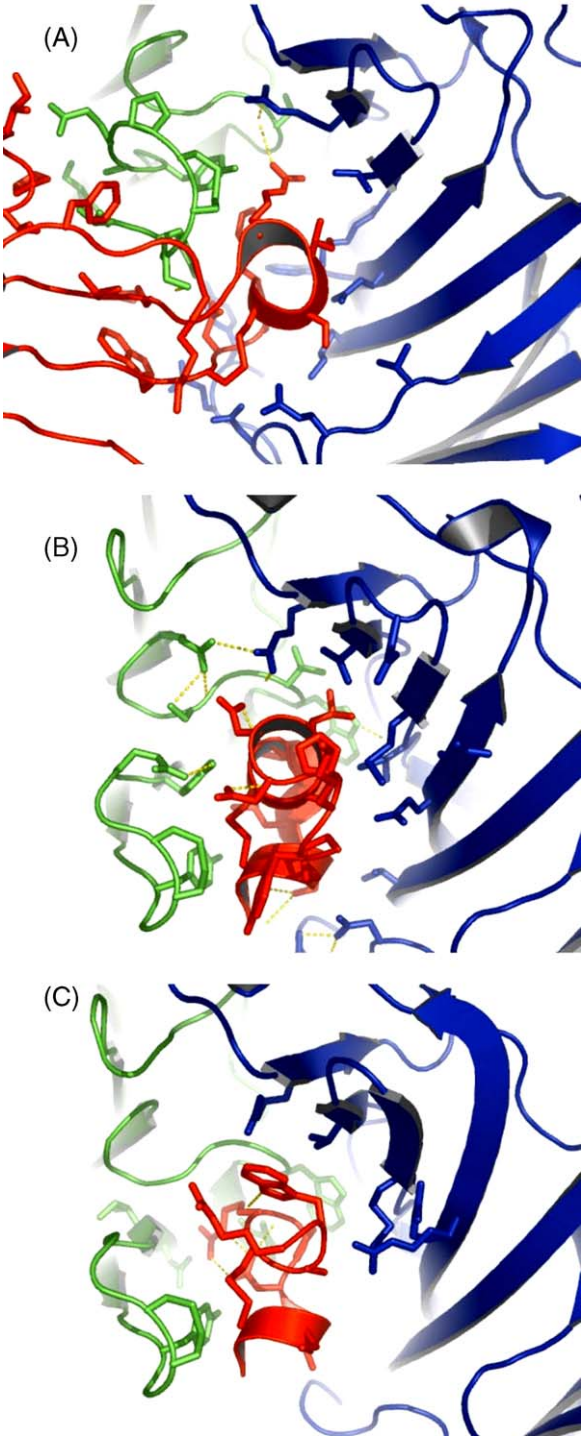

Fig. 1 – Details of the toxin–AChBP complexes. (A) α-Cobratoxin bound to Ls-AChBP; (B) α-conotoxin A10L-D14K-PnIA bound to Ac-AChBP; (C) α-conotoxin ImI bound to Ac-AChBP. Principal subunit is in green, complementary subunit in blue, and toxins in red.

Table 1 – AChBP-toxin interactions

Cbtx	Ls-AChBP		PnIA	Ac-AChBP		ImI	Ac-AChBP[a]	
	(+)	(−)		(+)	(−)		(+)	(−)
T6	T184		G1	Y186		G1	Y186	
P7	S32, T184, E190, A191		C2–C8	C188–189		C2–C8	C188–C189, Y186, Y193	
			S4		T34, D162, S164	S4		S164
I9	E190							
W25		E163	L5	Y186, Y193		D5	Y186	
C26–C30		E157, D160	P6	W145	I116, Y53	P6	W145, Y91	I116, Y53
D27	Y185		P7	W145, Y193		R7	D195, Y91	
A28		K34, Y164					I194	
F29	Y185, Y192	W53	A9		I116, Q55	A9		M114, I116
S31		Q55, T155	L10	V146	V106, M114	W10		V106, D75, T108
I32		Q55, L112			I116			
		M114	N11	E151, S148	R77	R11	E191	
R33	T144, C187, Y192	R104	N12	E191, C189		C12		R57
			P13		V106, M114			
G34	S186		K14		D110			
K35	S186		Y15	C188				
R36	Y185							
V37	T184, S186							
F65	T184, C187 P189							
R68	S186							

[a] Ac-AChBP numbering follows that of Celie et al. [8].

