4

Directed Mutagenesis of Nicotinic Receptors to Investigate Receptor Function

Jürgen Ludwig, Holger Rabe, Anja Höffle-Maas,
Marek Samochocki, Alfred Maelicke and Titus Kaletta
Galantos Pharma GmbH
Germany

1. Introduction

Nicotinic acetylcholine receptors (nAChR) are the archetypes of drug receptors. In 1905, John Newport Langley introduced the concept of a receptive substance on the surface of skeletal muscle that mediated the action of a drug, such as nicotine and curare (a neurotoxin made from a plant) (Langley et al., 1905). He also proposed that these receptive substances were different in different species and tissues, and they undergo conformational changes in response to the respective drug. Today nAChRs are considered prototypes of receptors that function as integral signal transducers. They have the response element and the ion channel domain within the same molecular entity as the ligand-binding domain that is activated by acetylcholine. In contrast, muscarinic acetylcholine receptors (mAChR) are prototypic G protein-coupled receptors. They also sense molecules outside the cell, but they require the G proteins to induce cellular responses by coupling to intracellular signalling pathways. More important than their historical role, nicotinic receptors continue to be at the forefront of science, as they are drug targets for muscle and nerve diseases, such as Alzheimer's and Parkinson's diseases, Schizophrenia and Myasthenia gravis. Therefore, this receptor family serves as an excellent example for demonstrating the suitability of site-directed mutagenesis for investigating receptor function and exploring drug action.

The neurotransmitter acetylcholine binds to the extracellular domain of the nAChR and, consequently, opens the receptor-integral membrane channel for Na^+, K^+ and Ca^{2+} ions. The channel can close in two ways. (1) The acetylcholine dissociates from its extracellular binding site, a process that is enhanced by rapid cleavage of the neurotransmitter by the acetylcholine esterase in the synaptic cleft. Thus released, the neurotransmitter has only a short time to act on nAChRs before the signal is terminated again. (2) The channel closes spontaneously despite the presence of transmitter, a process called desensitization. Desensitization is a protective mechanism against exposure to acetylcholine and its agonists that is too long or too strong. Desensitization thus avoids excessive influx of ions into the cell, which can result in impairment of cellular function and cell death.

Ligand binding to nAChR usually occurs in the submillisecond range. The receptor-integral channel is opened only for a few milliseconds and is then closed. Nicotinic receptors are therefore extremely fast and efficient signal transducers. They play key roles in such life-important properties as muscle contraction and brain action.

1.1 Structure

A nAChR is composed of five subunits that form a central pore. The extracellular domain contains the binding site for the neurotransmitter acetylcholine (ACh). The four transmembrane helices from each subunit make up the integral ion channel. Depicted are some of the features described in this section. The C-loop is an important part of the acetylcholine binding site. The binding site of the modulating substance galantamine (GAL) is situated at another part of the C-loop. A phosphorylation site (P) is important for modulating the activity of the receptor. A glycosylation site (G) seems to play a role in cobratoxin resistance. Ivermectin and PNU-120596 are other substances that modulate the activity of the receptor, but they have different locations (IVE and PNU). The cytoplasmatic loop is important for receptor targeting.

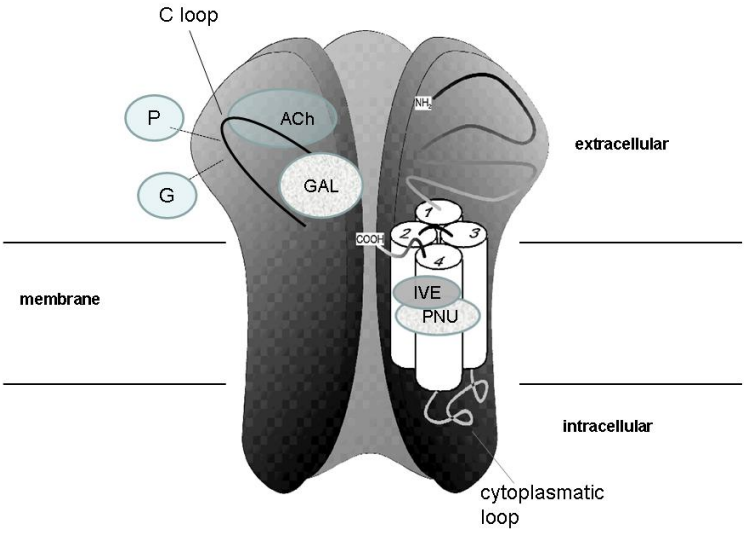

Fig. 1. Schematic representation of a nicotinic Acetylcholine Receptor (nAChR)

The nAChR is assembled from five subunits that are organized around a central pore. Seventeen homologous subunits (α1 – α10, β1 – β4, γ, δ and ε) are known in vertebrates (Zouridakis et al., 2009). This pool of subunits accounts for the vast number of nACh receptor subtypes that exhibit extensive functional diversity with respect to their pharmacological profile, spatiotemporal expression patterns and kinetic properties.

All subunits share the same architectural blueprint, *i.e.*, they consist of a large aminoterminal extracellular domain containing the name-giving cysteine loop, followed by a transmembrane domain and a small intracellular domain. The transmembrane domain consists of four transmembrane regions (TM1 to TM4). TM1, TM2, and TM3 are linked by two short loops. A long and highly variable intracellular loop occurs between TM3 and TM4. Except for those of the long intracellular loop, the amino acid residues are

substantially conserved. This pool of subunits accounts for the vast number of nACh receptor subtypes that exhibit extensive functional diversity with respect to their pharmacological profile, spatiotemporal expression patterns and kinetic properties.

1.2 Use of directed mutagenesis in nicotinic acetylcholine receptor research

Site-directed mutagenesis is a powerful tool to investigate the role of individual amino acids within a protein, to understand the function of a given protein or to understand pharmacological interactions between the protein and compounds. However, modifying individual amino acids or larger parts of a protein with unknown function bears a number of risks. (1) The impact of a particular mutation might appear to be subtle, but it could also lead to a dysfunctional receptor or to the failure of receptor assembly. (2) A mutation might exert an effect on a distant site of the molecule due to conformational changes. This might be interpreted wrongly. (3) The mutated protein might adopt new properties that are unrelated to the natural protein. Hence, it is advisable always to design a battery of receptor mutants or chimeras. It is also important to include similar mutants, either by mutating an amino acid to several different amino acids or by creating similar chimeras. If the results of these mutants are consistent, the risk of erroneously assigning a wrong function can be reduced. Combining mutagenesis studies with other approaches, such as molecular modelling, will further substantiate a hypothesis. A number of such cases will be described in Section 2. Here, the two major mutant types will be described.

1.2.1 Single amino acid changes

Single amino acid changes can be used to investigate the role of amino acids in the binding of the natural ligands and drugs. Furthermore, this type of mutagenesis can be applied to cases in which computer algorithms have predicted motifs, such as glycosylation or phosphorylation sites. Other possible applications are assembly, the targeting of the receptor, or the identification of signals for expression.

A common way to designate a mutant is to use a letter-number-letter scheme. The first letter indicates the wild-type amino acid, using the one-letter code (see Table 1 in the chapter by Figurski et al. for the amino acid codes); the number refers to the position of the amino acid in the protein; and the final letter designates the amino acid that now occupies the position of the original amino acid (*e.g.*, T197A refers to a mutant in which threonine at position 197 was changed to alanine).

1.2.2 Chimeric receptors

Chimeric receptors combine parts of different receptors. This type of mutagenesis is useful in cases in which, instead of a single amino acid, a whole region is in the centre of interest. Examples are functional domains, like the ligand-binding domain or the channel domain, and segments critical for protein signalling, sorting or targeting.