Overall, these interactions agreed reasonably well with previous structure–activity studies, and clearly explain the antagonist mode of action of α-neurotoxin [12].

Soon after this first three-dimensional insight into the nAChR–toxin complex at the molecular level, two structures of *Aplysia californica* AChBP in complex with α-conotoxins were published [8,10]. Celie et al. co-crystallised a variant of α-conotoxin PnIA (A10L-D14K-PnIA) with AChBP (Fig. 1B) and Hansen et al. obtained the structure of the AChBP–ImI complex (Fig. 1C). ImI and PnIA–PnIB are well studied α-conotoxins and there is considerable interest in understanding their mode of action. As seen for the AChBP–cobratoxin complex, all five binding sites were occupied by an α-conotoxin molecule and the loop C was stabilised in ''open'' conformation (Fig. 3B and C). However, in both AChBP–conotoxin complexes loop F did not undergo significant movement upon conotoxin binding, probably because the α-conotoxins are much smaller ligands than the α-neurotoxins and/or bind in a different manner. PnIA variant and ImI α-conotoxins bind deeply into AChBP ligand binding pocket (PnIA variant binds only 1 Å deeper than ImI), burying 60% and 75 % of their solvent accessible surface area, respectively. In particular, loop I is orientated similarly in the cleft and interacts with the same AChBP residues in both peptides, in agreement with mutagenesis data (Table 1 [40,41]). The N-terminus part of α-conotoxins contains the small α-helix that appears to be a major element for binding to AChBP and nAChRs. Since it is also the most conserved sequence in neuronally active α-conotoxins, its role can be probably extended to the whole family (see next section). From residue 7, however, PnIA variant and ImI use different interactions to create a high affinity and/or selective binding (Table 1). Indeed, the PnIA variant makes mainly hydrophobic contacts in the ACh pocket, using P7, A9 and L10 as important anchoring points, while ImI forms a salt bridge (R7 with D197) and more hydrogen bonds.

If we compare α-neurotoxin and α-conotoxin interactions with AChBP, a small α-helix structure appears as a major determinant for binding, even if the orientations and specific interactions employed differ significantly. Clearly, with side chains protruding at 360°, this structure is ideally suited to allow multiple contacts on both side of a binding pocket located at the interface of two subunits. In both AChBP–conotoxin structures, the disulfide bond C2–C8 of α-conotoxins interacts with the vicinal disulfide bond in loop C of AChBP. The crucial role of this loop C double cysteine for α-conotoxin binding could then explain why no potent conotoxins have been found at other Cys-loop receptors that do not contain this vicinal disulfide bond. α-Conotoxin stabilisation of the C loop conformation in an ''open'' state, which approximates the resting state of the nAChR receptor, appears to be an essential ingredient for antagonist activity. Indeed, other structures of antagonist or large buffer molecules (e.g. MLA and PEG) bound to AChBP adopt the same conformation (Fig. 3D and H).

4. AChBP–toxin crystal structures versus nAChR–toxin computational models

Prior to the AChBP–toxin crystal structures, several groups have attempted to model the toxin–nAChR interaction using AChBP-based nAChR homology models in combination with crystal and NMR structures of toxins and mutagenesis-based distance restrains. To determine the predictive value of these models it is instructive to compare these with the more recent AChBP co-crystal structures. In 2001, shortly after the publication of the first AChBP structure, Harel et al. modelled the interaction of a toxin with the nAChR [42]. This work was

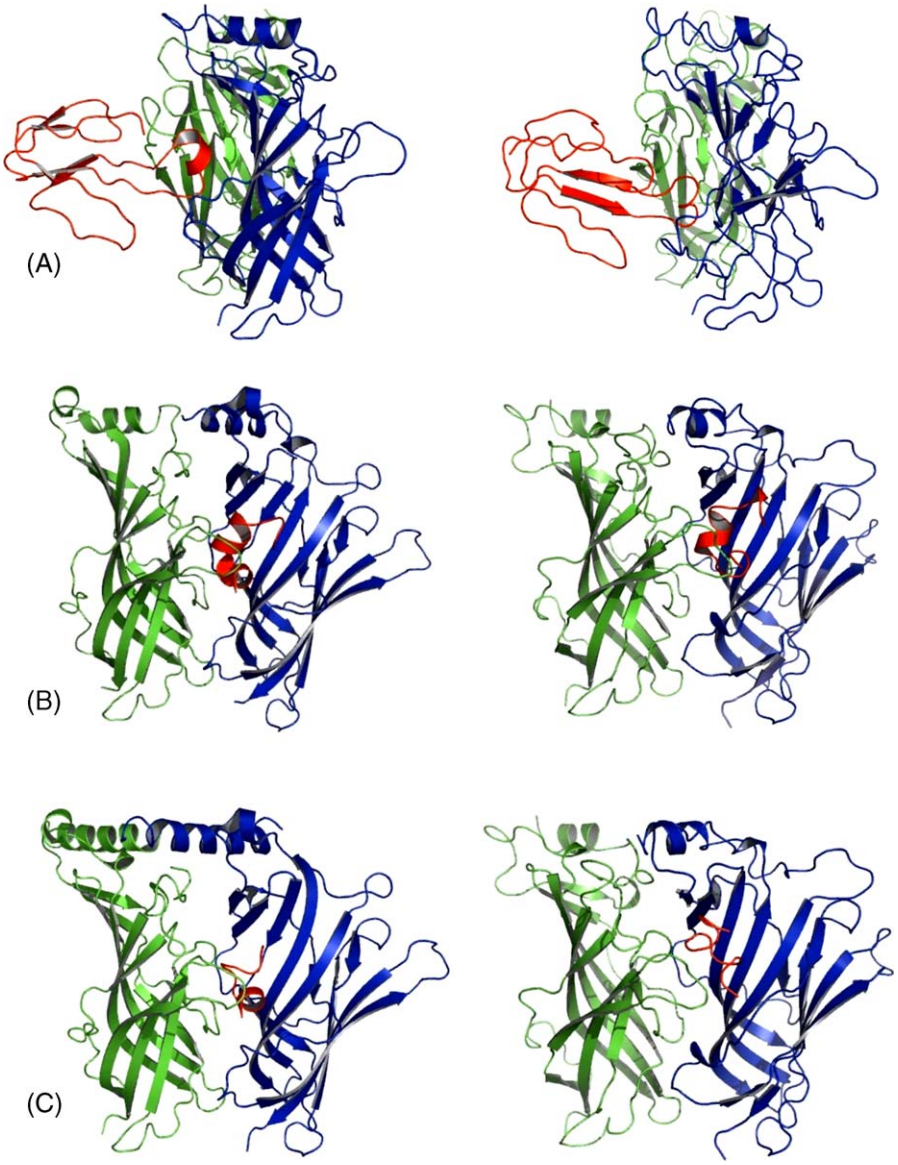

Fig. 2 – AChBP–toxin crystal structures vs. computational models. (A) α-Cobratoxin bound to AChBP (left) and a α7 nAChR homology model (right); (B) α-conotoxin A10L-D14K-PnIA bound to AChBP (left) and α-conotoxin PnIB bound to a α7 nAChR homology model (right); (C) α-conotoxin ImI bound to AChBP (left) and a α7 nAChR homology model (right). Principal subunit is in green, complementary subunit in blue, and toxins in red.