A common way to designate a receptor chimera is to use the names of the receptor types and the name of the joining amino acid (*e.g.*, alpha7-V201-5HT$_3$ refers to a chimeric receptor with the N-terminal part of the α7 nAChR joined with the C-terminal part of the 5HT$_3$ receptor at the amino acid valine 201.

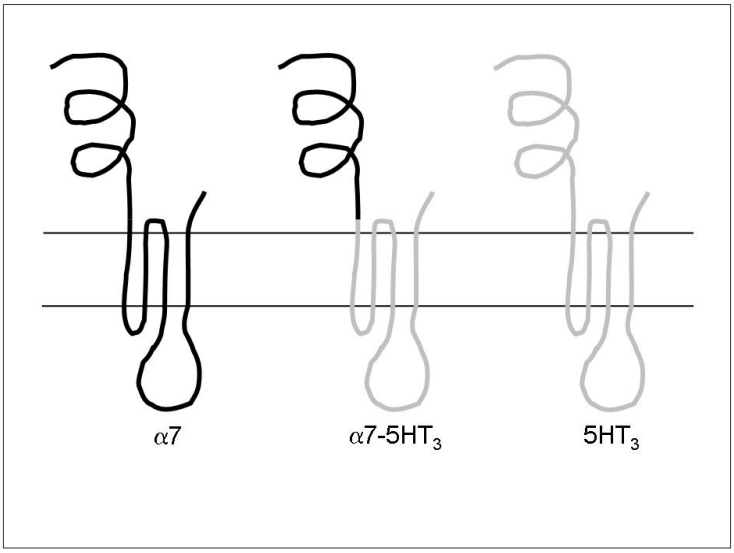

Fig. 2. Schematic representation of a single subunit each from the α7 receptor, the a7-$5HT_3$ chimera, and the $5HT_3$ receptor. The black line denotes the α7 protein; the grey line denotes the $5HT_3$ protein.

1.3 In vitro systems to test functional properties of mutated receptors

Electrophysiology is a critical tool for receptor research. This section gives a basic introduction to the reader not familiar with this technique. Two methods are commonly used: (1) the two-electrode voltage clamp method using *Xenopus* oocytes and (2) the whole-cell voltage clamp method amenable for cell lines.

1. Oocytes from the frog *Xenopus laevis* produce almost all ion channel receptor types in high amounts upon injection of their mRNAs. Since only transient protein expression is possible in this system, oocytes can be used only for a short period of a few days. As the name suggests, two sharp microelectrodes filled with a high molarity potassium chloride solution are pricked into 0.6 to 1 mm oocytes to initiate the recording of channel activity. Fortunately an oocyte can be used for several hours. This is enough time to allow the study of a series of agonists and antagonists.
2. The whole-cell patch-clamp technique is the most common electrophysiological method used for many mammalian cell lines such as HEK-293, CHO, GH4C1 or PC-12 cells. These cells can be easily transfected with nAChR expression vectors. It is possible to generate stable cell lines that have integrated the nAChR sequence into the genomic DNA. The whole-cell voltage clamp method employs thin pipettes, which enable good electrical access to the interior of a cell combined with full external electrical insulation. This technique allows measuring small electric currents generated by ion flow through a single receptor molecule or through a couple of receptors. The fast gating and desensitizing channels require fast and direct drug application methods, such as U-tubes and Y-tubes. These systems can only be used with the small mammalian cells (10

– 20 μm). Heterologously expressed receptors in mammalian cell lines might differ in their biophysical and pharmacological characteristics as compared to those analyzed in *Xenopus laevis* oocytes. Therefore, results from the two systems are not always in agreement.

2. Lessons learned from directed mutagenesis

2.1 Identification of intramolecular signals for nicotinic receptor targeting

The physiological role of nAChRs depends on their localisation in specific regions of the cell. For example, presynaptic receptors regulate the transmitter release from synaptic vesicles into the synaptic cleft. Postsynaptic receptors modulate the postsynaptic potential that stimulates action potential formation in neurons or at the neuromuscular junction (Wonnacott et al., 1997; Albuquerque et al., 2009). The observation of differential expression of nAChRs at pre- and postsynaptic sites triggered the search for specific cellular localization signals. This section illustrates mutagenesis approaches to investigate receptor targeting.

Chicken ciliary neurons proofed to be an excellent tool to study receptor targeting (Williams et al., 1998; Temburni et al., 2000). Ciliary ganglion neurons express two nAChR subtypes: (1) α7 nAChRs, for which localisation is restricted to the perisynaptic dendritic membrane and (2) heteromeric nAChRs consisting of α3, α5 and β4 subunits, which are expressed primarily in postsynaptic membranes (Jacob et al., 1986; Conroy and Berg, 1995). The α3 and α7 nAChR subunits are highly homologous to each other, with one exception: the long cytoplasmatic loop shows great diversity in sequence and length (Lindstrom et al. 1996). It also has been shown that this loop is required for the cellular sorting and trafficking machinery. Therefore, it might be the candidate domain for subcellular targeting. Chimeric α7 nAChR subunits were constructed, in which the cytoplasmatic loop was replaced by the homologous region of the α3 nAChR subunit. Furthermore, a myc-epitope tag was added at the C-terminus to allow detection of the receptor without affecting function. Then the chimaeric α7 nAChR subunit with the α3 nAChR cytoplasmatic loop was ectopically expressed in chicken ciliary neurons. Indeed this chimeric receptor was targeted to the postsynaptic membrane, as shown by antibody staining of the myc-epitope tag. This result demonstrates that the cytoplasmatic loop of the α3 nAChR governs the subcellular targeting of the receptor. Other endogenous nAChR subunits do not play this role because the nAChR subunit does not co-assemble with the α3 nAChR subunit (Conroy and Berg, 1995). In addition, α7 nAChR chimeric subunits containing the cytoplasmatic loop of the α5 or β4 nAChR subunit were designed and expressed. These chimeras were targeted to the perisynaptic site (Temburni et al., 2000). This result means that not only the α7 nAChR subunit contains signals for perisynaptic localisation, but that the α5 and β4 nAChR subunits also do.

Interestingly, in the case of the α3α5β4 heteromer, in which perisynaptic and postsynaptic signals are both present, it is the cytoplasmatic loop of the α3 nAChR subunit that determines the targeting to the postsynaptic site of ciliary ganglion neurons. Taken together the results show that the cytoplasmatic loop contains the cellular localisation signal.

The next step was to determine the exact signal peptide within the cytoplasmatic loop. Before initiating the studies, it was tried to transfer the described approach to hippocampal

neurons (Xu et al., 2006). Again the cytoplasmatic loops between the α4 and α7 nAChR subunits were swapped and ectopically expressed in various combinations in hippocampal neurons. Unfortunately, no surface expression could be detected in cells expressing these chimeric nAChR subunits, either alone or in various combinations. It was possible that the design of the chimera was too aggressive. Possibly a critical peptide sequence needed to enable proper receptor assembly and expression in hippocampal cells was accidentally removed.

Therefore, another mutagenesis strategy was chosen. Swapping internal protein domains may affect receptor assembly. Instead model proteins were used. They were left intact, but they were tagged with putative signal peptides from the nAChR. Two non-neuronal transmembrane proteins were chosen. CD4 and the Interleukin 2 receptor when heterologously expressed in neurons are evenly distributed (Gu et al., 2003). It was tested whether the intracellular loops of the α4 and α7 nAChR subunits are able to target these proteins to specific sites in neurons. When the cytoplasmatic loop of the α7 nAChR was fused to the intracellular-oriented C-terminus of CD4, the chimeric protein was only detected in the dendrites. In contrast, the homologous α4 nAChR cytoplasmatic loop leads to axonal expression.