driven by NMR data on a complex between α-bungarotoxin and a nAChR peptide mimotope. The complex was then simply superimposed to the AChBP crystal structure to reveal several important clashes with AChBP loops and side chains. A model of α7 nAChR-α-cobratoxin based on extensive mutagenesis was then presented by Fruchart-Gaillard et al. [12]. Based on the rather close conformation of a HEPES-bound AChBP structure, molecular dynamics were applied on loop F of a α7 nAChR homology model to accommodate the bulky α-neurotoxin and avoid clashes. When compared to the crystal structure, the model appears gratifyingly similar (Fig. 2A) with the only major difference seen in the conformation of loop C.

The first model of the interaction between an α-conotoxin and nAChRs was presented by Dutertre et al., based on docking simulations and distance restrains obtained from previous mutagenesis data [13]. PnIB (only two residues different with A10L-D14K-PnIA) docked to a homology model of the α7 nAChR resulted in a complex remarkably close to the crystal structure (Fig. 2B). Indeed, despite having used an AChBP structure bound to HEPES as the template for interactions at the mammalian α7 nAChR, the orientation and contacts established were conserved between the two approaches. The absence of clashes in this instance can be explained by the fact that loop F does not move upon conotoxin binding [8]. Recently, these predictions were tested through mutagenesis on α3β2 nAChR, and results confirmed that this model can be extended to all 4/7 α-conotoxins interacting with α7, α3β2 and α4β2 nAChRs [11]. Finally, Dutertre et al. and Ellison et al. reported two very similar models for ImI interaction with α7 nAChR [13,43]. In this case,

however, ImI was docked in a clearly different mode compared to the AChBP crystal structure (Fig. 2C). Indeed, ImI was predicted to have a much shallower binding site in the ACh binding pocket of mammalian nAChRs. There are a number of factors that might contribute to the differences observed. First, in contrary to α-conotoxins PnIA–PnIB, ImI displays a very different dissociation constant for AChBP compared to nAChRs. Indeed, ImI binds with sub-nanomolar (K_d of 0.88 nM) affinity to Ac-AChBP, while it has only modest affinity for α7 nAChR (IC_{50} of 250–500 nM). Therefore, a shallower binding site for ImI in the binding pocket as seen in the model would be consistent with the modest affinity and fast off-rate observed at the α7 nAChR. This difference should not be completely surprising, as AChBP–toxin co-crystal structures are only a model of the mammalian nAChR ligand binding domain and far from an equivalent structure beyond the ACh binding pocket. While it would have been particularly difficult, if not impossible, to correctly guess the conformational changes that were seen in loops (especially C and F) upon binding of the different toxins, overall, the predictions obtained for α-neurotoxin and α-conotoxin interactions with nAChRs are in good agreement with the AChBP crystal structures.

5. Structures of the resting, activated and desensitised states of the nAChR

Nicotinic receptors from the Cys-loop family are allosteric proteins [14]. It is thought that spontaneously, these proteins can transition rapidly between different conformational states known as resting, activated and desensitised states [15]. Agonists stabilise the activated state (conducting), while the antagonists, such as toxins, stabilise the receptor in the resting state (non-conducting) [16,44]. The desensitised state (non-conducting) is reached after prolonged application of agonist. The properties that direct the level of energy necessary to reach a given state differ from a receptor subtype to another. For instance, the α7 nAChR subtype is known to desensitise rapidly upon agonist application from electrophysiology studies, as opposed to the α3β2 nAChR subtype, which desensitises slowly. The molecular mechanisms that allow such re-arrangements are not understood, and AChBP structures may help in identifying each nAChR allosteric entity. AChBP lacks a transmembrane domain, but results obtained from tryptophan fluorescence experiments have shown that AChBP undergoes conformational changes (or conformational stabilisation) upon the binding of ligands [45,46]. Moreover, AChBP coupled to the transmembrane span of the 5-HT_3 receptor enabled channel gating and a similar chimera was found to stabilise the receptor in a state close to the desensitised form of a ligand-gated ion channel [47,48]. Therefore, it seems that AChBP has retained the ability to induce allosteric transitions in the membrane spanning portion of this class of receptors. In addition, AChBP-based models were recently utilised to probe the allosteric structural transitions in several ligand-gated ion channels. Indeed, two groups have attempted to dissect the molecular events that link agonist binding to channel gating in 5-HT_3 receptor and muscle nAChR [49,50]. In both studies, residues located at the apex of M2–M3 transmembrane helices were shown to interact with the extracellular Cys-loop, propagating the signal to the pore domain. However, different mechanisms were proposed to trigger the opening of a 5-HT_3 and nAChR channel; a *cis–trans* isomerization of a proline residue and electrostatic and hydrophobic interactions, respectively.