In order to narrow down the precise localisation signals in the cytoplasmatic loop of the nAChR subunits, the following strategy was chosen. Various overlapping fragments covering the loop from its N-terminal region to its C-terminal region were fused to the C-terminus of the Interleukin-2 receptor. The chimeric receptors were expressed in hippocampal neurons. A specific 25-amino acid-fragment (residue positions 30-54) of the α4 nAChR cytoplasmatic loop targeted the chimera to axons. A 48-residue fragment (positions 33-80) of the α7 nAChR cytoplasmatic loop targeted the chimaera to dendrites (Xu J. et al. 2006).

In a last step, site-directed mutagenesis of specific amino acid residues identified in the targeting sequences a leucine motif (DEXXXLLI) in the α4 nAChR cytoplasmatic loop and a tyrosine motif (YXXx) in the α7 nAChR loop.

In conclusion, an iterative approach of chimera design has pin-pointed the precise targeting sequence of a receptor. It is important to note that chimera design may destroy receptor expression or function. Therefore, it is advisable to generate a set of chimeras and to recognize that the change of an expression system may require an adaption of chimera design.

2.2 Confirming computer-based predictions for posttranslational modifications

Posttranslational modifications are important mechanisms for regulating protein expression and protein activity in eukaryotes. Three posttranslational modifications are known for the nicotinic acetylcholine receptor family: glycosylation, phosphorylation and palmitoylation (Albuquerque et al., 2009). Modern computational algorithms effectively help to identify sequence motifs for putative posttranslational modifications. However, experimental approaches are needed to confirm that the site is actually used for posttranslational modifications and to understand its physiological role. By illustrating the role of phosphorylation, this section exemplifies how to combine computational tools with mutagenesis strategies.

Phosphorylation of α7 nAChR negatively regulates its activity. For example, tyrosine kinase inhibition by genistein, a kinase inhibitor, decreases α7 nAChR phosphorylation and, as a consequence, strongly increases acetylcholine-evoked currents (Charpantier et al., 2005). Therefore, it is interesting to know where and which phosporylation sites are present in the protein sequence. Computer analysis predicts two putative phosphorylation sites (tyrosines at residues 386 and 442) in the long cytoplasmatic loop between TM3 and TM4 of the human α7 nAChR. A site-directed mutagenesis that replaced the tyrosines with alanines (Y386A and Y442A) was carried out, and a receptor double mutant with both mutated phosphorylation sites was tested in *Xenopus* oocytes. Indeed the activity of the receptor double mutant was increased to an extent comparable to inhibition of the wild-type receptor by genistein (a kinase inhibitor). This result confirmed that at least one of the two sites is a physiological phosphorylation site. As the receptor double mutant was insensitive to genistein, it can also be assumed that there are no additional physiologically relevant phosphorylation sites in the protein sequence, which might have been overlooked by the computer algorithm. We note that the value of the receptor mutant not only lies in confirming the phosphorylation site, but also in establishing a physiological role for this posttranslational modification. It is primarily required for the regulation of receptor activity, rather than for receptor expression.

2.3 Receptor chimeras demonstrate the modular domain structure of nAChRs

As mentioned in the introduction, nAChRs are integral signal transducers in which the signalling domain and the response element are within one protein. A key question for understanding the molecular design of a receptor family is whether this is achieved through a modular architecture with functionally independent and separable elements.

In 1993 it was impressively demonstrated that domains are interchangeable between different ligand-gated ion channels. This was exemplified with chimeras made from the α7 nAChR and the $5HT_3$ receptors. The $5HT_3$ receptor is modulated by the ligand serotonin (5HT), permeable for Na^+ and K^+ ions, but it is blocked by Ca^{2+} ions. The authors constructed recombinant chimeric receptors with the N-terminal part of the α7 type nAChR, containing the ligand-binding site, and the C-terminal part of the $5HT_3$ receptor, containing the ion channel domain. They constructed five different chimeras with different junction points for the two receptor parts, using conserved residues W173, Y194, V201, L208 and P217 (the numbers refer to the residue of the α7 receptor). Four of the five chimeric subunit constructs produced properly assembled membrane receptors with an intact extracellular ligand-binding domain, as confirmed by radioactive α-bungarotoxin-binding assays. (α–bungarotoxin is a competitive inhibitor of acetylcholine and binds with high affinity to the acetylcholine-binding site.) Two of the five chimaeras were functional, as shown by the two-electrode voltage clamp technique in *Xenopus* oocytes. One chimera (V201) was able to form large acetylcholine-evoked currents; another (Y194) was able to form small currents.

The V201 chimera was obviously the most interesting chimera and, therefore, was investigated thoroughly by electrophysiological methods and compared to the respective wild-type receptors.

First the ligand-binding properties of the chimaera were pharmacologically studied with a set of modulators and ligands. The agonists were acetylcholine and nicotine, and the

antagonists were α-bungarotoxin and curare, both of which have little or no effect on the $5HT_3$ receptor. In contrast 5HT, the natural ligand of the $5HT_3$ receptor, has no effect on the α7 nAChR receptor. In the *Xenopus* oocyte system, the chimera responded to these ligands in a manner similar to the response of the α7 nAChR wild-type receptor, *i.e.*, acetylcholine increased the current; curare inhibited the currents; and 5HT had no effect.

Second the ion channel properties of the chimera were investigated. α7 type nAChRs and $5HT_3$ receptors differ in their sensitivity to external calcium. While α7 type nAChRs currents increase with higher calcium concentrations, $5HT_3$ receptor currents decrease. The α7 nAChR channel domain is highly conductive for calcium ions, whereas the $5HT_3$ receptor channel domain is blocked by calcium. In addition, external calcium ions have a potentiating effect of the acetylcholine action on α7 nAChRs. In *Xenopus* oocyte studies, the α7 nACh-V201-$5HT_3$ receptor chimera behaved in a manner similar to the response of the $5HT_3$ receptor towards the external calcium concentration. This means that the ion channel properties of the $5HT_3$ receptor are independent of its ligand-binding properties. It appears that, by swapping the ligand-binding domains, a $5HT_3$ receptor can be engineered to respond to acetylcholine and other nAChR modulators like a natural nAChR.

Interestingly, the onset and desensitization kinetics of the current of the chimera is in between the rapid kinetics of wild-type α7 nAChR and the slow kinetics of the $5HT_3$ receptor. This suggests that not all properties of a receptor can be exchanged by simply swapping the ligand-bind domains. The specific kinetic properties of a receptor apparently require interplay between different domains.

In conclusion, computer-based predictions and traditional biochemistry can certainly suggest the domain structure of a protein. However, in order to distinguish whether domains are functionally independent or just building blocks of a larger functional unit, chimeras are invaluable tools.

2.4 Concatemeric nACh receptors revealed the subunit order of nAChRs

The principal architecture of heteromeric nAChRs allows for, at least theoretically, numerous combinatorial arrangements of the various α, β, γ, δ and ε subunit types around the central pore. These arrangements could result in many different receptors with different properties. Which of the possible arrangements are realized in nature, and what distinct properties might they have? For example, it is conceivable that different arrangements of the subunits have different acetylcholine-binding properties because the binding site is located at the interface of two subunits.

In neuronal tissue, many different nAChR combinations are usually present. It can be difficult to study individual nAChR types. In addition the actual arrangement of the subtypes cannot easily be assessed. Directed mutagenesis can be used to force subtypes to form specific nAChR arrangements. Thus, it can be a powerful tool for investigating the roles of different nAChR arrangements. This section focuses on the α4β2 type nAChR, which is the most abundant form of heteropentameric nACh receptors in the mammalian brain.

A pentameric α4β2 nAChR could consist of three α4 and two β2 subunits, or vice versa. Earlier research using *Xenopus laevis* oocytes has shown that the use of equal amounts of α4

and β2 subunit mRNAs generates an $(\alpha4)_2(\beta2)_3$ nAChR (*i. e.*, the α4 and β2 subunits are in a 2:3 stoichiometry, respectively) (Anand et al., 1991; Cooper et al., 1991). In contrast, under conditions in which the α4 subunit mRNA was in excess, an $(\alpha4)_3(\beta2)_2$ nAChR was obtained (3:2 stoichiometry) (Zwart and Vijverberg, 1998). The two different α4β2 nAChRs differ in their pharmacological properties. The $(\alpha4)_2(\beta2)_3$ nAChR is highly sensitive to acetylcholine, whereas the $(\alpha4)_3(\beta2)_2$ nAChR is less sensitive. Since both receptor forms are present in the brain (Marks et al., 1999; Gotti et al., 2008), it has been suggested that the ratio of the two nAChRs is part of a regulatory mechanism for neuronal cell response to nicotine (Tritto et al., 2002; Kim et al., 2003). In addition to their differential sensitivity to acetylcholine and nicotine, the two receptor forms differ in other properties, such as desensitization kinetics and Ca^{2+} permeability (Nelson et al., 2003; Zwart et al., 2008; Moroni et al., 2006; Tapia et al., 2007; Moroni et al., 2008).

For each stoichiometric nAChR, there exist two possible orders of the subunits. The $(\alpha4)_2(\beta2)_3$ nAChR can be α4–α4–β2–β2–β2 (1.1) or α4–β2–α4–β2–β2 (1.2). The $(\alpha4)_3(\beta2)_2$ nAChR can be α4–α4–α4–β2–β2 (2.1) or α4–α4–β2–α4–β2 (2.2). Which of the possible arrangements are realized and what functional significance is conferred by specific positions of each subunit within the receptor complex? Insight into this issue has been gained by using a specific type of directed mutagenesis, *i.e.*, concatemers. A concatemer is a long synthetic gene that contains smaller genes (*e.g.*, the subunit genes) linked in series. In this case, the resulting protein consists of a defined sequence of subunits in which the carboxyl-terminus of the preceding subunit is covalently linked with the amino-terminus of the following subunit (Zhou et al., 2003; Nelson et al, 2003). This technique allows one to enforce a predefined subunit order for the receptor. Studies employing this technique with tandem and triple concatemers of the α4 and β2 subunits showed that only two arrangements were functional. For the $(\alpha4)_2(\beta2)_3$ nAChR, it was the α4–β2–α4–β2–β2 (1.2) arrangement; and for the $(\alpha4)_3(\beta2)_2$ nAChR, it was the α4–α4–β2–α4–β2 (2.2) arrangement (Carbone et al., 2009). Thus, in both receptor forms a triplet of the same subunit was avoided. In summary, the mutagenesis studies described improved our understanding of how the subunit types are arranged (Zhou et al., 2003; Nelson et al, 2003).

Recently, the use of a pentameric nAChR concatemer revealed that there exists also at the α4/α4 interface a functional acetylcholine-binding site (Mazzaferro et al., 2011). Originally, the acetylcholine-binding sites were thought to be located at the α4/β2 subunit interfaces. As these interfaces are present in both receptor isoforms, it is unlikely that they account for differences in acetylcholine sensitivities. In the new study the authors clearly identified the α4/α4 interface in α4–α4–β2–α4–β2 receptors as an additional acetylcholine-binding site. They used a combined approach of a pentameric nAChR concatemer, chimeric α/β subunits with mutagenesis of loop C and structural modelling to determine that this α4/α4 interface accounts for isoform-specific characteristics, *i.e.*, for the low acetylcholine sensitivity. In conclusion, directed mutagenesis permitted a defining of the order of the nAChR subunits and, thus, allowed a determination of the way agonist-binding sites are formed.

2.5 Cobratoxin

The α-neurotoxins from snake venoms are potent antagonists of nicotinic acetylcholine receptors. In mouse some of them are over ten times more toxic than nicotine (*e.g.*, the LD_{50}

of α-cobratoxin from *Naja naja* in mouse is 0.4 mg/kg versus 7.1 mg/kg for nicotine). Despite the high toxicity of the snake toxins, some animals are resistant to α-neurotoxins. This is the case for animals that feed on cobras, such as the mongoose, and, of course, the snake itself. A substantial effort using directed mutagenesis has been made to characterize the interaction between α-neurotoxins and the nicotinic acetylcholine receptors to identify the mechanism of this resistance.

2.5.1 Understanding the cobratoxin – α7 nicotinic acetylcholine receptor interaction

The binding site of the α7 nAChR is composed of six loops, A to F. In order to identify which of these loops interact(s) with α-cobratoxin (α-Cbtx), extensive site-directed mutagenesis was carried out to generate 40 receptor mutants (Fruchart-Gaillard et al., 2002). The possible role of a given amino acid in a α7 nAChR loop thought to interact with α-Cbtx was determined by comparing a mutant receptor with a changed amino acid to the wild-type receptor using a competition binding assay with radioactive iodide-labelled α-bungarotoxin. Only mutations in loops C, D and F reduced the affinity to α-Cbtx. Hence, these loops may be critical for α7 nAChR - α-Cbtx interaction. Mutations of loop C at residues F186 and Y187 showed the greatest effect. They reduced the affinity by 100- to 200-fold. It is important to notice that not every mutation at these two positions reduced affinity. For example, F186R reduced affinity by a factor of 100; in contrast, F186A reduced affinity by a factor of only 4; and F186T, not at all. Hence, when determining a possible role of an amino acid by site-directed mutagenesis, amino acid properties may matter (*e.g.*, size or charge). It is often important to test several amino acid exchanges for a full understanding.

Similarly, a site-directed mutagenesis approach was taken to generate 36 toxin mutants. The objective was to identify the interaction sites of α-Cbtx with the receptor (Antil-Delbeke et al., 2000). This study found loop II and the C-terminal tail of α-Cbtx to interact with α7 nAChR.

To determine which amino acid(s) of the receptor interacts with which amino acid(s) of the toxin, α7 nAChR receptor mutants were tested with α-Cbtx mutants in the competition assay. The studies revealed that the amino acid R33 in loop II of α-Cbtx interacts with a number of amino acids in loop C of the α7 nAChR, such as Y187, W148, P193, and Y194 (Fruchart-Gaillard et al., 2002). Another example is the amino acid K35 of α-Cbtx that interacts with the amino acids F186 and D163 of the α7 nAChR.

This information was then used in a computational 3D model to orient α-Cbtx in the binding pore of α7 nAChR and to help understand the mechanism of the antagonistic action of α-Cbtx. How can the large α-Cbtx molecule exert its antagonistic action on a binding site that is configured to fit small ligands, such as acetylcholine or nicotine? The docking study revealed that only the tip of loop II of the toxin plugs into the cavity between two receptor subunits. About 75% of the remaining surface of the toxin stays outside the toxin-receptor complex. In this way, α-Cbtx behaves like a small ligand and effectively antagonizes α7 nAChR.

2.5.2 Establishing resistance against snake toxin

A snake is usually resistant to its own venom. Hence, it is interesting to find out whether it is resistant from a specific difference in the target molecule of its toxin. In this case, the

target molecule is the nicotinic acetylcholine receptor. There are considerable differences in the protein sequences of the nAChR ligand-binding domains of snakes (*Naja spes*) and mammals. The differences may suggest the different sensitivities. It is possible to make the snake α1 nAChR sensitive to α-Bungarotoxin, a venom of the elapid family. It has been shown by site-directed mutagenesis that introducing the mutation N189F, which substitutes the asparagine in the snake protein sequence with the phenylalanine in the mouse sequence, abolishes resistance (Takacs et al., 2001). This suggests not only that the snake α1 nAChR contains a ligand-binding domain for snake toxins, but also that a single amino acid can cause sensitivity or resistance. In order to test whether the asparagine indeed confers the resistance, a F189N mutation was introduced into the nAChR of the mouse. Two-electrode voltage clamp analysis in *Xenopus* oocytes showed that this mouse receptor mutant was resistant to α-Bungarotoxin. This means that a single amino acid substitution can determine sensitivity or resistance to a snake venom.