As ligands often display different affinity for the different structural states, a property that might be relevant and under-utilised in nAChR drug design, it is of interest to consider these different conformations (resting, activated and desensitised states) in the context of the current AChBP crystal structures. Fig. 3 shows all currently available structures of AChBP subunit interfaces comprising 13 different ligands with various properties (agonists, partial agonist, antagonists and buffer molecules) co-crystallised with AChBP. A rapid examination of these different structures reveals they can be classified into only two categories, either ''open'' or ''closed'' with respect to the C loop conformation. This observation does not directly fit with the three allosteric states of the nAChRs.

Grutter et al. first suggested that the nAChR resting state likely resembles the HEPES bound AChBP structure but with the loop C in an open conformation [51]. Toxins are known to bind to the resting state of nAChR, and accordingly, the AChBP–toxin complexes show that loop C is frozen with its anti-parallel β-sheet arm open (Fig. 3A–C). In addition, the antagonist MLA, as well as a relatively large buffer molecule, PEG, also stabilised the loop C in the same conformation (Fig. 3D and H), supporting the view that these structures represent a resting state of the nAChR. On two occasions the AChBP subunit interface has been crystallised in its ligand-free form (Fig. 3F and G) and again loop C appears in an ''open'' conformation similar to the antagonist-bound AChBP, indicating that these ''lucky'' crystallographic events may have trapped the protein in the ''true'' resting state. Indeed, even if antagonists stabilise the resting state, the interactions that take place during binding invariably induce a structural perturbation (e.g. loop F in the α-cobratoxin–AChBP complex). Therefore, the unliganded AChBP structures are more likely to represent the true nAChR resting state.

As expected, the activated state of nAChR most likely resembles the agonist-bound AChBP structures (Fig. 3K–N). These structures are all similar to the buffer-molecule bound AChBP structures (Fig. 3E, I, J, and O) with a loop C in a ''closed'' conformation. In the buffer (HEPES) bound AChBP structures, loop C is slightly more open and thus could represent a resting or inactivated state, consistent with the lack of agonist action associated with HEPES addition to α7 nAChR [13]. If, as suggested by others, the buffer molecules-bound AChBP represent the desensitised state of the nAChR [51], then its structure is not fundamentally different from the agonist-bound structures (Fig. 3). To explain this controversial interpretation, we suggest that the desensitisation may not be associated with an easily detectable change in the extracellular domain compared to the activated state, but rather a conformational rearrangement associated with this loss of ion channel conductance that occurs in the pore domain. In support of this hypothesis, single point mutations in the channel-lining regions of muscle or neuronal nAChRs produce a loss of desensitisation and convert α-conotoxin

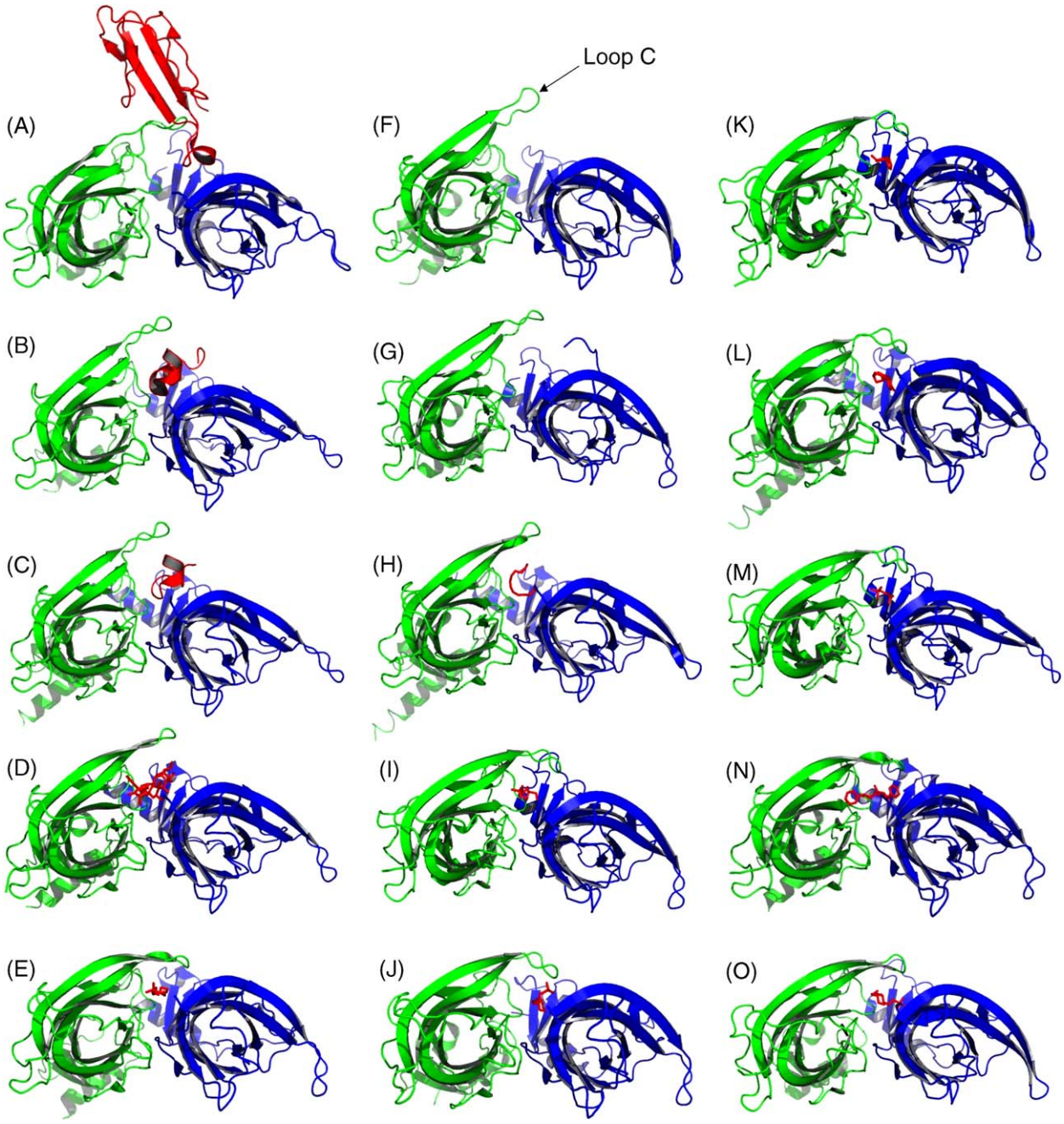

Fig. 3 – AChBP structures. All different AChBP subunit interfaces available to date are shown with a clockwise rotation of 90°x from Fig. 2. Principal subunit is in green, complementary subunit in blue, and ligands and buffer molecules in red. Loop F has been removed for clarity. Loop C of the principal subunit is indicated. Structures are identified as follow: PDB code number, AChBP species (Ac: *Alpysia californica*; Ls: *Lymnaea stagnalis*; Bt: *Bulinus truncatus*) and ligand co-crystallised. (A) 1YI5-Ls-AChBP-Cbtx; (B) 2BR8-Ac-AChBP-PnIA; (C) 2BYP-Ac-AChBP-ImI; (D) 2BYR-Ac-AChBP-MLA; (E) 2BR7-Ac-AChBP-HEPES; (F) 2BR7-Ac-AChBP-HEPES free; (G) 1UV6-Ls-AChBP-carbamylcholine free; (H) 2BYN-Ac-AChBP-PEG; (I) 1UX2-Ls-AChBP-HEPES; (J) 2BJ0-Bt-AChBP-CAPS; (K) 1UW6-Ls-AChBP-nicotine; (L) 2BYQ-Ac-AChBP-EPI; (M) 1UV6-Ls-AChBP-carbamylcholine; (N) 2BYS-Ac-AChBP-LOB; (O) 1I9B-Ls-AChBP-HEPES.

A10L-PnIA into an agonist [52,53]. In addition, residues involved in the desensitised closed gate have been located between αG240 and αL251 of the muscle nAChR using the substituted-cysteine-accessibility method [54]. This region encompasses the closed resting gate but extends further into M2 segment, indicating that even if the channel is closed in both the desensitised and resting states, the structure of the pore is different. Finally, phosphorylation of residues of the M3–M4 cytoplasmic loop were also found to affect desensitisation [54].

In conclusion, to model nAChR in the resting (or antagonist-bound), activated or desensitised states, several sets of molecular coordinates are now available. These structures allow the design or in silico screening of novel ligands using AChBP structures bound to a ligands of diverse size. Using AChBP structures in complex with similar sized ligands of equivalent function (agonist or antagonist) will enhance the predictability achievable from these approaches. This can be best illustrated with the three AChBP–toxin complexes: the two conotoxin-bound AChBPs have almost superimposable structure, while the cobratoxin-bound AChBP exhibits significant differences.

6. Future directions