Interestingly, N189 is an N-glycosylation site, and it has been postulated that the bulky glycosyl residue may prevent the toxin from entering the binding site (Barchan et al., 1992). This hypothesis might explain the resistance of the mongoose to snake venoms and the sensitivity of mammals. In fact, the mongoose nAChR has an N-glycosylation site at N187, whereas other mammals, such as mouse, cat and humans, lack an N-glycosylation site in the ligand-binding domain.

Another resistance mechanism may be deduced from the receptor-toxin interaction, as described in the previous section. The F189 residue in the α1 nAChR is analogous to F186 in the α7 nAChR. F186 was identified to be critical for the receptor-binding interaction. Therefore, it is conceivable that two mechanisms in the snake receptor confer resistance to α-neurotoxins: (1) disruption of a critical protein-protein interaction and (2) steric hindrance via glycosylation.

2.6 Mapping of the binding sites of allosteric potentiating ligands

Modulators of ligand-gated ion channels (LGICs) have become therapeutically important because, in contrast to traditional agonists or antagonists, these substances change receptor activity only in the presence of the natural ligand. This allows a more physiological control of a LGIC. This concept has been broadly applied for the treatment of epilepsy. For example, benzodiazepines, such as diazepam ("Valium"), present a major class of allosteric modulators of the $GABA_A$-receptors.

Cholinergic neurotransmission is a prominent therapeutic target for the treatment of diseases like Alzheimer's disease. The drug galantamine exerts its therapeutic action by allosteric modulation of the nAChRs (Bertrand and Gopalakrishnan, 2007; Maelicke et al., 2001). Understanding the mechanism of allosteric modulation is therefore important for developing novel drugs for treating Alzheimer´s disease (Faghih et al., 2007).

Today a range of allosteric modulators is known. Synonymously used terms are "allosteric potentiating ligands" (APL) and "positive allosteric modulators" (PAM). They fall into different classes. Galantamine is a representative of the type I class of PAMs (PAM I), which enhance nAChR activity by increasing the current without affecting receptor desensitization. In contrast, members of the type II class of PAMs (PAM II) increase the current of nAChRs, but also reduce their desensitization (Hurst et al., 2005). Hence, a

number of studies were directed to the identification of possible different binding sites, which would help to explain the mechanistic differences of the two classes.

2.6.1 PNU-120596

The group around Neil Millar ran a combined approach to locate the binding site of the type II modulator PNU-120596 a well-studied developmental compound. In a first round of experiments, they compared the action of PNU-120596 on the α7 nAChR and the $5HT_3$ receptor with a set of α7 nACh/$5HT_3$ receptor chimeras (Young et al., 2008). In these chimeras, principally the extracellular part of the α7 nAChR was combined with at least the first three transmembrane domains from the $5HT_3$ receptor. PNU-120596 could, of course, potentiate α7 nAChR. However it could potentiate neither the original $5HT_3$ receptor nor any of the receptor chimeras. This indicated that the PNU-120596 binding site is located in the transmembrane part of the nACh receptor.

In a second round of mutant receptor designs, the amino acid sequence of the transmembrane part of the α7 nAChR was compared with the one from the $5HT_3$ receptor. A number of differences were identified. Amino acids that are not conserved in the α7-nAChR (which is potentiated by PNU-120596) and the $5HT_3$ receptor (which is not potentiated by PNU-120596) formed the basis for a set of α7 nAChR mutants. Amino acids in the nAChR were mutated to the corresponding amino acids of the $5HT_3$ receptor. As expected, some of the mutants were simply not functional, whereas others showed responses to PNU-120596 that were similar to that of wild-type nAChR. Five mutants having otherwise normal function were less responsive than wild type to the potentiating activity of PNU-120596. Two of them (A225D and M253L) were nearly resistant to PNU-120596.

In order to understand why these five amino acids of wild-type nAChR conferred sensitivity to PNU-120596 modulation, the authors investigated several computer models of the nAChR. It turned out that the five amino acids were part of an intra-subunit cavity. Docking simulations revealed that the most favourable docking position of PNU-120596 was at a location very near to the locations of the five amino acids.

Complementary studies using a set of different α7/5HT3 receptor chimeras showed that the α7 nAChR ion channel domain is essential for the action of PNU-120596 (Bertrand et al., 2008). This has led to the model that PNU-120596 exerts its function by stabilizing the cavity in an agonist-like fashion (Barron et al., 2009).

In summary, a combined approach of site-directed mutagenesis experiments, molecular modelling and docking studies was needed to identify the binding site of PNU-120596.

2.6.2 Galantamine

The following studies revealed a totally different location for the binding site of galantamine. They offer an explanation of why the mechanisms of action of the two allosteric modulating ligands are substantially different.

The identification of the galantamine binding site on the extracellular domain close to the acetylcholine binding site has taken more than a decade. Several different approaches were required to finally locate it precisely (Schröder et al., 1994; Ludwig et al., 2010).

A first step was the combination of results from earlier studies about an antibody called FK1 (Schröder et al., 1994; Brejc et al., 2001; Luttmann et al., 2009). An important feature of antibody FK1 is its ability to block the potentiating effect of galantamine on the nAChR without affecting the response to acetylcholine or agonists. The strategy described in this paper helped to identify two stretches (27 and 28 amino acids in length) that contain part of the binding site of galantamine. However, the question remained as to which amino acids participate in the interaction (Schröder et al., 1994). A protein from a freshwater snail assisted in narrowing the range of possible amino acids (Brejc et al., 2001; Smit et al., 2001). The freshwater snail *Lymnaea stagnalis* produces a protein that is homologous to the ligand-binding domain of nAChRs. It is called Acetyl Choline Binding Protein, and it assembles as a homopentamer amenable to X-ray crystallography. A 2.7 Å resolution structure was used as a template to model the ligand-binding domains of α7 and α4β2 nAChRs (Luttmann et al., 2009). In these models only a small proportion of the amino acid stretches of the receptor lie in a position at the outer surface that would be accessible to the FK1 antibody (Figure 3). As both amino acid stretches contribute to the FK1 epitope, it seems highly probable that the epitope is located at the junction of the two stretches.

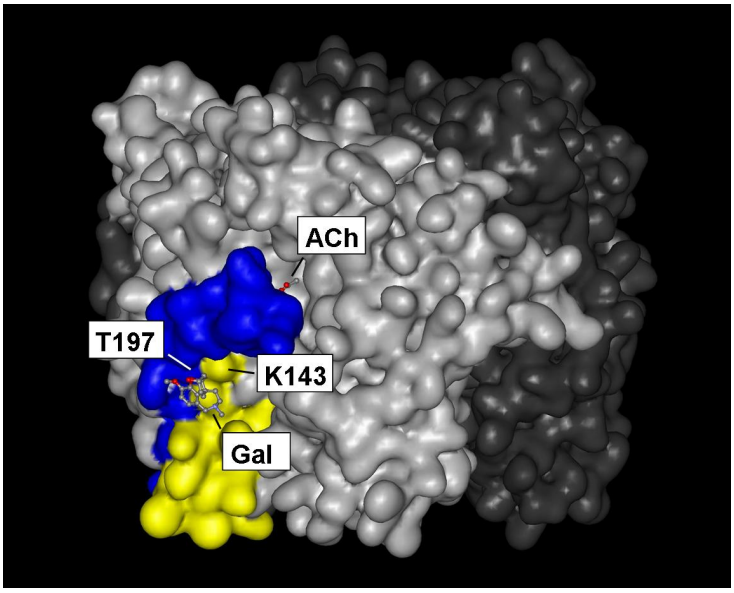

Fig. 3. Surface model of the α7 nAChR ligand-binding domain. Only the ligand-binding domain (LBD) is shown. The cell membrane and channel domain would be beneath the LBD. Two of the five subunits are shown in light grey, with the other ones depicted in dark grey. One molecule of acetylcholine (ACh) is bound. It is almost buried inside the binding site. The amino acids in yellow and blue belong to amino acid stretches identified in Schröder et al., 1994 as contributing to the epitope for the galantamine blocking antibody FK1. Mutation of the amino acids T197 and K143 showed no (T197) or reduced (K143) galantamine effect when stimulated with acetylcholine. This is in line with the assumption that the galantamine binding site is at the junction of these two amino acid stretches. The amino acids T197 and K143 are possible binding sites for galantamine (Gal), as predicted by docking studies.

These insights led to a hypothetical binding site, which was proven with a set of eight different α7 nAChR mutants (Ludwig et al., 2010). All of the mutants were functional, albeit two had much lower affinities for acetylcholine and agonists than wild type. Four of the mutants, all of which showed a normal response to agonists, had no or a reduced response to galantamine. These four mutants were altered in amino acids at the borders of the two stretches mentioned above and were adjacent in the α7 nAChR model (Ludwig et al., 2010). Docking studies pinpointed two amino acids, threonine 197 and lysine 143, as the galantamine binding site (Luttmann et al., 2009). The two amino acids that were replaced in the other two mutants were shown to be oriented in a way in which they would be unlikely to interact with galantamine directly.

A mechanistic model to explain how the binding of acetylcholine opens the ion channel proposes that the C-loop (Fig. 1) acts as a lever that moves upon binding of acetylcholine. Since the galantamine binding site is located at the lower part of the C-loop, it is assumed that binding of galantamine enhances the action of this lever.

2.6.3 Ivermectin

Ivermectin is a member of the PAM I class of modulators. It has a potentiating action on the nAChR activation without affecting the desensitization. Collins and Millar performed an approach similar to the one described for PNU-120596 to identify the amino acids that play a critical role in the interaction with ivermectin (Collins and Millar, 2010).

The authors conducted experiments with α7 nAChRs, $5HT_3$ receptors and the already described α7-$5HT_3$ chimeric receptors in *Xenopus* oocytes. The experiments led to the conclusion that the transmembrane domain plays a critical role in the allosteric modulation by ivermectin. The results showed that, while ivermectin potentiated the effect of acetylcholine on the α7 nAChR, it had no effect on the $5HT_3$ receptor. In contrast, the α7-$5HT_3$ chimeric receptor, which contains the ligand-binding domain of the α7 nAChR and the ion channel domain of the $5HT_3$ receptor, was surprisingly inhibited by ivermectin. The reason for this unexepected response of the chimeric receptor to ivermectin is not known, but it might be that the $5HT_3$ receptor extracellular domain blocks the access of ivermectin to a transmembrane domain binding site that is accessible in the chimeric receptor.

In subsequent experiments, the authors changed selected amino acids in the α7 nAChR. The mutations A225D, Q272V, T456Y, and C459Y almost completely prevented allosteric modulation by ivermectin. It is interesting to note that some of these mutants react similarly to PNU-120596 and to ivermectin. The A225D and C459Y mutants showed a reduced response to both compounds. In contrast, mutations at Q272V and T456V reduced the allosteric potentiation of ivermectin, but not of PNU-120596. This suggests that the amino acids responsible for the allosteric potentiating action of ivermectin partially overlap the ones responsible for the action of PNU-120596.

2.6.4 Conclusion

Section 2.6 describes the use of directed mutagenesis for the identification and investigation of the binding sites of the three nAChR modulators PNU-120596, galantamine and ivermectin. Galantamine and ivermectin are classified as PAM I

modulators, while PNU-120596 is a PAM II class allosteric modulator, based on electrophysiological properties. However, the binding site of galantamine is on the extracellular ligand-binding domain, while ivermectin and PNU-120596 bind to the channel domain of the receptor. Apparently similar electrophysiological properties do not reflect similar binding sites.

3. Concluding remarks

This chapter has highlighted milestones of the directed mutagenesis research performed on nAChRs. It includes research about receptor targeting, the modular domain architecture, the order of the heteromeric subunit assembly, the mechanism of toxin resistance and the receptor interaction with modulatory ligands. Future research will continue to investigate the nicotinic receptor family and its role in diseases, such as Alzheimer's or Parkinson's. The interaction between α7 nAChR and β-amyloid is just one of the many urgent questions that need to be resolved (Tong et al., 2011). Directed mutagenesis will remain one of the most powerful tools on the journey towards the full understanding of this molecular machine.

4. References

Anand R., Conroy, W.G., Schoepfer, R., Whiting, P., & Lindstrom, J. (1991). Neuronal nicotinic acetylcholine receptors expressed *Xenopus* oocytes have a pentameric quaternary structure. *Journal of Biological Chemistry*, Vol.266, No.17 (June 1991), pp. 11192-11198, ISSN 0021-9258.

Antil-Delbeke, S., Gaillard, C., Tamiya, T., Corringer, P., Corringer, P., Servent, D., & Ménez, A. (2000). Molecular determinants by which a long chain toxin from snake venom interacts with the neuronal alpha7-nicotinic acetylcholine receptor. *Journal of Biological Chemistry* Vol.275, No.38 (September 22), pp.29594–29601 ISSN 1083-351X

Albuquerque, E., Pereira, E., Alkondon, M., & Rogers, S. (2009). Mammalian nicotinic acetylcholine receptors: from structure to function. *Physiological Reviews*, Vol.89, No.1, (January 2009), pp. 73-120. ISSN 1522-1210

Barchan, D., Kachalsky, S., Neumann, D., Vogel, Z., Ovadia, M., Kochava, E., & Fuchs, S. (1992). How the mongoose can fight the snake: The binding site of the mongoose acetylcholine receptor. *PNAS* Vol. 89, No.16 (August 15), pp.7717-7721, ISSN 0027-8424

Barron, S.C., McLaughlin, J.T., See, J.A., Richards, V.L., & Rosenberg, R.L., (2009). An allosteric modulator of alpha7 nicotinic receptors, N-(5-Chloro-2,4-dimethoxyphenyl)-N'-(5-methyl-3-isoxazolyl)-urea (PNU-120596), causes conformational changes in the extracellular ligand binding domain similar to those caused by acetylcholine. *Molecular Pharmacology*, Vol.76, No.2 (August 2009), pp. 253-263, ISSN 0026-895X

Bertrand, D., Bertrand, S., Cassar, S., Gubbins, E., Li, J., & Gopalakrishnan, M. (2008). Positive allosteric modulation of the alpha7 nicotinic acetylcholine receptor: ligand interactions with distinct binding sites and evidence for a prominent role of the M2-M3 segment. *Molecular Pharmacology*, Vol.74, Nr.5, (November 2008), pp.1407-1416, ISSN 0026-895X

Brejc, K., van Dijk, W., Klaassen, R., Schuurmans, M., van Der Oost, J., Smit, A. and Sixma, T. (2001). Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature*, Vol.411, No.6835, (May 2001), pp.269-276, ISSN 0028-0836

Carbone, A.L., Moroni, L.M., Groot-Kormelink, P.J. & Bermudez, I. (2009). Pentameric concatenated $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ nicotinic acetylcholine receptors: subunit arrangement determines functional expression. *British Journal of Pharmacology*, Vol. 156, No.6 (March 2009), pp. 970–981, ISSN 0007-1188.