Thanks to the different AChBP structures now available, we can start to develop rational approaches to the design of nAChR ligands with novel selectivity. Indeed, key interactions as seen in AChBP-ligand co-crystal structures give a clear view of the minimum pharmacophore required for binding. It appears that agonists make few contacts with conserved receptor residues and therefore subtype selectivity might be difficult to achieve, as already evident from the existing molecules. In contrast, antagonists make extensive contacts with receptor residues located outside the conserved pocket, allowing the design of specific interactions with unique amino acids. For instance, it is surprising that no potent conotoxin active at the α4β2 nAChR subtype has been found, while very potent conotoxins (PnIA and MII) target the closely related α3β2 nAChR subtype. Therefore, α4β2 nAChR selective ligands could be engineered based on the structure of a α3β2 selective conotoxin (or a small helix scaffold) and predicted complimentary interactions with the α4 subunit. Of particular interests, the α4 residues T147, R185 and A191 (S148, I186 and E192 in α3, respectively), could be coupled to specific conotoxin residues in order to generate pairwise interactions that may produce active ligands. An ideal starting scaffold would be α-conotoxin GID, which binds with high affinity to α7 and α3β2 nAChRs but also displays modest affinity for α4β2 nAChR. GID has a flexible N-terminal tail composed of four residues, which has been shown to be important for the α4 selectivity [55]. From a preliminary model of the interaction of GID with α4β2 nAChR, it appears that D3 of GID interacts with R185 of α4. Then, homology models of nAChRs based on AChBP will be used to screen in silico these novel ligands before synthesising and testing them in vitro. A similar approach based on ω-conotoxin pharmacophore has successfully led to the design of small voltage-gated calcium channel inhibitors [56]. Finally, it would be interesting to get additional structural data of the same toxins or similar toxins bound to *Lymnaea* or *Bullatus* AChBP to obtain additional clue to the molecular basis of nAChR selectivity.

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