Charpantier, E., Wiesner, A., Huh, K., Ogier, R., Hoda J., Allaman, G., Raggenbass, M., Feuerbach, D., Bertrand, D., & Fuhrer, C. (2005). α7 Neuronal nicotinic acetylcholine receptors are negatively regulated by tyrosine phosphorylation and src-family kinases. *The Journal of Neuroscience*, Vol.25, No.43, (October 26), pp.9836 – 9849, ISSN: 1529-2401

Collins, T., and Millar, N.S. (2010). Nicotinic acetylcholine receptor transmembrane mutations convert ivermectin from a positive to a negative allosteric modulator. Molecular Pharmacology, Vol.78, No.2, (August 2010), pp. 198-204, ISSN 0026-895X

Conroy, W.G., & Berg D.K. (1995). Neurons can maintain multiple classes of nicotinic acetylcholine receptors distinguished by different subunit compositions. *The Journal of Biological Chemistry,* Vol.270, No. 9, (March 1995), pp. 4424- 4431, ISSN 0021-9258

Cooper, E., Couturier, S. & Ballivet, M. (1991). Pentameric structure and subunit stoichiometry of a neuronal nicotinic acetylcholine receptor. *Nature*, Vol.350, No.6315 (March 1991), pp. 235-238, ISSN 0028-0836.

Drisdel, R., Manzana, M. & Green, W (2004). The role of palmitoylation in functional expression of nicotinic α7 receptors. *The Journal of Neuroscience*, Vol.24 No.46, (November 17), pp. 10502–10510, ISSN: 1529-2401

Eiselé, J., Bertrand, S., Galzi, J., Devillers-Thiéry, A., Changeux J., & Bertrand D. (1993) Chimaeric nicotinic-serotonergic receptor combines distinct ligand binding and channel specificities. *Nature*, Vol.366, No.6454, (December 1993), pp.479-483, ISSN: 0028-0836

Fruchart-Gaillard, C., Gilquin, B., Antil-Delbeke, S., Le Novère, N., Tamiya, T., Corringer, P., Changeux, J., Me´nez, A., & Serven, D. (2002). Experimentally based model of a complex between a snake toxin and the α7 nicotinic receptor. *PNAS*, Vol.99, No.5, (March 5), pp.3216–3221, ISSN 0027-8424

Faghih, R., Gfesser, G., & Gopalakrishnan, M. (2007). Advances in the discovery of novel positive allosteric modulators of the alpha7 nicotinic acetylcholine receptor. *Recent Patents on CNS Drug Discovery*, Vol.2, No.2 (June 2007), pp. 99-106, ISSN 1574-8898

Gahring, L., & Rogers, S. (2006). Neuronal nicotinic acetylcholine receptor expression and function on nonneuronal cells. *AAPS Journal*, Vol.7, No.4, (January 2006), pp. E885-894, ISSN ISSN 1550-7416

Geerts, H., Guillaumat, P., Grantham, C., Bode, W., Anciaux, K., & Sachak, S. (2005), Brain levels and acetylcholinesterase inhibition with galantamine and donepezil in rats, mice, and rabbits. *Brain Research*, Vol.1033, No.2, (February 2005), pp. 186-193, ISSN 0006-8993

Gotti, C., Zoli, M., & Clementi, F. (2006). Brain nicotinic acetylcholine receptors: native subtypes and their relevance. *Trends In Pharmacological Science*, Vol.27, No.9 (September 2006), pp. 482–491, ISSN 0165-6147.

Gotti C., Clementi F., Fornari A., Gaimarri A., Guiducci S., Manfredi I., Moretti M., Pedrazzi P., Pucci L., & Zoli M. (2009). Structural and functional diversity of native brain neuronal nicotinic receptors. *Biochemical Pharmacology,* Vol.78, No.7, (October 2009), pp. 703-711. ISSN 0006-2952

Hucho, F., Tsetlin, V. & Machold, J. (1996). The emerging three-dimensional structure of a receptor. The nicotinic acetylcholine receptor. European Journal of Biochemistry, Vol.239, No.3, (August 1996), pp.539-557, ISSN 1432-1033

Hurst, R., Hajós, M., Raggenbass, M., Wall, T., Higdon, N., Lawson, J., Rutherford-Root, K., Berkenpas, M., Hoffmann, W., Piotrowski, D., Groppi, V., Allaman, G., Ogier, R., Bertrand, S., Bertrand, D. & Arneric, S. (2005). A novel positive allosteric modulator of the alpha7 neuronal nicotinic acetylcholine receptor: in vitro and in vivo characterization. *Journal of Neuroscience*, Vol.25, No.17, pp. 4396-4405, ISSN 1529-2401

Jacob, M.H., Lindstrom, J.M., & Berg, D.K. (1986). Surface and intracellular distribution of a putative neuronal nicotinic acetylcholine receptor. *The journal of cell biology,* Vol. 103, No. 1, (July 1986), pp. 205-214, ISSN 0021-9525

Kim H., Flanagin B.A., Qin C., Macdonald R.L., & Stitzel J.A. (2003). The mouse Chrna4 A529T polymorphism alters the ratio of high to low affinity α4β2 nAChRs. *Neuropharmacology* Vol.45, No.3 (September 2003), pp. 345–354, ISSN 0028-3908.

Langley, J. (1905). On the reaction of cells and of nerve-endings to certain poisons, chiefly as regards the reaction of striated muscle to nicotine and to curari.. *The Journal of Physiology*, Vol.33, No.4-5 (December 1905), pp. 374-413, ISSN 1469- 7793

Lindstrom, J., Anand, R., Gerzanich, V., Peng, X., Wang, F. and Wells, G. (1996). Structure and function of neuronal nicotinic acetylcholine receptors. *Progress in Brain Research,* Vol.109, No.1 (1996), pp. 125-137, ISSN 0079-6123

Ludwig J., Höffle-Maas A., Samochocki M., Luttmann E., Albuquerque E., Fels G., & Maelicke A. (2010). Localization by site-directed mutagenesis of a galantamine binding site on α7 nicotinic acetylcholine receptor extracellular domain. *Journal of Receptors and Signal Transduction*, Vol.30, No.6, (December 2006), pp. 469-483, ISSN 1532-4281

Luttmann, E., Ludwig, J., Höffle-Maas, A., Samochocki, M., Maelicke, A., & Fels G. (2009). Structural model for the binding sites of allosterically potentiating ligands on nicotinic acetylcholine receptors. *ChemMedChem*, Vol.4, No.11, (November 2009), pp. 1874-1882, ISSN 1860-7187

Macklin, K., Maus, A., Pereira, E., Albuquerque, E., & Conti-Fine, B. (1998). Human vascular endothelial cells express functional nicotinic acetylcholine receptors. *Journal of Pharmacology and Experimental Therapeutics,* Vol.287, No.1, (October 1998), pp. 435-439. ISSN 1521-0103

Maelicke, A., & Albuquerque, E. (2000). Allosteric modulation of nicotinic acetylcholine receptors as a treatment strategy for Alzheimer's disease. *European Journal of Pharmacology*, Vol.393, No.1-3, pp.165-170, ISSN 0014-2999

Maelicke, A., Samochocki, M., Jostock, R., Fehrenbacher, A., Ludwig, J., Albuquerque E., & Zerlin, M. (2001) Allosteric sensitization of nicotinic receptors by galantamine, a new treatment strategy for Alzheimer's disease. *Biological Psychiatry*, Vol.49, No.3, (February 2001), pp. 279-288, ISSN 0006-3223

Marks, M.J., Whiteaker, P., Calcaterra, J., Stitzel, J.A., Bullock, A.E., Grady, S.R. et al. (1999). Two pharmacologically distinct components of nicotinic receptor-mediated rubidium efflux in mouse brain require the β2 subunit. *The Journal of Pharmacology and Experimental Therapeutics* Vol.289, No.2 (May 1999), pp. 1090–1103, ISSN 0022-3565.

Maus, A., Pereira, E., Karachunski, P., Horton, R., Navaneetham, D., Macklin, K., Cortes, W., Albuquerque, E., & Conti-Fine, B. (1998). Human and rodent bronchial epithelial cells express functional nicotinic acetylcholine receptors. *Molecular Pharmacology*, Vol.54, No.5, (November 1998), pp. 779-788. ISSN 0026-895X

Mazzaferro, S., Benallegue, N., Carbone, A., Gasparri, F., Vijayan, R., Biggin, P.C., Moroni, M., & Bermudez, I. (2011). An additional ACh binding site at the α4/α4 interface of the $(\alpha4\beta2)_2\alpha4$ nicotinic receptor influences agonist sensitivity. *Journal of Biological Chemistry*, Vol.286, No.35 (September 2011), pp. 31043-31054, ISSN 0021-9258.

Miwa, J., Stevens, T., King, S., Caldarone, B., Ibanez-Tallon, I., Xiao, C., Fitzsimonds, R., Pavlides, C., Lester, H., Picciotto, M., & Heintz, N. (2006) The prototoxin lynx1 acts on nicotinic acetylcholine receptors to balance neuronal activity and survival in vivo. *Neuron*, Vol.51, No.5, (September 2006), pp. 587-600. ISSN 0896-6273

Moroni, M, Zwart, R., Sher, E., Cassels, B.K., & Bermudez, I. (2006). α4β2 nicotinic receptors with high and low acetylcholine sensitivity: pharmacology, stoichiometry, and sensitivity to long-term exposure to nicotine. *Molecular Pharmacology* Vol.70 No.2 (August 2006), pp. 755–768, ISSN 0026-895X

Nelson, M.E., Kuryatov, A., Choi, C., Zhou, Y., & Lindstrom, J. (2003). Alternate stoichiometries of α4β2 nicotinic acetylcholine receptors. *Molecular Pharmacology*, Vol.63, No.2 (February 2003), pp. 332-341, ISSN 0026-895X

Sambrook, J., & Russell, D. (2000). *Molecular Cloning: A Laboratory Manual, 3 Vol.* 3rd edition, Cold Spring Harbor Laboratory, ISBN 0879695773, New York

Schröder, B., Reinhardt-Maelicke, S., Schrattenholz, A., McLane, K., Kretschmer, A., Conti-Tronconi, B., & Maelicke, A. (1994). Monoclonal antibodies FK1 and WF6 define two neighboring ligand binding sites on Torpedo acetylcholine receptor alpha-polypeptide. *Journal of Biological Chemistry*, Vol.269, No.14 (April 1994), pp. 10407-10416, ISSN 0021-9258

Smit, A., Syed, N., Schaap, D., van Minnen, J., Klumperman, J., Kits, K., Lodder, H., van der Schors, R., van Elk, R., Sorgedrager, B., Brejc, K., Sixma, T., & Geraerts, W. (2001) A glia-derived acetylcholine-binding protein that modulates synaptic transmission. Nature, Vol.411, No.6835, (May 2001), pp. 261-268, ISSN 0028-0836

Storch, A., Schrattenholz, A., Cooper, J., Abdel Ghani, E., Gutbrod, O., Weber K., Reinhardt, S., Lobron, C., Hermsen, B., Soskiç, V., Pereira, E., Albuquerque, E., Methfessel, C., & Maelicke, A. (1995) Physostigmine, galanthamine and codeine act as 'noncompetitive nicotinic receptor agonists' on clonal rat pheochromocytoma cells.

European Journal of Pharmacology, Vol.290, No.3, (August 1995), pp. 207-219, ISSN 0014-2999

Takacs, Z., Wilhelmsen, K., & Sorota, S. (2001). Snake a-neurotoxin binding site on the Egyptian cobra (*Naja haje*) nicotinic acetylcholine receptor is conserved. *Mol. Biol. Evol.* Vol.18, No.9, pp.1800–1809, ISSN: 0737-4038

Tapia, L., Kuryatov, A., & Lindstrom, J. (2007). Ca^{2+} permeability of the $(\alpha4)_3(\beta2)_2$ stoichiometry exceeds that of $(\alpha4)_2(\beta2)_3$ human acetylcholine receptors. *Molecular Pharmacology*, Vol.71, No.3, (March 2007), pp. 769-776, ISSN 0026-895X

Temburni, M., Blitzblau, R., & Jacob M. (2000). Receptor targeting and heterogeneity at interneuronal nicotinic cholinergic synapses in vivo. *Journal of Physiology,* Vol.525.1 (March 2000), pp. 21-29, ISSN 0022-3751

Thomsen, T., Kaden, B., Fischer, J., Bickel, U., Barz, H., Gusztony, G., Cervos-Navarro, J., & Kewitz, H. (1991). Inhibition of acetylcholinesterase activity in human brain tissue and erythrocytes by galanthamine, physostigmine and tacrine. *European Journal of Clinical Chemistry and Clinical Biochemistry*, Vol.29, No.8, (August 1991), pp. 487-492, ISSN 1437-4331

Tritto, T., Stitzel, J.A., Marks, M.J., Romm, E., & Collins, A.C. (2002). Variability in response to nicotine in the LSXSS RI strains: potential role of polymorphism in alpha4 and alpha6 nicotinic receptor genes. *Pharmacogenetics* Vol.12, No.3 (April 2002), pp. 197–208, ISSN 0960-314X.

Tong, M., Arora, K., White, M. M., & Nichols, R. A. (2011). Role of key aromatic residues in the ligand-binding domain of alpha7 nicotinic receptors in the agonist action of beta-amyloid. *Journal of Biological Chemistry*, Vol.286, No.31, (September 2011), pp. 34373-34381, ISSN 0021-9258

Unwin, N. (2005). Refined structure of the nicotinic acetylcholine receptor at 4A resolution. Journal of Molecular Biology, Vol.346, No.4, (March 2005), pp.967-989, ISSN 0022-2836

Williams, B., Temburni, M., Levey, M., Bertrand, S., Bertrand, D., & Jacob, M. (1998). The long internal loop of the α3 subunit targets nAChR to subdomains within individual synapses on neurones in vivo. *Nature Neuroscience,* Vol. 1 (November 1998), pp. 557-562 ISSN, 1097-6256

Wonnacott, S. (1997). Presynaptic nicotinic ACH receptors. *Trends in Neurosciences,* Vol. 20, No.2 (February 1997), pp. 92-98, ISSN 0166-2236

Xu, J., Zhu, Y., & Heinemann, S. (2006). Identification of sequence motifs that target neuronal nicotinic receptors to dendrites and axons. *The journal of neuroscience,* Vol.26, No.38, (September 2006), pp. 9780- 9793, ISSN 0270-6474

Young, G., Zwart, R., Walker, A., Sher, E., & Millar, N. (2008). Potentiation of alpha7 nicotinic acetylcholine receptors via an allosteric transmembrane site. *Proceedings of the National Acadademy of Sciences USA,* Vol.105, No.38, (September 2008) pp. 14686-1491, ISSN 1091-6490

Zhou, Y., Nelson, M.E., Kuryatov, A., Choi, C., Cooper, J., & Lindstrom, J. (2003). Human α4 β2 acetylcholine receptors formed from linked subunits. *The Journal of Neuroscience*, Vol.23, Nr.27 (October 2003), pp. 9004 –9015, ISSN 0270-6474.

Zouridakis, M., Zisimopoulou, P., Poulas, K., & Tzartos, S. (2009). Recent advances in understanding the structure of nicotinic acetylcholine receptors. *IUBMB Life*, Vol.61, No.4, (April 2009), pp. 407-423, ISSN 1521-6551